XXVIIIth International Conference on Magnetic Resonance in Biological Systems

Frontier of magnetic resonance in biological systems

University College Dublin

Ireland

August 19-24, 2018

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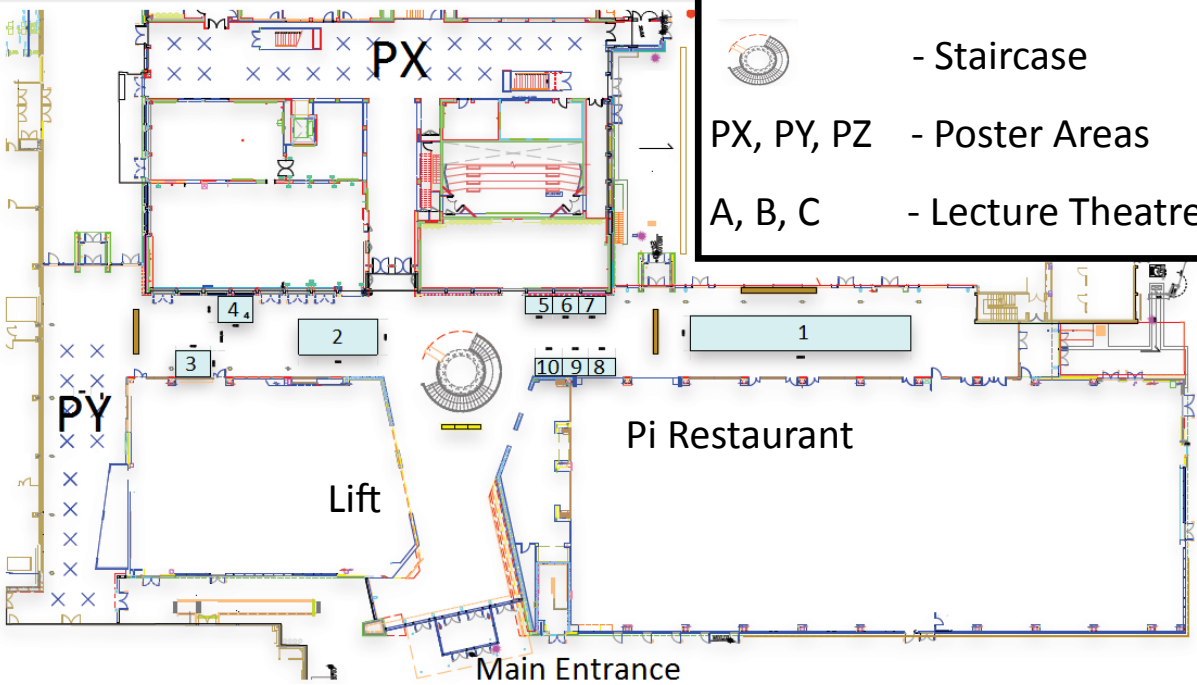
O'Brien Centre for Science

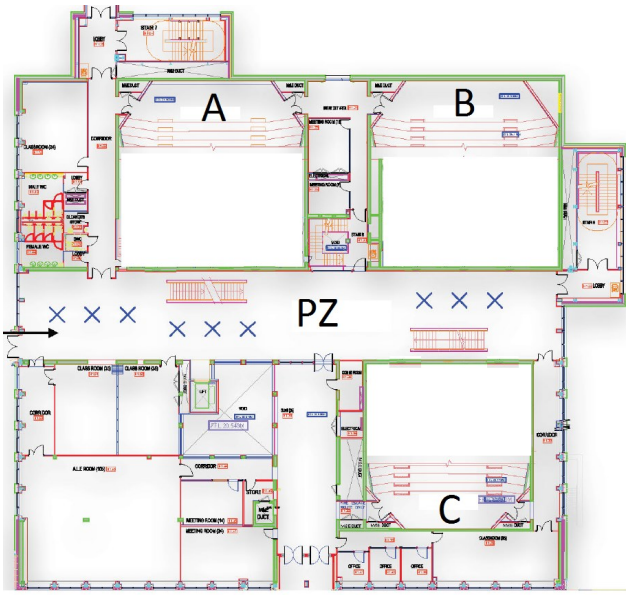
Level 0



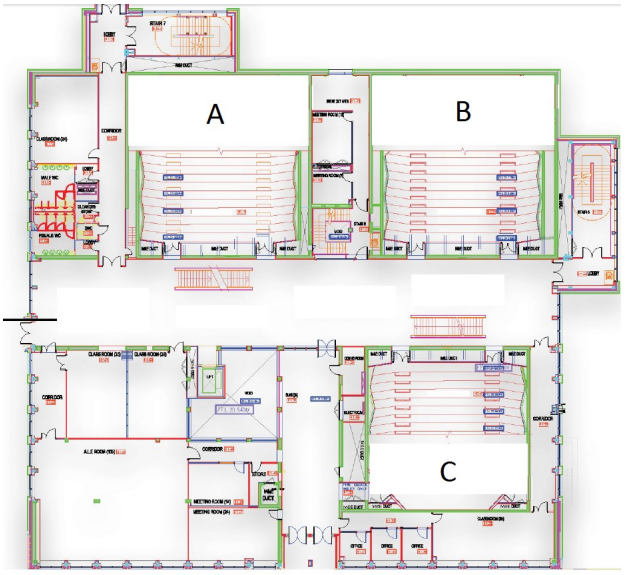
- Staircase
PX, PY, PZ - Poster Areas
A, B, C - Lecture Theatres

1—BRUKER	3—CIL	5—Cortecnet	7—Silantes	9— SIGNALS
2—JOEL	4—Merck	6—STELAR	8—FCNMR	10—MR Resources

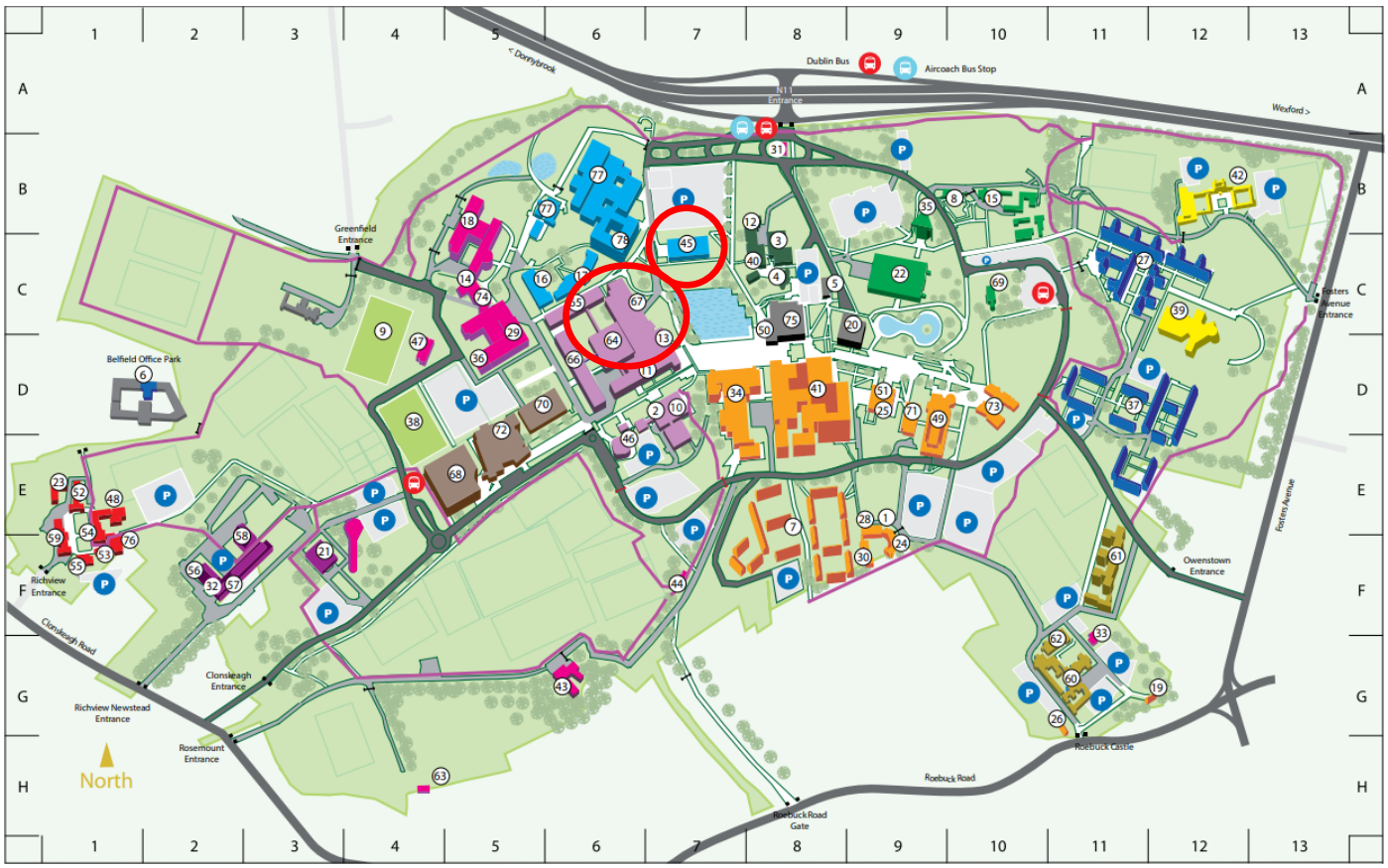
Level 1



Level 2



University College Dublin



O'Brien Centre for Science — 13, 64, 67 (C6, D7)

O'Reilly Hall — 45 (C7)

Services

Bank	5	C8
Bicycle Shop	8	B10
Campus Bookshop	34	D7
Centra Supermarket	37	D11
Copi-Print	34,41,49	D7,D8,D9
Laundry	27, 61	C11, F11
Pharmacy	70	D5
Post Office	51	D9
Sports Centre Barber	68	E5
Student Desk	74	C8
Student Health Service	70	D5
Students' Union	70	D5
Students' Union Shop	22,34,64	C9,D7,D6

Welcome to ICMRBS 2018 in Dublin

On behalf of the organising committee, it is my pleasure to invite you to join in the International Conference on Magnetic Resonance in Biological Systems, ICMRBS, 2018 on 19-24 August at the University College Dublin, Dublin, Ireland. Ireland has been known as the land of Saints and Scholars. The capital city of Dublin lies at the mouth of the river Liffey embracing the Irish Sea. There is evidence of a community living in Dublin since fifth century. It is a city rich in historical and cultural attractions including the unique Book of Kells, the famous Guinness brewery, the neighbouring hills of Tara and the monastic village of Glendalough.

ICMRBS is a well-established biennial NMR conference that has grown substantially since the initial meeting dating back to 1964. It has been well attended by Nobel Laureates, world-class scientists, leading scientific investigators, decision makers, world-renowned commercial partners, publishers and leading companies in the area of nuclear magnetic resonance research and its applications. In addition to covering novel developments in the use of magnetic resonance in biological systems, the ICMRBS meeting is considered one of the major conferences in the rapidly growing field of macromolecular structure characterisation by various magnetic resonance techniques. This conference includes sessions on solution-state NMR, solid-state NMR, EPR, MR imaging, Metabolomics and all other related areas.

The conference will take place at University College Dublin (UCD) one of the largest academic institutions in Ireland. UCD is located approximately 4 km south of Dublin city centre on green leafy grounds. The scientific sessions will take place in the O'Reilly Hall, and in the neighbouring O'Brien Science Centre both of which is surround a beautiful lake. University College Dublin has state-of-the-art on-site accommodation and multi-purpose restaurants and cafes which will cater for conference delegates.

The ICMRBS 2018 will start on 19th August with plenary and prize giving lectures and a welcome reception. In addition to plenary speaker presentations, there will be three parallel sessions and poster presentations for each day covering every aspects of magnetic resonance on biological systems. The farewell reception will take place on 23rd August and the excursions are planned throughout the week for sampling the history and culture of Dublin and its surroundings towns.

The international advisory committee, the scientific program committee and the local organising committee hope to make the ICMRBS 2018 conference an exciting and memorable scientific event. You will also have the opportunity to visit a hospitable and attractive city with many cultural attractions. It is our pleasure to welcome you to Dublin, and we hope that you enjoy the conference and your visit.

2018 ICMRBS Organising Committee
Chandralal Hewage, University College Dublin (Chairman)

Welcome

Organisation

Organising Committee

Chandralal Hewage University College Dublin
Kenneth Mok Trinity College Dublin
Lorraine Brennan University College Dublin
Paul Malthouse University College Dublin
Mike Williamson University of Sheffield
Dermot Brougham University College Dublin
John Parkinson University of Strathclyde
Patrick Timmons University College Dublin

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Wolfgang Jahnke Novartis International Switzerland
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Yunyu Shi University of Science and Technology of China
Kevin Gardner The City University of New York USA
Christian Griesinger Max Planck Institute for Biophysical Chemistry
Mitsu Ikura University of Toronto
Michael Sattler HZ München and TU München

Current ICMRBS Council Members

Current Council Membership (2016-2018)

Chairperson: **Peter Wright**, The Scripps Research Institute
Secretary General: **Ray Norton**, Monash University
Treasurer: **John Markley**, University of Wisconsin-Madison

2018-2028 (Dublin)

Chandralal Hewage University College Dublin
Kenneth Mok Trinity College Dublin
Mike Williamson University of Sheffield

2016-2026 (Kyoto)

Ichio Shimada Tokyo University
Hideo Akutsu Yokohama City University
Masatsune Kainosho Tokyo Metropolitan University

2014-2024 (Dallas)

Kevin Gardner CUNY Advanced Science Research Center
Mei Hong Massachusetts Institute of Technology
Jose Rizo-Rey University of Texas Southwestern Medical Center

2012-2022 (Lyon)

Muriel Delepierre Institut Pasteur
Carine van Heijenoort ICSN
Nadia Izadi-Pruneyre CNRS

2010-2020 (Cairns)

Ray Norton Monash University
Gottfried Otting Australian National University
Joel Mackay The University of Sydney

2008-2018 (San Diego)

Peter E. Wright The Scripps Research Institute
H. Jane Dyson The Scripps Research Institute
Arthur G. Palmer III Columbia University

Organisation

General Information

Registration desk and conference office

The registration desk and conference office are located in the O'Reilly Hall and will be open Sunday the 19th August and remain open until the end of the conference. The registration desk opening hours are as follows:

Sunday 19th	13:00 - 18:00	O'Reilly Hall
Monday 20th	07:30 - 18:30	O'Reilly Hall
Tuesday 21st	08:00 - 15:30	O'Brien Science Centre
Wednesday 22nd	08:00 - 15:30	O'Brien Science Centre
Thursday 23rd	08:00 - 15:30	O'Brien Science Centre

Locations of sessions

All sessions will take place in University College Dublin, Dublin 4. The plenary sessions will be held in the O'Reilly Hall. The parallel sessions will be held in the O'Brien Science Centre (Theatres A, B and C). The poster sessions will be held in the O'Brien Science Centre. Odd numbered posters will present on Monday and Wednesday, and even numbered posters will present on Tuesday and Thursday.

Name Badges

All delegates are kindly asked to wear the provided badges through the conference when on campus.

Speakers

Stewards will be available to assist speakers in transferring their presentations to provided computers or setting up personal computers. Speakers are kindly asked to be present in the lecture hall 30 minutes before the session starts.

Speaker Rooms

The O'Reilly Hall conference room will be available for speakers only; the room is located on the first floor on the O'Reilly Hall.
Please note computers are not provided in these rooms.

Poster Presentations

Categories

New Methodologies	P1 - P25
Pharmaceutical & Drug Development	P26 - P41
Membrane Proteins	P42 - P65
Biomolecular Structure & Function	P66 - P115
Structural Biology	P116 - P151
Nucleic Acids	P152 - P170
Protein-DNA/RNA Interactions	P171 - P186
Biomolecular Interactions	P187 - P215
Protein-Protein Interactions	P216 - P236
Disordered Proteins - Interactions	P237 - P248
Disordered Proteins - Aggregation	P249 - P261
Relaxation & Dynamics	P262 - P284
Computational NMR	P285 - P293
Biological Solids	P294 - P301
Metabolism & In-Cell NMR	P302 - P306
Metabolomics	P307 - P315
Biological NMR & Imaging	P316 - P318
Paramagnetic Systems	P319 - P321
EPR/ESR	P322 - P325

Set-up and removal

Authors are kindly asked to have their posters put up before 13:00 on Monday the 20th of August, and leave them on display for the duration of the conference.
Poster areas are separated according to category and all boards will be labelled with the individual abstract code which can be found in this book in the list of abstracts.
Posters should be removed before 18:00 on Thursday the 23th of August; any unclaimed posters will be discarded.

Sessions

There are four poster sessions, from Monday the 20th of August to Thursday the 23rd of August each day between 13:50 and 15:50. Authors are kindly asked to be present at their posters on the day they have been allocated.
Poster session allocations are as follows:
Odd Numbers: Monday and Wednesday
Even Numbers: Tuesday and Thursday

Lunch, Tea/Coffee, and Water

Lunch, tea, coffee and water will be available in the O'Brien Science Centre.

Welcome mixer and farewell reception

The welcome mixer will be held in the O'Brien Science Centre on Sunday, August 19th at 18:15 and we invite you to join us for some refreshments.
The farewell reception will be held in the O'Brien Science Centre on Thursday, August 23rd at 18:30.

Public transportation

UCD accommodation is located on campus; however several bus routes serve the university. A bus stop located at the main entrance of the University on the N11 is serviced by bus numbers 39a, 145 and 46a which arrive every 5/15 minutes and all travel through Dublin city centre. A single ticket from UCD to St Stephen's Green

(central location) costs €2.15 (Leap Card) or €2.80 (cash, coins only, no change given). Visitor Leap Cards are available for purchase at Dublin Airport. Additional information can be found at www.dublinbus.ie.

Vendor user meetings and activities

Bruker will host their user meeting in the O'Brien Science Centre (First Floor, Theatre E) on Sunday 19th at 12:00-14:00, following brunch at 11:00.
JEOL will host their seminar in the O'Brien Science Centre (Second Floor, Theatre B) on Tuesday 21st at 13:00-13:50.
Bruker will host their hospitality suites in the O'Brien Science Centre (Ground Floor) on Monday 20th at 19:00-21:45.
Merck will host their hospitality suite in the O'Brien Science Centre (Ground Floor) on Tuesday 21st at 19:00-20:30.

Students' Night Out

A students' night out will be held at the Russell Court Hotel on Harcourt Street on Wednesday 22nd starting at 20:00. Transport to the venue is not provided; public transport travels between UCD and the city centre.

Awards

Founders' Medal Award

In 2002, the ICMRBS Council established the Founders' Medal to recognize exceptional contributions by young scientists to the development and/or progress of the field of magnetic resonance in biological systems. The 2018 awardees are **Sebastian Hiller** (University of Basel, Switzerland) and **Lynette Cegelski** (Stanford University, USA).

Poster Prizes

Suraj Manrao Student Science Fund is contributing 10 poster awards of €100 each to deserving student posters at ICMRBS 2018 in Dublin. The posters will be reviewed during the sessions and the winners will be announced during the farewell reception. The Suraj Manrao Student Science Fund for poster prizes is an ongoing ICMRBS event from Suraj Manrao.

Internet

UCD free WiFi service, "UCD Wireless" will be accessible throughout the UCD buildings and residences during the conference period.

Next ICMRBS Conference

The XXIX ICMRBS Meeting will be held in Boston, Massachusetts, USA on August 23-28, 2020

Regulations

Name badges are required for all of the conference sessions, including the poster session and hospitality suites. No smoking is permitted on the UCD campus grounds. Mobile phones must be turned off or silenced during the conference. No photography or recording is permitted in any session, including the poster session.

Gen Info

Tours

Registration for these tours is required through the conference organisers, Keynote PCO.

Pre-Conference Full Day Tour - Glendalough and the Dublin Mountains

This tour will travel to the south of Dublin city to rural county Wicklow where delegates will visit the important ecclesiastical settlement of Glendalough (Gleann Dá Loch) which may be translated as 'The Valley of the Two Lakes'. The valley (glen) was formed through glacial processes over 20,000 years ago. In the 6th century St Kevin established a monastic settlement in this remote area and remains of much of the 'monastic city' including the 30 meter high Round Tower, the impressive Gateway, elaborately decorated crosses and various churches and cathedrals dating from the 12th century are maintained to the present day. During the visit to Glendalough delegates will have an opportunity to explore the monastic city, enjoy the spectacular scenery of the area, experience the visitors centre, partake in some refreshments and hear from an expert site guide on mathematics in Glendalough.
Date: Sunday, 19th August
Departure Time: 07:30
Departure Location: UCD

Post-Conference Full Day Tour - Cliffs of Moher and Galway

Travel with us to the Cliffs of Moher in County Clare located on Ireland's wild rugged Atlantic coast. The cliffs are the highest cliffs in Europe at 214 meters/700 feet high and were recently voted as the seventh most wonderful heritage site in the world, a global poll conducted by UNESCO. Arriving at the Cliffs of Moher make sure to visit the Atlantic Edge - an exciting interpretive centre and a new Visitor Experience. Situated in a large domed cave, see photographs, exhibits, displays and experiences that will enchant all who visit. Continue the tour through the dome and experience a virtual reality Cliff face adventure known as THE LEDGE, shown in the audio visual theatre allowing you to experience life at the cliff face both above and below sea level. Moving outside the visitor centre enjoy a walk along the Cliff face and experience for yourself the exhilarating freedom and the power of nature at work. The Cliffs are 214m high at the highest point and range for 8 kilometres along the western seaboard of County Clare. Visit O'Brien's Tower which stands proudly on a headland off these wonderful Cliffs and see the Aran Islands and Galway Bay.
Date: Friday, 24th August
Departure Time: 07:30
Departure Location: UCD

Half Day Tour - National Stud and Japanese Gardens Tour

This tour includes a nice mixture of culture and nature in Co. Kildare. Visiting the National Stud and Japanese Gardens. The Irish National Stud is home to some of Ireland's finest and most famous racehorses. Join a guided tour of the farm, then pop into the state-of-the-art Horse Museum where the nation's long love affair with horse racing is brought to life. Wander around the internationally acclaimed Japanese Gardens, created in the early 20th century by Japanese gardener Tassa Eida and his son. Finally, take a stroll through Saint Fiachra's Garden, dedicated to the patron saint of gardeners and designed to capture the main elements of the Irish landscape - rock and water
Date: Monday, 20th August
Departure Time: 13:00
Departure Location: UCD

Half Day Tour - Dublin City and Guinness Storehouse Tour

Participants will be collected by private coach and will enjoy a sightseeing tour of Dublin City. The tour will provide an introduction to Dublin taking in its many sights and attractions, including O'Connell Street, the Georgian squares of Merrion Square and Fitzwilliam Square, Christchurch Cathedral, St. Patrick's Cathedral and, Government Buildings. The highlight of the tour will be a visit to the world famous Guinness Storehouse, Ireland's most visited tourist attraction. It was here in 1908 that statistician, W.S. Gosset devised his famous t-test. Participants will partake in the Guinness Experience and enjoy a pint of the black stuff at the iconic Gravity Bar while taking in a 360deg view of the city.
Date: Tuesday, 21st August
Departure Time: 13:00
Departure Location: UCD

Half Day Tour - Coastal Scenic Tour with Malahide Castle and Gardens Tour

Delegates will be brought along the North shore of Dublin, where a view of Howth Head, Bull Island Bird Sanctuary and other sights can be seen. Malahide Castle and Gardens is one of the oldest castles in Ireland, set on 260 acres, this magnificent & historic 12th century castle has been home to the Talbot family for over 800 years.
Date: Wednesday, 22nd August
Departure Time: 13:00
Departure Location: UCD

Half Day Tour - Dublin Tour with Trinity College & Book of Kells

Participants will be collected by private coach and will enjoy a sightseeing tour of Dublin City. The tour will provide an introduction to Dublin taking in its many sights and attractions, including O'Connell Street, the Georgian squares of Merrion Square and Fitzwilliam Square, Christchurch Cathedral, St. Patrick's Cathedral and, Government Buildings. The highlight of the tour will be a visit to Trinity College Dublin where you will be welcomed at the Old Library and the Book of Kells Exhibition – a "must see" on the itinerary of all visitors to Dublin. A walk through the cobbled stones of Trinity College Dublin will bring visitors back to the 18th century, when the magnificent Old Library building was constructed. Inside is housed the Book of Kells – a 9th century gospel manuscript famous throughout the world.
Date: Thursday, 23rd August
Departure Time: 13:00
Departure Location: UCD

Satellite Workshop

Isotopic-labelling Approaches for Biomolecular NMR spectroscopy

This workshop will showcase some of the latest developments in stable isotopic labelling and their application to biomolecular NMR spectroscopy. The two sessions will feature talks from leading scientists in protein and nucleic acid structural biology. Early career researchers will have the opportunity to present their latest results in short talks, which will be selected from submitted abstracts.

Organisers

Dr Michael Plevin
Department of Biology
University of York
York, UK

Dr Anastasia Zhuravleva
Astbury Centre for Structural Molecular Biology
University of Leeds
Leeds, UK

Workshop Schedule

The workshop will take place in the O'Brien Science Centre on Sunday 19th of August. Registration, coffee, lunch and sponsor stands will take place on the ground floor, while sessions 1 & 2 will take place in Theatre F on the first floor.

09h30 - Registration, Coffee and Sponsor Stands

10h30 - Session 1 – Large Protein Machines
Prof John Christodoulou, Professor in Biological NMR Spectroscopy, Department of Structural and Molecular Biology, University College London, UK
Prof Charalampos Kalodimos, Chair, Structural Biology Department, St. Jude Memphis, USA

12h30 - Buffet Lunch and Sponsor Stands

14h00 - Session 2 – Nucleic acids: structure, function and interactions
Prof Teresa Carlomagno, Professor of Structural Chemistry, Leibniz University Hannover, Germany
Prof Hashim al-Hashimi, Director, Duke Center for RNA Biology, Duke University, North Carolina, USA

15h45 - Closing Remarks and Registration for ICMRBS 2018

Programme

	Aug 19 Sun
	O'Reilly Hall
13.00-18.00	**Registration**
16.00-16.10	**ICMRBS Opening** **Chandralal Hewage**
16.10-17.00	**Chair: Peter Wright** **Keynote Lecture** **KL1-Lewis Kay** NMR, Why Bother?
17.00-18.00	**Chair: Mei Hong** **Founders' Medal Lecture** **ML1-Sebastian Hiller** Dynamic chaperone–client-interactions studied by solution NMR spectroscopy **ML2-Lynette Cegelski** New ways of looking at bacterial cell walls and biofilms
18.15-20.15	**O'Brien Science Centre**
	Welcome Reception

	Aug 20 Mon AM		
	O'Reilly Hall		
08.30-09.15	**Chair: Kevin Gardner** **PL1- Ann McDermott** Structure and Dynamics of Protein Complexes		
09.15-10.00	**Chair: Bob Griffin** **PL2-Guido Pintacuda** Fast magic-angle spinning NMR of membrane proteins		
10.00-10.50	**COFFEE** **O'Brien Science Centre**		
	O'Brien Science Centre		
	O'CONNOR - Theatre A	**ELAN - Theatre B**	**ACCENTURE - Theatre C**
	Chair: Fabien Ferrage **New Methodologies I**	**Chair: Vladimir Sklenar** **Protein DNA/RNA Interactions**	**Chair: Helen Mott** **Biomolecular Interactions I**
10.50-11.20	**IL1-Gerhard Wagner** Methods for studying membrane proteins and other complex systems	**IL4-Frederic Allain** Hybrid structural approaches to solve structures of protein-nucleic acid complexes	**IL7-Paul Gooley** The complex mode of agonist binding and activation of the relaxin GPCR, RXFP1
11.20-11.40	**SL1-Beat Vögeli** Exact distance measurement in RNA and large proteins	**SL3-Masato Katahira** Protein-quadruplex DNA/RNA interactions in telomere regulation and anti-prion activity, and the first successful observation of in-cell NMR signals of DNA/RNA in human cells	**SL5-Heiko Möller** Substrate and Inhibitor binding to the 216 kDa transmembrane enzyme complex Na+-NQR from V. cholerae
11.40-12.10	**IL2-Gunnar Jeschke** Rigid or flexible or rather in between – What distance distributions tell on ensemble width	**IL5-Junji Iwahara** Dynamics of basic side chains in protein-DNA interactions	**IL8-Birthe Kragelund** Rethinking Protein Interactions by Disorder
12.10-12.30	**SL2-Theodore Kwaku Dayie** Structural dynamics and interaction studies of large RNAs facilitated by selective Isotope labeling	**SL4-David Neuhaus** Structural basis of activation of human PARP-1 by DNA single-strand breaks	**SL6-Björn Burmann** Chaperone–client interactions: From basic principles to roles in health and disease
12.30-13.00	**IL3-Chun Tang** Probing conformational dynamics on the basis of solvent paramagnetic relaxation enhancement	**IL6-Janez Plavec** Structural variability of G-rich DNA regions	**IL9-Bob Griffin** Structural Studies of beta-2-microglobulin fibrils: MAS NMR and cryoEM

	Aug 20 Mon PM		
	O'Brien Science Centre		
	O'CONNOR - Theatre A	**ELAN - Theatre B**	**ACCENTURE - Theatre C**
	Chair: Lorraine Brennan	**Chair: Mitsu Ikura**	**Chair: Gerhard Wagner**
	Dis. Proteins & Imaging	**EPR/ESR**	**Paramagnetic Systems**
15.50-16.20	**IL10-Kevin Brindle** Metabolic imaging with hyperpolarized 13C-labelled cell substrates – from mouse to man	**IL13-Daniella Goldfarb** The structural dynamics of calmodulin in vitro, in cell extracts and in cells	**IL16-Lucia Banci** Metal-dependent functional processes elucidated through paramagnetic NMR
16.20-16.40	**SL7-Kyouhoon Han** PreSMos (Pre-Structured Motifs) as mediators of IDP-target binding and aggregation	**SL9-Tatyana Smirnova** DEER and smFRET Distance Measurements as Applied to Intrinsically Disordered Proteins	**SL11-Yin Yang** New Gd(Ⅲ) spin labels for DEER distance measurements in-vitro and in-cell
16.40-17.10	**IL11-Lucio Frydman** Progress in High-Definition ADC mapping by Spatiotemporally Encoded MRI	**IL-14-Gary Lorigan** EPR Structural Studies of Membrane Proteins	**IL17-Dermot Brougham** NMR relaxometry and the influence of solvent and ligand dynamics on function of nano-probes designed for bio-applications
17.10-17.30	**SL8-David Cowburn** Deciphering the functional role of 'fuzziness' for IDPs in the nuclear pore complex	**SL10-Mithun Mahawaththa** Studying conformational changes of proteins using a small clickable Gd(III) tag	**SL12-Denis Lacabanne** Mechanistic information on the multidrug ATP-Binding Cassette (ABC) transporter BmrA revealed by solid-state NMR
17.30-18.00	**IL12-Silvio Aime** Design and testing of Frequency-encoding MRI reporters	**IL15-David Norman** The Effect of Perdeuteration on Spin Relaxation and Distance Measurement by EPR	**IL18-Marcellus Ubbink** Protein methyl pseudocontact shifts for assignment and detection of subtle structural changes
	O'Reilly Hall		
18.15-19.00	**Chair: Daniella Goldfarb** **Plenary: Marina Bennati** Progress in ENDOR and Overhauser DNP at High Magnetic Fields to Study Biomolecules		
	O'Brien Science Centre		
19.00-21.45	**BRUKER Hospitality**		

	Aug 21 Tue AM		
	O'Reilly Hall		
08.30-09.15	**Chair: Ken Mok** **PL4- Martin Caffrey** Mesophase Mirabilis. The Lipid Cubic Phase as a System for Investigating Membrane Proteins		
09.15-10.00	**Chair: Masatsune Kainosho** **PL5- Ichio Shimada** Function-related Dynamics of Membrane Proteins		
10.00-10.50	**COFFEE**	**O'Brien Science Centre**	
	O'Brien Science Centre		
	O'CONNOR - Theatre A	**ELAN - Theatre B**	**ACCENTURE - Theatre C**
	Chair: Mike Williamson	**Chair: Janez Plavec**	**Chair: Matthias Buck**
	New Methodologies II	**Nucleic Acids**	**Biomolecular Interactions II**
10.50-11.20	**IL19-Ad Bax** Protein folding monitored by real time pressure-jump NMR	**IL22-Bin Xia** How Bacterial Xenogeneic Silencers Selectively Recognize Foreign DNA in The Resident Genome?	**IL25-Weontae Lee** Dissecting Protein-Protein Interactions for Drug Development by NMR Spectroscopy
11.20-11.40	**SL13-Haribabu Arthanari** 15N-Detection: Harmonizing the sensitivity-resolution conundrum	**SL15-Jan Marchant** Accurate Measurement of Residual Dipolar Couplings in Large RNAs by Variable Flip Angle NMR	**SL17-David Fushman** NMR studies revealed structural basis for the inhibitory effects of ubistatins in the ubiquitin-mediated signaling pathways
11.40-12.10	**IL20-Fabien Ferrage** Protein dynamics with high-resolution field-dependent NMR: from high fields down to 0.33 T	**IL23-Teresa Carlomagno** RNP Complexes in RNA metabolism: a view by integrative structural biology	**IL26-Christian Griesinger** Structure dynamics and kinetics of folding and recognition in proteins by NMR
12.10-12.30	**SL14-Magdalena Kowalska** Ultrasensitive beta-detected NMR to study interactions of metal ions with biomolecules	**SL16-Cyril Dominguez** STAR WARS Mission: destroy the STAR protein Sam68 in the cancer galaxy	**SL18-Rina Rosenzweig** Molecular Chaperones in the Human Protein Disaggregation System
12.30-13.00	**IL21-Ryo Kitahara** High-pressure NMR spectroscopy: Tools for studying protein dynamics	**IL24-Hashim Al-Hashimi** Dynamic Basis for DG•DT Misincorporation via Tautomerization and ionization	**IL27-Ray Norton** Antibody interactions of intrinsically disordered antigens: implications for vaccine development

	Aug 21 Tue PM		
	O'Brien Science Centre		
	O'CONNOR - Theatre A	**ELAN - Theatre B**	**ACCENTURE - Theatre C**
	Chair: Steve Matthews	**Chair: Marc Baldus**	**Chair: Paul Malthouse**
	Structural & Integrated Biology	**Metabolomics**	**Pharmaceuticals & Drug Development**
15.50-16.20	**IL28-Michael Sattler** NMR to study functional important conformational dynamics in biomolecular recognition	**IL31-Hector Keun** Determinants of the urinary and serum metabolome in children from six European populations	**IL34-Martin Scanlon** NMR in the changing landscape of fragment-based drug design
16.20-16.40	**SL19-James Prestegard** Structural Characterization of Methyl-Labeled Glycoproteins Using 13C-Observation	**SL21-John Markley** Tools and Resources for NMR-Based Metabolomics	**SL23-Hans Robert Kalbitzer** Inhibition of Amyloid Aβ Aggregation by High Pressures or Specific D-Enantiomeric Peptides
16.40-17.10	**IL29-Angela Gronenborn** Sweet entanglements probing protein:glycan interactions by NMR	**IL32-Lorraine Brennan** Metabolomics- what can we learn from it?	**IL35-Bong-Jin Lee** Structural study on toxin-antitoxin systems in Mycobacterium Tuberculosis : a target for developing antimicrobial agents
17.10-17.30	**SL20-Christopher Waudby** High-resolution 2D NMR spectroscopy of patient-derived glycoproteins at natural isotopic abundance	**SL22-Michael Kennedy** NMR spectroscopy and electron microscopy identification of metabolic and ultrastructural changes to the kidney following ischemia-reperfusion injury	**SL24-Mark McCoy** NMR Applications for Biologics Discovery & Development
17.30-18.00	**IL30-Peter Crowley** Supramolecular Scaffolds for Protein Assembly	**IL33-David Wishart** NMR in Metabolomics: The Future is Bright	**IL36-Douglas Kojetin** A structural mechanism for directing corepressor-selective inverse agonism of PPARγ
	O'Reilly Hall		
18.15-19.00	**Chair: Ichio Shimada** **Plenary: Andrew Webb** High Field MRI		
	O'Brien Science Centre		
19.00-20.30	**MERCK Hospitality**		

	Aug 22 Wed AM		
	O'Reilly Hall		
08.30-09.15	**Chair: Muriel Delepierre** **PL7-Harald Schwalbe** NMR and Cryo-EM studies of peptide nascent chain complexes in the exit tunnel of the ribosome		
09.15-10.00	**Chair: Jane Dyson** **PL8-Song-I Han** State of tau protein upon liquid-liquid phase separation		
10.00-10.50	**COFFEE** **O'Brien Science Centre**		
	O'Brien Science Centre		
	O'CONNOR - Theatre A	**ELAN - Theatre B**	**ACCENTURE - Theatre C**
	Chair: Anja Böckmann	**Chair: Angela Gronenborn**	**Chair: Daniel Nietlispach**
	Biological Solids I	**Disordered Proteins - Aggregation**	**Macromolecular Complexes**
10.50-11.20	**IL37-Hartmut Oschkinat** Structural Investigations and Experiences with 110 kHz spinning as well as Dynamic Nuclear Polarization (DNP)	**IL40-Peter Wright** NMR characterization of intrinsically disordered regulatory proteins	**IL43-Charalampos Kalodimos** New structural insights into complexes between chaperones and non-native proteins
11.20-11.40	**SL25-Timothy Cross** Solid State NMR Spectroscopy at 1500 MHz in a 35.2 T Hybrid Magnet	**SL27-Alfonso De Simone** Structural Basis of the Neurotoxicity of α-Synuclein Oligomers	**SL29-Beate Bersch** Structural Basis of Membrane Protein Translocation Through the Mitochondrial Intermembrane Space by Small, Hexameric TIM Chaperones
11.40-12.10	**IL38-Melinda Duer** Heavy mice and light things: using solid-state NMR spectroscopy to understand biological tissues in health and disease	**IL41-Roland Riek** Amyloids: From the origin to the end of life	**IL44-Jerome Boisbouvier** Self-assembly, Structure and Functional Studies of Protein Machineries with Molecular Weights of up to 1 MDa
12.10-12.30	**SL26-Tuo Wang** Determine the Supramolecular Architecture of Pathogenic Fungal Cell Walls Using DNP Solid-State NMR	**SL28-Kendra Frederick** DNP-assisted solid state NMR spectroscopy for structure determination in biological environments	**SL30-Nikolaos Sgourakis** Getting in the Groove: Chaperone-assisted peptide exchange dynamics in the Major Histocompatibility Complex
12.30-13.00	**IL39-Tatyana Polenova** Structure and Dynamics of HIV-1 Capsid Protein Assemblies by an Integrated Approach	**IL42-David Eliezer** Linking membrane binding, function and aggregation: tau and alpha-synuclein	**IL45-Roberto De Guzman** NMR of translocon of bacterial nanoinjectors

	Aug 22 Wed PM		
	O'Brien Science Centre		
	O'CONNOR - Theatre A	**ELAN - Theatre B**	**ACCENTURE - Theatre C**
	Chair: Arthur Palmer	**Chair: Michael Overduin**	**Chair: Teresa Carlomagno**
	Computational NMR	**Biomolecular Structure & Function**	**Protein Interactions & Folding**
15.50-16.20	**IL46-Peter Guntert** Structure-based methyl resonance assignment and multi-state eNOE analysis with CYANA	**IL49-Carrie Partch** Morning larks vs. night owls: NMR insights into multisite phosphorylation and control of circadian timing in humans	**IL52-Anthony Mittermaier** Interplay between charge and conformational sampling in the disordered carboxyl terminus of yeast gamma tubulin
16.20-16.40	**SL31-Tobias Madl** De novo structure prediction of biomolecules using solvent-accessibility data	**SL33-Ewen Lescop** Redox control of the human iron-sulfur repair protein MitoNEET activity via its iron-sulfur cluster.	**SL35-Franz Hagn** Probing allosteric structural changes of neurotensin receptor by solution-state NMR
16.40-17.10	**IL47-Wiktor Kozminski** High dimensionality and high resolution NMR experiments for IDPs	**IL50-Rafael Bruschweiler** Advanced complex mixture analysis by NMR and NMR/MS for metabolomics	**IL53-Marius Clore** Uncovering invisible dark states of biological macromolecules and their complexes by magnetic resonance
17.10-17.30	**SL32-Frans Mulder** New computational tools to study Intrinsically Disordered Proteins	**SL34-Michael Latham** Disparate effects of adjacent mutations in the ABC-ATPase Rad50 D-loop	**SL36-Gianluigi Veglia** Role of conformational dynamics on protein kinase A function and dysfunction
17.30-18.00	**IL48-Alexandre Bonvin** High-resolution, integrative modelling of biomolecular complexes from fuzzy data.	**IL51-Isabelle Landrieu** Modulation by Phosphorylation of Tau Protein Interaction with Protein Partners	**IL54-John Christodoulou** Protein Folding on the Ribosome
	O'Reilly Hall		
18.15-19.00	**Chair: Ray Norton** **PL9-Anthony Watts** Pushing the limits of solid state NMR for biomembranes: DNP and fast spinning		
20.00-23.00	**Student Nightout in Downtown Dublin - Russell Court Hotel**		

	Aug 23 Thu AM		
	O'Reilly Hall		
08.30-09.15	**Chair: Nadia Izadi-Pruneyre** **PL10- Isabella Felli** New methods to study intrinsically disordered proteins		
09.15-10.00	**Chair: John Markley** **PL11-Kevin Gardner** Nature's Switches, Scientists' Tools: Mechanisms and Applications of Environmentally-Switched Protein/Protein Interactions		
10.00-10.50	**COFFEE** **O'Brien Science Centre**		
	O'Brien Science Centre		
	O'CONNOR - Theatre A	**ELAN - Theatre B**	**ACCENTURE - Theatre C**
	Chair: Guido Pintacuda	**Chair: Charalampos Kalodimos**	**Chair: Jerome Boisbouvier**
	Biological Solids II	**Disordered Proteins - Interactions**	**Membrane Proteins**
10.50-11.20	**IL55-Mei Hong** Solid-State NMR Determination of the Cholesterol-Binding Structure of Membrane Proteins Using Long-Range Distances	**IL58-Julie Forman-Kay** Non-cooperative folding tuned by phosphorylation of an intrinsically disordered protein to regulate translation initiation	**IL61-Scott Prosser** Molecular and Mechanistic Underpinnings of Signal Transduction - NMR Inspired Studies of the Conformational Landscape of GPCRs
11.20-11.40	**SL37-Yoshitaka Ishii** Prion-like Propagation and Structural Conversion of Alzheimer's Amyloid-β: Solid-state NMR Studies	**SL39-Francois-Xavier Theillet** Improved 13C-detection enables studies of IDPs in physiological conditions and concentrations	**SL41-Daniel Nietlispach** Investigating the dynamic landscape of G-protein-coupled receptors (GPCRs)
11.40-12.10	**IL56-Alexander Barnes** Magic Angle Spinning Spheres, Electron Decoupling with CPMAS Below 6 K, and DNP within Human Cells Using Fluorescent Polarizing Agents	**IL59-Tanja Mittag** Phase separation and mesoscale assembly for functional compartmentalization	**IL62-Mehdi Mobli** Structural characterisation of bioactive disulfide-rich peptides and their interactions with lipid membranes and ion channels in solution
12.10-12.30	**SL38-Angelo Gallo** Structural basis for the interaction between a peptidyl carrier protein and condensation domain in the enacyloxin hybrid PKS-NRPS	**SL40-Jordan Chill** Insights into T cell regulation from NMR-based monitoring of WIP conformational ensembles	**SL42-Adam Lange** A single NaK channel conformation is not enough for non-selective ion conduction
12.30-13.00	**IL57-Anja Böckmann** Solid-state NMR going viral: investigating capsids and envelopes from the Hepatitis B particle	**IL60-Martin Blackledge** Dynamic complexes and complex dynamics: Large scale molecular motion in biological function	**IL63-Francesca Marassi** Probing the structures and functions of membrane proteins in membranes

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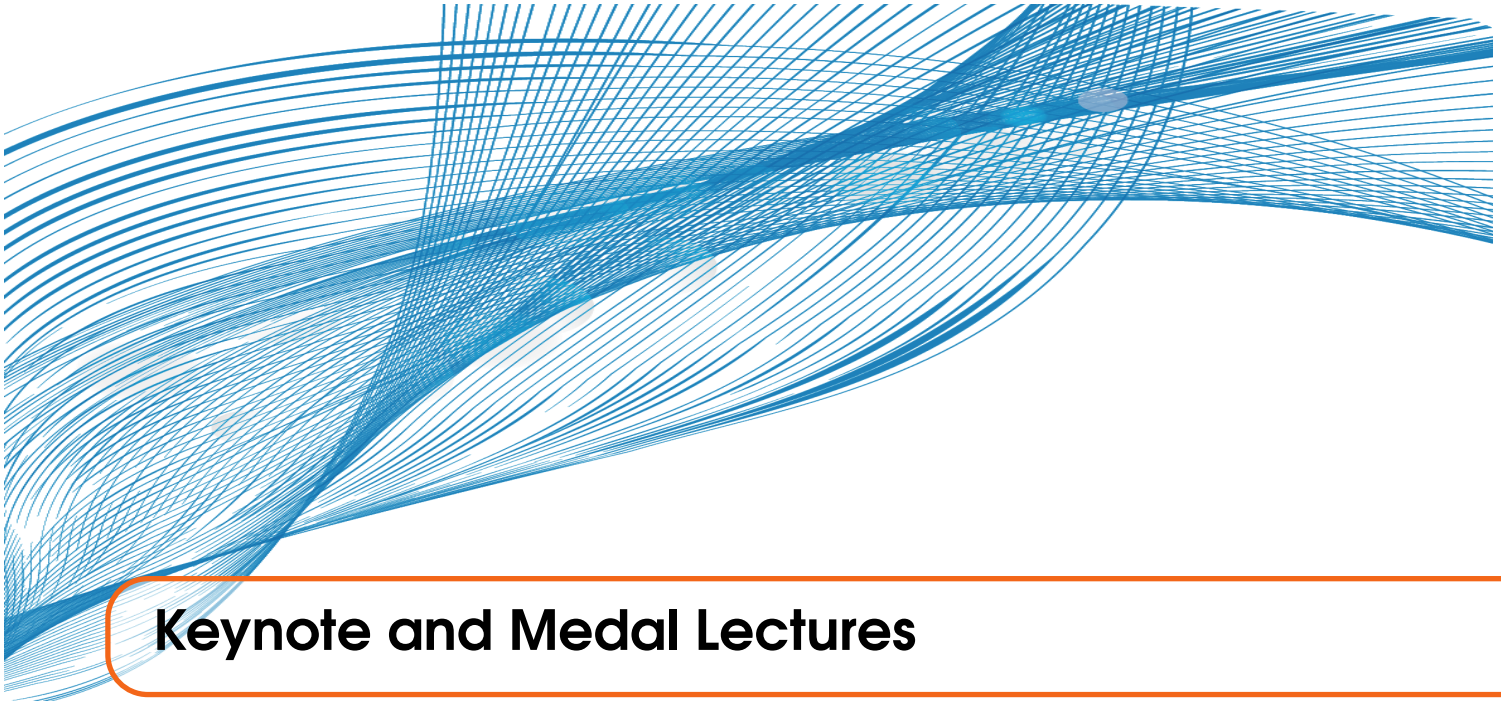
	O'Brien Science Centre		
	O'CONNOR - Theatre A	ELAN - Theatre B	ACCENTURE - Theatre C
	Chair: Peter Guntert	Chair: Carine van Heijenoort	Chair: Peter Crowley
	Relaxation & Dynamics	Enhanced Polarisation	Metabolism & In-Cell NMR
15.50-16.20	**IL64-Joseph Lewandowski** Dynamics of molecular assemblies in the solid state	**IL67-Björn Corzilius** DNP-enhanced MAS NMR enables the selective probing of functional conformational changes	**IL70-Patrick Giraudeau** Fast quantitative 2D NMR for targeted and untargeted metabolomics
16.20-16.40	**SL43-Koh Takeuchi** Dynamic structure guided optimization of ligand using forbidden coherence transfer method	**SL45-Stefan Bibow** Lipid- and cholesterol-mediated timescale-specific modulation of membrane protein dynamics in nanodiscs	**SL47-Harindranath Kadavath** Transient Interactions of Folded and Intrinsically Disordered Proteins Revealed by In-cell NMR
16.40-17.10	**IL65-Daiwen Yang** Dengue Virus NS2B-NS3 Protease Activity Modulated by Dynamics of NS2B	**IL68-Anne Lesage** DNP enhanced biomolecular NMR spectroscopy at high magnetic field and fast magic-angle spinning	**IL71-Yutaka Ito** NMR approaches to investigating protein 3D structures in living eukaryotic cells
17.10-17.30	**SL44-Katja Petzold** MicroRNA's Dynamics Influence Targeting of mRNA by Relaxation Dispersion NMR	**SL46-Dmitry Shishmarev** Methylglyoxal - a 'faulty metabolite' and a probe for rapid-dissolution dynamic nuclear polarisation	**SL48-Alexander Shekhtman** Real-Time In-Cell Nuclear Magnetic Resonance Spectroscopy to study Quinary Protein Interactions
17.30-18.00	**IL66-Flemming Hansen** Using 13C direct-detected NMR to characterise side-chain dynamics and interactions in proteins	**IL69-Xin Zhou** Plumonary Microstructure, Function MRI and Molecular Imaging With Hyperpolarized 129Xe	**IL72-Marc Baldus** Cellular solid-state NMR applied to bacterial and human cells
18.30-20.30	O'Brien Science Centre		
	Farewell Reception		
Aug 24 Fri	**Conference Tours**		



Keynote and Medal Lectures

KL1

NMR, Why Bother?

Lewis Kay

University of Toronto, Canada

With the ever-evolving development of new biophysical tools and increasingly powerful techniques for biomolecular structural studies it is reasonable to contemplate the role of solution based NMR spectroscopy in going forward. In this talk I will describe a number of studies on molecular machines from my laboratory, emphasizing the unique role that NMR can play in providing quantitative descriptions of molecular dynamics and how such motion relates to function. The complementarity of NMR to other structural techniques is such that as they continue to advance so to will the utility of NMR. If anything NMR is far more valuable today than it was even a decade ago. This will be emphasized through a number of examples.

ML1

Dynamic chaperone–client-interactions studied by solution NMR spectroscopy

Sebastian Hiller[1], Björn Burmann[2], Guillaume Mas[1], Irena Matečko-Burmann[1], Roland Riek[3], Juan Gerez[3], Silvia Campioni[3], Stefan G.D. Rüdiger[4], Magdalena Wawrzyniuk[4]

[1]Biozentrum, University of Basel, Switzerland
[2]Biozentrum, University of Basel and University of Gothenborg, Switzerland
[3]ETH Zurich, Switzerland
[4]Utrecht University, Netherlands

Molecular chaperones are essential in cellular protein homeostasis. Central mechanistic aspects of chaperone function are however not well understood at the atomic level, including how chaperones recognize clients, in which conformational states clients are bound, and how chaperone–client interactions are integrated into functional cycles. I will summarize our recent contributions to understand such aspects by high-resolution NMR spectroscopy and present our ongoing work on this topic. Our initial work on the homotrimeric periplasmic holdase Skp with bound outer membrane proteins provided the first atomic-level description of a natural full-length chaperone–client complex [1, 2]. Subsequent work showed how periplasmic chaperones shape individual client folding trajectories at the membrane bilayer [3]. We now find that the monomeric state of Skp is intrinsically disordered, and that oligomerization initiates folding of the chaperone via a “stapling” mechanism. Skp possesses a unique mechanism of coupled oligomerization, folding and client-binding mechanism, showing how an ATP-independent chaperone can modulate its activity in function of the presence of client proteins. An atomic-resolution characterization had revealed how the chaperone Spy selectively recognizes the flexible, locally frustrated regions of partially folded client Im7 in a highly dynamic fashion. The interaction sites are identical for further chaperones, highlighting that general principles govern client recognition [4,5]. These insights could nown be fruitfully used to investigate mechanistic details of the functional role of chaperones in Parkinson’s disease. Parkinson’s is one of the most common neurodegenerative disorders, pathologically manifested by intracellular accumulation of aggregates of the intrinsically disordered protein α-Synuclein. Systematic investigations on an array of six different chaperones identified a general chaperone interaction motive at the α-Synuclein amino-terminus. A dominant role of chaperones among the interactions of cytosolic α-Synuclein was validated with in-cell NMR spectroscopy and the functional basis for the effects of known post-translational modifications on the α-Synuclein-chaperone interaction could be reconstituted in vitro. The data reveal the mechanism how molecular chaperones control the state and function of α-Synuclein in vivo and how the disturbance of these interactions leads to progression of pathologically relevant aggregates.

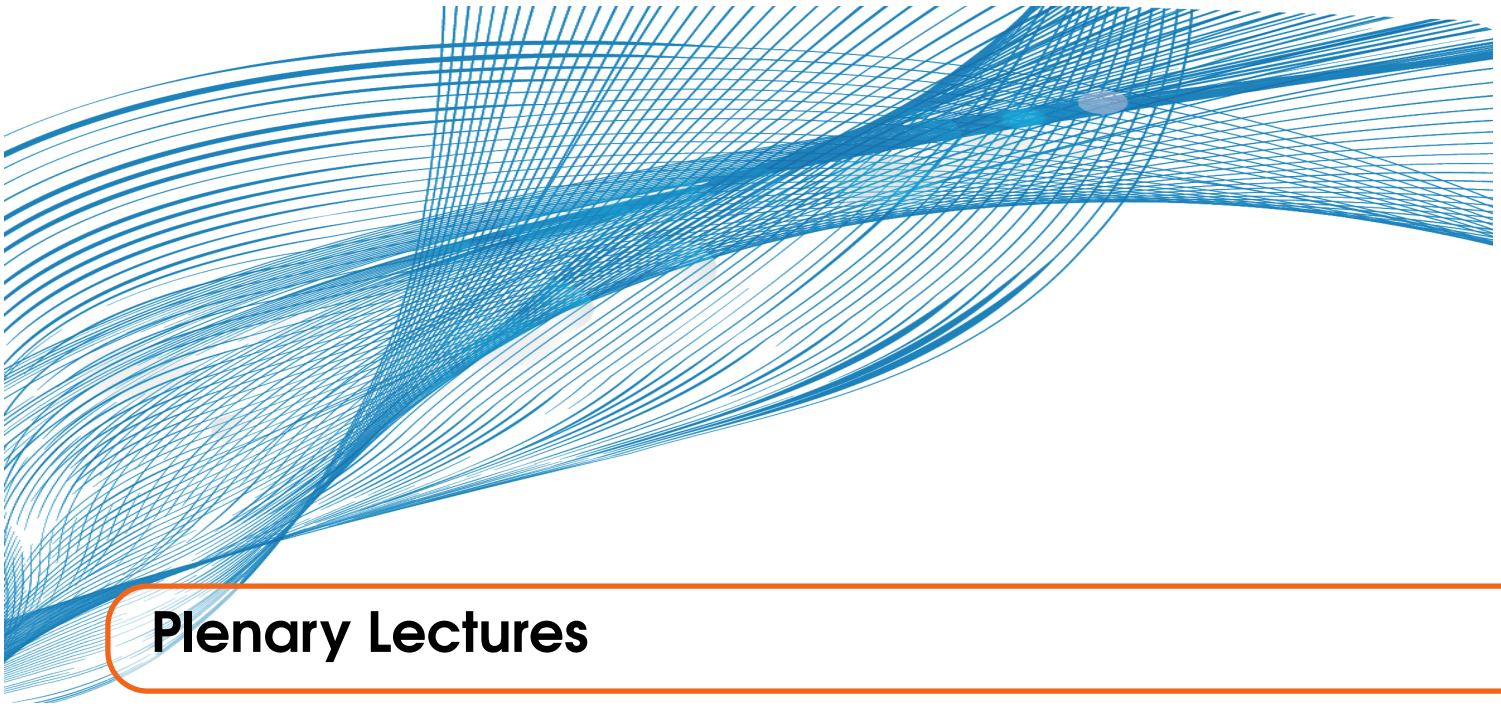
ML2

New ways of looking at bacterial cell walls and biofilms

Lynette Cegelski

Stanford University, United States

The bacterial cell wall is essential to cell survival and is a major target of antibiotics. Beyond the cell surface, bacteria assemble macromolecular architectures during biofilm formation. Biofilms are implicated in serious infectious diseases and have emerged as a target for anti-infectives. Our research program is inspired by the challenge and importance of elucidating chemical structure and function in these complex biological systems and we strive to transform our discoveries into new therapeutic strategies. We have introduced new approaches using whole-cell and cell-wall solid-state NMR, integrated with biochemical and other biophysical analysis, to reveal how the biological functions of cell walls and biofilms depend on their chemical composition and architecture. I will report on our recent discoveries including: (i) the determination of cell-wall compositional changes due to antibiotics in the context of intact unperturbed cells by whole-cell NMR and (ii) the composition and function of an amyloid-associated biofilm matrix for uropathogenic E. coli, including the unprecedented discovery of a naturally produced chemically modified cellulose and our identification of the molecular basis for its assembly. Solid-state NMR of the intact cellulosic material, in particular, enabled the elucidation of the zwitterionic phosphoethanolamine modification that had evaded detection by conventional methods from one of the most commonly studied bacterial biofilm systems.



Plenary Lectures

PL1

Structure and Dynamics of Protein Complexes

Ann McDermott

Columbia University, United States

Allostery in ion channels controls activation coupled inactivation and partly controls mean open time. Solid state NMR experiments on full length wild type channel in proteoliposomes , provide evidence for evacuation of ions from the selectivity filter during inactivation and strong coupling between opening and ion affinity. Furthermore, a number of site specific mutants altered in their inactivation properties in the hinge of the inner helix (e.g. F103A) suggest that a group of bulky residues serve as "hotspots" for allostery. We will discuss applications of new methods for characterizing conformational exchange.

The talk will also discuss structures of amyloids involved in human biology, and new NMR methods to sensitize detection of signals. RIPK1:RIPK3 core complex of the necrosome, which initiates TNF-induced necroptosis in the context of immune defense, cancer and neurodegenerative diseases. Using solid-state NMR, we determined the high-resolution structure of the core. RIPK1 and RIPK3 assume serpentine conformations, with short β-segments. Packing analogous to other amyloids results in a hydrophobic core with both hetero and homo hydrophobic contacts, and unusual exposed "ladders" of interacting amino acids. The molecularly detailed structure of a hetero-oligomeric amyloid and provides insights into the mechanisms of signal transduction and of inhibition of necroptosis.

PL2

Fast magic-angle spinning NMR of membrane proteins

Guido Pintacuda

CRMN (CNRS/ENS Lyon/UCBL), France

Building on a decade of continuous advances of the community, the recent development of very fast (60 kHz and above) magic-angle spinning (MAS) probes has revolutionised the field of solid-state NMR. Today, rapid "fingerprinting" of proteins is possible with a ten-fold reduction of the required sample amounts with respect to conventional approaches, not only in deuterated molecules but also in fully-protonated substrates. Extensive and robust resonance assignments can be derived rapidly for small-to-medium sized proteins (up to ca. 300 residues), opening the way to the determination of inter-nuclear proximities, relative orientations of secondary structural elements, protein-cofactor interactions, local and global dynamics. Fast MAS and 1H detection techniques have nowadays been shown to be applicable to membrane-bound systems. This talk reviews the strategies underlying this recent leap forward in sensitivity and resolution, describing its potential for the detailed characterization of membrane proteins in lipid bilayers.

PL3

Progress in ENDOR and Overhauser DNP at High Magnetic Fields to Study Biomolecules

Marina Bennati

Max Planck Institute for Biophysical Chemistry & University of Göttingen, Germany

Electron-nuclear double resonance (ENDOR) and dynamic nuclear polarization (DNP) are two techniques based on polarization transfer between electron and nuclear spins. Despite differences, they both rely on the subtle mechanism of hyperfine interaction at the atomic scale. This lecture will give an overview on our recent insights and the application potential of these two methods in solids (ENDOR) and solution (DNP) for studies of biological systems. We have introduced high frequency (94 and 263 GHz) ENDOR spectroscopy to elucidate proton-coupled electron transfer (PCET) in biological transformations involving amino acid radical intermediates. Using 2H-Mims and cross-polarisation edited ENDOR in conjunction with quantum chemical calculations, we determined the hydrogen bond network around intermediates formed in the long-range PCET of ribonucleotide reductase. In studies of the Overhauser DNP effect in the liquid state, we have recently observed 13C NMR signal enhancements up to three orders of magnitude at various magnetic fields. These enhancements are consistent with theoretical predictions based on the available theory. Possible developments and applications of scalar Overhauser DNP as a tool to enhance sensitivity in liquid NMR will be discussed.

PL4

Mesophase Mirabilis. The Lipid Cubic Phase as a System for Investigating Membrane Proteins

Martin Caffrey

Trinity College Dublin, Ireland

The lipid cubic phase (in meso) method for crystallizing membrane proteins has to its credit over 500 structure records in the Protein Data Bank. Progress in applying the method reflects innovations that relate to protein enrichment, direct biophysical and biochemical characterization and refolding in the mesophase and in situ data collection at X-ray synchrotrons and free electron lasers using microcrystals. These varied methods will be reviewed and examples will be provided where they have contributed to the determination of high-resolution crystal structures of membrane integral enzymes, receptors, transporters and carriers with insights into mechanism of action and the prospect of drug discovery.

Funded in part by Science Foundation Ireland

PL5

Function-related Dynamics of Membrane Proteins

Ichio Shimada

The University of Tokyo, Japan

Membrane proteins play fundamental roles in many physiological processes and are target proteins for drug development. For better understanding of the functions of the membrane proteins, not only precise

static three-dimensional structures determined by X-ray crystallography and cryo-electron microscopy methodologies, but also dynamical nature are required. NMR provides us information about membrane proteins dynamics, including conformation equilibrium related to functions. However, it is frequently difficult to obtain information about the membrane protein dynamics related to the functions, due to the molecular weight limitation in NMR. We have recently developed novel NMR methods for characterizing protein dynamics utilizing multiple quantum relaxation rates of side-chain methyl groups, which can be sensitively observed in high molecular weight proteins. In this paper, we will show our recent results of function-related dynamics of membrane proteins.

PL6

High Field MRI

Andrew Webb

Dept Radiology, Leiden University Medical Center, Netherlands

This talk will describe recent developments in several areas of magnetic resonance hardware and sequences which have been applied to clinical research and patient scanning at field strengths between 1.5 and 7 Tesla. Topics will include the design of very high permittivity materials/metamaterials for improved magnetic field homogeneity and lower power deposition, new ceramic-based resonators for multi-element transmit arrays, methods for the rapid non-invasive estimation of tissue conductivity, high resolution motion-free imaging of the eye, and whole-body optical-based measurement of temperature changes. Clinical applications include studies of patients with eye tumours, epilepsy and neuromuscular dystrophies.

PL7

NMR and Cryo-EM studies of peptide nascent chain complexes in the exit tunnel of the ribosome

Harald Schwalbe, Linda Schulte, Jeifei Mao, Clemens Glaubitz, Achillleas Frangakis

Goethe-University Frankfurt, Germany

We recently demonstrated the influence of translation kinetics on the native state of the protein gamma B crystallin analysing proteins with identical sequence but synonomous mRNA. Here, using (DNP-enhanced) NMR and cryo-EM studies, we show that disulfide bond formation can occur in the exit tunnel of the ribosome, yielding heterogeneous polypeptide chain configurations. Thus, the nascent chain can access different, native and non-native fold topologies within the exit tunnel.

(1) Synonymous Codons Direct Cotranslational Folding toward Different Protein Conformations. Buhr F, Jha S, Thommen M, Mittelstaet J, Kutz F, Schwalbe H*, Rodnina MV*, Komar AA* Mol Cell. 2016 61:341-351.

PL8

State of tau protein upon liquid-liquid phase separation

Songi Han, Yann Fichou, Yanxian Lin

University of California Santa Barbara, United States

Non-membrane bound organelles that behave like liquid droplets are widespread among eukaryotic cells. Their dysregulation appears to be a critical step in several neurodegenerative conditions. Here we report that tau protein, the primary constituent of Alzheimer neurofibrillary tangles, can form liquid droplets upon liquid-liquid phase separation (LLPS) in vitro and in extracellular medium, and hence the necessary biophysical properties to undergo LLPS in live cells. Tau is an intrinsically disordered protein that complexes with RNA to undergo LLPS that we determined to be an entropy driven complex coacervation process. The question is what the dynamic and structural property of tau is upon LLPS formation and as a function of time, and what the role of this dense liquid phase state is in mediating, facilitating or impeding pathological aggregation of tau – an unanswered yet hotly debated topic. We will rely on electron paramagnetic resonance (EPR) lineshape analysis, EPR dipolar spectroscopy and Overhauser dynamic nuclear polarization (ODNP) relaxometry studies to differentially characterize the state of tau in solution state, the LLPS state, and en route to fibrillar aggregates.

We find that tau is locally freely tumbling and capable of diffusing through the droplet interior despite the high protein concentration within the complex coacervate phase. In fact, tau in the condensed phase state does not reveal any immediate changes in local protein packing, local conformations and local protein dynamics from that of tau in the dilute solution state [1]. In contrast, the population of aggregation-prone tau as induced by the complexation with heparin is accompanied by large changes in local tau conformations and irreversible aggregation [2,3]. However, prolonged residency of tau within the droplet state, the presence of disease mutations, and the presence of aggregation promoting cofactors leads to the emergence of detectable beta-sheet structures according to thioflavin-T assay and imaging. One take home message is that the droplet state can incubate tau and pre-dispose the protein toward the formation of insoluble fibrils. The other take home message is that EPR and ODNP techniques relying on spin labels are uniquely suitable approaches to delineate the dynamic, conformational and fibrillar state of tau within LLPS or en route to tau fibrils that are inherently complex states of matter.

[1] RNA Stores Tau Reversibly in Complex Coacervates, X. Zhang, N.E. Eschmann, Y. Lin, H. Zhou, J. Rauch, I. Hernandez, E. Guzman, K.S. Kosik, S. Han, PLOS Biol., 15 (7) (2017). [2] Signature of an aggregation-prone conformation of tau, N.A. Eschmann, E.R. Georgieva, P. Ganguly, P.P. Borbat, M.D. Rappaport, Y. Akdogan, J.H. Freed, J.E. Shea, S. Han, Sci. Rep., 7 (2017). [3] Heparin-induced tau filaments are structurally heterogeneous and differ from Alzheimer's disease filaments, Y. Fichou and S. Han et al, Chem. Comm., 54 (2018).

PL9

Pushing the limits of solid state NMR for biomembranes: DNP and fast spinning

Anthony Watts

University of Oxford, United Kingdom

Solid state NMR methods can provide dynamic and structural information for both purified and heterogeneous and biologically fully functioning systems, at sub-Å resolution and in a wide dynamic range (ms – ps), providing new insights into biomolecular assembly and function. Recent advances in-

clude the application of novel fast (60kHz) magic angle spinning [1], DNP [2] and NMR crystallography [3].

One major advantage of ssNMR is the ability to resolve ligand (or drug) conformation, the binding site environment and local dynamics within a membrane bound target at near physiological and functionally relevant conditions in natural membranes, to inform design and mode of action, using solid state NMR approaches [4, 5]. This information is obtained by isotopically (2H, 13C, 19F, 15N, 17O) labelling selective parts of either a ligand or the protein under study, and observing the nucleus in non-crystalline, macromolecular complexes [6, 7, 8]. Conformational information and details comes from recoupling data at high resolution between defined sites within the bound ligand. A novel observation is that ligands with complex structure have differential mobility at their binding sites, which has implications for efficacy and action. Substituted imidazole pyridines, for example, which inhibit the H+/K+-ATPase and have therapeutic use, are constrained in the imidazole moiety, but shows significant flexibility at the pyridine group [9]. It is this mobile group which has a direct interaction with an aromatic (Phe198) residue, with concomitant π-electron sharing [10]. Similarly, the steroid moiety of ouabain undergoes motions that are similar to those of the target protein, the Na+/K+-ATPase, but the rhamnose undergoes a high degree of flexibility at fast rates of motion whilst interacting with Tyr198 [11]. For acetyl choline when bound in the nicotinic acetyl choline receptor (nAChR), the quaternary ammonium group undergoes fast rotation at an aromatic binding site, driven by thermal fluctuations that are functionally significant [11]. Differential dynamics of bound ligands may explain their poor resolution in the residual electron density of ligands in target proteins.

[1]. Dannatt et al., (2015) J. of Biomolecular NMR, 62; 17-23 [2]. Pike, et al., (2012) J. Mag. Res., 215, 1-9. [3]. Higman, et al., (2011) Angew. Chem. Int. Ed. 2011, 50:1 – 5 [4]. Watts, A. (2005) Nature Drug Discovery, 4, 555-568 [5]. Ding, et al., (2013) Biochem. J. 450, 443-457. [6]. Sun, et al., (2018) Angew. Chemie. (Intl). (in press) [7]. Watts, A. (1999) Curr Op in Biotechn, 10, 48-53. [8]. Judge et al., (2015) Meth. in Mol. Biol., vol. 1261 [9]. Watts, et al., (2001) J. Biol. Chem. 276, 43197-43204. [10]. Kim, et al., (2005) J. Med Chem. 48, 7145-7152 [11]. Middleton et al., (2000) Proc. Natl. Acad. Sci, 97, 13602-13607 [11]. Williamson et al., (2007) Proc. Natl. Acad. Sci, 104, 18031-18036.

PL10

New methods to study intrinsically disordered proteins

Isabella Felli

CERM and Department of Chemistry “Ugo Schiff”, University of Florence, Italy, Italy

A variety of interesting intrinsically disordered proteins (IDPs) or protein regions (IDRs) with important, yet unexplored, functional and regulatory roles is emerging. NMR represents the only method to access atomic resolution structural and dynamic information on these highly flexible, disordered proteins. The impact of these properties on NMR parameters will be discussed as well as important aspects to be considered in the design of optimal NMR experiments for the study of IDPs/IDRs[1-4]. Several examples of particular features or motives that often occur in IDPs/IDRs will be presented, revealing novel structural and dynamic modules not yet described in the PDB[5-8].

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PL11

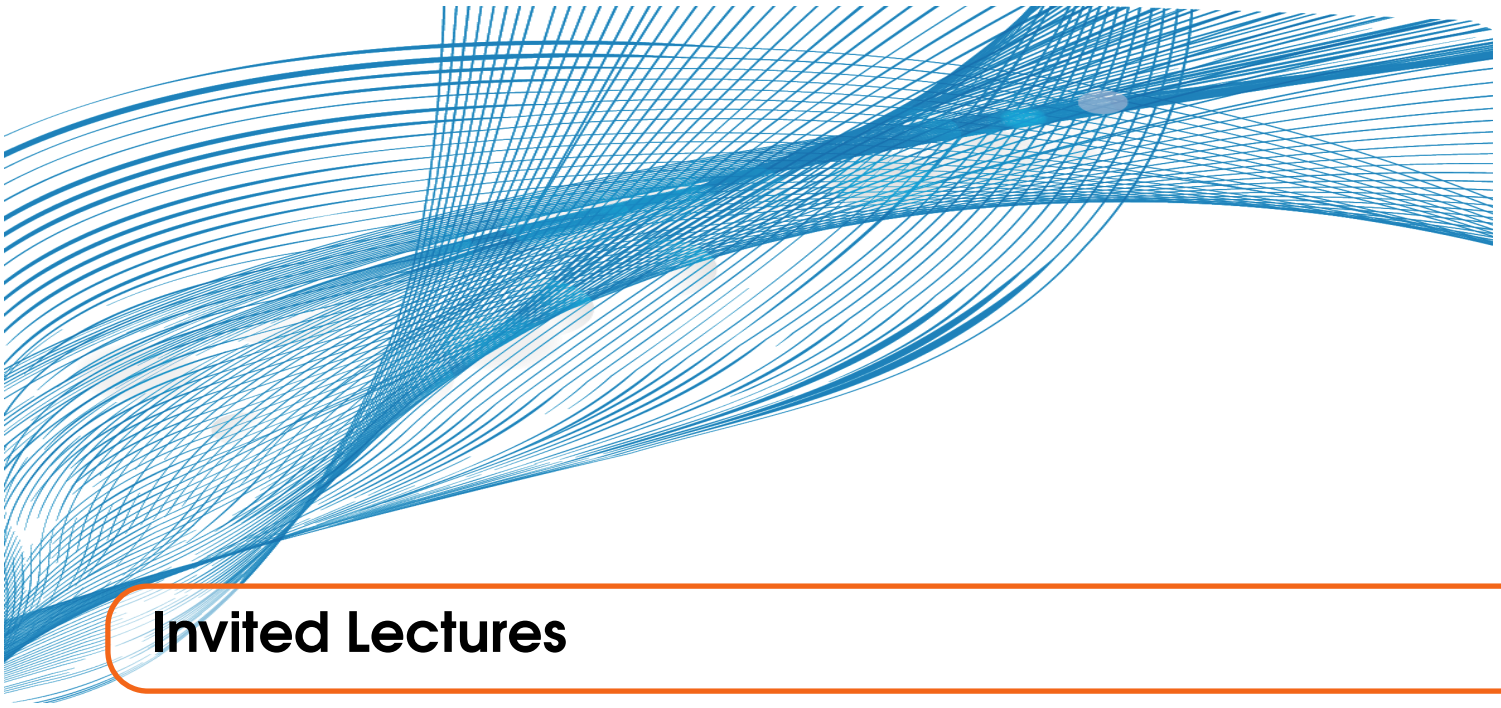
Nature’s Switches, Scientists’ Tools: Mechanisms and Applications of Environmentally-Switched Protein/Protein Interactions

Kevin Gardner

CUNY Advanced Science Research Center, United States

Environmental cues regulate many biological processes, linking the activities of cellular pathways to changing conditions. Such regulation is often initiated by sensory protein domains which use small molecule ligands to convert environmentally-triggered changes into altered protein/protein interactions. Several families of these domains have evolved with remarkable diversity in their inputs and outputs. Using a combination of biophysics, biochemistry and synthetic chemistry, we seek to gain insight into the mechanistic controls of such environmental sensing domains for both fundamental understanding and subsequent artificial control.

Here I will discuss examples of our work that show how one such family of protein domains convert changes in environmental stimuli – blue light or small molecule cofactors – into control of a wide range of output functions. Our primary tool for understanding the mechanisms of this control has been a powerful combination of magnetic resonance techniques: solution NMR studies of protein structure, dynamics and kinetics; NMR-based ligand screening; laser- and pressure-enhanced NMR to trigger conformational changes on demand; EPR-based distance measurements of dynamic ensembles of proteins interconverting among conformations. When used in conjunction with several biophysical and biochemical approaches, my group has demonstrated how this control is triggered by changes in the occupancy or configuration of bound cofactors, leading to conformational changes in the surrounding protein that alter subsequent protein/protein interactions. I will highlight data collected from parallel studies of diverse systems, from the human hypoxia inducible factor (HIF) system to plant & bacterial photosensors, sharing insights we have gained into conserved signaling mechanisms and developed methods to artificially regulate such proteins. Taken together, our work provides an integrated view of a fascinating class of natural switches and lays the foundations for routes by which these can be manipulated to achieve desired therapeutic and/or technological outcomes.



Invited Lectures

IL1

Methods for studying membrane proteins and other complex systems

Gerhard Wagner[1], Haribabu Arthanari[2], Koh Takeuchi[3], Sven Hyberts[2], Mahmoud Nasr[2], Meng Zhang[2], Zhen-Yu Sun[2], James Yu[2], Wolfgang Bermel[4], Abhinav Dubey[2], Joshua Ziarek[5], Franz Hagn[6], Andreas Plückthun[6]

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The complexity of larger proteins and protein membrane complexes creates problems that motivate search for new and improved NMR techniques. We were particularly concerned with studies of membrane proteins, which ideally should be studied in real membranes, should be correctly folded and be suitable for specific isotope labeling to enable NMR assignments. This is crucial for collecting structural parameters, monitor dynamics and mobility changes upon ligand binding. To address that proteins should be in a real membrane we decided on using bilayers in covalently circularized nanodiscs (cNDs), such as the neurotensin 1 receptor, NTR1, and the cannabinoid receptor CB2, and their complexes with heterotrimeric G protein Gi, as well as the voltage-dependent anion channel VDAC1 and its complex with hexokinase. To address the challenges we are facing we explored techniques of 15N, 13C and 19F detection. Furthermore, we rely heavily on non-uniform sampling. For the largest systems we use NMR to select good conditions and employ negative stain and cryo EM microscopy and NMR detection of ILV methyl side chain signals.

IL2

Rigid or flexible or rather in between – What distance distributions tell on ensemble width

Gunnar Jeschke

ETH Zurich, Switzerland

Proteins and their domains are traditionally classified into structured or intrinsically disordered. The former class is considered to conform to the Anfinsen dogma, stating that sequence encodes a single representative conformation with lowest free energy. The latter class is considered to be describable by random-coil models. It is known that some intrinsically disordered proteins and domains can undergo disorder-order transitions and these are often approximated as random coil to single conformation transitions. By now the community is well aware that this dichotomy is an approximation, but it is still considered as a rather good approximation.

Distance distributions between spin labels measured by us and many other EPR groups demonstrate that many proteins of interest exist in intermediate-order states and can undergo partial order-disorder or disorder-order transitions. I discuss what can happen in structure determination of such systems if the Anfinsen/complete disorder dichotomy is presumed. Availability of distance distributions allows for a different approach to ensemble modelling. I discuss the basic assumptions and algorithm of this RigiFlex approach and illustrate it on recent application examples.

IL3

Probing conformational dynamics on the basis of solvent paramagnetic relaxation enhancement

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Paramagnetic relaxation enhancement (PRE) has been established as a powerful tool in NMR for probing protein structure and dynamics. The PRE is usually measured with a paramagnetic tag covalently attached at a specific site of an otherwise diamagnetic protein, which may pose some adverse effect on protein structure and dynamics. Alternatively, as a label-free method, the protein structure can be characterized using the solvent PRE (sPRE); with an inert paramagnetic cosolute randomly colliding with the subject protein, the resulting sPRE should manifest the relative solvent exposure of protein nuclei. When a protein fluctuates among multiple conformations at μs-ms timescale, the observed sPRE becomes the ensemble average of the sPRE values of all constituting conformers. We have thus devised methods to deconvolute the relative contributions to the observed sPRE values. I will demonstrate how our method can be employed to probe protein and RNA dynamics using several examples. The sPRE can be computed with a stand-alone program for rapid evaluation, or with the invocation of a module in the latest release of the structure calculation software Xplor-NIH. In conjunction with other biophysical/biochemical techniques, the sPRE measurement can be readily used to visualize conformational ensembles of biological macromolecules.

IL4

Hybrid structural approaches to solve structures of protein-nucleic acid complexes

Frédéric Allain[1], Julien Boudet[1], Georg Dorn[1], Christoph Gmeiner[1], Thomas Wiegand[1], Alexander Leitner[1], Ruedi Aebersold[1], Beat H Meier[1], Maxim Yulikov[1], Gunnar Jeschke[1], Georg Lipps[2]

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Protein-RNA-complexes are central for the regulation of gene expression and thus crucial for cellular function. While solving structures of protein-RNA complex with NMR has been very successful owing to the low affinity of many RNA-protein interactions, the size limitation of the complexes that could be tackled structurally solely by NMR has remained an issue. Using EPR, Mass-spectrometry (MS) and more recently solid-state NMR in combination with liquid-state NMR has allowed us to investigate the structures of several protein-nucleic acid complexes of size and complexity that we could not tackle solely with solution-state NMR. The structures as well as their functional biological relevance will be discussed.

IL5

Dynamics of basic side chains in protein-DNA interactions

Junji Iwahara

University of Texas Medical Branch, United States

Positively charged side chains play major roles in recognition of nucleic acids by proteins. However, dynamic properties of these basic side chains are not well understood. Over the past decade, primarily to obtain a better understanding of protein-DNA interactions, we have made progress in NMR methodologies that can characterize internal motions, hydrogen bonds, and electrostatic interactions of basic side chains of proteins. In this talk, I will present our recent NMR studies on the dynamics of Lys, Arg, and His side chains at the protein-DNA interfaces. Results for the Egr-1 zinc fingers and the Antp homeodomain in the free and DNA-bound states will be presented. Our NMR data on these two protein-DNA complexes indicate that the Arg side chains interacting with DNA bases are strongly immobilized, forming rigid interfaces. In contrast, despite strong short-range electrostatic interactions, many basic side chains interacting with the DNA phosphates exhibited high mobility, forming dynamic interfaces. This high mobility seems to arise from dynamic transitions between distinct states in which the charged moieties are either in direct contact (i.e., contact ion pair [CIP]) or separated by water (i.e., solvent-separated ion pair [SIP]). The high mobility of the interfacial Arg and Lys side chains interacting with DNA phosphates should mitigate the overall loss of conformational entropy in the protein-DNA association and allow dynamic recognition of DNA by the proteins.

IL6

Structural variability of G-rich DNA regions

Janez Plavec

National Institute of Chemistry, Ljubljana, Slovenia, Slovenia

Nucleic acids can in addition to a duplex structure adopt various four-stranded secondary structures such as G-quadruplexes and i-motifs. The canonical Watson-Crick paired DNA duplexes play major roles in genetic inheritance and gene expression. On the other hand, multi-stranded structures have been associated with many different biological functions of DNA. G-rich sequences have been identified in numerous regions of human genome including chromosomal telomeres and many gene promoters, which play important roles in DNA recombination, replication, transcription, translation and many other critical biological processes. G-quadruplexes are formed by G-rich sequences with repeat units being tracts of three to five guanine residues separated by different number of residues with consensus motif of 5'-Gj(No)Gk(Np)Gl(Nr)Gm-3'. Their basic structural unit is a G-quartet, where four guanine residues associate into a cyclic structural element through Hoogsteen-type hydrogen-bonds. In a unimolecular G-quadruplex four strands of columnar structures are connected with loops. Loop residues contribute to stabilization of G-quadruplex or form secondary structural elements such as a hairpin and base triples themselves. Our laboratory has been using NMR to uncover structural details of G-quadruplexes in relation to sequence details [1-3], cation and pH changes [4], presence of cosolutes [5] and interaction with ligands [6-7]. In the case of GGGAGCG repeats we have observed reversal of roles; structure formed is four-stranded, but is stabilized by GCGC- and GAGA-quartets, while GGG tracts form loops [8]. No G-quartets or Hoogsteen-type hydrogen-bonded guanine residues are present in AGCGA-quadruplexes, and the overall topology is conserved in the presence of Li+, Na+, K+ and NH4+ ions. AGCGA-quadruplexes exhibit lower sensitivity to presence of cations such as potassium or sodium with respect to G-quadruplexes.

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IL7

The complex mode of agonist binding and activation of the relaxin GPCR, RXFP1.

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The ectodomain of the relaxin GPCR receptor, RXFP1, comprises an N-terminal LDLa module, essential for activation, tethered to a leucine-rich repeat (LRR) domain by a 32-residue linker. Activation is proposed to proceed by relaxin binding with strong affinity to the LRR domain and then, through an unknown process, enable the LDLa module, proposed to be a tethered agonist, to bind and activate the transmembrane domain. As the linker shows poor sequence identity amongst homologues, it has been thought of as simply a disordered region to tether the LDLa module to the receptor. We have found mutations within a conserved region of the linker immediately C-terminal to the LDLa module (GDNNGW, residues 41-46) significantly weaken relaxin affinity, suggesting an additional binding site. Using NMR spectroscopy and titrations of 15N-labelled LDLa-linker with relaxin or a paramagnetic (Mn2+) labelled relaxin, we have elucidated a discrete relaxin-binding site (residues 46-63) on the linker. Additional NMR experiments show residues 49-52 of the linker have a weak propensity for helix which on relaxin titration stabilizes. While a lack of 1H-1H NOEs suggest that the linker extends away from the LDLa module, characterization of 15N-relaxation parameters in the absence and presence of relaxin suggests that the linker weakly associates with the LDLa module. These experiments suggest that the role of the LDLa module, along with relaxin, is to stabilize a conformation of the linker, especially the residues GDNNGW, which then serves as the true agonist.

IL8

Rethinking Protein Interactions by Disorder

Birthe B Kragelund

Department of Biology, University of Copenhagen, Denmark

Intrinsically disordered proteins (IDPs) (or –regions (IDRs)) are functional while existing in broad ensembles of near iso-energetic conformations. Despite their lack of tertiary structure, IDPs are involved in communication with other molecules forming associations ranging from binary, discrete complexes to large multicomponent assemblies. Similar to globular proteins their complexes serve structural, functional and regulatory roles, but due to their dynamic nature, they expand the types of association possible, further enabling functional regulations by very different mechanisms.

The fast dynamics characteristic of IDPs may persist in their complexes and the degrees of disorder within the complex can therefore vary greatly. We have been exploring the role of disorder in cellular control processes including pH homeostasis, cytokine signalling, transcriptional regulation, and DNA metabolism, combining NMR spectroscopy with other biophysical methods as SAXS, neutron diffraction, single-molecule FRET and cell biology [1–4]. In one end of the scale we observe folding-upon-binding forming nearly globular-like complexes with little disorder while at the other end, disorder may persist and results in complexes where both binding partners stay disordered despite high-affinity binding [3]. Still, the kinetics combined with higher order complex formation allows regulation on biologically relevant timescales. Between these extremes, a continuum of dynamic complexes is possible. The characterisation and functional decoding of dynamic complexes challenges the methodological toolbox, but NMR spectroscopy continues to be a critical contributor in the understanding of disorder dependent biology.

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IL9

Structural Studies of beta-2-microglobulin fibrils: MAS NMR and cryoEM

Robert Griffin

MIT, United States

Magic angle spinning (MAS) NMR and cryoEM have emerged as important complementary techniques for structural biology, in particular for insoluble systems including amyloid fibrils and membrane proteins. In this presentation, we utilize multidimensional MAS NMR and selectively isotopic labeling to investigate amyloid fibrils, with a focus on β2-microglobulin (β2m) and its truncation variant, ΔN6-beta-2m, two major protein components in DRA fibrils. Our studies of these pathological fibrils illustrate effective resonance assignment strategies for large β-assembles. The distribution of seven β-strands in the central domain of β2m fibrils are identified and indicate an approximately 70-residue fibril core with dynamic C and N terminal segments of 10-20 residues. Two extra β-strands at both termini of ΔN6-beta-2m indicate an extended fibril core compared to β2m. The relatively more rigid termini of ΔN6-beta-2m, together with the finding of its natively trans-Pro32 in monomeric and fibril proteins, in contrast to the cis-to-trans isomerization accompanied in the fibril formation of β2m, rationalize its enhanced potential of fibril formation. Intermolecular 13C-15N correlation experiments have identified a parallel-in-register packing of the two fibrils, suggesting a generic feature of intermolecular packing in various fibrils. The measurement of 1200 13C-13C and 13C-15N distances in beta-2m permits an assessment of the structure of the monomer in the fibril. The preliminary conformation is consistent with cryoEM electron density maps which also elucidate the interface between the monomers in the fibril.

IL10

Metabolic imaging with hyperpolarized 13C-labelled cell substrates – from mouse to man

Kevin Brindle

University of Cambridge, United Kingdom

Molecular imaging is likely to play an increasingly important role in predicting and detecting tumour responses to treatment and thus in guiding treatment in individual patients [1]. We have been developing methods for detecting the early responses of tumours to therapy, including metabolic imaging with hyperpolarized 13C-labelled substrates. We have been using this technique to detect tumour treatment response, to monitor disease progression and to investigate the tumour microenvironment (reviewed in [2]). Exchange of hyperpolarized 13C label between lactate and pyruvate and net flux of label between glucose and lactate have been shown to decrease following treatment. We have compared the effectiveness of this technique for detecting early evidence of treatment response with similar 18FDG-PET measurements. Exchange of hyperpolarized 13C label between injected [1-13C]pyruvate and endogenous lactate can also be used to monitor disease progression. In a genetically engineered mouse model of pancreatic cancer we showed increased lactate labelling as the disease progressed, which potentially could be used clinically to guide earlier intervention. We have also used hyperpolarized [1-13C]pyruvate to investigate glycolytic metabolism in patient derived xenograft (PDX) models of glioblastoma, which showed significant metabolic heterogeneity between tumours derived from different patients, and to monitor response and resistance to PI3K inhibitors in genetically engineered models of breast cancer. We have shown that the capacity of tumours to resist oxidative stress can be determined by monitoring the reduction of [1-13C]dehydroascorbate and by monitoring flux of hyperpolarized 13C label from glucose into an intermediate in the pentose phosphate pathway. Although hyperpolarization of the 13C nucleus produces a massive gain in sensitivity further potential gains in sensitivity are possible if the polarization is transferred to adjacent spin-coupled protons in the molecule. We have recently demonstrated the feasibility of this approach by imaging hyperpolarized [1-13C]lactate via its methyl protons. Metabolic imaging with hyperpolarized 13C-labelled cell substrates has translated to the clinic and we conducted our first clinical study in Cambridge in 2016. Some early results in breast cancer and in glioma will be presented.

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IL11

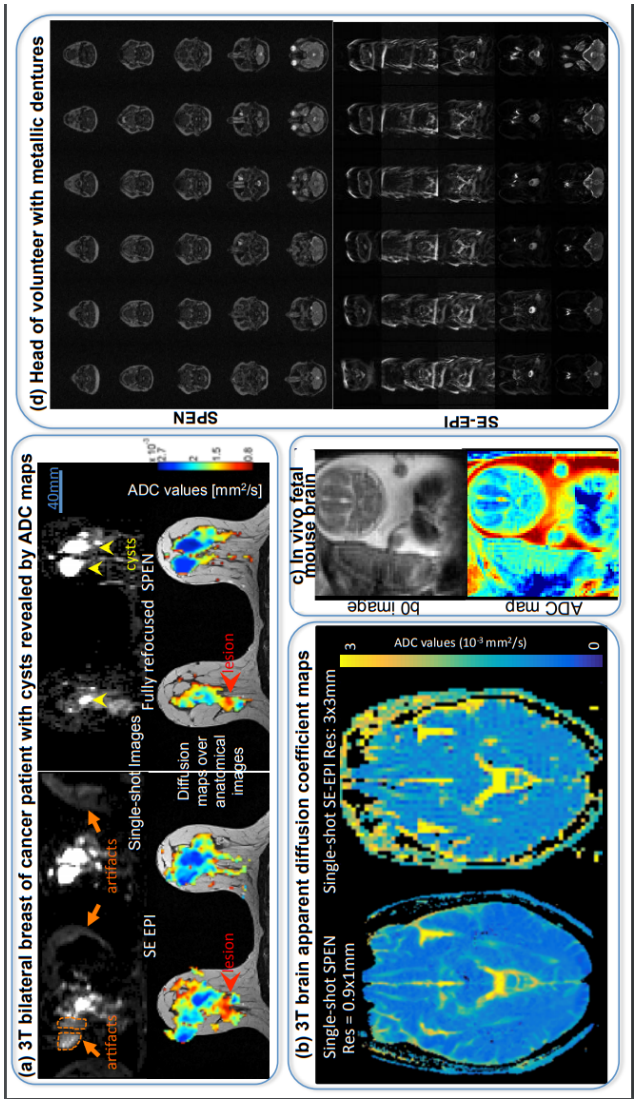
Progress in High-Definition ADC mapping by Spatiotemporally Encoded MRI

Lucio Frydman

Weizmann Institute, Israel

Over the last decade we and others have introduced and perfected a so-called spatiotemporal encoding (SPEN) methodology to collect multidimensional NMR spectra and images in a single scan. This talk will focus on introducing this technique and showing its potential to deliver superior imaging information, particularly in comparison with established methods such as spin-echo EPI in the realm of single-shot MRI, and fast-spin-echo/RARE in multi-shot anatomical MRI. The figure below illustrates some of our ongoing work in the former area including (a) Breast-cancer diffusion-based studies executed on a patient with both cysts and a lesion. In the brain cases the SPEN/PE axes run along the vertical (RO horizontal), whereas in the breast scan SPEN/PE was imparted horizontally. (b) Single-shot diffusion brain characterizations. (c) ADC maps collected inside a

live pregnant mouse for a 14-day-old fetus at 7T. (d) Single-shot 3T data collected on a volunteer's head with non-ferromagnetic dentures. Notice in (a) the difficulties to deal with fat-derived replicas that complicate the identification of cysts and lesions, in (b) the much better resolution, in (c) the quality of the features, and in (d) EPI's complication to deal with the implant-related field inhomogeneities.



IL12

Design and testing of Frequency-encoding MRI reporters

Silvio Aime

University of Torino, Italy

MRI suffers from an intrinsic insensitivity for which the in vivo detection of specific molecules has to be overcome by designing suitable amplification procedures. One possibility relies on the the use of CEST agents (CEST= Chemical Exchange Saturation Transfer). Upon applying a second rf field at the absorption frequency of an exchangeable protons pool, a net saturation effect is detected on the 1H-water signal. These are frequency encoding systems that allow multiple agents detection in the same anatomical region as well as they offer the possibility of designing innovative responsive probes that report on specific parameters (e.g. pH) of the microenvironment in which they distribute. To overcome sensitivity issues, the use of Liposomes (LipoCEST) and RBCs (ErythroCEST) appear to offer valuable solutions. Another approach deals with the access to hyperpolarized (HP) molecules. The use of HP molecules has opened new horizons providing the possibility of investigating in vivo metabolic processes. It will be shown how a level of hyperpolarization sufficient for in vivo studies can be obtained for pyruvate and lactate through the application of a procedure based on the use of para-Hydrogen and magnetic field cycling. MRI suffers from an intrinsic insensitivity for which the in vivo detection of specific molecules has to be overcome by designing suitable amplification procedures. One possibility relies on the the use of CEST agents (CEST= Chemical Exchange Saturation Transfer). Upon applying a second rf field at the absorption frequency of an exchangeable protons pool, a net saturation effect is detected on the 1H-water signal. These are frequency encoding systems that allow multiple agents detection in the same anatomical region as well as they offer the possibility of designing innovative responsive probes that report on specific parameters (e.g. pH) of the microenvironment in which they distribute. To overcome sensitivity issues, the use of Liposomes (LipoCEST) and RBCs (ErythroCEST) appear to offer valuable solutions. Another approach deals with the access to hyperpolarized (HP) molecules. The use of HP molecules has opened new horizons providing the possibility of investigating in vivo metabolic processes. It will be shown how a level of hyperpolarization sufficient for in vivo studies can be obtained for pyruvate and lactate through the application of a procedure based on the use of para-Hydrogen and magnetic field cycling.

IL13

The structural dynamics of calmodulin in vitro, in cell extracts and in cells

Daniella Goldfarb

Weizmann Institute of Science, Israel

Tracking the structural dynamics of proteins in the cell is challenging because of the difficulties imposed by the cellular environment on most of the biophysical methods used to explore protein structures. We use high-frequency, double electron-electron resonance (DEER) distance measurements employing Gd3+ spin labels, which features high sensitivity and high redox stability essential for in cell measurements. We demonstrate the feasibility of the methodology on the Ca2+ binding protein calmodulin (CaM), which is a dumbbell-shaped protein comprising globular N- and C- domains connected by a flexible linker. As a result of Ca2+ binding it undergoes a conformational change, which enables binding to target proteins and thereby modulating their activity. We have prepared two mutants, N53C/T110C and T34C/T117C, where one Gd3+ tag is situated at the N-lobe and the other in the C-lobe and followed the CaM conformational transitions upon Ca2+ and IQ peptide in-vitro, in cell extract and in Hela cells. In-vitro we clearly detect the conformational transitions upon Ca2+ and IQ binding for both mutates, although the protein flexibility is high in all states. The behavior was similar in cell extracts showing that in the cell extract, in the presence of Ca2+, CaM is bound to a substrate. A different situation was encountered in Hela cells where we observed the conformation transition associated with Ca2+ binding for the N53C/T110C mutant but we did not detect the binding to the substrate. For the second mutant, T34C/T117C, the distance distribution indicated differences in the apo-CaM conformation and the change upon binding Ca2+ was not obvious.

IL14

EPR Structural Studies of Membrane Proteins

Gary Lorigan, Indra Sahu, Robert McCarrick

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CW and pulsed Electron Paramagnetic Resonance (EPR) spectroscopic techniques coupled with site-directed spin-labeling (SDSL) can provide important structural information on complicated biological systems such as membrane proteins. Strategically placed spin-labels alter relaxation times of NMR active nuclei and yield pertinent structural information. EPR techniques such as Double Electron-Electron Resonance (DEER) and Electron Spin Echo Envelope Modulation (ESEEM) are powerful structural biology tools. The DEER technique can be used to measure distances between 2 spin labels from 20 to 70 Å. However, the application of DEER spectroscopy to study membrane proteins can be difficult due to short phase memory times (Tm) and weak DEER modulation in more biologically relevant proteoliposomes when compared to water soluble proteins or membrane proteins in detergent micelles. The combination of these factors often leads to broad distance distributions, poor signal to noise, and limitations in the determination of longer distances. The short phase memory times are typically due to uneven distributions of spin-labeled protein within the lipid bilayer, which creates local inhomogeneous pockets of high spin concentrations. Approaches to overcome these limitations and improve the quality of DEER measurements for membrane proteins will be discussed: lipodisq nanoparticles, bi-functional spin labels (BSL), and Q-band pulsed EPR spectroscopy. ESEEM data will be shown to probe the secondary structure of membrane proteins. CW-EPR spectra of spin-labeled membrane proteins will be used to investigate dynamics and the immersion depth in a lipid bilayer.

IL15

The Effect of Perdeuteration on Spin Relaxation and Distance Measurement by EPR

David Norman, Michael Stevens

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Distance measurement obtained using EPR methodologies such as PELDOR or DEER on spin labelled macromolecules has been limited to a maximum of approximately 80Å. The use of deuterated buffers has been routine and is essential to enabling such measurements. We have demonstrated that full protein deuteration has a highly significant effect on decreasing the dephasing rate of excited electron spins, thus extending the main limitation to long distance measurement by EPR. We report a number of experiments in which the use of perdeuterated systems has enabled the measurement of precise, long-distance measurements that were previously inaccessible. We also describe and discuss associated effects of deuteration such as sensitivity and temperature response. We will also describe other factors that become significant in the measurement of perdeuterated systems using as examples measurements made on very extended helical systems. References Hammond, C. M., Sundaramoorthy, R., Larance, M., Lamond, A., Stevens, M. A., El-Mkami, H., Norman, D. G., and Owen-Hughes, T. (2016) The histone chaperone Vps75 forms multiple oligomeric assemblies capable of mediating exchange between histone H3–H4 tetramers and Asf1–H3–H4 complexes, Nucleic Acids Res 44, 6157-6172. El Mkami, H., and Norman, D. G. (2015) Chapter Five-EPR Distance Measurements in Deuterated Proteins, Methods in enzymology 564, 125-152. El Mkami, H., Ward, R., Bowman, A., Owen-Hughes, T., and Norman, D. G. (2014) The spatial effect of protein deuteration on nitroxide spin-label relaxation: Implications for EPR distance measurement, Journal of Magnetic Resonance 248, 36-41. Bowman, A., Hammond, C. M., Stirling, A., Ward, R., Shang, W., El-Mkami, H., Robinson, D. A., Svergun, D. I., Norman, D. G., and Owen-Hughes, T. (2014) The histone chaperones Vps75 and Nap1 form ring-like, tetrameric structures in solution, Nucleic Acids Res 42, 6038-6051. Bowman, A., Ward, R., Wiechens, N., Singh, V., El-Mkami, H., Norman, D. G., and Owen-Hughes, T. (2011) The histone chaperones Nap1 and Vps75 bind histones H3 and H4 in a tetrameric conformation, Molecular Cell 41, 398-408.

IL16

Metal-dependent functional processes elucidated through paramagnetic NMR

Lucia Banci

CERM & Dept. of Chemistry, University of Florence, Italy

Metalloproteins constitute a large share of the human genome and of eukaryotic organisms in general, with a significant number of them binding paramagnetic metal ions or metal cofactors. In the majority of the cases these metal ions and cofactors are the site of the functional process. NMR can provide not only a detailed description of the structural feature of the functional sites, but more significantly, of the interactions patterns and of the steps of the reactions they are involved in. However, a paramagnetic center can dramatically affect the NMR signals at various extent depending on its nature. Therefore, specifically tailored experiments should be developed. Furthermore, NMR characterization can be effectively integrated with EPR data so to have a detailed understanding of the cellular processes. A few examples will be presented for the processes responsible of the biogenesis of iron-sulfur proteins and of the transfer of iron-sulfur clusters to the recipient proteins.

IL17

NMR relaxometry and the influence of solvent and ligand dynamics on function of nano-probes designed for bio-applications

Dermot Brougham, Jenny Merlin, Eoin McKiernan, Eoghan MacMahon

University College Dublin, Ireland

Controlling the density and functionality of molecules adsorbed onto nanoparticles is critical for the development of smart nanomaterials for biomedicine. Surface chemistry strongly influences drug loading, the in vitro and in vivo colloidal stability and the blood circulation time. In the case of magnetic iron-oxide nanoparticles (MNPs) the solvent interaction with the magnetic moments, mediated by the surface chemistry, generates changes in the local NMR signal intensity which can be used to create image contrast in MRI. Hence 1H NMR relaxation can be used to study dynamics of magnetic moments [1-2] for dispersed particles (fast dynamics) and for nanoparticle assemblies (slow dynamics). We will describe how NMR measurements of MNP suspensions can provide fundamental insights into ligand-ligand, solvent-particle and particle-particle interactions. We will then show how this information can be exploited to develop approaches to particle dispersion, [3-4] to particle assembly into functional clusters, [5-6] and to understand nanoparticle interactions with bio-systems.

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IL18

Protein methyl pseudocontact shifts for assignment and detection of subtle structural changes

Marcellus Ubbink

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Pseudocontact shifts (PCS) are convenient sources of information to probe protein structures using NMR spectroscopy. We have used sets of methyl PCS generated with up to three positions of the Caged Lanthanoid NMR Probe (CLaNP) #5 [1] on the N-terminal domain of HSP90 to test to what extend methyl resonances can be assigned solely on the basis of PCS information. An new and extended version of the software Parassign [2] was used. With a combination of the PCS from two tags, up to 60% out of the 76 ILV methyls could assigned reliably and providing useful suggestions, with the correct assignment present among 2 or 3 possibilities, for most of the remaining resonances. Several important considerations of how to place the tags were found and will be discussed. The same system was used to probe subtle movements of methyl groups in the binding pocket of a small and weakly binding ligand. Using a triangulation approach the new position of the methyl group upon ligand binding can be restrained to a limited area [3]. The movements are only of the order of 1 – 3 Å, yet can be detected by changes in PCS. It is important to consider the effect of tagging, because tag attachment can also result in subtle methyl rearrangements. This approach can provide restraints for ligand docking studies.

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IL19

Protein folding monitored by real time pressure-jump NMR

Ad Bax

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The equilibrium between a protein's folded and unfolded state is strongly impacted by hydrostatic pressure, to an extent determined by the difference in volume of the unfolded and folded states. Many proteins, in particular larger proteins, can be unfolded by applying a modest amount ($\leq$3 kbar) of hydrostatic pressure. Smaller proteins often can be mutated to generate small internal cavities, such that they also have a large volume difference between the folded and denatured states, and can be unfolded at pressures that are within reach inside an NMR spectrometer. Rapidly decompression inside the NMR magnet enables a range of different experiments to monitor the actual folding process under native conditions by two- and three-dimensional NMR. Novel NMR experiments permit probing of the structural properties of these intermediates and show evidence for a state with non-native contacts on the pathway towards the folded state. This observation goes against the widely held concept that native contacts define the path towards the folded structure, a hypothesis that formed the basis for many hundreds of protein folding studies.

IL20

Protein dynamics with high-resolution field-dependent NMR: from high fields down to 0.33 T.

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Understanding the physics and chemistry that underlie the function of biological macromolecules requires an atomic-resolution description of their conformational space and the timescales of their motions. NMR is a powerful experimental technique to characterize dynamics of biomolecules. In particular, nuclear spin relaxation provides access to motions on picosecond to nanosecond timescales. However, high-field relaxation only provides limited sampling of motions and has led so far to simple models of motions. Can we do better? We explore low magnetic fields while preserving the high resolution and sensitivity of high-field NMR thanks to a sample shuttle prototype. High-resolution relaxometry allows us to quantify relaxation over a broad range of magnetic fields and better quantify protein motions in the ps-ns range.2,3 Here, we present carbon-13 relaxation rates recorded between 0.3 and 22.3 T on methyl groups in ubiquitin. This ensemble of rates allows the analysis of motions with up to three correlation times spanning three orders of magnitude from picoseconds to nanoseconds. Experimental results were interpreted with the help of a 1 microsecond trajectory simulated by molecular dynamics (MD). We have used our sample shuttle to build a unique two-field NMR spectrometer that permits the excitation and observation of 1H, 13C and 15N spins at two magnetic fields. We have used this system to measure accurate relaxation rates at low field and thus validate our analysis of relaxometry. We have measured chemical shifts at low field with a two-field spectrometer and shown that signals broadened beyond detection by chemical exchange at high field can be recovered in a two-field correlation. This approach allows the observation of molecular systems prone to enhanced chemical dynamics and paves the way for performing NMR on very high field spectrometers.

IL21

High-pressure NMR spectroscopy: Tools for studying protein dynamics

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High-pressure-NMR, including hydrostatic- and gas-pressure NMR, are useful tools for studying protein structure and dynamics. Hydrostatic-pressure NMR allows structural characterization of less ordered conformers (i.e., high-energy conformers) of proteins in the range of 0.1–250 MPa. Gas-pressure NMR at 1 MPa O2 is used to investigate the location of and ligand accessibility to internal cavities of proteins. Based on studies of

protein structure and dynamics using both pressure NMR techniques, we suggest that internal cavities are sources of protein conformational fluctuations. We have investigated the conformational fluctuations of outer surface protein A (OspA) by high-pressure NMR techniques. OspA is crucial in the infection of Borrelia Burgdorferi, which causes Lyme disease, and has large internal cavities in the C-terminal domain. In a previous study, pressure-induced changes in the NMR spectra indicated that the intermediate conformer of wild-type OspA has a folded N-terminal domain but disordered central and C-terminal domains (Kitahara et al., Biophys. J. 2012). In addition, we suggested that the selective unfolding of the C-terminal domain exposes the receptor binding sites of OspA to interact efficiently with the tick receptor for OspA (TROSPA) in the tick gut. Here, we report on the structural characteristics of the pressure-stabilized intermediate using paramagnetic relaxation enhancements (PREs). PREs for backbone amide protons of the E128C and A140C mutants, in which the paramagnetic probe S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate (MTSL) was covalently attached to C128 and C140, respectively, were measured at 950 MHz. Although the amide group signals of the disordered region showed new cross-peaks in a narrow spectral region, which is typical for a disordered and mobile polypeptide chain, the structural characteristics of the central and C-terminal domains at 250 MPa, inferred from the PRE data, did not match the highly extended conformation. However, the disordered region showed sub-conformations in the unfolded ensemble. Moreover, using gas-pressure NMR, we observed significant O2-induced PREs for the amide protons located around the internal cavities of the C-terminal domain, showing that O2 could access the protein interior even in the native state ensemble (Kawamura et al., Biophys. J. 2017). Similar results were obtained for T4-lysozyme and its cavity-enlarged mutant L99A (Kitahara et al., Sci. Rep. 2016, Protein Sci. 2018). Internal cavities might not be just packing defects but structural elements to produce conformational fluctuations and enable ligand-binding.

IL22

How Bacterial Xenogeneic Silencers Selectively Recognize Foreign DNA in The Resident Genome?

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Horizontal gene transfer plays an important role in the evolution of bacteria, such as the acquisition of antibiotic resistance or novel metabolic capacity by the recipient bacteria. Bacterial xenogeneic silencing proteins selectively bind to AT-rich regions of the chromosome and repress the transcription of horizontally acquired DNA, including a large number of virulence genes. Up to date, four distinct families of xenogeneic silencers have been identified: H-NS of Proteobacteria, Lsr2 of the Actinomycetes, MvaT of Pseudomonas, and Rok of Bacillus. They all have an N-terminal oligomerization domain and a C-terminal DNA-binding domain, and all exhibit similar AT-rich DNA binding preferences, but clearly with subtle differences. The solution structures of the DNA binding domains of H-NS, Lsr2, and MvaT, reveal that MvaTctd and H-NSctd have similar overall folds, while the structure of Lsr2ctd is distinct. Interestingly, H-NS and Lsr2 share a common DNA binding mechanism to recognize AT-rich sequences through binding the minor groove of DNA with an AT-hook like motif. The conserved "Q/RGR" sequences of the AT-hook like motifs adopt an extended conformation which is inserted into the DNA minor groove. However, the solution structure of the DNA-binding domain of MvaT in complex with a high affinity DNA dodecamer reveals that MvaT recognizes the AT-rich DNA both through base readout by an "AT-pincer" motif inserted into the minor groove and through shape readout by multiple lysine side chains interacting with the DNA sugar-phosphate backbone. This novel DNA recognition mechanism enables MvaT to better tolerate GC-base pair interruptions in the binding site. The DNA binding domain of Rok adopts a typical winged helix fold, but with a novel DNA recognition mechanism, different from the other xenogeneic silencers. Rok binds DNA minor groove by forming hydrogen bonds to bases through N154, T156 at the N-terminal of α3 helix and R174 of wing W1, assisted by four lysine residues interacting electrostatically with DNA backbone phosphate groups. The structural features endows Rok with preference towards DNA sequences harboring AACTA, TACTA, and flexible multiple TpA steps, while rigid A-tracts are disfavored. Correspondingly, the Bacillus genomes containing Rok show a dramatic underrepresentation of AACTA and TACTA, which are enriched significantly in Rok binding regions, along with multiple TpA steps. Our studies demonstrated that nature has evolved unique molecular solutions for different bacterial genera to distinguish foreign from self DNA, related to the characteristics of individual genome. The abilities of xenogeneic silencers from different bacteria to distinguish foreign from self DNA should be dependent on the mean AT-contents of their corresponding genomes, and it is even likely that these xenogeneic silencers have to fine-tune their DNA binding properties to efficiently silence foreign genes against the backdrop of the self genome.

IL23

RNP Complexes in RNA metabolism: a view by integrative structural biology

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Recent technical advancements have overcome the molecular-weight limits of traditional NMR methods. The most prominent example is the use of isoleucine, leucine and valine (ILV-) methyl groups as probes, which can provide inter-subunit distances even for molecular machines as large as hundreds of kDa. During the past decade, a handful of integrative computational tools have been introduced that can accurately translate such sparse information into structures [1]. Though, as most of these tools were developed in a case-specific manner, there is still an ongoing demand for a standardized integrative computational method. Here we present a general integrative modeling framework that can deal with molecules of different nature (e.g. proteins, nucleic acids) and make use of diverse distance and/or proximity information to determine the structures of supramolecular assemblies [2]. Our framework includes (i) extension of the well-known integrative computational tool HADDOCK, such that it can address supramolecular complexes having Fig. 1 Integrative structural biology scheme »6 components; (ii) proof-of-concept incorporation of various (CSP, NOE, PRE) ILV-methyl distances, which is demonstrated on a realistic benchmark spanning from 85kDa to 450kDa; (iii) a new approach to score different conformers of large assemblies with respect to the experimental data; (iv) a statistical analysis to evaluate the fitness of the data. This approach will be demonstrated on the example of protein-protein and protein-RNA complexes, whose structure is obtained by a powerful combination of solution-state NMR and small angle neutron scattering (SANS). In the second part of the talk, I will show the application of ssNMR to study large RNP complexes. I will present the ssNMR-based structure of the 26mer box C/D RNA in complex with the protein L7Ae [3], together with the experimental strategy that we used to obtain it. Lastly I will present first ssNMR spectra for the RNA part of a 400 kDa RNP complex.

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Invited

IL24

Dynamic Basis for DG•DT Misincorporation via Tautomerization and ionization

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Tautomeric and anionic Watson-Crick-like mismatches play important roles in replication and translation errors through mechanisms that are not fully understood. Using NMR relaxation dispersion, we resolved a sequence-dependent kinetic network connecting G•T/U wobbles with three distinct Watson-Crick mismatches consisting of two rapidly exchanging tautomeric species (Genol•T/U⇌G•Tenol/Uenol; population <0.4%) and one anionic species (G•T–/U–; population ≈0.001% at neutral pH). Inserting the sequence-dependent tautomerization/ionization step into a minimal kinetic mechanism for correct incorporation during replication following initial nucleotide binding leads to accurate predictions of dG•dT misincorporation probability across different polymerases, pH conditions, and for a chemically modified nucleotide, and provides mechanisms for sequence-dependent misincorporation. Our results indicate that the energetic penalty for tautomerization/ionization accounts for ≈10-2-10-3–fold discrimination against misincorporation, which proceeds primarily via tautomeric dGenol•dT and dG•dTenol with contributions from anionic dG•dT– dominating at pH ≥8.4 or for some mutagenic nucleotides.

IL25

Dissecting Protein-Protein Interactions for Drug Development by NMR Spectroscopy

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Protein–protein interactions play critical roles in regulating a variety of cellular functions, including many diseases. Therefore, inhibiting and/or controlling of protein–protein interactions in atomic level have been considered as a key technique for developing therapeutic drugs. Particularly, Wnt/b-catenin signaling is aberrantly activated in most human colorectal cancers (CRCs) and many proteins interact each other cooperatively in tumor promotion. Destabilization of both b-catenin and Ras signaling via targeting axin is a potential therapeutic strategy for treatment of CRC and other type cancers activated Wnt/b-catenin and Ras pathways. We identified axin as a direct target through in vitro binding studies, and uncovered details of the interaction between a novel drug compound and regulators of the G-protein signaling (RGS) domain of axin using NMR spectroscopy. Other example is targeting for Dishevelled (Dvl)/CXXC5 interaction in regulation of the Wnt/β-catenin pathway in osteoblast differentiation. We previously identified that CXXC5 is a negative feedback regulator of the Wnt/b-catenin pathway via its interaction with Dvl and suggested the inhibiting Dvl–CXXC5 interaction as a potential target for anabolic therapy of osteoporosis. Nuclear magnetic resonance (NMR) and functional analysis confirmed inter-molecular interaction between Dvl protein and screened compounds. In conclusion, small-molecule inhibitors developed to block Wnt/b-catenin signaling by dissecting PPI are potential candidates for both anti-cancer and anti-osteoporosis drugs.

IL26

Structure dynamics and kinetics of folding and recognition in proteins by NMR

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Kinetics of protein-protein interactions (protein-protein recognition) will be described with a new mathematical method to distinguish conformational selection and induced fit (1) which includes a concept for the measurement. Further, the role of partially disordered proteins in droplet formation is investigated. The adaptor protein SLP65 which interacts with CIN85 (2). The two proteins are essential for B cell activation. The protein is found to be mainly unstructured and its various segments entertain different functions or interact with membranes, SH3 domains and forming coiled coils. Based on the structures, a molecular lego will be described that reduces the SLP65/CIN85 interaction to its absolutely necessary essentials. The two proteins can perform phase separation which is essential for function. We are additionally interested in the sites of interactions of small molecules and oligomers of IDPs that are important in neuro- and cellular degeneration. Interference with these aggregates specifically on the oligomer level proves to be a valid concept for treatment of devastating diseases such as Parkinson's, Alzheimer's, Creutzfeldt Jacob disease and Type II diabetes mellitus (3).

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IL27

Antibody interactions of intrinsically disordered antigens: implications for vaccine development

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Intrinsically disordered proteins are highly abundant in the malaria parasite Plasmodium and related pathogens.[1] Merozoite surface protein 2 (MSP2) is one such antigen from P. falciparum [2] that is highly abundant on the surface of merozoites and elicits a protective response in humans. Indeed, our recent analysis of antibody recognition of disordered antigens shows that disordered antigens are excellent vaccine candidates.[3] Crystallographic and NMR studies show[4] that recognition of a conserved N-terminal epitope from MSP2 by the mAb 6D8 is incompatible with

the membrane-bound conformation of this epitope,[5] suggesting a mechanism by which parasite MSP2 escapes 6D8 recognition. Intriguingly, NMR also identifies transient, strain-specific interactions between the 6D8 mAb and regions of MSP2 beyond the conserved epitope. Even though these interactions are transient, they nonetheless modulate the binding of these epitopes, either as peptides or full-length antigens, to the antibody.[6] In contrast, the conserved C-terminal region of MSP2 is recognised by mAbs 4D11 and 9H4. 4D11 binds to merozoites much more strongly than 9H4. A crystal structure of 4D11 Fv bound to the epitope NKENCGAA reveals the possible conformation of the C-terminal region of MSP2 on the parasite.[7] The results of these studies underpin ongoing efforts to optimize recombinant MSP2 constructs for development as malaria vaccine candidates and to develop peptide-based antigens. [1] Z. P. Feng, et al., Mol. Biochem. Parasitol. 2006, 150, 256. [2] X. Zhang, et al., J. Mol. Biol. 2008, 379, 105. [3] C. A. MacRaild, et al., Structure 2016, 24, 148. [4] R. A. V. Morales, et al., Sci Rep 2015, 5, 10103. [5] C. A. MacRaild, et al., Biochim. Biophys. Acta 2012, 1818, 2572. [6] B. Krishnarjuna, et al., Commun. Biol. 2018, in press. [7] J. Seow, et al., J. Mol. Biol. 2017, 429, 836.

IL28

NMR to study functional important conformational dynamics in biomolecular recognition

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We study molecular mechanisms and dynamics of multidomain proteins and regulatory protein-RNA and protein-protein complexes by combining solution NMR with complementary techniques, such as SAXS, SANS or FRET. Solution NMR-spectroscopy and SAXS/SANS provide unique information on functionally important dynamics and are combined with structural information from X-ray crystallography or electron microscopy. Together these data provide unique information for defining domain or subunit arrangements in multidomain proteins and protein complexes and for characterizing intrinsically disordered regulatory regions.

Examples will be presented, that highlight the role of conformational dynamics and population shifts in protein-RNA recognition linked to alternative splicing and miRNA biogenesis.

IL29

Sweet entanglements probing protein:glycan interactions by NMR

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Carbohydrate-protein interactions play a pivotal role in the entry of viruses into their cellular hosts with carbohydrate-binding proteins, such as antibodies and lectins, being developed as potential antivirals. They block virus entry into host target cells and halt virus transmission from virus-infected cells to non-infected cells, thereby preventing infection. Their mode of action entails binding to carbohydrates on cell surface receptors or viral attachment proteins. Binding between carbohydrates and proteins can be studied by NMR spectroscopy, either by monitoring changes in the protein or the carbohydrate spectra. Titration of a carbohydrate into a 15N-labeled protein sample and observation of amide backbone chemical shift changes (chemical shift mapping) is commonly used to identify the sugar binding pocket on a protein. However, such protein-based approaches are blind to any conformational effects mediated by the sugar and ligand-based NMR approaches are necessary to this end. We have developed several suitable NMR spectroscopic approaches for detailed analysis of the sugar conformation. We will present the application of uniformly 13C-labeled carbohydrates to investigate carbohydrate-protein interactions, taking advantage of the 13C spectral dispersion for the sugar in isotope-filtered experiments. Chemical shifts for the bound sugar signals are easily extracted from 1H,13C-HSQC experiments. 2D 13C-filtered NOESY-1H,13C-HSQC experiments permit the identification of protein contact sites on the sugar and carbohydrate contact sites on the protein are identified from 2D CNH-NOESY experiments.

IL30

Supramolecular Scaffolds for Protein Assembly

Peter Crowley

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The spontaneous assembly of proteins into soluble oligomers is widespread in biology. Oligomers composed of two or more subunits can afford new functionality with assembly / disassembly enabling the regulation of function. Supramolecular building blocks such as synthetic macrocycles are proving to be versatile triggers of protein assembly. By using a combined approach of NMR spectroscopy, X-ray crystallography, size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) and small angle X-ray scattering (SAXS) we provide convincing evidence that calixarenes and cucurbiturils act as “molecular glues” for protein recognition and assembly.[1-4] We demonstrate that sulfonatocalix[8]arene mediates the assembly and disassembly of cytochrome c tetramers, circumventing the requirement for a competitive inhibitor. Furthermore, the data point to protein encapsulation by a flexible macrocycle.

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IL31

Determinants of the urinary and serum metabolome in children from six European populations

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Metabolic phenotyping of urine and serum can help deconvolve the link between early life exposure and disease risk predisposition, yet to date we lack large-scale metabolome studies in children that combine analyses of these biological fluids. We sought to address this and to understand the major determinants of the metabolome in children by exploiting a unique biobank established as part of the HELIX early-life exposome project (http://www.projecthelix.eu)

Methods: Metabolic phenotypes of matched urine and serum samples from 1,192 children recruited from birth cohorts in six European countries (aged 6 – 11) were measured by 1H nuclear magnetic resonance (NMR) spectroscopy and a targeted LC-MS/MS metabolomic assay (Biocrates AbsoluteIDQ p180 kit).

Results: Metabolic associations to BMI z-score included a novel association with urinary 4-deoxyerythreonic acid (from threonine catabolism), valine, p-cresol sulphate, pantothenate, serum carnitine and serum branched-chain amino acids. Dietary-metabolite associations were identified including urinary creatine with meat intake, urinary hippurate with vegetables and fruits, and urinary proline betaine and scyllo-inositol with fruit intake. Metabolic pathway correlations were also identified including a link between serum threonine and production of urinary 4-deoxyerythronic acid.

Conclusions: Using a multi-platform metabolic phenotyping approach, we have established a pan-European reference metabolome for urine and serum from healthy children, identifying a novel metabolic association between threonine catabolism and BMI. The six European populations studied share common metabolic characteristics and metabolic associations with age, gender, BMI z-score and diet. This study provides a novel resource for investigating the links between the exposome and child health.

IL32

Metabolomics- what can we learn from it?

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Metabolomics is the study of small molecules called metabolites. Patterns of these metabolites have been associated with metabolic health disease, drug response and food intake. Advancements in analytical techniques to measure metabolites has ensured an ever increasing coverage of metabolites. In the field of nutrition and health applications of metabolomics has increased in recent years. Of particular note is the increased interested in the assessment of Food Intake biomarkers. The potential of these biomarkers is related to their ability to provide objective measures of food intake. This has arisen due to limitations associated with current approaches: Dietary assessment methods including food-frequency questionnaires and weighed food diaries are associated with many measurement errors including energy under-reporting and incorrect estimation of portion sizes. While biomarkers have great potential, there are a number of challenges associated with them. These will be discussed and potential areas that need addressing highlighted. Finally, metabolomics is also set to play a critical role in the development of the areas of Precision Medicine and Precision Nutrition. Personalising medical decisions based on metabolic profiles is becoming a reality. Similarly, personalising dietary strategies based on dietary biomarkers and metabolic profiles is gaining momentum. Critical advancements still need to be achieved for metabolomics to realise its full potential.

IL33

NMR in Metabolomics: The Future is Bright

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In principle, NMR is an ideal technique for metabolomics. It is non-destructive, non-biased, highly quantitative, requires no prior separation, permits the identification of novel compounds and needs no chemical derivatization. However, relative to other analytical techniques NMR is slow and relatively insensitive. Furthermore the identification and quantification of compounds in mixtures by NMR is manually intensive and often error-prone. Because of these limitations, NMR is being supplanted by mass spectrometry for many metabolomic applications. In this presentation I will describe a number of innovations that both my lab and other NMR spectroscopists have developed that are allowing NMR to match or even exceed the speed, sensitivity and metabolite coverage claimed by various mass spectrometry methods. In particular, I will describe efforts to increase NMR sensitivity to nanomolar levels through the use of higher magnetic fields, better probes and Dynamic Nuclear Polarization (DNP), to greatly reduce sample size requirements through the development of microprobes and tube-in-tube NMR, and to reduce signal complexity through the application of selective isotopic labeling and improved pulse sequences as well as improvements to extract "invisible" metabolite information from NMR spectra. Finally I will describe our recent efforts to completely automate NMR-based metabolomics using software programs such as MagMet and Bayesil. MagMet, in particular, is more than 95% accurate in terms of compound identification and is able to identify and quantify up to 60 compounds from an NMR spectrum in less than 2 CPU minutes. I will outline our efforts to extend MagMet to the quantitative analysis of beverages (such as beer and wine) and how MagMet has been integrated into inexpensive metabolomics kits, which should make NMR-based metabolomics accessible to anyone with an NMR spectrometer. These and other developments suggest the future of NMR in metabolomics is looking very promising.

IL34

NMR in the changing landscape of fragment-based drug design

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Fragment-based drug design (FBDD) has become an established and successful approach to the identification of very small molecules that bind selectively to a target of interest. However, fragment hits identified in primary screens usually have low affinities, with KD values in the high μM to mM range, and structural data on protein-fragment complexes are almost always required to develop fragments into high affinity ligands.(1) Where a system is not amenable to X-ray crystallography, NMR is currently the only viable alternative to generate structural data. The length of time that it takes to generate the structure of a protein-ligand complex from NMR data presents a challenge that limits the use of this approach. We have developed a number of methods employing sparse methyl-labeling strategies to accelerate the process of generating structures of weakly binding fragments in complex with their protein targets.(2)

Another complication that can arise is where fragment binding induces a conformational change in the protein, and binds to a pocket that is not observed in the structure of the apo protein. Such "cryptic pockets" have been found in an increasing number of protein targets, although they have almost always been found serendipitously.(3) We have attempted to develop an approach to identify cryptic pockets from analysis of sparsely populated states that are evident in NMR relaxation data.(4)

In this presentation I will describe a number of examples where we have generated data using NMR spectroscopy to support programs of structure-guided FBDD.

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IL35

Structural study on toxin-antitoxin systems in Mycobacterium Tuberculosis : a target for developing antimicrobial agents

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The bacterial toxin–antitoxin (TA) system is a module that may play a role in cell survival under stress conditions. Generally, toxin molecules act as negative regulators in cell survival and antitoxin molecules as positive regulators. Thus, the expression levels and interactions between toxins and antitoxins should be systematically harmonized so that bacteria can escape such harmful conditions. Since TA systems are able to control the fate of bacteria, they are considered as potent targets for the development of new antimicrobial agents. TA systems are widely prevalent with a variety of systems existing in bacteria: there are three types of bacterial TA systems depending on the property of the antitoxin which binds either the protein toxin or mRNA coding the toxin protein. Moreover, the multiplicity of TA genes has been observed even in species of bacteria. Therefore, knowledge on TA systems such as the individual characteristics of TA systems, integrative working mechanisms of various TA systems in bacteria, interactions between toxin molecules and cellular targets, and so on is currently limited due to their complexity. In this regard, it would be helpful to know the structural characteristics of TA modules for understanding TA systems in bacteria. Here, we present the structural information of TA systems by using NMR & X-ray crystallography and suggest antibiotics candidates which inhibit the interaction between Toxin and Antitoxin proteins from infectious bacteria, especially focusing on the TA modules of Mycobacterium Tuberculosis.

IL36

A structural mechanism for directing corepressor-selective inverse agonism of PPARγ

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Small chemical modifications can have significant effects on ligand efficacy and receptor activity, but the underlying structural mechanisms can be difficult to predict from static crystal structures alone. Here we show how a simple phenyl-to-pyridyl substitution between two common covalent orthosteric ligands targeting peroxisome proliferator-activated receptor gamma (PPARγ) converts a transcriptionally neutral antagonist into an inverse agonist. X-ray crystallography, molecular dynamics simulations, and mutagenesis coupled to activity assays reveal a water-mediated hydrogen bond network linking the pyridyl group of the inverse agonist to Arg288 that is essential for inverse agonism. NMR spectroscopy reveals that PPARγ exchanges between two long-lived conformations when bound to the inverse agonist containing the pyridyl group, but not the neutral antagonist with the phenyl group, including a conformation that prepopulates a corepressor-bound state, priming PPARγ for high affinity corepressor binding. Our findings demonstrate that ligand engagement of Arg288 may provide new routes for developing PPARγ inverse agonists. This is an important finding because although the structural mechanisms affording activation of PPARγ are well understood, it remains poorly understood how to design transcriptionally repressive corepressor-selective PPARγ inverse agonists.

IL37

Structural Investigations and Experiences with 110 kHz spinning as well as Dynamic Nuclear Polarization (DNP)

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Aiming at the design of an allosteric modulator of the neonatal Fc receptor (FcRn)-Immunoglobulin G (IgG) interaction, a new methodology including NMR fragment screening, X-ray crystallography, and magic-angle-spinning (MAS) NMR at 100 kHz after sedimentation was developed, exploiting very fast spinning of the nondeuterated soluble 42 kDa receptor construct to obtain resolved proton-detected 2D and 3D NMR spectra. A small molecule is presented that binds into a conserved cavity of the heterodimeric, extracellular domain composed of an α-chain and β2-microglobulin (373 residues). Proton-detected MAS NMR experiments on fully protonated [13C,15N]-labeled FcRn yielded ligand-induced chemical-shift perturbations for residues in the binding pocket and allosteric changes close to the interface of the two receptor heterodimers present in the asymmetric unit as well as potentially in the albumin interaction site. In future, a major factor facilitating investigations on large protein complexes and native samples will be dynamic nuclear polarization (DNP), which was introduced to increase signal-to-noise by one or two orders of magnitude. In order to improve the quality of DNP spectra and to obtain maximum signal-to-noise, new radicals were synthesized and employed in measurements of protein samples around 190K. Enhancements in the range of 15-20 were observed in this temperature range while acceptable spectral resolution is observed. A comparison of signal-to-noise per unit time and enhancements of several radicals is presented, showing deviations concerning radical performance when proton T1 of the compared samples is affected differently by the radical. DNP is applied in investigations of collagen structure and related diseases. The rare but functionally essential post-translational collagen modification 5-hydroxylysine can undergo further transformations, including crosslinking, O-glycosylation, and glycation. DNP and lysine labelling provide sufficient NMR sensitivity to identify these adducts in skin and vascular smooth muscle cell extracellular matrix. In the longer term this combination promises elucidation of structural consequences of collagen modifications in intact tissue. Alkaptonuria (AKU) is a rare disease where patients suffer from an elevated level of plasma homogentisic acid, which over the course of decades accumulate in cartilaginous tissues to form a striking, dark pigmentation, often accompanied by acute loss of joint function and severe arthritic symptoms. Detection of the pigment in the intact extracellular matrix is challenging; however, using DNP-enhanced solid-state NMR spectroscopy, we have demonstrated that a HGA-derived pigment 1H-13C correlation signal can be detected from a sample of formalin-fixed human cartilage. Moreover, widespread disruption of the interstrand hydrogen bonding in the collagen proteins is observed in the spectra obtained from pigmented AKU cartilage. By applying the same technique to osteoarthritic (OA) human cartilage, we are able to compare the hydrogen bonding distribution of collagen proteins in OA and AKU cartilage, supporting the hypothesis that AKU represents an extreme form of OA at a molecular level.

Invited

IL38

Heavy mice and light things: using solid-state NMR spectroscopy to understand biological tissues in health and disease

Melinda Duer

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The extracellular matrix (ECM) forms the bulk of our structural tissues and provides them with their particular mechanical properties. At the microscopic level, it provides the scaffold which supports cells but more intriguingly, at the molecular level, it provides the communication system between the cells in the tissue and the signals that drives the individual behaviour of cells. Ultimately, if we can understand how the extracellular matrix structure dictates the behaviour of cells, then we can develop ways to treat diseases such as cancer, by changing the extracellular matrix to drive the necessary change in cell behaviour. However, understanding the molecular level properties of the extracellular matrix has been hampered by the lack of methods to study tissues at the atomic scale. In this talk, I will describe the various solid-state NMR spectroscopy approaches my group has taken over the last decade to tackle these complex questions. The first requirement is native-like tissues in which we can control isotope labelling patterns so that we can record assignable multidimensional NMR spectra. Using multidimensional solid-state correlation NMR spectra (13C-13C, 13C-15N) as fingerprints of the underlying molecular structures in isotope-labelled native tissues has allowed us to develop laboratory-grown tissues that have very similar molecular structures to native tissues, and thus represent demonstrably good models of native tissue.[1] The refined laboratory-grown tissues can then be manipulated by growing them with isotope labels in specific components to allow detailed study of structure and function of the various extracellular matrix components. For instance, 13C, 15N labelling Gly and Pro in the collagen component has led to the unexpected conclusion that Gly-Pro-Hyp triplets in collagen act like springs allowing the collagen helix to flex, rather than these triplets being rigid structures as they have long been assumed to be.[2] We have now coupled this approach with NMR methods to examine calcified tissues in health, such as bone, and in diseases such as vascular calcification (hardening of the arteries). In calcified tissues, the extracellular matrix incorporates stacks of ordered nanocrystals of a complex calcium phosphate phase. Using an NMR crystallography approach, we have put forward a new model for the structure of bone mineral,[3] and now have an understanding of what chemical species control bone mineral strength. In combination with the methods described above, we now also have an intriguing insight into what initiates hardening of the arteries, which leads to a potential route to prevent this condition, the major cause of cardiovascular disease worldwide.

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IL39

Structure and Dynamics of HIV-1 Capsid Protein Assemblies by an Integrated Approach

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Recent methodological advances will be presented that enable atomic-level characterization of structure and dynamics of large biological assemblies of HIV-1 capsid protein. HIV-1 capsids, assembled from 1,500 copies of the capsid (CA) protein, are an integral part of mature virions. Conical in shape, capsids enclose the viral genetic material (two copies of RNA) together with several proteins that are essential for viral replication. In the assembled state, capsids are remarkably dynamic, with the CA residue motions occurring over a range of timescales from nano- to milliseconds. These motions are functionally important for capsid's assembly, viral maturation, and interactions with host factors. In this talk, I will present an integrated MAS NMR, DNP, MD, and DFT approach to probe the structure and functionally important motions in assemblies of CA and their complexes with host factors Cyclophilin A (CypA) and TRIM5α, as well as assemblies of CA-SP1 maturation intermediates. The role of dynamic allosteric regulation in capsid's assembly, maturation, and escape from the CypA dependence will be discussed. It will be demonstrated that the integration of experimental NMR and DNP methods and theory, at classical and quantum mechanical levels, yields quantitative, atomic-level insights into the dynamic processes that govern the capsid's function.

IL40

NMR characterization of intrinsically disordered regulatory proteins

Peter Wright

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Intrinsically disordered proteins (IDPs) are highly abundant in the human proteome and are strongly associated with numerous devastating diseases, including cancers, age-related neurodegenerative disorders, diabetes, cardiovascular and infectious diseases. IDPs mediate critical regulatory functions in the cell, including transcription, translation, the cell cycle, and numerous signal transduction events. The lack of stable globular structure confers numerous functional advantages on IDPs, allowing them to exert an exquisite level of control over cellular signaling processes, but poses a major challenge to which traditional structural biology approaches are poorly suited. Many regulatory IDPs contain multiple interaction motifs. The intermolecular interface between such IDPs and their targets is energetically heterogeneous and is characterized by both static and dynamic interactions that mediate crosstalk between signaling pathways and lead to unique allosteric switches. NMR has emerged as the primary tool for elucidation of the structural ensembles, dynamics, interactions, posttranslational modifications, and functional mechanisms of IDPs. Relaxation measurements are especially important for characterization of IDP complexes, providing novel insights into the dynamic processes that mediate binding, competition for a common target, and allostery. The applications of NMR to elucidate the role of IDPs in dynamic cellular signaling will be illustrated by reference to pathways regulated by the tumor suppressor p53 and by the general transcriptional coactivators CBP (CREB binding protein) and p300, with particular reference to the mechanism of action of a unidirectional, hypersensitive switch that downregulates the hypoxic response by displacing the hypoxia inducible factor HIF-1α from CBP/p300.

IL41

Amyloids: From the origin to the end of life

Roland Riek, Marielle Wälti, Carolin Seuring, Dhiman Ghosh, Beat H Meier, Jason Greenwald, Juan Gerez, Saroj Rout, Anja Böckmann

ETH Zurich, Switzerland

Protein aggregation into amyloids is both associated with neurodegenerative diseases as well as function such as hormone storage in the pituitary. The structure determinations of amyloid fibrils by solid state NMR at atomic resolution establishes both a structure-function as well as a structure-disease relationship as demonstrated with the 3D NMR structures of the HET-s prion, Abeta(1-42) fibrils associated with Alzheimer's disease, and of the hormone beta-endorphin stored in secretory granules in an amyloid state.

Based on the structure-activity relationships of amyloid, we and others developed to so called amyloid world hypothesis, which states that peptide amyloids may have played an important role in the origin of life. Experimental support in the area of enzymatic active peptide amyloids, amyloid generation under prebiotic condition and template-assisted peptide synthesis in the amyloid are presented.

IL42

Linking membrane binding, function and aggregation: tau and alpha-synuclein

David Eliezer

Cornell University, United States

The proteins alpha-synuclein and tau are linked to Parkinson's and Alzheimer's disease, respectively, yet little is known about their normal physiological functions. Alpha-synuclein aggregation is considered key to its role in PD. Despite this, the physiological context for pathological aS assembly remains poorly understood. Understanding the earliest steps leading to aS aggregation, which likely constitute the best targets for preventing aggregation, will require a detailed understanding of the normal function of aS at the levels of both molecular structure and basic cell biology. An example of the utility of structural investigations of aS function is the discovery of Y39 as a novel phosphorylation site, and of the potential role of this phosphorylation event in driving membrane-induced synuclein aggregation, an area of rapidly growing importance. We are also utilizing cellular assays of synuclein function to enable structure-function studies and to reveal novel aspects of synuclein function in regulation vesicle exocytosis. Tau is normally a microtubule binding protein, but can also bind to membrane surfaces, an interaction that may be either physiologically or pathologically relevant. Work in the lab has demonstrated that tau-membrane interactions are mediated by short amphipathic helices, which also play a role in mediating membrane-induced aggregation to tau into novel oligomeric protein-phospholipid complexes. The core of these complexes is comprised of the PHF6 and PHF6* hexapeptide motifs, but surprisingly, only the former is found in a beta-strand conformation, while the remainder of the core is ordered but does not exhibit regular secondary structure. The properties of these oligomers bear some resemblance to recently characterized membrane-induced oligomers of alpha-synuclein.

IL43

New structural insights into complexes between chaperones and non-native proteins

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Molecular chaperones are necessary for maintaining a functional proteome in the cell by preventing the aggregation of unfolded proteins and/or assisting with their folding. Despite the central importance of the binding of chaperones to unfolded substrates, the structural basis of their interaction remains poorly understood. The scarcity of structural data on complexes between chaperones and unfolded client proteins is primarily due to technical challenges originating in the dynamic nature of these complexes.

I will discuss how NMR spectroscopy can be used as an extremely powerful tool to determine the structural and dynamic basis for the recognition and interaction of unfolded proteins by molecular chaperones.

IL44

Self-assembly, Structure and Functional Studies of Protein Machineries with Molecular Weights of up to 1 MDa

Jerome Boisbouvier

Institut de Biologie Structurale (CEA/CNRS/UGA), France

NMR spectroscopy offers a unique ability to monitor structural and dynamic changes in real-time and at atomic resolution. Historically, the application of real-time 2D NMR techniques has been limited to the study of small proteins. In this presentation, I will show that a combination of methyl specific labeling approaches, optimized NMR spectroscopy, electron microscopy (EM) and mass-spectrometry (MS) can be used to decipher, at the structural level, the self-assembly process of the half-megadalton dodecameric TET2 proteolytic machinery. While complementary time-resolved EM and native MS approaches were used to characterize the low-resolution structures of oligomerization intermediates, NMR was used to obtain individual kinetics data on the different transient intermediates and the formation of final TET2 particle.

In order to determine the structure of the TET2 machinery at atomic resolution, we set up a novel calculation approach integrating cryo-EM with solid-state and solution NMR. The developed approach allowed us to solve the structure of the 12 x 39 kDa-large TET2 assembly to a precision below 1 Å, exceeding by far current standards of NMR and EM. This new approach provides high precision structures even in case where only medium to low resolution cryo-EM density maps are available.

Finally, I will show how NMR can provide functional insight into the 1 MDa large hsp60 chaperonin, while the machinery is processing client proteins. Although the structures of fully open and closed forms of chaperonins were solved by X-ray crystallography or EM, elucidating the mechanisms of such ATP-driven molecular events requires studying the proteins at the structural level under working conditions. In order to study the chaperonin in action, we introduced an approach that combines site-specific NMR observation of very large proteins, with an in situ ATP-regeneration system. Using this method, we reveal how nucleotide binding, hydrolysis and release control switching between closed and open states. While the open conformation stabilizes the unfolded state of client proteins, the internalization of the client protein inside the chaperonin cavity speeds up its functional cycle. This approach opens new perspectives to study structures and mechanisms of various ATP-driven biological machineries in the heat of action.

IL45

NMR of translocon of bacterial nanoinjectors

Roberto De Guzman

University of Kansas, United States

Nanoinjectors of the type III secretion system (T3SS) are 4 megadalton macromolecular complexes assembled by many pathogenic bacteria to in-

ject virulence proteins into human cells to cause infectious diseases such as dysentery, food poisoning, bubonic plague, and lung infections. All bacteria that require nanoinjectors for virulence have developed antibiotic resistance, and there are no approved vaccines for widespread use for any of these bacteria. Because nanoinjectors are essential for virulence, surface exposed, and found only among pathogens, disrupting their assembly is an attractive strategy for developing new antibiotics. We use NMR to study the assembly of nanoinjectors, which consist of a needle, a tip and a translocon – a pore-like structure on the host cell membrane that allows the entry of virulence proteins into the host. A major unknown in this field is the atomic structure of the translocon. We show that the dearth of atomic-level structural data on the translocon is because one of its constituent membrane proteins – represented by the BipC family of translocon proteins – is predominantly disordered with a small portion of alpha helices. BipC is currently the only translocon protein that can be expressed and purified under native condition. Further, this 419-residue membrane protein yields excellent quality NMR data, allowing us to determine its molecular interactions with its binding partners – such as its chaperone, the tip protein, and the BipB family of translocon proteins. Because of the intrinsically disordered nature of BipC, NMR becomes essential in elucidating atomic-level data on the translocon that would not be possible by crystallography or cryoEM.

IL46

Structure-based methyl resonance assignment and multi-state eNOE analysis with CYANA

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Specifically labelled methyl groups are the best accessible NMR probes in proteins that are too large for backbone or complete assignment. However, their assignment in the absence of usable through-bond spectra is challenging. In general, only through-space NOESY spectra are informative and applicable for these systems. Several computational methods for NOESY- and structure-based methyl assignment have recently been published and compared (1). While these algorithms are capable to find methyl assignments, they may suffer from either long computation times or a low completeness/reliability of the methyl assignments. We have therefore adapted the FLYA automated assignment algorithm in CYANA (2) for this purpose. Structure-based methyl assignment with FLYA relies on an evolutionary algorithm to find an optimal mapping between NOESY peaks expected based on the given 3D structure and the measured NOESY peaks. We show that FLYA is a valuable alternative to existing approaches for structure-based methyl assignment in that it usually assigns more methyl groups, has a predictable runtime, and flexibility to include other types of information, such as arbitrary isotope labeling patterns, HN-methyl NOEs, or data from other types of spectra.

The "exact" interpretation of NOESY peak build-ups (eNOEs) allows the calculation of multi-state structural ensembles that can reveal spatial sampling and long-range correlations (3). The determination of eNOE distance restraints for structure calculation requires an understanding of the underlying spin dynamics and involves the multi-step eNORA protocol, which has so far been performed with a stand-alone MATLAB script (4). We have integrated the complete eNORA protocol, the multi-state structure calculation, in which restraints have to be fulfilled only on average over an ensemble of multiple structural states, and automatic sorting/clustering of structural states from different conformers calculated independently, into CYANA. This greatly facilitates the application of the eNOE method for multi-state structure determination.

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IL47

High dimensionality and high resolution NMR experiments for IDPs

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Studies of biomolecular structure and dynamics by NMR spectroscopy at atomic resolution require acquisition of multidimensional spectra. However, the recording time of sufficiently resolved multidimensional spectra is often very long due to the sampling limitations. A variety of different methods, mostly based on non-uniform sampling, were proposed to overcome this limitation in multidimensional NMR spectroscopy. They could be utilized in two different ways, either to shorten the experiment duration without loss of resolution, or to perform experiments that are not obtainable conventionally, i.e. with significantly improved resolution and/or of high dimensionality. Most often first of these two, so called "Fast NMR" approach, is shown as the example of the utility of these methods, as it saves expensive spectrometer time. However, in many cases spectra which are not possible to record conventionally, featuring extraordinary resolution and high number of dimensions may be more interesting from scientific point of view as they reveal effects that are hidden, when spectral lines are broad, or enable resolving spectral ambiguities when peaks are overlapped. This second approach we refer to as "Accurate NMR". Its full potential is manifested when the overall experiment time is less important than a new information available from spectra of high dimensionality (4-6D) or of high resolution approaching natural line-width. The new methods were applied for NMR studies of intrinsically disordered proteins, where the structural disorder in combination with highly repetitive amino-acid sequences causes severe peak overlap in the spectra. Several novel 4-7D pulse sequences are proposed. The new experiments employ non-uniform sampling that enables achieving high resolution in indirectly detected dimensions.

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IL48

High-resolution, integrative modelling of biomolecular complexes from fuzzy data.

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The prediction of the quaternary structure of biomolecular macromolecules is of paramount importance for fundamental understanding of cellular pro-

cesses and drug design. In the era of integrative structural biology, one way of increasing the accuracy of modelling methods used to predict the structure of biomolecular complexes is to include as much experimental or predictive information as possible in the process.

We have developed for this purpose a versatile information-driven docking approach HADDOCK (http://haddock.science.uu.nl) [1,2]. HADDOCK can integrate information derived from biochemical, biophysical or bioinformatics methods to enhance sampling, scoring, or both [3]. The information that can be integrated is quite diverse with as most recent additon cryo-EM maps [4].

In my talk, I will illustrate HADDOCK's capabilities with various examples and describe some recent developments around the modelling of membrane protein complexes and large assemblies. I will also introduce the concept of explorative modelling in which the interaction space defined by a limited number of restraints is systematically mapped [5].

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IL49

Morning larks vs. night owls: NMR insights into multisite phosphorylation and control of circadian timing in humans

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Circadian rhythms coordinate the timing of nearly all behavioral and physiological processes with approximately 24-hour periodicity. One clock protein, Period 2 (PER2), functions as the defining state variable in this genetically encoded clock through the oscillation of its abundance and subcellular localization, which is regulated by phosphorylation predominantly through the casein kinase 1 isoforms δ and ε (CK1δ/ε). Phosphorylation at S479 promotes the proteasomal degradation of PER2, whereas phosphorylation of a competing site, S662, results in the initiation of multisite phosphorylation that stabilizes PER2. A serine to glycine mutation at the priming site (S662G) leads to premature PER2 degradation and shortens circadian period, causing familial advanced sleep phase (FASP) syndrome. People with FASP syndrome are intrinsically driven to wake up and fall asleep much earlier than the general population (morning larks), while other mutations in Period and CK1 genes lengthen circadian period and lead to delayed sleep phase syndrome (night owls). Using NMR spectroscopy coupled with cellular assays, we identified a new and unexpected role for CK1δ/ε that is dictated by the slow phosphorylation of a key non-consensus site. Time-resolved NMR studies were used to monitor phosphorylation by CK1δ at single-residue resolution and demonstrate the sequential nature of its multisite phosphorylation. NMR also provides a robust approach to analyze the effects of newly identified substrate-competitive small molecule inhibitors of CK1δ/ε and explore their regulation of PER2 phosphorylation. Understanding the molecular basis for control of circadian timing by PER2-CK1δ/ε signaling will aid in the development of pharmaceuticals to treat sleep phase syndromes, as well as ameliorate circadian disruption resulting from shift work and jet lag.

IL50

Advanced complex mixture analysis by NMR and NMR/MS for metabolomics

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The Ohio State University, United States

The rapid, reliable and comprehensive identification and quantitation of a large number of organic molecules in complex mixtures, such as metabolites in biological systems in the context of metabolomics, will be discussed using multidimensional NMR tools. They include curated databases of known metabolites, multidimensional spectral query for identification and quantitation, and rapid non-uniformly sampled 2D 1H-1H TOCSY collection and spectral reconstruction. Many of these developments are well-suitable for automation and have been integrated into our COLMAR suite of web servers and databases (http://spin.ccic.ohio-state.edu/index.php/colmar). Since for most biological samples a large number of spectral features belong to unknown metabolites, there is a pressing need to identify them accurately and efficiently. An approach will be described that synergistically combines multiple sources of information, including NMR, cheminformatics, and/or high-resolution mass spectrometry.

IL51

Modulation by Phosphorylation of Tau Protein Interaction with Protein Partners

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In the course of our molecular investigation of Tau functions and dysfunctions in AD, nuclear magnetic resonance (NMR) spectroscopy is used to identify the multiple phosphorylations of Tau and to characterize Tau interactions with its molecular partners. These tasks remain challenging due to Tau highly dynamical character and its 80 Ser/Thr residues, potential sites of phosphorylation that can be combined to give a multiphosphorylated Tau, leading to a very complex regulation of Tau interactions. It has proven crucial to identify phosphorylation sites to be able to link specific phosphorylations with structural or functional modifications. Functional aspects include the regulation by Tau phosphorylation of both interaction of Tau with protein partners and aggregation.

This study was supported by grants from the LabEx DISTALZ, ANR BinAlz and EU ITN TASPPI. We acknowledge support from TGE RMN THC (FR-3050, France) and FRABio (FR 3688, France).

IL52

Interplay between charge and conformational sampling in the disordered carboxyl terminus of yeast gamma tubulin

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Tubulins are an ancient family of eukaryotic proteins characterized by an amino-terminal globular domain and disordered carboxyl terminus. These carboxyl termini play important roles in modulating the behavior of microtubules in living cells. However, the atomic-level basis of their function is not well understood. These regions contain multiple acidic residues and their overall charges are modulated in vivo by post-translational modifications, e.g. phosphorylation. We used a combination of NMR and computer simulations to investigate the interplay between the abundance of negative charge and conformational sampling in the disordered carboxyl terminus of γ-tubulin (γ-CT). NMR-based measurements of Asp and Glu side chain pKa values revealed large perturbations of up to 0.4 pH units. We used these values to calculate the net charge of the polypeptide as a function of pH and compared the result with PFG-NMR diffusion measurements. We found that the hydrodynamic radius of the γ-CT expands substantially more with increasing charge than one might expect, based on previous analysis of a database of disordered proteins. Finally, we examined a γ-CT with a a Y>D mutation at Y11 in the polypeptide (Y445 in the full protein). This mutation introduces additional negative charge at a site that is phosphorylated in vivo and produces a phenotype with perturbed microtubule function. NMR relaxation measurements show that the Y11D mutation produces dramatic changes in the millisecond-timescale motions of the entire polypeptide. This observation is supported by Monte Carlo simulations that, like NMR, predict the WT γ-CT is largely unstructured and that the substitution of Tyr 11 with Asp causes the sampling of extended conformations that are unique to the Y11D polypeptide. Together, these observations suggest that the highly acidic character of the γ-CT may assist in coupling the phosphorylation state at a single site to global conformational sampling properties.

IL53

Uncovering invisible dark states of biological macromolecules and their complexes by magnetic resonance

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Sparsely-populated, transient, “dark” states of macromolecules, characterized by short lifetimes, play a key role in many biological processes including macromolecular recognition, conformational rearrangements and assembly. Yet, these species are invisible to conventional structural and biophysical techniques, including crystallography and conventional NMR. We will briefly summarize new developments involving the application of lifetime line broadening (ΔR2) and Dark-state Exchange Saturation Transfer (DEST) spectroscopy, combined where appropriate with relaxation dispersion and very small exchange-induced shifts, to probe exchange processes (with lifetimes ranging from about 20 s to 10 ms) between NMR-visible and very high-molecular weight (1 to >100 MDa) NMR-invisible (“dark”) macromolecular assemblies and to elucidate atomic resolution structure and dynamics within these dark states. We will illustrate these approaches to the study of interactions of intrinsically disordered proteins and various folding intermediates with the chaperone GroEL. We will also illustrate how intermolecular paramagnetic relaxation enhancement measurements can be used to asses the extent of sampling of the cavity of GroEL by protein substrates.

IL54

Protein Folding on the Ribosome

John Christodoulou

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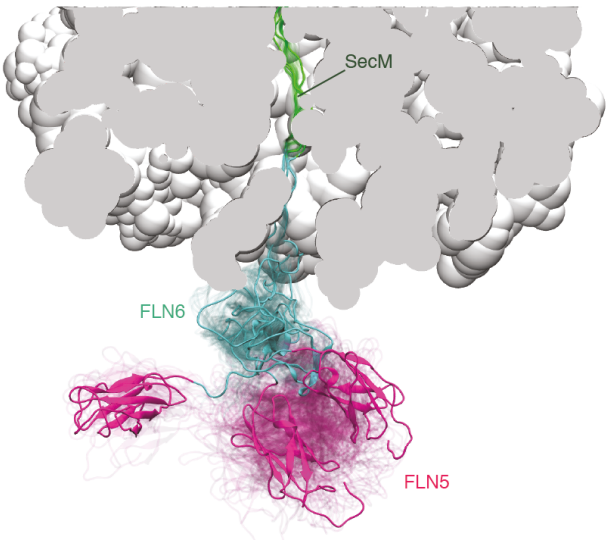
The ability for proteins to successful acquire their 3D structures is an essential requisite for biological activity within all cells. Our understanding of the fundamental principles underlying the chemical kinetics of protein folding has been derived almost exclusively from the study of renatured isolated proteins using both experimental and theoretical approaches. However, in vivo, protein folding can begin at the earliest stages of biosynthesis, which is carried out by the ribosome. Co-translational folding of nascent polypeptides is a fundamental process that is now beginning to be explored at atomic resolution through improvements in preparative biochemistry and physical and computational techniques. The ability of NMR spectroscopy to provide a simultaneous description of the structure and dynamics of the fledgling nascent polypeptide will be described.



IL55

Solid-State NMR Determination of the Cholesterol-Binding Structure of Membrane Proteins Using Long-Range Distances

Mei Hong, Matthew Elkins, Matthias Roos, Venkata Mandala, Alexander Shcherbakov

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Membrane protein structure and function are intimately dependent on the lipid environment. Cholesterol is an essential component in eukaryotic membranes, but membrane protein interaction with cholesterol has been difficult to elucidate by any structural technique. We have developed two solid-state NMR approaches to elucidate the cholesterol-binding site in membrane proteins and demonstrate these on the influenza M2 protein.

While M2 is well known to be a proton channel, it also has a second function of mediating Influenza virus budding from host cells by causing membrane scission in a cholesterol-dependent manner. To understand how cholesterol interacts with M2 to generate the necessary membrane curvature for scission, we have determined the cholesterol-complexed structure of M2 using 13C-19F REDOR distance measurements and 2H NMR orientation measurements. Our results give unexpected information about the stoichiometry and location of bound cholesterol to the M2 tetramer, and explain how M2 is attracted to the neck of the budding virus to cause membrane scission. Crucial for constraining the M2-cholesterol complex structure are long-range 13C-19F distances between 13C-labeled protein and fluorinated cholesterol. To further harvest the power of the high gyromagnetic ratio of 19F for distance determination, we have developed robust 19F-19F, 13C-19F and 1H-19F dipolar recoupling and spin diffusion techniques under high-field and fast MAS conditions, and show that distances up to 1.6 nm can now be measured reliably. As an alternative approach for determining cholesterol-membrane-protein interactions, we have developed a novel 2D 13C DNP-NMR technique to probe cholesterol-protein proximity using biosynthetically labeled cholesterol.

IL56

Magic Angle Spinning Spheres, Electron Decoupling with CPMAS Below 6 K, and DNP within Human Cells Using Fluorescent Polarizing Agents

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We demonstrate that spheres, rather than cylinders, can be employed as rotors in magic angle spinning experiments. Spheres spinning at the magic angle have significant advantages over cylinders, including simplicity and favorable scaling to sub-millimeter scales. We show initial experiments employing spheres for MAS experiments and observe rotational echoes from KBr, demonstrating stable spinning at the magic angle. We also describe the first MAS DNP experiments performed colder than 6 Kelvin, yielding DNP enhancements from biradicals of 242 and longitudinal magnetization recovery times < 2 s.[1,2] Furthermore, we show that microwave driven electron decoupling effectively attenuates detrimental interactions between electron and nuclear spins to increase the resolution and signal intensity in cross polarization (CP) MAS experiments.[2,3] Frequency chirped microwave pulses from custom-developed frequency agile gyrotrons are employed for electron decoupling.[4] Electron spin control is further improved using teflon lenses to focus microwave intensity and increase the electron spin Rabi frequency. Experiments on model systems are extended to intact human cells in the first demonstration of in-cell DNP, using both fluorescent trimodal DNP polarizing agents, and also abbreviated biradicals and sterically protected monoradicals. We show DNP NMR signal enhancements within HEK293 cells of >50, and together with cryogenic MAS <6 K, our experimental platform yields overall increases of NMR signals of >2500 within cryoprotected human cells. Time constants to replenish the DNP enhanced NMR signal within cells are <3 seconds even at sample temperature <6 K, enabling rapid signal averaging and yet further gains in NMR sensitivity. In-cell NMR results are further supported with in vitro MAS DNP experiments of isotope enriched protein kinase C (PKC) modulators actively being developed as viral latency reversal agents for HIV cure research.

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IL57

Solid-state NMR going viral: investigating capsids and envelopes from the Hepatitis B particle

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We will present proton and carbon-detected solid-state NMR spectra, and sample preparation methods from classical bacterial to more complex cell-free for analysis of large protein assemblies like viral capsids and envelopes. We will highlight the high sensitivity of NMR to the subtle conformational differences induced on particle formation, which are very hard to see by x-ray and EM. We will show how the membrane envelope proteins from the duck hepatitis B virus can be synthesized in vitro as sub-viral particles, and will discuss fast MAS (110kHz) spectra recorded on < 1 mg of sample, for the envelopes as well as for the capsid.

IL58

Non-cooperative folding tuned by phosphorylation of an intrinsically disordered protein to regulate translation initiation

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Cap-dependent translation is regulated by phosphorylation of the intrinsically disordered eIF4E-binding proteins (4E-BPs). The eukaryotic translation initiation factor 4E (eIF4E), or cap-binding protein, interacts with eIF4G to form a platform for the translation initiation complex, while 4E-BPs inhibit translation initiation by competing for the same eIF4E surface as eIF4G. The 4E-BP:eIF4E interaction becomes progressively weaker with increasing 4E-BP phosphorylation, with 3 nanomolar Kd for non-phosphorylated 4E-BP2 (the neural isoform), 270 nanomolar for pT37/pT46 4E-BP2 and 15 micromolar for fully, 5-phospho 4E-BP2, sufficiently weak to enable eIF4G to compete for eIF4E binding and enable translation initiation. Phosphorylation of T37 and T46 in 4E-BP2 induces folding of residues P18 to R62 into a four-stranded beta-fold domain, partially sequestering the canonical eIF4E-binding helix (Bah et al, Nature

2015). The pT37 and pT46 are part of two conserved TPGGT motifs that form hairpin turns central to the structure. The C-terminal intrinsically disordered region (C-IDR), which remains disordered after phosphorylation, contains the secondary eIF4E-binding site and other three phosphorylation sites (S65, T70 and S83). Current NMR data demonstrate the non-cooperativity of the folded domain, with beta structure in fast exchange with helical conformations. C-IDR phosphorylation sites modulate the equilibrium between these conformations, enabling tuned stability to tune access to eIF4E binding sites. The two hairpin turns formed by phosphorylated TPGGT motifs are remarkably stable, acting as molecular staples, and can function as transplantable units for phospho-regulation of stability. Our results demonstrate how translational regulation depends on non-cooperative folding with dynamic exchange of conformational states leading to graded inhibition of eIF4E binding by hierarchical phosphorylation of 4E-BP2, converting it into an eIF4E binding-incompatible conformation.

IL59

Phase separation and mesoscale assembly for functional compartmentalization

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Liquid-liquid phase separation of proteins leads to demixing from solution and results in a dense, protein-rich phase, which co-exists with a light phase depleted of protein. Recent findings support a model in which phase separation is the biophysical driving force for the formation of membrane-less organelles in cells, such as stress granules, nucleoli and nuclear speckles. Current open questions are: (i) How is phase separation propensity encoded in the protein sequence, (ii) are dense liquid droplets used as reaction compartments in the cell, and (iii) is physiological phase separation disrupted in disease states? To address these, we study two systems, the tumor suppressor Speckle-type POZ protein (SPOP) and the RNA-binding protein hnRNPA1. SPOP, a substrate adaptor of a ubiquitin ligase, localizes to different liquid membrane-less organelles in the cell nucleus, where it encounters its substrates, but it is never found diffuse in the cell. However, its recruitment mechanism to these organelles is not understood. Here, we show that SPOP undergoes LLPS with substrate proteins, and that this mechanism underlies its recruitment to membrane-less organelles. Multivalency of SPOP and substrate for each other drive their ability to phase separate. We present evidence that the SPOP/substrate assemblies are active ubiquitination compartments in vitro and in cells. SPOP cancer mutations reduce the propensity for phase separation. We propose that SPOP has evolved a propensity for phase separation in order to target substrates localized in membrane-less compartments. Recent mutagenesis experiments have revealed the importance of aromatic residues for the ability of low-complexity regions (LCRs) of RNA-binding proteins to undergo LLPS. Here, we investigate the interactions that mediate phase separation of the intrinsically disordered LCR of hnRNPA1. Phase separation of hnRNPA1 promotes the fibrillization of mutants of hnRNPA1 that cause ALS and other neurodegenerative diseases. We find that aromatic side-chains cluster and lead to compaction of the LCR, and that this compaction is coupled to LLPS. Understanding the interactions that mediate phase separation has the potential to provide mechanistic insight into membrane-less compartmentalization in cells.

IL60

Dynamic complexes and complex dynamics: Large scale molecular motion in biological function

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Proteins are inherently dynamic, exhibiting conformational freedom on many timescales (1); implicating structural rearrangements that play keys role in molecular interaction and thermodynamic stability. Intrinsically disordered proteins (IDPs) represent extreme examples where flexibility defines molecular function. IDPs exhibit highly heterogeneous local and long-range structural and dynamic propensities, allowing inter-conversion between a quasi-continuum of accessible conformations. While considerable effort has been devoted to describing the conformational space sampled by IDPs,2 little is known about the timescale of their intrinsic dynamics. We use NMR to describe the conformational space sampled by IDPs (2) to investigate the characteristic timescales of their intrinsic dynamics (3,4) and to map their complex molecular recognition trajectories. Examples include the replication machinery of paramyxoviruses, where the highly disordered phospho- and nucleoproteins interact to initiate transcription and replication (5) the JNK signalling pathway, where specificity is controlled by disordered domains of MAP kinases (6) or the nuclear pore, where weak interactions between nuclear transporters and highly flexible chains containing multiple short recognition motifs, facilitate fast passage into the nucleus (7). Finally, a combination of solution techniques reveals large-scale domain dynamics in Influenza polymerase that are essential for import into the nucleus of the infected cell (8).

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IL61

Molecular and Mechanistic Underpinnings of Signal Transduction - NMR Inspired Studies of the Conformational Landscape of GPCRs

R. Scott Prosser, Shuya Kate Huang, Libin Ye, Dmitry Pichugin, Aditya Pandey, Ned Van Eps, Oliver Ernst, Christopher Di Pietrantonio

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In the last decade, X-ray crystallography and cryo-electron microscopy have brought about a renaissance in GPCR structural biology. Recent atomic-resolution structures provide the possibility of mechanistic explanations of function and have renewed efforts in drug discovery. However, the "structure-function" perspective ignores the reality of protein dynamics and the fact that most proteins adopt a fluid ensemble of functional conformers (states). We are interested in the role of this ensemble associated with receptor activation and response to ligand. Recent NMR data in HDL nanodisks highlights key functional states associated with the adensoine A2A G-Protein-Coupled Receptor, which have parallels with other class A receptors. Much of this talk will focus on allosteric regulation of GPCRs and prospects towards drug discovery.

IL62

Structural characterisation of bioactive disulfide-rich peptides and their interactions with lipid membranes and ion channels in solution

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Disulfide-rich peptides including hormones, toxins and antimicrobial peptides, are short polypeptide chains (peptides) that fold into defined three-dimensional structures. They are secreted molecules that have naturally evolved high potency and selectivity, to regulate biological processes while remaining stable in the harsh extracellular milieu. These peptides owe their remarkable stability to formation of multiple, covalent, disulfide bonds between the side chains of different cysteine residues that act to "tie" the molecule into well-defined "scaffolds".

I will present work on (1) the production and isotope labeling of disulfide rich peptides, including the production of segmentally labelled two domain peptides, (2) application of advanced NMR methods in structural characterisation of these peptides and finally the interaction of these peptides with both their receptors (3) and to membrane bilayers in (4) in solution using circularised nanodiscs.

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IL63

Probing the structures and functions of membrane proteins in membranes.

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Membrane proteins are among the most attractive targets for molecular structure determination and drug development. Their localization at biological membranes places them at the forefront of all interactions of the human body with the outside world and their malfunction is linked with major human diseases. Because the structures and functions of membrane proteins are highly directional, and intimately associated with the lipids that make up the membranes, their characterization necessitates the reconstitution of protein-lipid assemblies in samples that resemble the native membrane as closely as possible. NMR is well suited for this task, with complementary solution NMR and solid-state NMR approaches available for examining a wide range of membrane protein conformations and dynamics. Our NMR studies focus on two types of membrane proteins that play key roles in bacterial human infection and in regulation of human programmed cell death. Solid-state NMR studies of the outer membrane protein Ail, from Y. pestis, shed light on the influence of the outer membrane component lipopolysaccharide on the extracellular loops of Ail, which are important for ligand binding and human infection. Solution and solid-state NMR studies of the Bcl-2 family proteins embedded in membranes or associated with lipids illustrate a role for lipids as chaperones of these key proteins as they shuttle between cytoplasmic and intracellular membrane fractions. Optimized samples of both these proteins enable high resolution NMR spectra to be obtained in detergent-free membranes, and newly developed computational methods enhance protein structural accuracy, precision, and quality. In our view, NMR is the only technique capable of providing atomic-resolution information about functional membrane proteins in their native environment.

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IL64

Dynamics of molecular assemblies in the solid state

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Solid-state NMR is a powerful, complementary to other biophysical techniques, approach for studying at atomic resolution structures and dynamics of proteins in various forms from fibrils and membrane proteins to large protein complexes. In this presentation, I discuss several developments in methodology and applications concerning protein dynamics in the solid state with examples from both crystalline and non-crystalline systems illustrating advantages and challenges of the approach.

IL65

Dengue Virus NS2B-NS3 Protease Activity Modulated by Dynamics of NS2B

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Dengue virus, belonging to the Flaviviridae virus family, is a RNA virus responsible for the dengue fever, a tropical infectious disease whose incidence has increased drastically over the last decades. In the infection, the virus RNA genome is translated into a poly-protein containing structural and non-structural components. NS3 protease (NS3pro) is the N-terminal domain of the third non-structural protein. It is essential for the virus maturation and the cleavage of the poly-protein into functional individual proteins. NS3pro alone is unstable and needs its cofactor NS2B for protease activity. Previous crystal structures of NS2B-NS3pro from the Flaviviridae virus family suggest that the complex exists in two states, one "open" state corresponding to the inactive conformation and one "closed" state corresponding to the active conformation. Conformational exchange between different states in solution is evident from previous NMR studies, but no structural information is available for the "open" state and the kinetics for the conformational exchange process is unknown.

We characterized the conformational exchange using relaxation dispersion (RD) and chemical exchange saturation transfer (CEST) and correlated the dynamics of NS2B with the activity of NS3pro-NS2B through function and mutagnesis studies. We found that the NS2B in the complex exists in two conformers. The dominant conformer (96%) corresponds to the "closed" state, containing four β-strands (A50-K87) that each interact with NS3pro. The minor conformer (4%) corresponds to the "open" state, in which E66-K87 become unfolded and have no interactions with NS3pro. These two conformers undergo conformational exchange with an exchange rate of

3500 s-1. Deletion of the C-terminal disordered region (N88-R100) had no significant effect on the protease activity of NS2B-NS3pro. Further deletion of the last strand β4 (M84-K87) rendered it inactive. Destabilization of the β4 by mutating one residue to proline resulted in complete loss of the activity. We further showed that this mutant is unfolded in the region of E66-R100, and has a similar conformation to the minor conformer of the wild type NS3pro-NS2B. Stabilization of the "closed" conformation by introducing a disulfide linkage between residue 73 of NS2B and residue 115 of NS3pro enhanced the activity of NS3pro-NS2B. Our results demonstrate that the protease activity of NS3pro can be modulated by shifting the equilibrium of the two conformers of NS2B. This provides a new avenue for designing NS3 protease inhibitors by targeting the dyanmics of its cofactor NS2B.

IL66

Using 13C direct-detected NMR to characterise side-chain dynamics and interactions in proteins

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Proteins are dynamic entities and function is often related to motions and inter-conversions of diverse states. Most experimental studies of protein motions, to date, have focussed on the protein backbone and whilst knowledge of the protein backbone is essential to understand many aspects of protein function; generally understanding the dynamics and interactions of protein side chains is crucial. Specifically, it is the side chains that give the amino acids in proteins their unique chemical diversity and side chains play instrumental roles for nearly all functions of proteins.

New NMR-based methods, anchored in 13C-detection, to characterise the motions and interactions of functional side chains in large proteins will be presented. Specific focus will be on arginine side chains that are imperative for many active sites and protein-interaction interfaces since the terminal guanidinium group is perpetually positively charged and has five possible hydrogen bond formation sites. These features drive the formation of bidentate salt bridges, cation-π and π-π interactions, as well as hydrogen bonding networks.

Initially it will be shown how the strength of the interactions formed via the guanidinium group can be quantified. Arginine residues that form interactions of any kind via their guanidinium group experience a restriction of the rotational motion about the Ne-Cz bond as well as a decreased solvent exchange rate of the labile guanidinium protons. The rotational motion and the solvent exchange rates therefore serve as measures for the strength of the interactions formed by the arginine side chain. NMR methods to characterise both the solvent exchange rate of labile guanidinium protons and the dynamics of the guanidinium group will be presented.

Secondly, a new class of NMR experiments is presented, which allows a general quantification of motion and structure of side chains in large proteins. The new class of experiments allows a quantification of both non-methyl and methyl-bearing side chains and relies on 13C-13C correlation spectra. It is shown that intermediate chemical exchange of side chains can be probed using CEST experiments and also that long-range scalar couplings can be obtained. The new experiments are also applied to a 82 kDa protein, where well-resolved spectra with minimal overlap are obtained within a few hours.

Side chains cover protein surfaces and are imperative for substrate recognition and for most active sites in enzymes. The results presented promise a characterisation of side chains at a level that has hitherto been reserved for the protein backbone. It is envisaged that the new methods serve as particularly valuable tools to characterise active sites in enzymes, protein-protein or protein-nucleic acid interactions, where side chains and their interactions are expected to play crucial roles.

IL67

DNP-enhanced MAS NMR enables the selective probing of functional conformational changes

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NMR is a powerful and indispensable technique in structural biology. Nevertheless, the inherently low sensitivity towards nuclear spins is still one of the limiting factors in its application. Dynamic nuclear polarization (DNP) is a tool to overcome this problem by transferring the significantly larger spin polarization of unpaired electrons to the nuclei of interest during a typical solid-state NMR experiment. This allows the investigation of a wide range of sample systems and scientific problems where the analyte is in too low concentration to be amenable to standard MAS NMR techniques, for example in complex host-guest systems, in (near) native concentration, or where isotope labeling is impossible. In this presentation several scenarios will be presented where DNP enhancement plays a crucial role in the investigation of functional state conformations—or changes thereof—in biomolecules such as proteins and ribonucleic acids. First, we have utilized 1H-DNP-enhanced NMR in order to enlighten the catalytic mechanism of the hammerhead ribozyme. By a combination of nucleotide- as well as strand-selective isotope labeling and heteronuclear correlation-spectroscopy we have selectively probed interstrand contacts which allow us to elucidate the role of a divalent metal-ion co-factor in triggering functional conformational changes within the RNA molecule in frozen solution. Second, we have developed and employed a novel mechanism where heteronuclear cross relaxation effectively driven under DNP conditions can yield site-specific information by introduction of 13CH3 into biomolecular complexes. As will be demonstrated on a protein system, strategically employing isotope-labeling schemes potentially enables the extraction of long-range homonuclear distance information directly from DNP build-up dynamics. Furthermore, it will be shown that heteronuclear cross relaxation enables the selective observation of RNA aptamers in their ligand-bound complex, while signal from unbound biomolecules is effectively suppressed.

IL68

DNP enhanced biomolecular NMR spectroscopy at high magnetic field and fast magic-angle spinning

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Dynamic Nuclear Polarization (DNP) is an effective approach to alleviate the inherently low sensitivity of solid-state NMR under magic angle spinning (MAS), opening new possibilities for the investigation of the structure and dynamics of large-sized biomolecular assemblies. The signal enhancement comes, however, at the expense of a substantial signal broadening, associated with the necessity to perform experiments at cryogenic temperatures (at about 100 K), and with the presence of paramagnetic radicals. In this presentation, we will show that high quality DNP- enhanced NMR spectra of the Acinetobacterphage 205 (AP205) nucleocapsid can be obtained by combining high magnetic field (800 MHz) and fast MAS (40 kHz). In particular, we will show that these experimental conditions yield enhanced resolution, with observed carbon-13 NMR linewidths mainly governed by the inhomogeneous broadening. The increased refocused coherence lifetime allows the acquisition of scalar-based experiments, which in combination with dipolar spectra, enables the detection and assignment of long-range contacts, not observed at room temperature. The performance and limitations of current DNP polarizing agents with respect to

high magnetic field and fast MAS will be finally discussed.

IL69

Plumonary Microstructure, Function MRI and Molecular Imaging With Hyperpolarized 129Xe

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With the technique of spin-exchange optical pumping (SEOP), the spin polarization of hyperpolarized xenon can be enhanced four or five orders of magnitude, which makes feasible to obtain the gas phase signal, like in the lung's airspace morphology. We built a new designed 129Xe hyperpolarizer with a two-body optical cell, which can produce hyperpolarized 129Xe with 30% polarization for natural abundance xenon[1-2]. It enables us to image lung with hyperpolarized natural abundance xenon, showing a great potential for applying in clinics. By using hyperpolarized 129Xe ADC or ADK MRI, the microstructure of smoke-induced lung can be well visualized and evaluated [3-4]. Being a trace element in the atmosphere, xenon is soluble in water, blood and tissues. Therefore, dissolved phase xenon MRI could provide rich information related to the gas-exchange function of the lung. The morphological and physiological parameters of the radiation-induced lung injury (RILI), chronic obstructive pulmonary diseases (COPD) and emphysema can be non-radioactively and non-invasively obtained in vivo using hyperpolarized 129Xe diffusion, CSSR and CEST MRI [5-8], which could not be comprehensively achieved by the currently other imaging modalities. It demonstrates that such a new imaging technology is able to evaluate the microstructure and function of the lung, which paves a new way for the pulmonary disease research. Furthermore, different xenon biosensors were designed and developed to specifically detect ions, biothiols and H2S in living cell [9-12], showing the potential great applications of hyperpolarized xenon MRI in biomedicine.

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IL70

Fast quantitative 2D NMR for targeted and untargeted metabolomics

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NMR spectroscopy is a major analytical tool in metabolomics, thanks to its ability to provide repeatable and reliable information in a non-destructive fashion. However, one-dimensional (1D) 1H spectroscopy, the most widespread approach, is strongly limited by the numerous peak overlaps which hamper the analysis of complex biological samples. This limitation can be overcome by multi-dimensional NMR, and particularly by 2D NMR, albeit at the cost of a longer experiment time and of several specificities associated with the use of multi-pulse sequences.

In the last few years, we developed an ensemble of fast and quantitative 2D NMR approaches for the accurate analysis of complex mixtures [1,2]. These methods combine accelerated 2D pulse sequences such as ultrafast NMR with analytical chemistry methods such as external calibrations or standard additions. They provide efficient solutions for the accurate quantification of targeted compounds in mixtures, but also for the untargeted analysis of large sample collections.

We will illustrate the potential of these fast quantitative 2D NMR methods in omics sciences. Recent examples include the developments of untargeted lipid profling approaches at high field [3], but also on a benchtop spectrometer [4]. Targeted quantification approaches will also be discussed [5,6], as well as recent perspectives opened by promising preliminary results in the development of hyperpolarized metabolomics methods [7].

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IL71

NMR approaches to investigating protein 3D structures in living eukaryotic cells

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In vivo observations of 3D structures, structural changes, folding stability, dynamics or interactions of proteins are essential for the explicit understanding of the structural basis of their functions inside cells. In-cell NMR is currently the only approach that can provide structural information of proteins inside cells at atomic resolution. We have reported the first 3D protein structure calculated exclusively based on the information obtained in living E.coli cells [1]. Extending in-cell NMR to study proteins inside higher eukaryotic cells, and thus making this method more useful in medical and pharmaceutical researches, was another issue to be investigated. Currently in-cell NMR studies in various eukaryotic cells have become possible by either expressing target proteins inside cells or by introducing stable isotope-enriched proteins. Recently, protein global folds in Xenopus oocytes were obtained by exploiting paramagnetic NMR effects and NMR chemical shifts with the 3D protein structure prediction software Rosetta [2,3]. However, in order to elucidate the subtle difference between in vitro and in-cell structures, it remains necessary to achieve the de novo 3D protein structure determination from a sufficient number of NOE-derived distance restraints between side-chains. I will report our recent methodological developments which enabled the first high-resolution protein structure determinations in eukaryotes using the Sf9 insect cell line with the baculovirus protein expression system. Our method comprises (i) a bioreactor system [4] for extending the lifetime of Sf9 cells, (ii) advances in the processing of nonlinearly sampled NMR data, and (iii) structure refinement with Bayesian inference [5,6], which makes it possible to calculate accurate 3D protein structures from sparse data sets of conformational restraints that are based exclusively on NMR data from living cells. The method was applied to five proteins, rat calmodulin, human HRas, human ubiquitin, T. thermophilus HB8 TTHA1718, and Streptococcus protein G B1 domain. In all cases, we could observe well-resolved 3D NMR spectra and obtain structural information from in-cell NOESY data, suggesting

that our method can be a standard tool for protein structure determinations in living eukaryotic cells. For three proteins, we achieved well-converged 3D structures. Among these, the in-cell structure of protein G B1 domain was most accurately determined, demonstrating that a helix-loop region is tilted in a direction away from a beta-sheet compared to the conformation in diluted solution due to molecular crowding or other intracellular effects.

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IL72

Cellular solid-state NMR applied to bacterial and human cells

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For a long time, Solid-state NMR (ssNMR) has been used to study complex biomolecular systems. Over the last 15 years, ssNMR has seen strong methodological and instrumental developments that has allowed for the characterization of complex molecules including membrane proteins, amyloid fibrils or protein biopolymers with remarkable structural accuracy and comprehensiveness. More recently, ssNMR has profited from revolutionary enhancements in sensitivity, mainly due to the advent of Dynamic Nuclear Polarization (DNP) and it has seen significant progress in the field of ssNMR 1H detection. In our contribution we show how to make combined use of such methods to probe biomolecules in bacterial and human cells. Applications include protein translocation and insertion machines in bacteria and extend to membrane associated as well as soluble protein complexes in human cells. We also describe how to combine such ssNMR-based studies with modalities such as cryo-electron tomography (CET) and Fluorescence microscopy to obtain structural insight into cell organization from the atomic to sub-micrometer scale.

Selected Lectures

SL1

Exact distance measurement in RNA and large proteins

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In a string of recent studies, we have replaced the standard NOE-based procedure for structure determination by an approach that employs tight averaged distance restraints derived from exact NOEs (eNOEs) [Nichols et al. 2017]. So far, we have used the approach to calculate multi-state structural ensembles of various proteins of sizes up to 160 residues [Nichols et al. 2017]. Here, we present two novel ideas for taking the eNOE approach in unexplored directions, one being its application to nucleic acids and the other one tests on large proteins. RNA does not only translate the genetic code into proteins, but also carries out important cellular functions. As is the case for proteins, understanding such functions requires knowledge of the structure and dynamics at atomic resolution. Almost half of the published RNA structures have been solved by NMR. However, as a result of severe resonance overlap and low proton density, high-resolution RNA structures are rarely obtained from NOE data alone. Instead, additional semi-empirical restraints and labor-intensive techniques are required for structural averages, while there are only a few experimentally derived ensembles representing dynamics. Here we show that our eNOE-based structure-determination protocol is able to define a 14-mer UUCG tetraloop structure at high resolution without other restraints [Nichols et al., accepted]. Additionally, we use eNOEs to calculate a two-state structure which samples its conformational space. The protocol may open an avenue to obtain high-resolution structures of small RNA of unprecedented accuracy with moderate experimental efforts. A serious challenge for NMR spectroscopy is the size limit imposed on molecules to be studied. Standard studies are typically restricted to ca. 30-40 kDa. More recent developments lead to spin relaxation measurements in methyl groups in single proteins or protein complexes as large as a mega-Dalton, which directly allow the extraction of angular information or experiments with paramagnetic samples. However, these probes are all of indirect nature in contrast to the most intuitive and easy-to-interpret structural/dynamics restraint, the internuclear distance, which can be measured by NOE. Here, we demonstrate eNOE-based distance measurements on the 360 kDa half proteasome from Thermoplasma acidophilium [Chi et al. 2018]. Our findings open up an avenue for such measurements on very large molecules. These restraints will help in a detailed determination of conformational changes upon perturbation such as ligand binding.

Nichols, Born, Henen, Strotz, Orts, Olsson, Güntert, Chi, Vögeli, The exact NOE: Recent advances, 2017, Molecules 22, 1176

Nichols, Born, Henen, Strotz, Güntert, Vögeli, High-resolution small RNA structures from exact nuclear Overhauser enhancement measurements without additional restraints, 2018, Comm Biol accepted

Chi, Strotz, Riek, Vögeli, NOE-derived methyl distances from a 360 kDa proteasome complex, 2018, Chem Eur J 24, 2270-2276

SL2

Structural dynamics and interaction studies of large RNAs facilitated by selective Isotope labeling

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RNA controls various aspects of a virus's lifecycle and is, therefore, an exciting therapeutic target. Until recently, routinely targeting such RNAs with small molecules has been difficult. As a promising workaround, we have combined high-throughput small molecule microarrays (SMMs) with novel atom selective isotopic labeling strategies and NMR experiments to identify and validate candidate molecules that bind specifically. Our technologies also enable rapid assignments and structural characterizations of large RNAs (>50 nucleotides) using a combination of NMR and SAXS data. We will discuss how structural and dynamics measurements promise to lead to better understanding of drug-RNA interactions.

SL3

Protein-quadruplex DNA/RNA interactions in telomere regulation and anti-prion activity, and the first successful observation of in-cell NMR signals of DNA/RNA in human cells

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Telomeric DNA and telomeric repeat-containing RNA (TERRA) form G-quadruplex structures. Previously, we showed that the third RGG motif of translocated in liposarcoma (TLS) protein, also known as FUS, is responsible for a ternary complex formation with the G-quadruplex structures of telomeric DNA and TERRA. Here, we reveal the interactions in the binary and ternary complexes of RGG3 with telomeric DNA or/and TERRA. In the ternary complex, tyrosine bound exclusively to TERRA, while phenylalanine bound exclusively to telomeric DNA. Surprisingly in the binary complexes, RGG3 used both tyrosine and phenylalanine residues to bind to either TERRA or telomeric DNA. We propose that the plastic roles of tyrosine and phenylalanine are important for RGG3 to efficiently form the ternary complex, and thereby regulate the telomere shortening (1). We previously identified an RNA aptamer against a prion protein, r(GGAGGAGGAGGA) (R12). We showed that R12 forms a unique quadruplex structure with a tetrad and a hexad and reduces a level of the abnormal prion protein, PrPSc, in the mouse neuronal cells, implying its therapeutic potential as to prion diseases (2,3). Here, we reveal that RNA with analogous sequence to R12 can reduce the level of the abnormal prion protein more efficiently. Structure determination rationalizes the higher anti-prion activity of this new RNA (4). The other disease-related protein-DNA interactions will also be addressed (5-7). In order to understand intracellular biological events, information on the structure, dynamics and interaction of proteins and nucleic acids in living cells is of crucial importance. In-cell NMR is a promising method to obtain this information. Although NMR signals of proteins in human cells have been reported, those of nucleic acids were reported only in Xenopus laevis oocytes. Here, NMR signals of DNA and RNA were successfully observed for the first time in living human cells (8). The observed signals directly suggested the formation of DNA and RNA hairpin structures in living human cells.

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SL4

Structural basis of activation of human PARP-1 by DNA single-strand breaks.

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The highly abundant nuclear enzyme poly(ADP-ribose)polymerase-1 (PARP-1) is a key eukaryotic stress-sensor that detects DNA single-strand breaks (SSBs), the most frequent form of genomic damage; on binding an SSB, PARP-1 responds with an immediate burst of poly(ADP-ribose) synthesis that signals for assembly of DNA repair factors. PARP inhibitors hold great promise in cancer therapy as they can kill BRCA-deficient tumor cells selectively. However, the mechanism underlying PARP-1's function has long been obscure; inherent dynamics of SSBs and of PARP-1's modular six-domain architecture hindered structural studies. By combining solution NMR results with crystal structures of PARP domains on DNA double-strand breaks, we established in molecular detail how recognition and activation by SSBs occur in this key enzyme; PARP-1's combination of flexibly-linked structural elements makes an NMR-based strategy particularly well suited for studying this system. A simulated annealing approach yielded a solution structure for PARP-1's N-terminal F1 and F2 zinc-finger domains bound to a DNA dumbbell harbouring a single-nucleotide gap, showing how F1F2 bends and twists DNA at an SSB, creating a conformation inaccessible to undamaged DNA. A combination of chemical shift fingerprinting, 15N relaxation experiments and mutational analysis showed how assembly of further domains creates a composite interface for interaction with the catalytic domain, acting as an allosteric switch. Overall, these results allowed us to propose a mechanistic model for PARP-1 recruitment and activation that may aid understanding of the mechanism of PARP inhibition (Eustermann et al., Mol. Cell, 60, 742-754). Now, we are using NMR to investigate the detailed nature of changes in the 360-residue CAT domain that result from the DNA-damage-dependent domain assembly process and which lead to catalytic activation.

SL5

Substrate and Inhibitor binding to the 216 kDa transmembrane enzyme complex Na+-NQR from V. cholerae

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The first enzyme complex of the respiratory chain of the pathogenic bacterium V. cholerae is the Na+-translocating NADH:quinone oxidoreductase (Na+-NQR) consisting of six subunits and at least six redox-active cofactors. Being structurally unrelated to its eukaryotic counterpart, complex I, Na+-NQR's function is of significant interest from the perspective of basic research but also for the development of novel antibiotics. The Na+-NQR utilizes the free energy liberated during oxidation of NADH with ubiquinone to pump sodium ions across the cytoplasmic membrane. We investigated the physiological function of the Na+-NQR in vivo by 23Na NMR spectroscopy. Using shift reagents, we determined the kinetics of sodium ion translocation by living V. cholerae cells, and we could show that a strain lacking subunit NqrB shows the slowest kinetics of sodium ion pumping.[1] STD NMR spectroscopy in combination with other methods revealed that the NqrA subunit (50 kDa) harbors the catalytic quinone binding site.[2]. Furthermore, STD NMR, SPR experiments, and Trp fluorescence quenching titrations indicate that NqrA can bind two quinone-type molecules. We were able to record interligand NOEs between ubiquinone-1 and the inhibitor DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) providing direct experimental evidence that NqrA simultaneously interacts with two quinones in an extended binding site.[3] Our results obtained with the isolated NqrA subunit are supported by enzyme inhibition assays and NMR interactions studies with the holo-Na+-NQR complex (216 kDa) in detergent micelles and lipid nanodiscs. The quinone site was located by single-site mutation studies and by chemical shift perturbation mapping using either perdeuterated 15N- or epsilon-13C-methionine-labeled NqrA. We then mapped our results onto the X-ray structures of the individual NqrA subunit and the holo Na+-NQR complex.[4, 5] The binding site is situated in vicinity to the NqrB subunit that donates the electrons for quinone reduction. Remarkably, the inhibitor, DBMIB, acts as a covalent modifier of NqrA as well as a reversibly binding ligand. Our findings shed light onto mechanistic aspects of the Na+-NQR. Furthermore, chemical structures that specifically block its extended quinone site could have useful antibiotic properties.

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SL6

Chaperone–client interactions: From basic principles to roles in health and disease.

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Molecular chaperones are essential for maintaining a functional proteome in the cells. Nevertheless, central functional aspects of chaperones are still not well understood at the atomic level, including how chaperones recognize their clients, and in which conformational states clients are bound. I will describe recent research efforts to understand such aspects, employing high-resolution NMR spectroscopy as the main method. Initial work on the periplasmic holdase Skp with bound outer membrane proteins provided the first atomic-level description of a natural full-length chaperone¬¬–client complex [1, 2]. Subsequent work combining NMR spectroscopy with single-molecule force spectroscopy showed how periplasmic chaperones shape individual client folding trajectories, funneling the client polypeptide towards the native structure [3]. Based on these initial functional studies we subsequently utilized our mechanistic insights to investigate the functional role of chaperones in Parkinson's disease [4]. Parkinson's is one of the most common neurodegenerative disorders, pathologically manifested by intracellular accumulation of aggregates of the intrinsically disordered protein α-Synuclein. Systematic investigations on an array of chaperones identified a general chaperone interaction motive at the α-Synuclein amino-terminus. The dominant role of chaperone interactions for cytosolic α-Synuclein was validated with in-cell mass-spectrometry and NMR spectroscopy and the functional basis for the effects of several

known post-translational modifications of α-Synuclein could thus be reconstituted in vitro. Our data reveal how molecular chaperones in a direct manner control the state and function of α-Synuclein in vivo and how the disturbance of these interactions facilitates the progress of pathologically relevant α-Synuclein-states ultimately leading to the aggregates observed within Lewy bodies.

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SL7

PreSMos (Pre-Structured Motifs) as mediators of IDP-target binding and aggregation

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Intrinsically disordered proteins (IDPs) are highly unorthodox proteins that do not form three-dimensional structures under physiological conditions. Because IDPs even without well-folded 3-D structures are still capable of performing important biological functions and furthermore are associated with fatal diseases such as cancers, neurodegenerative diseases and viral pandemics discovery of IDPs has destroyed the classical structure-function paradigm in protein science, 3-D structure = function. Pre-Structured Motifs (PreSMos) refer to transient local secondary structural elements detected by high-resolution NMR or by other atomic-resolution techniques in the target-unbound state of IDPs. During the last two decades PreSMos have been steadily acknowledged as the critical determinants for target binding in dozens of IDPs. Currently, the PreSMo concept provides the most convincing structural rationale explaining the IDP-target binding behavior at an atomic resolution. In addition, PreSMos of certain IDPs seem to be implicated in the oligomerization (IDP-IDP self-association) process that may contribute to formation of amyloid fibrils. Here we present a brief developmental history, common characteristics of PreSMos along with an updated list of PreSMos.

SL8

Deciphering the functional role of 'fuzziness' for IDPs in the nuclear pore complex

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Disordered domains of Phe-Gly nucleoporins (FG-Nups) use multiple short linear motifs to provide rapid and specific diffusion of transport factors and their cargoes through the central channel of the nuclear pore complex. How this multivalent interaction can provide selectivity and avoid high avidity complexation and long residence times was previously unknown, and referred to as the 'transport paradox'. Using NMR and other methods, we decipher how the spacing and thermodynamic interactions permit selectivity compatible with rapid transport rates . (1) NMR titrations of multiple constructs of FG-Nups with the transport factor NTF2 showed HSQC peak changes associated with the -FSFG- motif, while intervening residues ('spacers') between the repeats were unaffected. Kd values between the interacting and non-interacting ensembles for increased numbers and spacing of motifs show a local concentration effect with very modest avidity, and fast exchange. The origin of limited avidity, from entropy/enthalpy compensation, was directly measured by isothermal titration calorimetry. NMR titration observing [2H, 15N] NTF2 HSQC shifts showed a constant area of surface perturbation independent of motif number. The derived Kd values differ slightly from those from the observation of FG-Nups, consistent with multiple contact sites as suggested from previous simulations . (2) These observations are consistent with fast, multi-site, multivalent interaction, and can be fully simulated in a multistate model. The transport paradox is thus resolved, by recognizing that a weak single FG motif affinity and enthalpy-entropy compensation operative at high valency states prevent strong avidity, enabling the required rapid and reversible interactions for diffusion. This study expands our mechanistic understanding of "fuzzy" interaction involving IDPs where the loss of conformational entropy on interaction is a feature of their function.

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SL9

DEER and smFRET Distance Measurements as Applied to Intrinsically Disordered Proteins

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Intrinsically disordered proteins (IDPs) are fascinating biomolecules that lack well-defined structures in their free states under native-like conditions. Nevertheless, IDPs are abundant in eukaryotes and are involved in critical cellular functions, including transcription, translation, and cell cycle control. Experiments provide substantial evidence that free IDPs in cells do not adopt defined structures and can be viewed as ensembles of freely interconvertible dynamic structures. Despite significant conceptual shifts in how we view the relationships between structure, disorder, and function, experimental characterization of the configuration ensembles of IDPs remains a challenge. Here we report on a combination of spin-labeling Q-band (35 GHz) Double Electron-Electron Resonance (DEER) experiments and smFRET measurements to characterize two IDPs by attaching molecular probes to unique cysteine pairs. While neuronal SNARE protein SNAP-25 is a highly disordered protein in isolation, it folds into a stable α-helix bundle upon forming the SNARE complex with syntaxin and synaptobrevin. Results of smFRET and DEER distances and distance distributions are compared for disordered SNAP-25 and folded SNAP-25 within the SNARE complex. In the rigid SNARE complex the mean distances determined by smFRET (4.7 nm) and DEER (4.3 nm) experiments agree well once the difference in size of the labels and the lengths of the molecular tethers is taken into account. For the disordered SNAP-25 DEER experiments show two distance distributions, the main one centered around 4.2 nm and a minor component centered around 2.3 nm, while smFRET detects only one population with the mean distance of 4.3 nm. It is suggested that the two populations are not detected by sm TIRFM FRET, because of a fast interconversion of the two forms whereas such an interconversion is frozen out in low temperature DEER measurements. Recent measurements using FRET in Multiparameter Fluorescence Detection (MFD) mode confirmed ms interconversion in S25. Glutamate receptor CTD2 cytoplasmic domain (N2B) is predicted to be ID. DEER measurements using two different spin labels, MTSL and IAP report on essentially iden-

tical distance distributions revealing one conformation with long distances centered at 7.3 nm and another conformation characterized by broader distribution at shorter distances, consistent with the roughed conformational energy landscape. These measurements are in a relatively good agreement with sm-FRET data as regarding the mean distance or the width of distributions. Using TIRFM FRET, a unique stochastic switching between two distinct disordered conformations on a second timescale was detected in N2B. FRET in MFD mode on the N2B(121) showed a complex dynamic exchange among at least three states. In summary, Q-band DEER measurements in frozen samples provide information on configurations in IDP consistent with results obtained by FRET measurements at room temperature. Supported by NSF 1508607 to TIS.

SL10

Studying conformational changes of proteins using a small clickable Gd(III) tag

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Measuring nanometre range distances in proteins using Gd(III) spin labels by double electron-electron resonance (DEER, also called PELDOR) spectroscopy at high fields is a powerful and sensitive tool in structural biology. It can be used to detect protein structural variability and conformational changes. In order to measure Gd(III)–Gd(III) DEER distances in proteins, it is necessary to tag the protein site-selectively with two Gd(III) ions. The propargyl-DO3A-Gd(III) tag is small and overall uncharged. It can be ligated to genetically encoded p-azido-phenylalanine residues by copper-catalyzed click chemistry via a relatively short and rigid tether. We measured DEER distance distributions in a series of proteins that were designed by ancestral reconstruction to follow the structural and dynamic properties of a protein evolving between different functions. Specifically, we attempted to follow the evolution of the enzyme cyclohexadienyl dehydratase (CDT) from an amino-acid binding ancestor. We attached the propargyl-DO3A tag to three ancestrally reconstructed proteins (AncCDT-1, AncCDT-3 and AncCDT-5) and wild-type CDT from Pseudomonas aeruginosa. AncCDT-1 binds arginine, ornithine, histidine and lysine. The DEER distance measurements conducted with AncCDT-1 showed two different distance distributions, with maxima at about 3 and 4.5 nm, which can be attributed to an open and a closed conformation. The ratio between the two changed in the absence of a ligand, leading to an increased population of the open conformation. This is the first time that two distinct distance distributions have been deduced from a single Gd(III)–Gd(III) DEER measurement, with relative populations shifted by the presence of a ligand. Importantly, the distance distributions were sufficiently narrow to resolve them. Intra-domain distances measured for the protein did not change significantly between samples with and without bound ligand, confirming that the structural equilibrium detected by inter-domain Gd(III)–Gd(III) distance measurements arises from domain motions while conserving their structure. No specific ligand molecules have been identified for AncCDT-3 and AncCDT-5. Nonetheless, the inter-domain DEER distance measurements in these proteins showed a change in the inter-domain distance by 0.2 nm following denaturation and refolding of the proteins to remove potential ligand molecules endogenous to E. coli. For wild-type CDT, the DEER distance analysis yielded a broad distance distribution, which may be due to trimer formation as reported by the crystal structure, and it also changed in response to substrate binding. In conclusion, the DEER experiments suggest that all proteins in the evolutionary path from AncCDT-1 to wild-type CDT undergo similar hinge motions between open and closed conformations. As all these proteins contain cysteine residues, DEER distance measurements with the propargyl-DO3A-Gd(III) tag offer a unique tool to investigate their structural changes in response to the presence of ligand molecules.

SL11

New Gd(III) spin labels for DEER distance measurements in-vitro and in-cell

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Pulse electron-paramagnetic resonance, particularly double electron-electron resonance (DEER), via site-directed spin-labeling, has developed as a powerful technique for exploring protein structure in frozen solutions. However, a clear understanding of the structure and dynamics of proteins under the cellular environment where molecular crowding, post-translational modification, and interaction with other biomolecules can affect proteins' conformations, is of great importance in delineating their unique functions. In this study, we present three new Gd(III) chelating tags, 4PhSO2-PyMTA, DO3MA-3BrPy and DO3A-3BrPy, for Gd(III)-Gd(III) distance measurements, based on the use of a stable C-S conjugation to the protein. The reaction of phenylsulfonated pyridine derivatives and protein thiols features high reactivity towards cysteine and a stable, short and rigid tether, which is valuable for distance measurements in-cell. Here we report the results of DEER distance measurement at W-band (94.9 GHz) on mutants of the proteins ubiquitin and GB1 doubly labeled with PyMTA-Gd(III), DO3MA-Gd(III) or DO3A-Gd(III), both in vitro and in HeLa cells. We delivered the labelled ubiquitin mutants into HeLa cells via electroporation or hypotonic swelling, and incubated the cells for different times after protein delivery. High-quality DEER data could be obtained up to 20 h incubated after cell delivery under in-cell reductive conditions at cellular protein concentrations of $\sim$ 5-10 μM. The DEER results of proteins labelled by these three tags showed narrower distance distribution in vitro, while DO3MA-Gd(III) and DO3A-Gd(III) samples presented high stability in living cells with a reasonable redution of modulation depth. The DO3A-Gd(III) spin label also featured the highest sensitivity (larger modulation depth and better SNR) in-cell, demonstrating great potential for future study of protein conformational changes in cellular environment.

SL12

Mechanistic information on the multidrug ATP-Binding Cassette (ABC) transporter BmrA revealed by solid-state NMR

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ABC transporters can translocate a variety of molecules by coupling drug efflux with ATP hydrolysis. The ATP hydrolysis is performed by a nucleotide-binding domain (NBD) whereas the drug translocation is operated by a transmembrane domain (TMD). ABC transporters are found in all forms of life and they are involved in a number of drug resistances. Despite this, the underlying mechanism of ABC transporters is still unknown.

Using solid-state NMR and a model system, we confirm and highlight different proprieties on ABC transporters mechanism. We report on conformational differences identified between two states of the protein adopted during the drug-export cycle: the inward-facing (IF) and outward-facing (OF) states. The observation of chemical shift perturbations (CSPs), as well as the apparition of new signals highlight the important changes in flexibility and conformation between the two states. We suggest that the change in dynamics might be central for transmitting the relevant conformational changes to the part of the protein driving transport, concomitant of an engine which is turning an input shaft, but which fails to connect in a rigid manner, trough adequate gears, with the output shaft driving the pump. From an NMR point of view, the study of BmrA from Bacillus subtilis (120 kDa) reconstituted in its natural lipids is a challenge due to the large number of resonances and signal-to-noise-ratio limitations. A full sequence specific assignment is extremely difficult. Still, we describe assignment approaches using selective labelling strategies and paramagnetic relaxation enhancement which allow to analyse the different fingerprints of different mutants and states of BmrA.

SL13

15N-Detection: Harmonizing the sensitivity-resolution conundrum.

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Sensitivity and resolution have been the two opposing yet important traits in NMR spectroscopy of biomolecules. With the advent of cryogenically cooled probed and non-uniform sampling methods, the battle between sensitivity and resolution has to be revisited. 15N detection has been largely ignored in the past due to the inherently low sensitivity of the 15N nuclei, that stems from it low gyromagnetic ratio (γ), which is about 1/10th that of 1H. However, this low γ translates to slower relaxation rates for the 15N nuclei, which results in narrow lines. Here we leverage this slow relaxation property of 15N and present a set of experimental paradigms for the direct detection this narrow, slow relaxing 15N resonances with applications to IDPs, large structured proteins and proteins that can be only expressed in eukaryotic media where deuteration is not possible. Each of these experimental paradigms has their unique advantages and limitations, which will be discussed. We have designed a suite of 3D and 4D experiments for 15N detection that either detects the 15N-D (attached to deuterium) or the TROSY component of 15N-H. Application of these experiments to answer key biological questions will be presented. For example, we have recently used the 15N-detection methods to reveal one of the mechanisms by which the nuclear localization of the transcription factor NFAT is regulated. We demonstrated that upon phosphorylation of NFAT by kinase PKA, chaperone protein 14-3-3 binds the disordered region of NFAT, thus obscuring the nuclear localization signal and preventing nuclear localization.

SL14

Ultrasensitive beta-detected NMR to study interactions of metal ions with biomolecules

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Our project aims at studying the interaction of essential metal ions with different biomolecules using the ultrasensitive beta-NMR technique [1], which until now has been used only for nuclear structure and material science in solid samples. Because in β-NMR the resonances are observed as changes in beta-decay anisotropy of hyperpolarized nuclei, the approach is up to 10 orders of magnitude more sensitive than conventional NMR. Our experimental setup [2] is located at the CERN-ISOLDE facility, where over 1000 different radioactive nuclei can be produced. We use optical pumping with lasers to polarize isotopes of different metallic elements [2]. The decrease in anisotropic emission of beta radiation from such hyperpolarized nuclei is then used to detect the NMR response, leading to the extreme sensitivity of beta-NMR.

Metal isotopes which have been already used for beta-NMR studies in solid samples include 8,9,11Li, 11Be, 25-28Na, and 23,29,31Mg. First studies on 26Na in liquid samples were performed by our team in December 2017. Soon to be polarized are several isotopes of K, Ca, Rb, Cu and Zn isotopes [1]. Our first biological studies took place in May 2018, where we probed the interaction of Na cations with DNA G-quadruplexes, present for example in telomers [3]. At a later stage we plan to investigate the interaction of Cu and Zn with different proteins.

This contribution will include an introduction of the beta-NMR technique and a description of the experimental setup, and will concentrate on the most recent results concerning the Na-G-quadruplex interaction.

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SL15

Accurate Measurement of Residual Dipolar Couplings in Large RNAs by Variable Flip Angle NMR

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The application of NMR spectroscopy to the study of large, biologically relevant RNAs is complicated by a number of factors, including limited chemical shift dispersion, undesirable relaxation parameters and a relative lack of long-range distance constraints. Recent approaches using nucleotide-specific deuterium labeling schemes have mitigated these difficulties, enabling structural studies of biologically relevant RNAs of increasing size and complexity. Although local structure is well-determined using these methods, definition of global structural features, including relative orientations of independent helices, remains a challenge. Residual dipolar couplings, a potential source of orientation information, have not been obtainable for large RNAs due to poor sensitivity resulting from rapid heteronuclear signal decay.

We will report on a novel multiple quantum NMR method for RDC determination that employs flip angle variation rather than a coupling evolution period for discrimination of couplings. The accuracy of the method and its utility for establishing interhelical orientations are demonstrated for a 36-nucleotide RNA derived from the 5'-Leader of MMLV, for which comparative data could be obtained. Applied to a 78 kDa Rev response element from the HIV-1 virus, which has an effective rotational correlation time of ca. 160 ns, the method yields sensitivity gains of an order of magnitude or greater over existing approaches. Solution-state access to structural organization in RNAs of at least 230 nucleotides is now possible.

SL16

STAR WARS Mission: destroy the STAR protein Sam68 in the cancer galaxy

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Sam68 and T-STAR are members of the STAR family of proteins that directly link signal transduction with post-transcriptional gene regulation. Sam68 controls the alternative splicing of many oncogenic proteins, such as CD44, cyclin D1, Bcl-x, SRSF1, HPV-16, and its overexpression is associated with poor prognosis in various cancers (1). T-STAR is a tissue-specific paralog that regulates the alternative splicing of the Neurexin1-3 pre-mRNAs important for neuron function.

Using a combination of NMR, X-ray crystallography, and SAXS, we have unraveled the structural basis of dimerization and RNA recognition by Sam68 and T-STAR (2). These structures reveal an unexpected and unique mode of dimerization and RNA binding that demarcates them as a distinct subfamily of STAR proteins. We demonstrate that this unique protein dimerization interface is crucial to recognize specifically a (A/U)AAN>15-(A/U)AA bipartite RNA motif and increase target specificity. Finally, we show that this dimerization interface is essential for biological activity in splicing control, and propose that Sam68 and T-STAR could affect some alternative splicing events by looping out regions of the pre-mRNA.

Because Sam68 is overexpressed in many types of cancers, we aim at identifying or designing drugs that specifically inhibit its function. We have screened a library of 450,000 compounds using a high-throughput RNA binding assay and identified compounds that interfere with Sam68 RNA binding ability. Using NMR, we have confirmed that these compounds bind directly Sam68. In addition, we have designed stapled peptides that mimic the dimerization interface of Sam68. The ability of these compounds and peptides to inhibit Sam68 functions will be further evaluated in cell-based assays.

References: 1- Frisone et al, Biomed Res Int. 2015:528954 (2015) 2- Feracci et al, Nat. Commun. 7:10355 (2016)

SL17

NMR studies revealed structural basis for the inhibitory effects of ubistatins in the ubiquitin-mediated signaling pathways

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Ubiquitination is a critical protein post-translational modification involved in a variety of vital processes in eukaryotic cells. The discovery of ubistatins [1], small molecules that impair proteasomal degradation of proteins by directly binding to (poly)ubiquitin upstream of the proteasome, makes ubiquitin itself a potential therapeutic target. Although ubistatins have the potential for drug development and clinical applications, the lack of structural details of ubiquitin-ubistatin interactions has impeded their development. To address this deficiency, a panel of new ubistatin derivatives was synthesized and characterized using functional and NMR-based binding assays [2]. We found that the most active compounds contain strongly acidic groups. We then used NMR and small-angle neutron scattering (SANS) to determine the structures of ubiquitin complexes with ubistatin B and hemi-ubistatin B. These structures revealed direct interactions of ubistatins with ubiquitin's hydrophobic surface-patch and the basic/polar residues surrounding it, which were confirmed by site-directed mutagenesis. Our results show that ubistatin B binds ubiquitin and di-ubiquitin tighter than a high-affinity ubiquitin-receptor, the UBA domain from the proteasomal shuttle protein ubiquilin-1, and shows clear preference for ubiquitin chains linked via K48 over those linked via K11 or K63. The 15N relaxation and SANS data revealed unexpected binding stoichiometries and structural arrangements of ubiquitin or di-ubiquitins in those complexes. Furthermore, through binding to ubiquitin, ubistatin B shields ubiquitin conjugates from disassembly by a range of deubiquitinases, including the 26S proteasome.

Finally, we found that ubistatin B penetrates human cancer cells and perturbs the cellular ubiquitin landscape. These findings highlight versatile properties of ubistatins and have implications for their future development and use in targeting ubiquitin-mediated signaling pathways. Combined with the earlier observations that ubistatins can arrest the cell cycle, producing effects similar to proteasome inhibitors [1]), our structural data suggest that the ubiquitin signal is a plausible candidate for therapeutic intervention in the ubiquitin-proteasome pathway.

References [1] Verma, et al., Science 306, 117-120 (2004). [2] Nakasone et al., Structure 25, 1839-1855 (2017)

SL18

Molecular Chaperones in the Human Protein Disaggregation System

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Accumulation of protein aggregates is associated with a wide array of human disorders, including neurodegeneration, diabetes, and even cancer. Our bodies, however, possess molecular chaperones which can both prevent such protein misfolding and aggregation, and also return proteins trapped in aggregates to their active state - thereby restoring lost function.

Very little is known, though, of how these chaperones dissolve protein aggregates or the mechanisms which enable this process.

The human disaggregation system is comprised of Hsp70, the major system ATPase, two classes of Hsp40 (DnaJ) co-chaperones (class A and class B), and Hsp110 nucleotide exchange factor, however the functions and mechanisms of action of these players is largely unknown. Furthermore, while a collaboration between class A and class B DnaJ chaperones was shown to be strictly required for disaggregation, the nature of this interaction and its effect on protein disaggregation has remained a mystery.

Here we focus on the ubiquitous and conserved Hsp70 chaperone and investigate Hsp70 interaction with client proteins, using solution advanced NMR spectroscopy techniques as well as selective isotope-labeling methodologies. Our results establish that both bacterial and human Hsp70 chaperones interact with clients by selecting the unfolded state from a pre-existing array of interconverting structures, suggesting a conserved mode of client recognition among Hsp70s and highlighting the importance of molecular dynamics in this recognition event.

In addition, by focusing on the DnaJA-DnaJB interaction, we have detected a transient forming complex between class A and class B chaperones and have mapped the interfaces of this complex to the DnaJA and DnaJB C-terminal client-binding domains. We have also, interestingly, detected different binding modes for DnaJA1 and DnaJA2 co-chaperones to DnaJB1, providing a potential explanation for higher potency of DnaJA2-DnaJB1 complex in protein disaggregation.

SL19

Structural Characterization of Methyl-Labeled Glycoproteins Using 13C-Observation

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Glycoproteins constitute the majority of all mammalian proteins. Yet, they are underrepresented in NMR structural studies due to challenges in preparing samples with homogeneous glycosylation and appropriate distributions of isotope labels. In particular, perdeuteration is difficult. Using two domain constructs of cell-surface signaling molecules, such as Robo1 and CEACAM1, we will demonstrate that incorporation of select amino acids carrying 13C-labeled methyl groups in mammalian cell expression media, combined with direct observation of 13C in fully-protonated samples, allows data acquisition with sensitivity and resolution superior to commonly used proton-detected experiments. Collection of residual dipolar couplings (RDCs), paramagnetic relaxation enhancements (PREs) and NOEs using specialized pulse sequences provides adequate data for assignment of resonances when structural models for individual domains exist. The data likewise allow determination of domain-domain orientation and bound-ligand geometry for these proteins.

SL20

High-resolution 2D NMR spectroscopy of patient-derived glycoproteins at natural isotopic abundance

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α-1 antitrypsin (α1AT) is a 52 kDa serine protease inhibitor abundant in human plasma. Many naturally occurring single amino acid substitutions, the most common of which is the Z (E342K) mutation, promote misfolding and aggregation into long, ordered polymer chains at the site of synthesis in the liver endoplasmic reticulum. Accumulation of polymers leads to liver cirrhosis and the reduced protection of the lungs against proteolytic degradation predisposes individuals to early-onset emphysema. The molecular defect that promotes polymerisation remains incompletely understood: a crystal structure of the Z variant is essentially identical to that of wild-type (WT) α1AT and therefore fails to encapsulate this aberrant behaviour [1]. Clearly it is therefore the solution structure and dynamics of α1AT variants that is key to elucidating the molecular basis for the enhanced polymerisation. However, the propensity of Z α1AT to misfold also precludes its recombinant expression. Here we have therefore carried out 2D NMR spectroscopy at natural isotopic abundance, to investigate for the first time the effect of mutations and post-translational modifications on the solution structure of patient-derived α1AT. We will describe the development of NMR strategies for the characterisation of α1AT at natural isotopic abundance – choice of spin system, optimisation of sample conditions, sample quality control, and pulse sequence design – and present high-quality 1H-13C NMR correlation spectra that have been acquired in this manner for patient-derived α1AT variants. We will report the assignment of ILVMA methyl resonances in recombinant α1AT and the transfer of these assignments to spectra observed at natural abundance, and discuss the consequences of glycosylation and inter-patient glycan polydispersity. Lastly, we will describe the chemical shift perturbations induced by the Z mutation E342K, and discuss these changes in relation to dynamics observed in recombinant wild type α1AT.

1. Huang, X. et al. Molecular Mechanism of Z α1-Antitrypsin Deficiency. J. Biol. Chem. 291, 15674-15686 (2016).

SL21

Tools and Resources for NMR-Based Metabolomics

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We recently developed an algorithm called ALATIS (Atom Label Assignment Tool using InChI String) and associated software tools for the unique and reproducible naming of compounds and their constituent atoms (1). We are applying this approach to construct a federated database of validated information from a large number of small molecule databases. This repository, called BMOD (Bimolecular MOdeling Database) now includes over 350,000 entries including natural products, metabolites, and drug-like molecular fragments. We have used our GISSMO (Guided Ideographic Spin System Model Optimization) tools (2) to create refined spin system matrices for a subset (over 1,175) of these compounds, which have enabled spectral simulations of their 1H NMR spectra at a variety of spectrometer frequencies utilized in imaging and biomolecular NMR (40, 100, 200, 300, 400, 500, 600, 700, 750, 800, 900, 950, 1000, and 1300 MHz). For the purposes of NMR-based metabolite identification or ligand screening, these simulated spectra of metabolites and drug-like fragments are superior to experimental spectra of compounds in that they are free of noise, spectral artifacts, and signals from contaminants. These magnetic field dependent spectra and their associated peaklists are accessible on the searchable GISSMO website. The website also offers a tool for simulating solution spectra of compound mixtures for comparison with experimental 1H NMR spectra (3). We have implemented an extensible software pipeline called RUNER (for Robust and Unique Nomenclature for Enhanced Reproducibility) that streamlines and standardizes the computational calculation of force field parameters for small molecules (4). The pipeline combines a web service and a graphical user interface (GUI) to enable seamless modifications and verified maintenance of atom force field parameters. The GUI supports implementation of the widely used molecular modeling software package Xplor-NIH. Supported by NIH grants P41GM103399, R01GM109046, and P41GM111135. 1. Dashti H, Westler WM, Markley JL, Eghbalnia HR. Unique identifiers for small molecules enable rigorous labeling of their atoms. Scientific data. 2017;4:170073. doi: 10.1038/sdata.2017.73. PubMed PMID: 28534867; PMCID: PMC5441290. 2. Dashti H, Westler WM, Tonelli M, Wedell JR, Markley JL, Eghbalnia HR. Spin system modeling of NMR spectra for applications in metabolomics and Small molecule screening. Anal Chem. 2017. Epub 2017/10/24. doi: 10.1021/acs.analchem.7b02884. PubMed PMID: 29058410. 3. Dashti H, Wedell, JR, Westler WM, Markley JL, Eghbalnia, HR, Applications of parameterized NMR spin systems of small molecules, submitted. 4. Dashti H, Wedell, JR, Cornilescu G, Schwieters CD, Westler WM, Tonelli M, Aceti DJ, Amarasinghe, GK, Markley JL, Eghbalnia, HR, Robust nomenclature and software for enhanced reproducibility in molecular modeling, submitted.

SL22

NMR spectroscopy and electron microscopy identification of metabolic and ultrastructural changes to the kidney following ischemia-reperfusion injury

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Cellular, molecular, and ultrastructural nephron changes associated with ischemia reperfusion injury-induced acute kidney injury (IRI-AKI) are not completely understood. Here, a multidisciplinary study was used to identify nephron changes in a mouse model of IRI-AKI. In the model, the kidneys were exposed using a surgical procedure and the renal pedicles occluded for 30 minutes after which the clamp was removed to allow the kidneys to reperfuse. At 24 hours later, the mice were placed in metabolism cages to collect urine for metabolic profiling analysis. The mice were then sacrificed and the kidneys harvested for histological analysis. Histological analyses of the cortical tissue of the damaged kidneys indicated distended Bowman's glomerular spaces and proximal and distal tubules. Increased filtrate volume was observed in the nephrons and it was concluded that this was caused by reduced water reabsorption by severely damaged proximal tubule brush borders and blocked flow of filtrate into collecting tubules by mucoprotein casts in distal tubules. Immunohistochemistry (IHC) of the cortical tissue of the damaged kidneys revealed protein AKI biomarkers (neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1) and cystatin C) in proximal tubules and glomeruli but not in distal tubules. Nuclear magnetic resonance (NMR) spectroscopy revealed that several urinary metabolites increased in concentration following IRI-AKI, including valine, alanine, and lactate, while other metabolites such as trigonelline, succinate, 2-oxoisocaproate, and 1- methyl-nicotinamide decreased or were absent in urine following IRI-AKI due to altered kidney function or metabolism. An important observation from the NMR-based metabolic profiling was an increase in urinary glucose following IRI-AKI that was caused by reduced reabsorption of glomerular-filtered glucose by damaged proximal tubule brush borders. Scanning electron microscopy revealed flattening of podocytes and pedicles surrounding glomerular capillaries, and transmission electron microscopy (TEM) revealed effacement of podocyte pedicles, both consistent with increased hydrostatic pressure in nephrons following IRI-AKI. TEM revealed shortened proximal tubule microvilli in IRI-AKI kidneys with diminished lamina propia, consistent with the histological observations. TEM unexpectedly showed a dramatic loss of mitochondria in distal tubule epithelia of IRI kidneys and emergence of multivesicular bodies of endosomes indicating ongoing cellular death, despite a lack of detection of protein AKI biomarkers in the distal tubules based on the IHC studies. Collectively, the data define ultrastructural changes to nephrons and altered kidney function and kidney metabolism associated with IRI-AKI. A self-consistent model of IRI-AKI is presented that explains all of the observations obtained from the histological, immunohistochemical, NMR spectroscopy and scanning and transmission electron microscopy observations.

SL23

Inhibition of Amyloid Aβ Aggregation by High Pressures or Specific D-Enantiomeric Peptides

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Pressure can shift the polymer-monomer equilibrium of Aβ, increasing pressure first leads to a release of Aβ-monomers, surprisingly at pressures higher than 180 MPa repolymerization is induced [1]. By high pressure NMR spectroscopy, differences of partial molar volumes ΔV0 and compressibility factors $\Delta\beta$' of polymerization were determined at different temperatures. The D-enantiomeric peptides RD2 and RD2D3 bind to monomeric Aβ with affinities substantially higher than those determined for fibril formation. By reducing the Aβ concentration below the critical concentration for polymerization they inhibit the formation of toxic oligomers. Chemical shift perturbation allows the identification of the binding sites. The D-peptides are candidates for drugs preventing Alzheimer's disease. We show that RD2D3 has a positive effect on the cognitive behaviour of transgenic (APPSwDI) mice prone to Alzheimer's disease. The heterodimer complexes have a smaller Stokes radius than Aβ alone indicating the recognition of a more compact conformation of Aβ identified by high pressure NMR before.

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SL24

NMR Applications for Biologics Discovery & Development

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Monoclonal antibodies (mAbs) and other therapeutic proteins are increasingly important drug discovery modalities. The high affinity, high specificity, long half-life and relatively low rates of adverse effects make mAbs attractive choices to bind to and modulate the activity of accessible targets. Critical antibody attributes include target-dependent factors such as affinity & epitope and target-independent factors such as good protein stability and low aggregation propensity. We routinely use NMR-data to differentiate the mechanism-of-action for a wide array of drug discovery molecules that range in size from 100 Da small molecule fragments to 150 kDa mAbs. The focus for this presentation is to highlight progress made toward differentiating therapeutic proteins by their solution behavior. We show that translational and rotational diffusion NMR measurements and structural fingerprinting are well-suited to capture structural changes and characterize intrinsic weak protein self-interactions that can lead to loss of function, aggregation, higher-order structure formation all of which can factor into developability decisions. Next, we show that in situ NMR measurements of formulated proteins can provide insights into complicated protein-ligand and protein-protein interactions that occur, especially at high (>160 mg/mg) protein concentrations. Finally, we use NMR and other biophysical techniques to study the solution behavior of novel, multivalent proteins. These include "next-generation" immune-modulators that aspire to improve the outcomes of clinically validated PD-1 based therapies.

SL25

Solid State NMR Spectroscopy at 1500 MHz in a 35.2 T Hybrid Magnet

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The Series Connected Hybrid (SCH) 35.2 T magnet has a 14 T superconducting outsert that is connected in series with a 21 T resistive insert, such that the large inductance of the outsert minimizes the multiples of 60 Hz noise that prohibits DC powered magnets from being used for NMR except for wideline condensed matter physics NMR spectroscopy. This 12.5 MWatt powered magnet is currently scheduled to operate 30 hours a week, most of the time for solid state NMR, but a portion of the time for various condensed matter physics experiments and in the near future there will be a THz ESR spectrometer available for users and also MRI instrumentation is under construction. Currently, we are using a Bruker Avance Neo console having an enhanced lock unit that uses a doped external 7Li lock signal with three probes: 1) an oriented sample (OS) double resonance probe; a quadrupolar single resonance 3.2 mm MAS probe; and a triple resonance spin ½ 2.0 mm MAS probe. A triple resonance 1H detection fast MAS probe is under development.

Initial stability of the SCH was measured at 0.2 ppm over a few minutes, and 0.5 ppm over a few hours. This capability is permitting unique spectroscopy for quadrupolar nuclei, and good preliminary spectra for spin ½ spectroscopy. The path forward for enhancing the spectroscopy is through Prof. Schiano's decade long research effort to develop a Cascade Compensation system for the SCH. This technology estimates fast and slow field fluctuations using an inductive pickup coil for fast fluctuations, and a field estimator based on NMR signals for slow fluctuations. Together, they form a two-loop cascade that reduces field fluctuations in the SCH more significantly than the Bruker lock system on short time scales. We hope to extend this timescale and have this technology implemented later this year providing better than a tenth of a ppm for signal averaging times. Additional probes and probe capabilities are under development including a 110 kHz fast MAS probe.

The majority of the presentation will focus on the science achieved in biological systems from both spin ½ and 17O spectroscopy. The dramatic gains in sensitivity and resolution with 17O spectra suggest a broad range of applications, including ion channels, and in these early stages illustrate novel chemistry that has not been accessible with superconducting magnetic fields and spin ½ nuclei. A variety of spin ½ spectra of proteins will also be shown from the oriented sample probe and triple resonance MAS probe.

SL26

Determine the Supramolecular Architecture of Pathogenic Fungal Cell Walls Using DNP Solid-State NMR

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Life-threatening invasive fungal infections affect more than two million patients worldwide. Its high mortality rate (20-95%) and the limited number and inefficacy of antifungals necessitate the development of new agents with novel mechanisms and targets. The fungal cell wall is a promising target as it contains polysaccharides absent in humans, however, its molecular structure remains elusive due to the difficulty in characterizing these complex biomaterials. By combining the resolution improvement from spectral editing and the sensitivity enhancement from MAS-DNP, we have revealed the cell wall architecture of a major pathogenic fungus Aspergillus fumigatus. In total, 65 intermolecular restraints have been obtained, which, assisted by the heterogeneous profile of molecular mobility and hydration, revealed a novel structure of fungal cell walls: chitin and α-1,3-glucan build a hydrophobic scaffold that is surrounded by a hydrated matrix of diversely linked β-glucans and capped by a dynamic layer of glycoproteins and α-1,3-glucan. The two-domain distribution of α-1,3-glucans signifies the dual functions of this molecule: contributing to cell wall rigidity and fungal virulence. This study provides the first high-resolution structural model of fungal cell walls and serves as the basis for assessing drug response to promote the development of wall-targeted antifungals.

SL27

Structural Basis of the Neurotoxicity of α-Synuclein Oligomers

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The aggregation of α-synuclein, a protein involved in neurotransmitter release at presynaptic terminals, is associated with a range of highly debilitating neurodegenerative conditions including Parkinson's disease. Fibrillar aggregates of α-synuclein are the major histopathological hallmarks of these disorders, although small oligomeric assemblies are believed to play a crucial role in neuronal impairment. Using biomolecular ssNMR in conjunction with FRET, FCS and solution NMR, we characterised the structural properties of toxic α-synuclein oligomers (Fusco et al, Science 2017, 358:1440-3). The study enabled the identification of the structural elements enabling toxic α-synuclein oligomers to perturb biological membranes and disrupt cellular function. Using neuronal cells and in vivo models of Parkinson's disease, we provide evidence that biomolecular NMR is a powerful technique to drive the rational design against the neurotoxicity of elusive α-synuclein oligomers retaining a significant level of structural disorder and conformational heterogeneity.

SL28

DNP-assisted solid state NMR spectroscopy for structure determination in biological environments

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Structural investigations of biomolecules are typically confined to in vitro systems under extremely limited conditions. These investigations yield invaluable insights, but such experiments cannot capture important structural features imposed by cellular environments. We recently demonstrated that structural studies of proteins in their native contexts are not only possible using state-of-the-art sensitivity-enhanced (dynamic nuclear polarization, DNP) solid-state nuclear magnetic resonance (NMR) techniques, but also that the native context can have a dramatic influence on protein structure. We aim to visualize such structural changes with atomic level resolution and understand how genetic background can influence protein folding. To do so, we have developed new biotechnology for sample preparation for DNP NMR to investigate the structure of a protein containing both an environmentally sensitive folding pathway and an intrinsically disordered region. We have 1) optimized experimental sensitivity of DNP NMR through modifications of the sample composition, 2) optimized experimental specificity of complex biological samples through careful use of isotopically enriched and depleted carbon, nitrogen and proton sources and 3) improved spectral resolution of DNP NMR through the reduction of chemical shift degeneracy via segmental and amino acid-specific isotopic labeling. Using a self-polymerizing yeast prion protein, Sup35, we are determining how native and mutant cellular environments affect amyloid structure for proteins at endogenous levels in biological contexts with atomic-level precision.

SL29

Structural Basis of Membrane Protein Translocation Through the Mitochondrial Intermembrane Space by Small, Hexameric TIM Chaperones

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Mitochondria are ubiquitous eukaryotic cell organelles that are surrounded by two membranes. 99% of all mitochondrial proteins are not synthesized in the mitochondrion, but in the cytosol and therefore need to be transported to their final location of action in the outer or inner mitochondrial membrane, the intermembrane space or the mitochondrial matrix. Mitochondria therefore possess different protein translocases that shuttle mitochondrial protein precursors to their particular destinations, depending on specific import signals. In this context, the transport of membrane proteins is particularly critical, due to their high propensity to precipitate in aqueous environments. In the cytosol, Hsp70 and Hsp90 shuttle these membrane protein precursors from the ribosome to the outer mitochondrial membrane. After translocation through the TOM complex, the precursors are taken over by ATP-independent complexes of small Tim proteins in the mitochondrial intermembrane space. Two hexameric complexes comprising Tim9/10 or Tim8/13 have been identified in yeast. Chaperoned by these Tim complexes, the precursors are then guided to their respective insertion machinery in the outer or inner mitochondrial membrane. We have developed an experimental approach for the reproducible formation of complexes between the aggregation-prone membrane protein precursors and the recombinant, hexameric TIM9/10 chaperone. An integrated structural biology approach using complementary techniques like NMR, SAXS, and data-guided molecular dynamics was used in order to identify structural and dynamical properties of chaperone-preprotein complexes as well as the preprotein binding site on the chaperone. TIM9/10-preprotein stoichiometry was determined from sedimentation and translational diffusion coefficients determined by analytical ultracentrifugation and DOSY experiments. All together, our findings decipher the molecular mechanism of how the small TIM9/10 hexameric complexes transport both α-helical and β-barrel proteins across the mitochondrial intermembrane space. Multiple clamp-like binding sites hold the mitochondrial membrane proteins in a translocation-competent elongated form, thus mimicking characteristics of co-translational membrane insertion. The bound preprotein undergoes conformational dynamics within the binding cleft of the chaperone, pointing to a multitude of dynamic local binding events. Our findings were further corroborated by in vivo yeast survival and transport assays with Tim9 or Tim10 proteins carrying mutations in the chaperone-preprotein binding sites.

SL30

Getting in the Groove: Chaperone-assisted peptide exchange dynamics in the Major Histocompatibility Complex

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Molecular chaperones TAPBPR (TAP-binding protein related) and tapasin associate with class-I major histocompatibility complex (MHC-I) molecules to promote optimization (editing) of peptide cargo. Here, we use methyl-based, solution NMR to investigate the molecular mechanism of peptide exchange performed by the 90 kDa chaperone protein complex. Our NMR data reveal TAPBPR-induced conformational changes on conserved MHC-I surfaces, consistent with our independently determined X-ray structure of the peptide-deficient complex. Conformational dynamics present in the empty MHC-I are stabilized by TAPBPR in a peptide-deficient complex, and become progressively dampened with increasing peptide occupancy. Incoming peptides are recognized by the chaperoned groove according to the global stability of the final pMHC-I product, and anneal in a native-like conformation. Our results demonstrate an inverse relationship between MHC-I occupancy by peptide and the affinity of TAPBPR for such pMHC-I molecules, where the lifetime of transiently bound peptides controls the dynamic regulation of a conformational switch, located near the TAPBPR binding site, which triggers TAPBPR release. Lastly, we discuss the role of protein dynamics in shaping chaperone specificity towards different human and murine class-I MHC alleles. In more recent work, we are performing an integrative structural modeling approach combining NMR and cryoEM data with computational modeling algorithms to understand the mechanism of peptide editing for a human Class-I system.

SL31

De novo structure prediction of biomolecules using solvent-accessibility data

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Since the late 90s, macromolecular modeling software packages, such as Rosetta, have been continuously developed and applied for protein de novo structure prediction. Using the protein primary sequence and corresponding short structural fragments from crystal structures deposited to the PDB, protein structure predictions for small proteins less than 100 amino acids can be achieved with reasonable accuracy. The performance of Rosetta has been improved continuously, for example by including experimental NMR-derived data, and bioinformatics-derived distance restraints based on evolutionary couplings. However, lack of sequence homology and the need for extensive NMR-based experimental data still limits the general applicability of this approach. Here we will present the applicability of NMR-derived surface accessibility data for protein de novo structure prediction in the framework of the de novo structure prediction package Rosetta. Surface-accessibility data is derived from paramagnetic relaxation enhancements (PRE) obtained from inert and soluble paramagnetic probes – termed solvent PRE (sPRE) - and provide detailed quantitative information about the solvent-accessibility of NMR-active nuclei. We show that high-quality sPRE data can be obtained in a straightforward manner without modification of the protein. sPRE data improves conformational sampling and scoring of CS-Rosetta, subsequently provides more accurate and better converged structural models, and thereby effectively shifts the size limitations of CS-Rosetta. We show that sPREs provide a new class of restraints that are easily accessible and applicable to proteins. Our observation that a restricted set of sPRE data is sufficient to improve structural quality indicates that this class of restraints will be particularly powerful for de novo structure prediction of larger proteins where complete chemical shift assignments are difficult to obtain. With this respect sPRE data can be used in combination with (sparse) restraints from conventional approaches and offer several benefits over conventional NMR-based approaches Our approach is open to complementary types of surface accessibility data such as for example bioinformatics and mass spectrometry (cross-linking, radical-mediated protein footprinting) data and will thereby allow integrating different techniques in one program.

SL32

New computational tools to study Intrinsically Disordered Proteins

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Intrinsically disordered proteins (IDPs) are a hitherto less recognized and less studied class of the proteome. In recent years it has become clear that protein intrinsic disorder is both highly prevalent and functionally relevant. In addition, many amyloid diseases are critically related to protein disorder. However, the structural investigation of IDPs is greatly hampered by their resistance to crystallization (for X-ray) and repetitive sequence and highly averaged spectroscopic signatures (NMR). It is therefore imperative that new and improved methods are developed that allow us to (1) efficiently infer IDP structure based on easily accessible information, such as chemical shifts [1]; (2) predict the electrostatics in IDPs that are crucial for predicting their interactions and other behavior [2]; (3) more accurately predict structure from sequence by guiding algorithm development by better validated data [3].

[1] POTENCI: prediction of temperature, neighbor and pH-corrected chemical shifts for intrinsically disordered proteins. Nielsen JT, Mulder FAA. J Biomol NMR. 2018;70(3):141-165. [2] pepKalc - scalable and comprehensive calculation of electrostatic interactions in random coil polypeptides. Tamiola K, Scheek RM, van der Meulen P, Mulder FAA. Bioinformatics. 2018. doi: 10.1093/bioinformatics/bty033. [3] Improved accuracy protein order/disorder prediction guided by NMR data. Nielsen JT, Dass R, Mulder FAA, 2018, in preparation

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SL33

Redox control of the human iron-sulfur repair protein MitoNEET activity via its iron-sulfur cluster.

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Human mitoNEET (mNT), the first identified Fe-S protein of the mammalian outer mitochondrial membrane (OMM), is composed of a 32 aa membrane anchoring N-terminus and a C-terminal cytosol-exposed folded domain. This domain dimerizes and each monomer binds one [2Fe-2S] cluster through three cysteines and one histidine. The lability of the cluster greatly depends on its redox state and on pH. Recently (1), we have proposed that mNT plays a specific role in Fe-S cluster repair of the cytosolic iron regulatory protein-1 (IRP-1), a key regulator of cellular iron homeostasis in mammalian cells. Interestingly, mNT is able to transfer its cluster in vitro to human IRP-1 but also to (cyano)bacterial apoferredoxin.

Here (2), we deciphered the mechanism by which mNT triggers in vitro its Fe-S repair capacity using complementary approaches including NMR, UV-visible, Mössbauer and Resonance Raman spectroscopies. We have determined the respective roles of the redox state of the mNT cluster and the presence of dioxygen both in cluster transfer and protein stability. We show that only the oxidized holo-mNT cluster triggers cluster transfer to a generic acceptor protein and that dioxygen has no effect on the cluster transfer reaction. In the absence of apo-acceptors, a large fraction of the oxidized holo-mNT form is converted back to reduced holo-mNT under low oxygen tension. By contrast, reduced holo-mNT, which holds a [2Fe-2S]+ with a global protein fold similar to oxidized holo-mNT, is resistant in losing its cluster or in transferring it. Our findings thus demonstrate that mNT uses an iron-based redox switch mechanism to regulate the transfer of its cluster. The oxidized state is the "active state" which initiates Fe-S transfer independently of dioxygen, whereas the reduced state is a "dormant form". We propose that the redox-sensing function of mNT is a key component of the cellular adaptive response to help stress-sensitive Fe-S proteins recover from oxidative injury.

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SL34

Disparate effects of adjacent mutations in the ABC-ATPase Rad50 D-loop

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Our genomic DNA is constantly experiencing stresses that can induce double-strand breaks (DSBs), which if not properly repaired can lead to cell death or cancer. Mre11-Rad50-Nbs1 (MRN) is a conserved, essential protein complex that initiates DNA DSB repair by preparing the damaged ends for repair, tethering the broken strands, and recruiting necessary downstream effectors to the site of the DNA DSB. Rad50 is a member of the ATP Binding Cassette (ABC) ATPase family; however, how ATP binding, ATP hydrolysis, and dimerization are related to Mre11 activities remains enigmatic. A recent screen of a combination chemotherapy revealed a curative outlier in a colorectal cancer tissue, which contained a mutation (L1240F) to the Rad50 D-loop - a motif that has been hypothesized to be critical for ATP hydrolysis. A mutation at the adjacent and conserved aspartate residue (D1241N) has been observed in breast cancer cells as well. We performed biochemical and structural studies on these two Rad50 D-loop mutations to elucidate the importance of this motif. 2D-methyl NMR correlation spectra of U-2H,12C,15N; Iled1-13CH3, Leud/Valg-[12CD3/13CH3], Mete-13CH3-labeled P. furiosus Rad50 nucleotide binding domain in complex with unlabeled Mre11 show chemical shift perturbations (CSPs) upon D-loop mutation. The CSPs arising from L828F (human L1240F) are wide ranging and directly linked to the Rad50 allosteric pathway, which connects the Walker A domain to the hinge motif and is correlated to Rad50 activities and fast timescale side chain methyl group dynamics. Interestingly, the mutation to the conserved aspartate in the D-loop, D829N (human D1241N), does not affect the Rad50 allosteric network. Moreover, both D-loop mutations show differences in ATP binding, hydrolysis, and ATP-induced dimerization relative to wildtype MR. Our studies reveal disparate mechanisms within adjacent D-loop residues L828F and D829N, which could lead to a further understanding of DNA DSB repair, the role of the Rad50 allosteric network in MR function, and a structural motif that could be an additional drug target for cancer treatments.

SL35

Probing allosteric structural changes of neurotensin receptor by solution-state NMR

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A crucial aspect of GPCR-mediated signaling are allosteric structural changes occurring upon stimulation by agonists and leading to downstream activation of associated G-proteins. However, these changes cannot be captured by crystallography without the use of additional tools such as nanobodies that stabilize an active-like state. Here, we use solution-state NMR spectroscopy to monitor structural changes within neutrotensin receptor induced by agonist and antagonists and a heterotrimeric inhibitory G-protein. We use methyl probes in Ile, Leu, Val and Ala residues, as well as backbone amides to monitor the global effects of GPCR activation. To achieve high-level and cutting-edge isotope labeling, we use an optimized neurotensin receptor that can be produced in E. coli in high yields. Our data provide high-resolution insights into the allosteric communication between the primary ligand and the G-protein binding sites, as well as novel information on crucial residues in the transmembrane helical part that are mediating this allosteric switching process. In addition, mutagenesis of a crucial residue in the so-called 'ionic-lock' motif located at the G-protein binding site corroborates a tight connection to the ligand binding site located at the opposite side of the protein. This study provides a detailed NMR picture of GPCR activation governed by structural and dynamical changes and thus will be helpful to better understand the correlation between the structural state of a GPCR and the corresponding signaling properties.

SL36

Role of conformational dynamics on protein kinase A function and dysfunction

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Eukaryotic protein kinases (EPKs) are phosphoryl transferase that mediate several signaling events and constitute major pharmaceutical targets. cAMP-dependent protein kinase A is a prototypical kinase of paramount biological importance as it is involved in a myriad of cellular processes. Using nuclear magnetic resonance (NMR) spectroscopy, we probed the enzyme's intramolecular allosteric network along the catalytic cycle. We discovered that kinase A conformational motions are highly organized and correlated during turnover. Fast dynamics in the ps-ns time scale are directly linked to the conformational entropy of binding, revealing the mechanisms for positive and negative allosteric cooperativity that drive both substrate binding and product release. Slow dynamics in the μs-ms time scale are responsible for the conformational transitions from catalytically incompetent to competent states. Disruption of these dynamics leads to dysfunctional signaling and disease. Since the C-subunit of protein kinase A is highly conserved within the kinase family, the present study offers unprecedented mechanistic insights into intramolecular signaling for designing novel kinase activators or inhibitors.

SL37

Prion-like Propagation and Structural Conversion of Alzheimer's Amyloid-β: Solid-state NMR Studies

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This work involves two separate topics on structures, kinetics, and functions of amyloid-β using solid-state NMR (SSNMR). Misfolded fibrillar aggregates of Aβ are a primary component of senile plaque, a hallmark of Alzheimer's disease (AD). Increasing evidence suggests that formation and propagation of misfolded aggregates of 42-residue Aβ42, rather than the more abundant 40-residue Aβ40, provokes the Alzheimer's cascade. Our group recently presented the first detailed atomic model of Aβ42 amyloid fibril based on SSNMR data.[1] The result revealed a unique structure that was not previously identified for Aβ40 fibril. Based on the results and additional SSNMR data, we first discuss how amyloid fibril structures affect "prion-like" propagation across different Aβ isoforms, including WT Aβ40 and E22G pathogenic mutant of Aβ40.[2] The results indicate drastic effects of minor sequence differences in the cross-propagation properties for the Aβ isoforms.

Secondly, we present our ongoing efforts to analyze a structural conversion from amyloid oligomer to fibril for Aβ42. Oligomeric amyloids of Aβ42 are believed to play important roles in AD. In this study, we isolated metastable oligomers of Aβ42 termed "SPA". 13C chemical-shift comparison and immunological analysis using a conformation specific antibody suggest that SPA has a similar conformation with amylospherioid (ASPD), toxic spherical assembly of Aβ that was identified from brains affected by AD.[3] The results provide insight into amyloid misfolding of Aβ42 in Alzheimer's disease.

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SL38

Structural basis for the interaction between a peptidyl carrier protein and condensation domain in the enacyloxin hybrid PKS-NRPS

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Modular polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) are giant bacterial multi-enzymes (1-3 MDa) which biosynthesize a wide range of structurally complex bioactive natural products including antibacterials, antifungals, anticancer and anti-parasitic agents, immunosuppressants and insecticides. Their modular nature makes them suitable as bioengineering scaffolds to produce new libraries of drugs and agrochemicals. However, in order for the engineering to be effective knowledge of factors responsible for control of the biosynthesis, such as protein-protein interactions, is indispensable. Here we consider protein-protein interactions implicated in the chain termination in the biosynthesis of enacyloxin; an antibiotic that is active against multidrug-resistant Acinetobacter baumannii, which is the number one on the list of WHO priority pathogens. Combining solution NMR, solid-state NMR, mass spectrometry based carbene footprinting, X-ray crystallography and molecular dynamics (MD) we are able to elucidate the interactions between a peptidyl carrier protein (Bamb_5917 PCP, 11 kDa) and a stand-alone condensation domain (Bamb_5915 C, 56 kDa). Solution NMR and carbene footprinting studies show a complex dynamic interaction between Bamb_5917 PCP and Bamb_5915 C that involve the C-terminal intrinsically disordered docking domain of Bamb_5917 PCP and β-hairpin docking domain of Bamb_5915 C and as well as globular segments of the proteins. 15N CEST of Bamb_5917 PCP in presence of 10% of Bamb_5915 C provides clues about changes in conformation of the C-terminal intrinsically disorded region upon binding. Overall all the data indicate large conformational changes of both Bamb_5917 PCP and Bamb_5915 C upon binding, which are consistent with correlated motions observed in MD simulations. Fast (60-100 kHz) magic angle spinning solid-state NMR of sedimented Bamb_5917 PCP:Bamb_5915 C complex is used to obtain an atomic resolution view of Bamb_5917 PCP within the 70 kDa complex. Docking calculations in HADDOCK provide insights into the interactions between the two proteins and biochemical assays demonstrate that the condensation reaction critically depends on the presence of the docking domains. Our results suggest an intriguing general allosteric regulation mechanism responsible for directionality of a condensation reaction and provide a basis for a synthetic biology approach to create hybrid PKS/NRPSs systems to produce new antibiotics.

SL39

Improved 13C-detection enables studies of IDPs in physiological conditions and concentrations

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Disordered proteins have essential functions in cell signalling. They act mainly through interactions that are most often modulated by post-translational modifications (PTMs) (1,2). We have reported novel, multiple phosphorylation mechanisms on oncogenic proteins established by "NMR-friendly" kinases, which permit NMR monitoring at 298K and pH<7 (3,4). However, most of PTMs occur in cells at micromolar concentrations, pH>7 and 310K; all these parameters affect drastically the specificity and the efficiency of the modifying enzymes. These conditions make the standard 1H/15N NMR experiments useless in the case of most IDPs because of very fast water-amide proton exchange. 13C-direct detection circumvents this drawback, but its sensitivity is often limiting. Here, we will present our improvements of 2D Ca/CO experiments to enable PTMs monitoring in physiological concentrations (<50 uM) and conditions (pH>7.5 and T>303K). We will show results obtained on important transcription factors and oncogenic proteins. We also show that recording NMR spectra in these physiological conditions allows to report new behaviors of lipid binding proteins such as alpha-synuclein, which opens new avenues for in-cell measurements.

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SL40

Insights into T cell regulation from NMR-based monitoring of WIP conformational ensembles

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WASP-Interacting Protein (WIP) is a multifunctional key participant in mediating actin-related cytoskeletal changes in human T cells. WIP is also an intrinsically disordered protein (IDP), lacking any significant secondary or tertiary structure across its 503 residues, and thus defies the ordinarily reliable structure-function paradigm. Our research focuses on how interactions between this 'hub' multi-tasker and its various structured binding partners control T cell destiny. In doing so we exploit the fact that solution NMR is now armed with IDP-optimized methodologies and well-suited for following and characterizing conformational ensembles that are typical of IDP behavior. Three such critical protein-protein contacts are of particular interest, the WIP N-terminal domain (with actin), a proline-rich central segment (with cortactin) and the C-terminal domain (with Wiskott-Aldrich syndrome protein, WASP). The first two are of intermediate binding energy (KD 50-3000 nM) and transiently modulate WIP interactions with the actin polymerization machinery. In contrast, the latter forms a tight complex with WASP and inhibits both its activity and eventual degradation in a phosphorylation-dependent manner, explaining why the hereditary Wiskott-Aldrich syndrome immunodeficiency results from WASP mutants unable to bind WIP. Having acquired extensive NMR data for these three interaction pairs – chemical shifts, temperature shifts, residual dipolar couplings, and relaxation rates, we can draw direct conclusions as to how WIP exerts its biological influence through disorder-to-order transitions. We established that transient structure in free WIP(N) and WIP(C) echoes their bound conformations, uncovering novel binding epitopes in the process, whereas WIP(mid) appears to exhibit a 'pre-cast' epitope for binding. We also utilized a novel J-modulated sequence to follow subtle ensemble shifts induced by environmental factors, such as temperature, denaturant or crowding agents, providing a new view of IDP biophysics in the cellular environment. We further investigated the largest conformational change, experienced by WIPC upon binding to WASP, by determining the contribution of various WIP epitopes to complex affinity, and eventually the structure of the WIP-WASP complex. This has led us to an unexpected structural explanation for phosphorylation-induced dissociation of this complex that may explain how this phospho-switch controls WASP degradation. Taken together our results provide a comprehensive map of WIP structure and dynamics and how these affect its interaction with T cell binding partners, and highlight the great impact of high-resolution NMR studies upon the field of biologically active unstructured proteins.

SL41

Investigating the dynamic landscape of G-protein-coupled receptors (GPCRs)

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G-protein-coupled receptors (GPCRs) are a large family of membrane embedded proteins that activate a multitude of signalling pathways triggering various cellular responses in health and disease. The last decade resulted in a surge of receptor structures mostly through crystallography, helping to shape a static view of these receptors in mostly inactive but also active states, including in complex with the cytoplasmic binding partners heterotrimeric G-proteins, β-arrestins and their mimetics. Despite this wealth of static information, key questions related to GPCR function and mechanism of action persist due to the intrinsically dynamic nature of these receptors.

We use 1H,13C NMR spectroscopy to investigate ligand based activation and basal activity of the β1 adrenergic receptor (β1AR).1,2 In our study we find evidence for the existence of novel active receptor states and a level of receptor plasticity in ternary complexes, which can be related to the pharmacological concept of partial agonism. Our NMR data connects receptor activity to the presence of μs-to-ms timescale dynamics and shows β1AR as a highly adaptable molecular signalling entity. Further investigations related to the influence of the membrane protein environment in receptor activation are presented.

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SL42

A single NaK channel conformation is not enough for non-selective ion conduction

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NaK and other non-selective channels are able to conduct both sodium (Na+) and potassium (K+) with equally high efficiency. In contrast to previous crystallographic results, we show that the selectivity filter (SF) of NaK in native-like lipid membranes adopts two distinct conformations that are stabilized by either Na+ or K+ ions. The atomic differences of these conformations are resolved by solid-state NMR (ssNMR) spectroscopy and molecular dynamics (MD) simulations. Besides the canonical K+ permeation pathway, we identify a side entry ion-conduction pathway for Na+ permeation unique to NaK. Moreover, under otherwise identical conditions ssNMR spectra of the K+ selective NaK mutant (NaK2K) reveal only a single conformational state. Therefore, we propose that structural plasticity within the SF and the selection of these conformations by different ions are key molecular determinants for highly efficient conduction of different ions in non-selective cation channels.

Shi et al., Nature Communications, 2018

SL43

Dynamic structure guided optimization of ligand using forbidden coherence transfer method

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Thermodynamic property of target protein-ligand interactions is a critical factor to define its binding specificity. Improved binding specificity can be achieved by introducing multiple spatially defined interactions, such as hydrogen bonds, aromatic-aromatic, and aromatic-methyl interactions, to the ligand-receptor interface. The introduction of these interactions results in restricted local dynamics and improved surface complementarity of the ligand in the bound state. Here, we present an NMR strategy to experimentally evaluate the local dynamics and the surface complementarity, using a single set of experiment based on forbidden coherence transfer (FCT). Using the interaction between a ligand, a myocyte-enhancer factor 2A (MEF2A) docking peptide, and a receptor, p38α, the FCT analyses successfully identified the site that shows a significant amplitude of motion and insufficient surface complementarity in the binding interface. More specifically, Val7 that is buried in the interface in the MEF2A-p38α complex showed the methyl axis order parameter smaller than 0.5 and less surface complementarity, as compared to the other buried residues. Asubstitution for Ile with a larger moiety at position 7 significantly improved the affinity and the enthalpic contribution of the interaction. The strategy could be further extended to the weak-affinity ligands under free-bound exchange. Furthermore, the dynamics of a small compounds in receptor-bound states was also quantitatively measured using the 19F-detected FCT strategy (CF3-FCT). By applying this CF3-FCT analysis to a model interaction system consisting of a CF_3-containing ligand, AST-487, and a receptor, p38α, we successfully quantified the amplitude of the CF_3 dynamics in the p38α-bound state. Thus the FCT analyses provide a useful local dynamics and structural information for the rational design of a ligand with higher affinity and preferable thermodynamic properties. (Mizukoshi et al, Angew Chemie, 55, 14606, (2016), Tokunaga et al, Molecules, 22, 1492, (2017))

SL44

MicroRNA's Dynamics Influence Targeting of mRNA by Relaxation Dispersion NMR

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MicroRNAs (miRNAs) are short, non-coding RNAs that regulates messenger RNA (mRNA) translation. At the core of miRNAs activity lays the base pairing between Argonaute associated miRNAs (miRISC) and their targets[1]. The dominant interaction in miRISC target recognition is the Watson-Crick base pairing of nucleotides (nts) 2-8, the seed sequence[1,2]. Beyond the seed, miRNA-mRNA complexes (nts 9-22) are predicted to contain non-canonical structural motifs[1]. Experimental observations of such motifs and their implications in target regulation have, however, remained elusive.

We employ 15N and 1H R1ρ relaxation dispersion NMR spectroscopy, where 1H probes were recently developed by us[3], and molecular dynamics to dissect the conformational flexibility of the miR-34a Sirtuin 1 (SIRT1) mRNA binding site at atomic resolution. R1ρ NMR data of the central bulge reveals that the 7nt long seed (ground state – GS) is in equilibrium with a transient and low populated excited state that elongates the seed with an additional GU base pair at its 3'-end (seed +1) to a complete 8nt seed. Trapping the excited state by stabilizing seed +1 with a two-point mutation, we quench the GS flexibility of the central bulge, without impairing Kd or Tm. 3D structures of GS and seed +1 constructs are calculated from NMR-informed molecular dynamics and successfully docked into the crystal structure of human Argonaute 2 (hAgo2). The complex reveals the biological relevance of the seed +1 excited state by showing that helix 7 in hAgo2 needs to move in order to accommodate the seed +1 state, but not the GS, which correlates with an activational switch in hAgo2[4]. The functional impact of the miRNA seed +1 excited state was studied human cells by luciferase reporter assay showing that the trapped excited state exhibits stronger repression, pointing towards the seed +1 state is guiding hAgo2 protein to its active state from the initial encounter complex.

This is the first time, that RNA dynamics are shown to influence microRNA targeting mRNAs and its efficiency. This finding can be exploited in cancer therapy, as the model used here is miR-34a targeting mSirt1, a potent cancer inhibitor.

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SL45

Lipid- and cholesterol-mediated timescale-specific modulation of membrane protein dynamics in nanodiscs

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Membrane protein function fundamentally depends on the surrounding lipid bilayer. In the past few years, it has become clear that this fundamental relationship is intricate and multilayered, going far beyond simple hydrophobic interactions (e.g. Autzen et al, Science, 2018). It has also become clear that mechanisms of membrane protein activation by temperature exist, likely involving lipid dynamics as a regulatory factor (e.g. Gao et al, Nature, 2016). Hence, atomic insights into lipid-mediated modulation of membrane protein dynamics would provide new insights with the potential to fundamentally extend our understanding on lipid-protein interdependencies. Although highly anticipated, such insights remained elusive due to its intrinsic difficulty. To elucidate the impact of lipid dynamics on a prototypical membrane protein, we reconstituted the outer membrane protein X (OmpX) into a saturated, unsaturated and cholesterol-containing lipid-bilayer using the nanodisc technology and recorded timescale-specific NMR relaxation experiments with residue-resolution. The relaxation data revealed that lipid order, modified either biochemically or biophysically, changes the dynamics of the immersed membrane protein in a specific and timescale-dependent manner. A temperature-dependent dynamics analysis furthermore suggests a direct coupling between lipid and protein dynamics in the picosecond, microsecond and millisecond timescale, caused by the lipid's trans-gauge isomerization, the rotational diffusion of lipids and the fluidity of the lipid phase, respectively. These observations evidence a direct modulatory capability of the membrane to regulate protein function through lipid dynamics. Strikingly, the available timescales for such coupling mechanism cover at least 6 orders of magnitude. The lipid bilayer environment thus provides a substantially larger repertoire to regulate functions of immersed proteins, as compared to its aqueous counterpart.

SL46

Methylglyoxal - a 'faulty metabolite' and a probe for rapid-dissolution dynamic nuclear polarisation

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Rapid-dissolution dynamic nuclear polarisation (RD-DNP) is an emerging technique in liquid-state NMR and MRI, which yields signal enhancements by up to 10,000-fold. Using RD-DNP methodology, we aim to develop novel molecular probes of cancer-related metabolic processes in cell suspensions and in vivo. Methylglyoxal is a highly-reactive keto-aldehyde, which is generated as a side product in the glycolytic pathway in cells, but quickly converted to D-lactate by two glyoxalase enzymes. It is considered to be a 'faulty metabolite', with its role in biology remaining enigmatic. Due to its high reactivity, it is capable of damaging cell proteins by non-specific chemical binding and cross linking, thus causing pathological changes. In 1967, Szent-Györgyi et al. postulated that methylglyoxal might be a regulator of cell division and development of cancer, however, despite this claim, only limited research has been done on this metabolite. We developed a RD-DNP method for hyperpolarisation of [2-13C]methylglyoxal that we had chemically synthesised. The glyoxalase reactions are known to be very rapid and they have not been previously amenable to be studied by NMR in whole cells. The RD-DNP approach enabled us to capture time courses of the glyoxalase reactions with high resolution and signal-to-noise ratios, as hyperpolarised methylglyoxal was quickly metabolised to D-lactate in suspensions of erythrocytes and EL-4 cells. Our kinetic data formed the basis of a quantitative description of solute flux via the glyoxalase pathway, thus probing the activity of the enzymes in situ. In addition, we demonstrated that RD-DNP is applicable to detecting glyoxalase activity in cancer cells, including in vivo in tumour-bearing mice, on the sub minute time scale. The developed approach has a potential to be used for detecting abnormal cells in the body, including those with inborn errors of metabolism, and their response to therapy, with expected translation into the clinic.

SL47

Transient Interactions of Folded and Intrinsically Disordered Proteins Revealed by In-cell NMR

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Structural biology of biomolecules is an important area of research due to the involvement of these proteins in several diseases and biological functions. While there is detailed structural and dynamic information of folded and intrinsically disordered proteins (IDPs) available from in-vitro experiments, little is known about the structure and dynamics in living cells. Understanding the structure-function-dynamics relationship of proteins is thereby crucial in living cells with a crowded environment and various potential binding partners is therefore crucial to establish the nature of both the function and disease progression of variety of proteins. In-cell NMR spectroscopy is a promising technique to reveal structural, dynamic and kinetic information of proteins at residue-resolution. Although different applications of in-cell NMR method have been conducted to date, either in prokaryotic or eukaryotic cells, only a few proteins allowed in-cell NMR observation.

We recently developed and optimized a novel method to electroporate 15N-labeled proteins into mammalian cells and successfully implemented in-cell NMR measurements. Here we present the in-cell NMR applications to derive atomic-resolution insights into the structure and dynamics of three folded model proteins and an IDP that is used in cancer diagnosis. We used various mammalian cell types such as HEK-293, HELA, Cos7 and A2780 and heteronuclear (1H-15N HSQC/HMQC) multidimensional in-cell NMR experiments were performed to monitor the amide finger print of proteins. To provide insights into the dynamic behavior of proteins in cells, we also performed 15N-relaxation measurements. Our data on the folded proteins revealed peak doubling/triplication and more for many residues, which are absent in the corresponding in vitro spectrum. This suggests that the selected folded proteins undergo transient interactions with other proteins or feels different environments in the cell. Of particular interest is that for certain folded proteins, we observed more complex behavior of the in-cell NMR resonances. Several residues, in particular those at the canonical binding pocket for the peptide ligands and partner proteins, showed perturbation in position and intensities. It suggests that intracellular ligands and proteins recognize their partner proteins in highly specific manner and the electroporated proteins retain their active and functional state. Our in-cell NMR data to address the structural and dynamic nature of the IDP related to cancer shows that the disordered nature of monomeric protein is stably preserved in both HEK and Cos7 cells.

In summary, by revealing the transient interactions of proteins within cells, we provide new insights into the high-resolution in-cell NMR measurements of folded and disordered proteins in various mammalian cell lines. Thus our novel approach will enable structural and dynamic analysis of

various other proteins associated with several diseases and biological activities inside living cells.

SL48

Real-Time In-Cell Nuclear Magnetic Resonance Spectroscopy to study Quinary Protein Interactions

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We developed real-time (RT) in-cell nuclear magnetic resonance (NMR) spectroscopy to monitor temporal changes in protein quinary structure, for $\geq$24 h, in response to external and internal stimuli. RT in-cell NMR consists of a bioreactor containing gel-encapsulated cells inside a 5 mm NMR tube, a gravity siphon for continuous exchange of medium, and a horizontal drip irrigation system to supply nutrients to the cells during the experiment. We showed that adding antibiotics that bind to the small ribosomal subunit results in more extensive quinary interactions between thioredoxin and mRNA. The results substantiate the idea that RNA-mediated modulation of quinary protein interactions may provide the physical basis for ribosome inhibition and other regulatory pathways.

Poster Presentations

P1

Towards biosynthetic stereospecific deuteration of all amino acid sidechains

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Introduction Protein structure determination by solution NMR has two major limitations: 1) lack of accurate sidechain information and 2) rapid signal decay in larger systems. These issues can be overcome through stereospecific deuteration, which slows signal decay and provides unambiguous sidechain chemical shift assignments. We propose that only isolated 1H-12C groups in a highly deuterated background possess sufficiently long-lived magnetization for non-methyl-containing amino acids in high molecular weight systems (>50 kDa). To achieve multidimensional NMR, magnetization from such groups can be transferred via nuclear Overhauser enhancements (NOEs) to transverse relaxation-optimized 1H-15N groups or 1H-13C methyl groups.

Methods We have developed a mutant human Pin1-WW domain system for quantifying isotopic labeling in deuterated E. coli growth media. The small size of the domain and its high expression levels (100 mg/L M9 minimal media) allow us to obtain well-resolved natural abundance 13C-HSQC spectra using protein purified from a single liter of bacterial culture.

Results Using D2O, unlabeled fumarate as carbon source, and oxalate and malonate to suppress endogenous fumarate production, we demonstrate stereoselective deuteration at the beta-methylene position (Hbeta3) in the oxaloacetate family of amino acids (Asp, Asn > Lys > Met). (The Hbeta2 position of Met was highly deuterated for unknown reasons.)

Stereospecific beta-hydrogen chemical shift assignments enhance the accuracy of sidechain chi1 dihedral angle determinations via 3-bond J-coupling measurements (Hbeta coupled to N, Halpha, or CO). This improves agreement with X-ray crystal structures, but also reveals highly populated alternative conformers that are present in solution at physiologically relevant temperatures.

We also demonstrate that our new isotope labeling technique yields rich new structural restraints in PagP, an integral membrane protein with an effective molecular weight 50 kDa within a DPC detergent micelle.

Conclusions We are currently developing biosynthetic methods for stereospecifically deuterating the sidechains of all amino acids. This overall approach will facilitate the determination of structural restraints for all amino acids in high molecular weight systems. Moreover, in low molecular weight systems, it will enable accurate determinations of sidechain conformations and dynamics.

P2

Molecular dynamics simulations of biological macromolecules in shear flow

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Biological macromolecules are exposed to a highly crowded environment in living cells. We now know that the cytoplasm, the nucleus and other organelles are highly viscous. These fluids markedly differ from dilute in vitro conditions. Viscosity is a measure of fluid internal friction. This parameter directly affects the hydrodynamic forces that act on immersed macromolecules. In addition, active motion of the viscous fluid has been observed in many plant and animal cells. This phenomenon is called cytoplasmic streaming. Nevertheless, the effect the motion of the fluid motion (i.e. flow) on biomolecules is not understood to sufficient detail. Active streaming is expected to generate forces due to local differences in the fluid pressure and velocity fields. We applied molecular dynamics simulations to study the motion of proteins of different size and shape in laminar flow. In our simulations, the proteins exhibit a superposition of random diffusion and shear flow-induced rotational motion. At lower shear stresses random diffusion dominates. Stronger shear enforces an active “rolling” motion along the axis of the applied flow. Based on our data, nearby molecules ought to undergo correlated motion in laminar flow. This in turn enhances the probability of molecular interactions and thus aggregation. We claim that biomolecular simulation in shear flow can make useful predictions regarding alignment, deformation, and dynamics. It is anticipated that the predictions made by our simulations can be compared against experiments that apply shear flow in situ such as Rheo-NMR techniques.

P3

A double-quantum approach to amine chemical shifts in arginine side-chains

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Arginine side-chains are often key for enzyme catalysis, protein-ligand and protein-protein interactions. The importance of arginine stems from the ability of the terminal guanidinium group to form many key interactions, such as hydrogen bonds and salt bridges, as well as its perpetual positive charge. We present here an arginine 13Cζ-detected NMR experiment in which a double-quantum coherence involving the two 15Nη nuclei is evolved during the indirect chemical shift evolution period. As the precession frequency of the double-quantum coherence is insensitive to exchange of the two 15Nη; this new approach is shown to eliminate the previously deleterious line broadenings of 15Nη resonances caused by the partially restricted rotation about the Cζ-Nε bond. Consequently, sharp and well-resolved 15Nη resonances can be observed. The utility of the presented method is demonstrated on the L99A mutant of the 19 kDa protein T4 lysozyme, where the measurement of small chemical shift perturbations, such as one-bond deuterium isotope shifts, of the arginine amine 15Nη nuclei becomes possible using the double-quantum experiment.

P4

Stripline microcoil methods for microfluidic NMR with high sensitivity

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In our lab a stripline microcoil NMR detector has been developed. Here we will present an overview of some of the various stripline NMR techniques.

The flat geometry of the stripline is ideal for NMR measurements in a microfluidic context. A simple capillary is placed on top of the stripline detector to contain the NMR samples. This makes it convenient to perform in-flow experiments. The stripline probe can easily be connected to a microreactor or other analytical instruments. The stripline is designed to maximize sensitivity while keeping a good resolution. To improve resolution a Shim-on-Chip technique has been developed, which is more suitable than conventional shimming methods for these microliter samples. The Shim-on-Chip consists of a series of parallel wires placed on top of the stripline chip. By controlling the currents through these wires independently an almost arbitrary magnetic field (in 1D) can be created to correct

for the inhomogeneity of the NMR magnet. Because the sample has a nearly one dimensional geometry the Shim-on-Chip is an ideal method to obtain good resolution for mass-limited samples. The stripline detector can also be manufactured with any desired shape. In our lab we have designed a tapered stripline which creates a well-defined gradient in the B1-field. This allows us to perform off-resonance rf pulses in a way similar to B0 Pulsed Field Gradients, without any additional hardware. With these pulses the time required for NMR experiments can be reduced as the B1 gradient pulses remove the need for phase cycling to perform coherence selection. For sensitive NMR measurements this technique increases throughput. Because the sample is contained within a capillary the stripline is ideal for NMR on high pressure (>100 bar) samples. Together with the small sample volume this is an ideal method to combine with high pressure chromatography. In our lab we have realized a hyphenation between Supercritical Fluid Chromatography (SFC) and NMR. The fast separations of complex mixtures with SFC combined with the powerful detection of NMR makes an ideal system for metabolomic studies. In this system supercritical CO2 (scCO2) can be used as an NMR solvent. Which has many advantages for NMR. There are no solvent protons, the relaxation is slower, but also scCO2 has a low viscosity. This low viscosity opens up the possibility of performing efficient Overhauser DNP at high magnetic fields (600 MHz). Here we will present our progress towards a SFC-DNP-NMR system aimed at high-throughput analysis of complex mixtures.

P5

Paramagnetic Relaxation Enhancement experiments with 13C-detection on Intrinsically Disordered Proteins. Deciphering the compaction nature of Osteopontin and the role of proline isomerization.

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Intrinsically disordered proteins (IDPs) carry out many biological functions. They act as hubs in intricate interaction networks that make living systems modular and functional. They lack a stable 3D structure and are able to adopt many different conformations in dynamic equilibrium. The interplay between local dynamics and global rearrangements is key for their function. Especially, the unique physicochemical and structural properties of proline residues raise the question to understand the structural role in folded and unstructured proteins[1,2]. In IDPs, proline positions are hypothesized to stabilize partially folded states in regions with residual structural propensity. NMR spectroscopy, and in particular 13C-detection[3], is exquisitely suitable to address these questions. A widely used NMR experimental approach to study long-range contacts in IDPs exploits paramagnetic effects. Due to their sensitivity, 1H detected experiments are generally used to determine paramagnetic relaxation enhancement (PRE) for amide protons (HN). However, under physiological conditions exchange broadening may hamper the detection of solvent exposed amide protons reducing the content of information available. Here we present an experimental approach based on direct carbon detection of PREs[4] that provides (1) improved resolution, (2) reduced sensitivity to exchange broadening and (3) complementary information deriving from the use of different starting polarization sources (HN, Hα and C'). Moreover, the C'-N correlation measured in the 2D CON spectra, allow direct observation of proline residues and their isomerization state. We applied this 13C-detected strategy to Osteopontin[4], a largely disordered IDP with a central compact region[5]. The combination of 15N relaxation experiments (T1, T2 and heteronuclear NOE) and PRE experiments allowed us to shed light on the local dynamics generated by proline-containing segments, the modulation of the cis-Pro population by their local environment and their role in long-range contacts. These methodological advances pave the way for a detailed characterization of compact states in IDPs and for the understanding of the role of proline residues. [1] T. R. Alderson, J. H. Lee, C. Charlier, J. Ying, A. Bax, ChemBioChem 2018, 19, 37–42. [2] E. B. Gibbs, F. Lu, B. Portz, M. J. Fisher, B. P. Medellin, T. N. Laremore, Y. J. Zhang, D. S. Gilmour, S. A. Showalter, Nat. Commun. 2017, 8, 1–11. [3] I. C. Felli, R. Pierattelli, J. Magn. Reson. 2014, 241, 115–125. [4] B. Mateos, R. Konrat, R. Pierattelli, I. C. Felli (submitted) [5] D. Kurzbach, T. C. Schwarz, G. Platzer, S. Höfler, D. Hinderberger, R. Konrat, Angew. Chemie - Int. Ed. 2014, 53, 3840–3843.

P6

Semi-automated chemical shift assignment methods in the latest version of NMRFAM-SPARKY

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Biomolecular NMR spectroscopy has evolved into a powerful tool for investigating molecular structures, dynamics, and protein-protein and protein-ligand interactions. NMR spectroscopy is used frequently as a component of integrative/hybrid methods for structure determination. Because the assignment of protein NMR spectra frequently is the slow step in the process, in particular with solid state NMR, it is important to develop ways around this bottleneck. We recently developed a plug-in for NMRFAM-SPARKY [1], called PINE-SPARKY.2 [2], which assists in automating backbone and sidechain assignments and has become widely used. We introduce here additional tools for semi-automated protein chemical shift assignment. They feature highly flexible and versatile computational methods that are applicable to a wide variety of solution- and solid-state NMR experiments. The methods are supported as interactive GUIs (graphical user interfaces) in NMRFAM-SPARKY and do not require any additional installation. These tools offer approaches to the assignment of challenging data sets from solid-state NMR and solution NMR of largely disordered proteins. The methods assist with selective assignment of NMR signals to residues undergoing dynamic transitions or involved in molecular interactions.

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[2] W Lee, JL Markley (2018) PINE-SPARKY.2 for automated NMR-based protein structure research. Bioinformatics 34(9):1586-8

P7

Proton-detected solid-state NMR studies of protein-protein and protein-ligand interactions in the HpDnaB helicase

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Solid-state NMR for protein structure research and other biological applications has traditionally been primarily concerned with carbon-detected

experiments. In recent years, the development of magic-angle spinning (MAS) frequencies exceeding 100 kHz has evoked further interest in proton-detected experiments since, in this MAS frequency regime, the 1H dipolar couplings begin to be averaged out sufficiently for spectral peaks of reasonable linewidth to be observed. Consequently, structure determination by site-specific chemical-shift assignment of protons in addition to carbon and nitrogen shifts is possible. Moreover, detection of protons potentially allows for investigation of the system in interaction with ligands, cofactors or other compounds of interest, where often protons are directly involved in said interactions, e.g. through hydrogen bonding.

HpDnaB helicase is involved in unwinding dsDNA ahead of the replication fork to prepare a ssDNA template for DNA replication. The full-length dodecameric complex contains 12x488 residues with a molecular weight of 672 kDa and its spectral resolution allows for efficient acquisition of 3D experiments.

An essential requirement for protein interaction studies with NMR spectra is chemical-shift assignment. Although assignment strategies both in solid- and solution-state NMR rest on decades of experience, proton detection in the solid state is relatively new and we would like to share some of the considerations that were made in the assignment process, especially bearing in mind the remarkable size of the protein. While assignment is often tedious and time-consuming, along with this "ugly face" of NMR comes its beauty: direct sensitivity to intermolecular interactions through chemical shift perturbations. Some of this beauty was caught in spectra which show e.g. protein complex formation and DNA binding, as well as paramagnetic relaxation enhancement and dynamics. In summary, both the beauty and the beast of this project are presented.

P8

Amino acid specific lineshapes in triple-resonance experiments

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Most investigations of proteins by NMR require assignment of backbone nuclei. This procedure is difficult for large proteins due to overlap and fast relaxation. For high molecular weight proteins at high field, the combination of deuteration, TROSY, and non-uniform sampling produces far higher resolution and sensitivity in the HNCA than for other backbone assignment experiments. This suggests that the ability to assign large proteins using only the HNCA is of considerable interest.

In practice, the HNCA suffers from degeneracies in peak position, which prevent unambiguous assignment. These ambiguities could be resolved using CB and/or CO chemical shifts, which are not available directly from HNCA spectra. However, CB and CO spins do affect the CA during indirect encoding, via their relatively strong J couplings and the resulting splitting patterns.

We have designed homonuclear decoupling pulses in which the resulting CA lineshape varies with chemical shift frequency of the decoupled spin. We can precisely tailor these splitting patterns using optimal control theory. These pulses allow indirect observation of the coupled/passive resonance frequency, while avoiding transfer delays and maintaining the HNCA sensitivity and resolution.

We have recently demonstrated how this can be used to access amino acid-specific lineshapes in the HNCA, which greatly simplifies the assignment process. Depending on CB and CO chemical shifts, clearly different CA lineshapes are observed. This has allowed assignment of a 30 kDa protein with only the HNCA experiment. Moreover, the decoupling strategy does not cause any off-resonance effects on the CA (such as Bloch-Siegert shifts), due to a new pulse design principle. Therefore, we observe a doubling of signal-to-noise ratio in the HNCA of a 30 kDa protein (in contrast to previous attempts, where the sensitivity gain was much more modest). Our strategy is plug-and-play into any spectrometer and requires no special reconstruction or analysis (other than paying attention to the lineshapes, i.e. singlet versus doublet). Therefore, anyone who works with the HNCA experiment can access the extra information and doubled signal-to-noise that our approach produces.

P9

A novel NMR method to determine fast hydrogen exchange rates in proteins

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Proteins have a dynamic and flexible structure that allows them to interact with the surrounding molecules. Various NMR based approaches exist that determine protein dynamics at both global and residue level. H-D exchange measurements is a popular method to map protons that exchange with the surroundings. Current models can measure exchange rates only up to a few seconds. We have developed a new method that can measure fast proton exchange rates up to approximately 10^5 s^{-1}. The method uses a series of CPMG pi pulses on the 15N channel, through which one can measure the decorrelation of the antiphase operator 2NxHz during the exchange event. We have applied the method on a sample of Calbindin and have observed exchange rates up to 600 s^{-1}. We have also presented with the help of simulations, the effect of experimental setups such as temperature, offset and the type of decoupling sequence used on the determined rates of exchange. The method gives residual level information and is applicable for both structured and disordered proteins.

P10

NMR2 for fast 3D structure determination of protein-ligand binding site without protein resonance assignment

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X-ray crystallography molecular replacement (MR) is a highly versatile tool for the detailed characterization of lead compound and binding modes in the pharmaceutical industry. The two major limitations of its application to drug research are (i) the availability of a similar protein structure, and (ii) obtaining well-diffracting crystals of the ligand-protein complexes of interest. While nowadays the first point is often not a limitation anymore, obtaining well-diffracting crystals might be difficult. In such situations structure determination of protein-ligand complexes by liquid-state NMR is a good option. Unfortunately, the established standard structure determination protocol is in general time-consuming, and a shortcut using available structural data as in the case of MR in X-ray crystallography is not available. Here, we present NMR2 (NMR Molecular Replacement), a MR-like approach in NMR to determine the structures of the binding pockets of ligands at atomic resolution. The calculation of structures of protein-ligand complexes relies on the collection of unassigned semi-quantitative intermolecular NOE distance restraints and on previously solved structures. The NMR2 method uses a high throughput structure calculation protocol, rather than a docking-scoring simulation. It is fast since it requires only a

few days of measuring time and bypasses the time-consuming sequential assignment steps for the protein. We will present multiple NMR2 applications covering several ligand topologies ranging from peptidomimetic to small molecules that bind strongly or weakly to protein receptors. We also report how NMR2 can make use of partially labelled protein using methyl-specific isotope labelling. Our findings demonstrate that NMR2 may open an avenue for the fast and robust determination of the binding pocket structure of ligand-protein complexes at atomic resolution.

P11

Design of Optimal Combinatorial Selective Labeling Schemes for Fast NMR Assignment of Proteins

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Assignment of backbone resonances is a crucial step in every protein NMR investigation. The conventional procedure of the backbone assignment requires acquisition of a set of 3D triple-resonance spectra for uniformly 13C,15N-labeled protein sample. The full set of 3D experiments for backbone assignment requires up to a week of measurements, thus limiting application of this method to the proteins with low stability. In case of large proteins the assignment procedure is also restricted by sensitivity of NMR experiments. Combinatorial selective 13C,15N-labeling (CSL) provides alternative fast approach for backbone resonance assignment. Analysis of simplified NMR spectra (e.g. 2D HSQC/HNCO) provides information about the residue types in each dipeptide. To obtain this information the CSL scheme is required, e.g. one needs to specify the number of labeled samples and the labeling pattern for each residue type in the samples. The CSL schemes can be either sequence-specific or universal. The sequence-specific schemes are designed for each individual protein. These schemes could be optimized to give maximum assignment information from as few labeled samples as possible. Contrarily, the universal schemes are initially calculated in such a way to be applicable for any protein sequence. We classified various CSL strategies that were previously proposed. To compare them we introduced the term NMR coding system (NCS) that is a combination of used amino acid labeling types and 2D NMR experiments. Here we present two novel deterministic algorithms for calculation of price-optimal sequence-specific and universal CSL schemes. The first algorithm 'ULabel' finds universal CSL schemes. A price-optimal scheme for each of the 8 NCSs and 20 amino acids was calculated based on the prices of commercially available 13C- and 15N-labeled amino acids. The calculated universal schemes contained from four to nine samples depending on the NCS used. This proves that the calculation of the sequence-specific labeling schemes is not an NP-hard problem. The second algorithm 'CombLabel' calculates sequence-specific schemes, based on the given stock of isotope-labeled AAs and the NCS that will be used. Theoretical calculations using literature examples revealed that CombLabel software outperformed the previously proposed algorithms by the amount of assignment information, which could be extracted from CSL and by the number of samples. The program robustly calculates CSL schemes containing up to 6 samples. The software was tested on the isolated voltage-sensing domain (VSD) of human Kv2.1 channel. The calculated price-optimal labeling schemes for the VSD (163 residues) contained five samples. Analysis of the 2D 1H,15N-TROSY and HNCO spectra measured for each sample in combination with the limited data from standard triple-resonance NMR spectra resulted in assignment of 70% of backbone resonances for the VSD. This work was funded by the Russian Science Foundation (#16-14-10338).

P12

Nanopore-confined Bilayers: Model Biomembranes with Defined Curvature and a Tool for Oriented Sample Magnetic Resonance

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Biological function of cellular membranes is primarily determined by both membrane proteins and the membrane biochemical composition. Recent evidence suggests that the membrane shape/local curvature may also play roles in modulating membrane properties and the proteins' function. While specific biomembrane compositions are more readily replicated in magnetic resonance experiments, formation of bilayers of specific shapes and curvature represents a more challenging task. Macroscopic alignment of such membranes – an important prerequisite of high resolution magnetic resonance and other studies – is even more difficult especially over a broad range of experimental conditions such pH, ionic strength, and temperature. We have developed methods for forming self-assembled lipid nanotubular bilayers inside cylindrical nanopores composed of anodic aluminum oxide (AAO). Such hybrid nanostructures, named lipid nanotube arrays, represent a new type of substrate-supported and macroscopically-aligned lipid bilayers that have many attractive features for both biotechnology and structure-function protein studies by magnetic resonance. Here we demonstrate a dramatic improvement in lipid nanotube technology vs. our previous studies by developing AAO substrates with exceptionally uniform high-density nanopore structure. This development allowed for employing oriented sample (OS) solid state NMR for a detailed examination of lipid-induced conformational changes of transmembrane helices over an exceptionally broad range of environmental conditions, including temperature, pH, and lipid composition. The exceptionally narrow linewidth of 2D NMR spectra demonstrate that the transmembrane helices maintain fast uniaxial rotational diffusion within the nanotubular bilayers membrane. This diffusion is sufficiently fast to average out chemical shift anisotropy even at 1.5 GHz 1H NMR frequency. We also extended the oriented sample (OS) lipid nanotube technology to spin labeling EPR including W-band (94.3 GHz) (HF EPR) spectroscopy. For the latter, a new photonic band-gap W-band resonator to accommodate planar AAO substrates has been developed and tested. Such a resonator/AAO system provides additional resolution for studies of conformational changes of gramicidin ion channel by spin labeling EPR. We also describe double-resonance (DEER) experiments from a membrane protein aligned by AAO system. Overall, lipid nanotube arrays provide a versatile platform for macroscopic alignment of membrane proteins - from small pore-forming and transmembrane peptides to large membrane protein complexes, such as bacterial reaction center (RC) protein from Rhodobacter sphaeroides and Anabaena sensory rhodopsin (ASR). Accessibility of either both or mainly inner leaflet of the nanotubular bilayers to water-soluble species enables studies of protein, peptides, and drug binding my magnetic resonance.

P13

Size determination of protein-ligand complexes and protein oligomer/aggregates with diffusion NMR spectroscopy

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Diffusion-ordered NMR spectroscopy (DOSY) is currently widely used

to identify components present in a sample mixture and provides information on the hydrodynamic radii of these components through the Stokes-Einstein equation (1-3). While used successfully with mixtures of small molecules (4), the application to biological macromolecules (5) has been hampered due to chemical shift overlap and shorter relaxation times. Herein we show new developments that allow DOSY to be applied for the size determination of larger species such as protein-ligand complexes and protein oligomers/aggregates. Proof-of-principle is first shown with a cross-linked oligomeric protein mixture where the hydrodynamic volumes of each component are estimated and subsequently verified with size-exclusion HPLC and SDS polyacrylamide gel electrophoresis. We then determine the sizes of (i) a peptide (39 AA)-ligand (fatty acid) complex and (ii) a sample of protein oligomers from a protein solution subjected under amyloid fibrillogenesis conditions. These studies aim to provide insight into the size of peptide-ligand complex and kinetics behind protein aggregation involved in amyloidosis as well as to determine the hydrodynamic radii of proteins within the mixture. All experiments were performed and optimized on an Agilent Technologies 18.8 T (800 MHz) DD2 Premium Compact spectrometer with a triple-resonance, 5 mm enhanced cold probe with Z-gradient coil, capable in producing gradient strength of 60 Gcm-1. In future, advancement in design of strong gradient coil strength (keeping the sample heating factor in account) and the development of new pulse sequences to suppress signal exchange (6) will be very useful to study the size and aggregation properties of biomolecules.

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P14

Probing mechanosensitive alterations in Na+ transport in red blood cells (RBCs) in physiological shear fields

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Optimal O2 and CO2 exchange is heavily dependent on the rheological behaviour of blood during flow and this is primarily influenced by the membrane deformability of individual red blood cells as the main cellular component of blood. Associations in impaired Na+ transport and increased blood viscosity have been reported in red blood cell senescence and pathologies of decreased perfusion such as chronic kidney disease. Na+ transport mediated through Na+ channels and transporters, is implicated strongly in mechanotransduction and are known to alter Na+ homeostasis in environments of plasma membrane deformability. The aim of this study was to investigate the dynamic changes in Na+ homeostasis in red blood cells under physiological shear fields experienced in the microvasculature using 23Na+ rheo multiple quantum filtered (MQF) NMR. Blood samples (n=5) were obtained from healthy male volunteers (age 27.6 $\pm$ 5.7 (mean$\pm$SD) years)) under full ethical approval, centrifuged, washed twice and resuspended in PBS in the presence and absence of Tm-DOTP5- shift reagent (SR). Haematocrit (Hct) was varied to 58, 70 & 90% to simulate increases in RBC density and subsequent cell packing exhibited as order of vascular branching increases. Hct dependent viscosity was confirmed using bulk rheology. To assess shear-induced Na+ dynamics, blood (800μl) was transferred into a custom-designed cone & plate rheo-NMR cell and measurements taken during static conditions and under shear rates experienced in the microvasculature (7.8 S¬-1) using a Bruker 9.4T spectrometer. Haemolysis was assessed in supernatants by absorbance at 540nm and compared against lysed cells. Triple-quantum-filtered (TQF) NMR pulse sequences with 48-step phase-cycling were employed in the absence and presence of SR to distinguish between intra and extracellular compartments. In the absence of SR, TQF magnitude demonstrated a Hct dependent decrease during shear relative to static conditions; 58% Hct = 1.454 $\pm$ 0.263 (TQFmag shear/ TQFmag static $\pm$ SD) (n=2), 70% Hct = 1.36 (n=1), 90% Hct = 0.92 (n=1). This observation of increased [Na]i with increasing Hct is correlated with single quantum measurements in the presence of SR; 58%static = 4.2mM $\pm$ 1.2 (mean$\pm$SD), 58%shear = 5.1mM$\pm$1.7(n=3), 70%static = 3.5mM $\pm$ 0.6, 70%shear = 5.3mM $\pm$ 0.6, 90%static = 15.6mM $\pm$ 1.3, 90%static = 19.6mM $\pm$ 1.6 (n=2), suggesting a link between cell packing and Na+ flux. Shear induced changes in TQFmag, relaxation rates and quadrupolar coupling were not statistically significant due to altered sodium relaxation in the presence of SR. However, the maximal creation time (T) demonstrated a change during shear Tstatic=9.2mS, Tshear=11.2mS (n=3). Thus, Na+ transport is altered in RBCs during flow, suggesting a facilitative role in the property of cell deformability due to a mechanosensitive mechanism; a property that is critically altered in diseases of poor perfusion.

P15

Determining isoleucine conformation distributions from chemical shift

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The molecular conformation and motions of proteins are at the heart of many biological processes. Obtaining parameters that describe structural and dynamic features in proteins, such as scalar-couplings, can be challenging particularly in large systems and those in chemical exchange. Relating chemical shifts to structural elements in proteins provides an alternative route to make significant in-roads in studying these systems. Describing side-chain structure is particularly important since they mediate numerous biological events and often behave independently of the backbone [1]. We have used both computational and experimental approaches to fully explore the relationship between chemical shift and side-chain conformation in isoleucine.

Density Functional Theory was used to characterise the dependence of side-chain chemical shifts on both χ-angles in isoleucine. These results showed that all the side-chain carbon atoms possess significant chemical shift variation depending on rotameric state and that each of the populated rotamers has a unique 'chemical shift profile'. These profiles act as a distinct maker for each state.

From these observations, we developed a method to determine isoleucine rotamer distributions from chemical shift. Unlike previous methods [2,3] we define each rotamer by both $\chi 1$ and $\chi 2$ angles, providing a near complete description of the side chain. This chemical-shift based method gives a notably more detailed description of the side-chain dynamics than what is readily available using scalar couplings. Additionally, the available nature of chemical shifts allows this method to be applied in situations where long-range scalar-couplings are impossible to obtain. We show the utility of this method by using it to evaluate a model of the 42kDa membrane protein complex, DsbA–DsbB, and to track changes between the L24A FF domain's ground state and an 'invisible' folding intermediate with a 2.6% solution population [4].

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P16

NMR characterization of a polysaccharide-protein conjugate

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Carbohydrate-based vaccines are among the safest and effective vaccines which prevent bacterial infectious diseases, like meningitis and pneumonia. Several methods have been used so far to characterize polysaccharide-protein conjugates. However, no accurate structural studies have ever been attained and a reduced number of methodologies has been proposed for measuring the degree of saccharide conjugation. This work instead shows that detailed information on large protein systems conjugated with polysaccharides can be achieved by a combination of solution and solid-state NMR spectroscopy. E. coli L-asparaginase II (ANSII) has been chosen to be conjugated with a polysaccharide even if it is not an immunogenic protein. ANSII is indeed a good protein model since its medical and favorable structural characteristics: it is in fact used as drug − mainly in its PEGylated form − for the treatment of acute lymphoblastic leukemia (ALL) and, moreover, shows very good spectral features, even when conjugated with PEG chains [1] or grafted onto gold nanoparticles [2]. Therefore, ANSII has been functionalized with Neisseria meningitidis serogroup C (MenC) capsular polysaccharide [3] to simulate a carbohydrate-based vaccine, then characterized by solution and solid-state NMR to assess the folding after glycosylation and estimate (semi)quantitatively the conjugation pattern. The characterization of large polysaccharide–protein conjugates is still a challenge in structural biology and an important goal for the development of vaccines. The NMR-based protocol showed in this study has the possibility to provide key information for rational design of novel products with improved efficacy.

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P17

Elucidation of the Fibrillization Mechanism of an ALS-Related Protein SOD1 by Using Novel Rheo-NMR Spectroscopy

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that leads to movement disorders. One of the causes of ALS is amyloid fibril formation of superoxide dismutase 1 (SOD1) inside motor neurons. SOD1 is an important enzyme that reduces oxidation stress inside cells; on the other hand, its intracellular fibrils display cytotoxicity, which leads to death of motor neurons and failure in neurotransmission. To date, the formation mechanism of SOD1 fibrils remains unclear. To reveal the fibrillization pathway in detail, we established a novel methodology, high-sensitivity Rheo-NMR spectroscopy. This methodology enables us to obtain atomic-level structural information on proteins under shear flow. Shear flow is known to induce fibril formation; therefore, Rheo-NMR can detect atomic-level structural changes during fibril formation in situ. We applied Rheo-NMR spectroscopy to the research on fibril formation of SOD1. First, we found that SOD1 formed amyloid fibrils by using Rheo-NMR instrument. During the fibrillization, we continuously acquired 1H-15N heteronuclear single quantum coherence (HSQC) spectra of SOD1. Although all cross-peak intensities decreased exponentially in a time-dependent manner, the decrease rates varied widely depending on the residues, implying that local structural changes occur and/or native SOD1 interacts with its fibril intermediates during fibril formation. In addition, after SOD1 fibrils were formed and the shear flow was terminated, in the spectrum we observed a lot of minor peaks that could not be detected under the static condition. Because the chemical shifts of some minor peaks almost correspond to those of peaks of SOD1 unfolded by the denaturant, SOD1 may be converted to its unfolded state in its amyloid formation pathway. Indeed, we observed that the structural destabilization caused by denaturant accelerated the fibril formation. Intriguingly, addition of the protein crowders destabilized the fold stability of SOD1 and thereby enhanced the fibrillization. Thus, these findings suggest that unfolding process is one of the limited steps in the fibril formation. In this study, we detected atomic-level structural changes of SOD1 during its fibril formation. These findings will lead to a further understanding of fibril formation of SOD1 and development of new medicine for ALS.

P18

Analysis of Phosphometabolites in Milk using 2D 1H-31P NMR Spectroscopy

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Phosphometabolites are important natural constituents of milk, they play important roles in health. Phospholipids are increasingly considered as nutrients with putative health benefits(1), Glucose-6-phosphate (G6P) and phosphate lactose are central metabolites in lactose synthesis, and glycolysis(2). Analyses of those metabolites in milk usually depend on several time consuming and troublesome procedures, because of the complexity of milk and their low concentrations. Herein, 2D 1H-31P SOFAST-HMQC has been used to detect the phosphometabolites in milk, it was found that phospholipds, G6P and phosphate lactose can be detected in a few minutes using the above mentioned method without any physical separation or treatment of the samples.

P19

NMR2 as a method for fast protein-fragment structure determination from semi-ambiguous restraints

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The early stages of drug discovery are often characterized by compounds with a weak affinity for their target (Kd from mM to uM). Therefore, these protein-ligand complexes are difficult to crystallize for x-ray crystallography structure determination. NMR spectroscopy enables the study of biomolecules in close to physiological conditions and provides distance restraints that are used for structure refinement such as Nuclear Overhauser Effect. However, a first time-consuming signal assignment step is often mandatory. The NMR2 method uses semi-ambiguous restraints ob-

tained from Nuclear Overhauser Effect SpectroscopY (NOESY) to refine the structure of a ligand in its binding site without the necessity of protein signal assignments. Therefore, NMR2 is excitingly fast and provides a first structure for a complex within a few days of measurement and a few hours of analysis. NMR2 has already demonstrated its effectiveness for several different complexes of a unassigned labeled protein and an assigned unlabeled ligand. Here, we report an in-silico study that demonstrates the potential application of NMR2 for Fragment Based Drug Discovery. The number of NOE restraints that are necessary to reach convergence for the simulated annealing algorithm, the effect of the error relative to these restraints and the impact of partial assignment are studied. Interestingly, the comparison with experimental data suggests that the ligand adopts at least two different poses in the binding site, thus emphasizing the interest of NMR spectroscopy for the study of such a dynamic system. The strategy for a routine investigation of fragments with weak affinity and potentially pose-exchange will be discussed in this poster.

P20

Stereoselective deuteration of amino acid residues using fumarate as a carbon source for E. coli in D2O

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Perdeuteration with selective methyl group protonation has had a significant impact on NMR studies of high-molecular-weight proteins and macromolecular complexes. However, rapid signal decay for large systems and lack of structural information from non-methyl-containing amino acid sidechains limit the utility of this approach for high resolution structure determination. To overcome this limitation, we present a new protocol for producing proteins in E.coli using inexpensive, non-isotopically enriched carbon sources, taking advantage of the native amino acid biosynthetic pathways in E.coli to stereospecifically incorporate protons into proteins produced in D2O-based media. Our approach produces isolated 1H-12C groups in a largely deuterated background, providing additional 1H magnetization that can be transferred via through space NOEs to nearby 1H-15N and 1H-13C methyl groups, which can then be resolved via multinuclear NMR to provide the necessary structural restraints for high resolution structure determination of large protein systems. To fully characterize 1H/2H-isotope incorporation by NMR, we applied our labelling strategy to a small WW domain from human Pin1 protein. Protein grown using glucose as the sole carbon source had high levels of protonation in aromatic rings and the Hβ positions of serine and tryptophan. In contrast, using our FROMP media (fumarate, rhamnose, oxalate, malonate, pyruvate), stereoselective protonation of Hβ2 with deuteration at Hα and Hβ3 was achieved in Asp, Asn, Lys, and Met residues. Our approach provides highly specific (1H-12C) labeling in an otherwise highly deuterated background. We anticipate that our approach can eventually be extended to produce stereo-selective isotope labeling in all 20 amino acids, facilitating NMR-based structure determinations of high molecular weight protein systems. When combined with methyl-specific protonation of aliphatic amino acids, it should be possible to obtain NOE-based distance restraints for all protein amino acid residues.

P21

Two approaches to studying temperature-induced changes in proteins with 3D NMR: time-resolved NUS and "Radon-like" transform.

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Changes in chemical shifts induced by a temperature are valuable source of information about protein structure and dynamics. For intrinsically disordered proteins (IDPs), these temperature coefficients (TC) can help to probe changes in structural propensities and shed light on the nature of unfolding process. However, the low dispersion of chemical shifts in IDPs, especially in HN dimension, hinders the measurement of TCs in a conventional manner i.e. with a series of 2D 15N HSQC spectra taken at various temperatures. In this study, we present two novel approaches allowing the determination of TCs from 3D temperature-swept HNCO technique. The traditional sampling of two indirect evolution times and a temperature "space" would take weeks and thus is infeasible. In our approach, we exploit the temperature-swept non-uniform sampling (NUS) where the temperature is co-incremented with an index of NUS point, allowing to complete measurement in a time similar to a single 3D HNCO experiment. Such data can be processed using two methods: time-resolved NUS (TR-NUS) or "Radon-like" transform. In TR-NUS we divide the dataset into hundreds of overlapping subsets and process them separately to obtain a movie-like 3D HNCO with a temporal pseudo-dimension, in addition to three "standard" frequency dimensions [1]. This provides "smooth" visualization of the changes that allows effective peak-tracking without assuming any model of chemical shift variations. However, the division into subsets leads to the sensitivity loss. In "Radon-like" transform the whole dataset is processed as one object and the algorithm "tests" the time-dependent frequency corrections for temperature-induced peak changes [2]. The model of the changes has to be assumed. The correction that leads to absorbtive Lorentzian line in all dimensions is the temperature coefficient for a given peak. The experiment has been performed on a sample of Osteopontin a 24kDa fully disordered protein involved in inflammation and metastasis. It contains a central compact state that may play an important role on ligand-binding processes. Notably, many peaks reveal non-linear changes which indicates interesting features of certain regions in OPN, also comprising the central compact state. Moreover, the different nuclei (HN, N and CO) report on different features of the protein based on the degree of the non-linear changes.

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P22

1H NMR Diffusion and CEST Methods for Characterisation of Cellular Systems

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We present simple 1H NMR methods that when combined with sophisticated analysis can provide significant insight into the behaviour of cellular systems. By precluding the need for isotopic labelling and using the intense water signal as the primary spectroscopic readout, the experiments proposed are straightforward to perform and readily extendible to MRI studies in-vivo.

Firstly, we demonstrate how variable diffusion time experiments in which the data is fit simultaneously can be used to measure a variety of biological properties reliably including cell number, membrane permeability and cell geometry. To do this, we develop a model to analyse the data including the effects of differential relaxation in the intra- and extra-cellular spaces, exchange across membranes and restriction within cells. We validate the use of this model using extensive Monte Carlo simulations of diffusion in restricted but partially permeable geometries. These show that the model is robust to a wide range of sample parameters without the need for significant prior knowledge about the system being studied. We perform this experiment to both yeast and mammalian cells and apply the experiment to create an assay that measures the uptake of Gadolinium based imaging agents by cells.

Secondly, we introduce the diffusion-weighted CEST (chemical exchange saturation transfer) experiment as a tool for measuring 1H CEST profiles associated with intra- and extra- cellular phases. In these 1H CEST experiments, molecules are identified through their interaction ¬with the dominant water signal via cross-relaxation or chemical exchange. As the translational diffusion properties of water associated with the intra- and extra-cellular water are significantly different, by combining the CEST experiment with a stimulated echo diffusion experiment we can differentiate between intra- and extra-cellular CEST effects. In a proof of principle study, the experiment is attempted on a system of giant unilamellar vesicles (GUVs), where the CEST signal associated with the two phases is known a priori and is shown to work correctly. The experiment is then used to investigate the intra-cellular CEST profiles of mammalian cell systems.

P23

Segmental isotopic labeling of challenging proteins by an asparaginyl endopeptidase and inteins mediated ligation

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Segmental isotopic labeling is a powerful tool for NMR studies of larger and multi-domain proteins. It facilitates NMR investigation of proteins with repetitive sequences and an individual domain in the context of a full-length protein by reducing signal overlaps in NMR spectra.

Despite several established methods such as Expressed Protein Ligation (EPL) (1, 2), protein trans-splicing (PTS) (3,4), and sortase-mediated protein ligation (5, 6), segmental isotopic labeling of proteins has been limited. This is because each method imposes certain requirements, thereby limiting its applications to specific cases and making the method technically challenging. For example, EPL requires an N-terminal cysteine residue in a C-terminal protein fragment for ligation. PTS approach needs association of two or more precursors fused with split intein fragments into the active intein for protein ligation. Enzymatic ligation typically leaves a recognition sequence in the ligated product.

Here, we present simple and traceless approaches for segmental isotopic labeling, which could overcome some of bottlenecks in the currently existing methods for segmental isotopic labeling (7, 8, 9). These approaches could potentially facilitate investigation of more challenging proteins using NMR.

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P24

1H15N - 1H15N correlation experiment for the assignment of slowly tumbling proteins

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Resonance assignment of proteins with long rotational correlation times remains a complicated and error-prone process due to limited dimensionality and resolution of experiments, brought on by the fast relaxation of coherences of interest. Here we demonstrate that a 4D HN(CA)NH [1, 2] experiment utilising transverse relaxation enhancement (TROSY) [3] in all dimensions and diagonal suppression offers excellent signal resolution when combined with non-uniform sampling and Signal separation algorithm[4, 5]-based processing. By avoiding the utilisation of transverse carbonyl coherences, the experiment doesn't suffer from sensitivity degradation at high magnetic fields, where the TROSY effect has the most beneficial results. To illustrate the experiment's usefulness, we acquired spectra for the 370-residue Maltose-binding protein (MBP) while lowering sample temperature to slow molecular tumbling. Direct correlation between 1H15N pairs leads to simple, direct and robust assignment process. The experiment is complementary to the traditional experiments correlating CA and CO chemical shifts with HN pairs, and both can be used concurrently to obtain and verify assignments.

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P25

Trifluoroleucine Incorporation into Proteins for 19F-NMR Using the Single-Protein Production (SPP) System in E. coli

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19F-NMR has tremendous potential for studies of macromolecular com-

plexes and integral membrane proteins (IMPs). Particularly interesting is the potential to utilize 19F-labeled methyl groups as site-specific probes in larger proteins. We have developed a system to efficiently incorporate 3'3'3'-trifluoroleucine (TFL) into proteins using the Single-Protein Production (SPP) system in E. coli. TFL is generally quite toxic to E. coli. Therefore, we created cells which are metabolically active but unable to grow by inducing MazF, an ACA-specific endoribonuclease in E. coli. Under this condition, using an mRNA transcript in which all ACA sequences are removed without altering the amino acid sequence of the protein, cells produce only a single target protein of interest. Using a Leu auxotrophic strain with the SPP system, TFL was efficiently incorporated into target proteins without discernible toxicity. We demonstrate TFL efficient incorporation into GB1 (6 kDa) and EnvZ-B domain (20 kDa), with high protein production efficiency. Although high TFL incorporation into larger proteins was found to destabilize these proteins, lower levels of TFL incorporation are being explored as a means of optimizing 19F-methyl labeling for studies of IMPs, protein complexes, and for in-cell NMR.

P26

Advancements in 2D NMR Methods to Characterize Monoclonal Antibody Therapeutics

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Protein therapeutics and especially monoclonal antibodies (mAbs) are clinically important, life-changing, life-saving medicines, and a rapidly expanding class of drugs. However, their development and manufacture presents many significant analytical challenges. In particular, there remains a need for technologies that can characterize the critical quality attribute of higher order structure (HOS) with high resolution, since even minor differences in conformation can affect the safety, efficacy and stability profile of a protein therapeutic. Two-dimensional (2D) heteronuclear 1H-13C-methyl correlated NMR is emerging as a viable technology for such characterization as it has been proven to be a robust and reproducible method to characterize biotherapeutic HOS at natural isotopic abundance. Thus 2D NMR can be a tool to establish consistency of protein folding for drug quality assessment as well as for structural comparability of related drug products (1–3). However, open questions persist about best practices for data acquisition and post-acquisition spectral analysis. Furthermore, there remains a major hindrance to recording 1H-13C methyl spectra on biotherapeutics when formulation components contain aliphatic resonances that interfere with the analyte signal of interest, thus limiting the full potential of 2D NMR methods in the biopharmaceutical industry. Here we will detail practical considerations for acquiring 2D 1H-13C methyl spectra on mAb therapeutic samples as well as describe best methods for employing multivariate analysis to analyze spectra in terms of structural similarity. We will further describe a novel selective double resonance technique that allows for the selective attenuation of unwanted signals arising from formulation components while minimizing losses in the sensitivity of the biotherapeutic signal of interest and detail how this may be combined with spectral reconstruction processing commonly employed with sparse sampling techniques to further attenuate unwanted signals post-acquisition. We will describe the experimental performance of these methods using the class-representative NIST standard monoclonal antibody (NISTmAb) in a variety of formulation.

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P27

Anti-leukemia activity of a Hsp70 inhibitor and its hybrid molecules

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The anti-leukemia activity of a small molecule inhibitor of Hsp70,apoptozole(Az), and hybrids in which it is linked to an inhibitor of either Hsp90 (geldanamycin) or Abl kinase (imatinib)were examined. NMR studies indicated that Az associates with an ATPase domain of Hsc70, resulting in blocking of ATP binding to the protein. The cell studies showed that Az treatment promotes leukemia cell death by activating caspase-dependent apoptosis without affecting the caspase-independent apoptotic pathway. Importantly, the hybrids composed of Az and geldanamycin, which have high inhibitory activities towards both Hsp70 and Hsp90, exhibit enhanced anti-leukemia activity relative to the individual inhibitors. However, the Az and imatinib hybrids have weak inhibitory activities towards Hsp70 and Abl, and display lower cytotoxicity against leukemia cells compared to those of the individual constituents. The results of a mechanistic study showed that the active hybrid molecules promote leukemia cell death through a caspase-dependent apoptotic pathway. Taken together, the findings suggest that Hsp70 inhibitors as well as their hybrids can serve as potential anti-leukemia agents.

P28

Speeding up the development of targeted precision therapies

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There is a growing interest in the development of nanotechnology-based medicines and diagnostics and intense research and patents, but all this research activity faces difficulties in the translational process and has produced only a limited number of new medicines on the market, partly due to the lack of appropriate characterization methods [1]. In this talk I will show how different NMR techniques can help in speeding the development of targeted-nanomedicines for treating tumors. In particularly I will show the characterization cascade for nanomedicines that we have developed at the Joint Research Center of the European Commission [2] and how we have applied it to the in-depth characterization of monodispersed PLGA-PEG nanoparticles functionalized with a biological macromolecule for the active targeting to neuroblastoma tumors.

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P29

NMR protocol of R&D of innovative pharmaceutics for targeted oncological chemotherapy

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Targeted chemotherapy is on a frontier of research in pharma, it is a key element of personalized medicine. It requires that a drug binds strongly and site specific to its biological target. In case of camptothecin family derivatives acting as the Top I inhibitors this site is defined as a nick in one strand of a duplex DNA in DNA/TopI complex.Using basic NMR experiments such as 1H NMR, NOESY and DOSY we have established two key elements required of compound to fulfill its biological role; selectivity and strong binding in a nick. Thus, using NOESY it was proved that camptothecin core is intercalating in a nick of model nicked DNA decamer in solution in the same geometry as found in a crystal structure of topotecan (TPT)/nicked twentymer DNA complex. Using basic chemical know-how we have chosen the camptothecin derivative SN38 and have introduced the substituent which was supposed and proved the covalent binding to nucleophiles in a nitrogen base in a nick of model DNA decamer. The formation of a bioconjugate was confirmed by 1H NMR, NOESY and DOSY. The selectivity of biding the nitrogen sites in four model nucleosides, dG,dC, dA, dT ,establishing two faces of a nick, was also performed. The novel derivatives prove their innovating nature because the covalent binding inside the nick was only evidenced in this project. They were patented in US: US 9,682,992B2 and in EU, EP2 912 039 B1,validated in PL,D,F, CH, GB. These compounds proved in vitro their several orders of magnitude higher activity for breast, colon,blood and lung cancer cell than presently used in clinic camptothecin derivatives. Even more importantly, they are much more tolerant to normal cells, a key virtue for patients in chemotherapy.Furthermore, applying simple NMR protocol of monitoring the kinetics of solvolysis in water of novel compounds we have established their half life time, t1/2, which is linked to a basic pharmacodynamic parameter defining the time required of a drug to reach the target in its original form. All the above NMR data give the basic set of information required for a further development of compounds in a preclinical and clinical stages. Research sponsored by National Medicines Institute and grant no.2017/27/B/ST4/00190

P30

Characterizing the Interactions of Anti-apoptotic Bcl-2 Family Protein, Bfl-1, with Low Molecular Weight Molecules identified by Fragment-based Screening

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Characterising interactions between the proteins and hit/lead compounds identified by fragment-based or high throughput compound screens at the level of individual amino acid residues is a critical step for lead/ligand optimization, and is at the heart of structure-based drug discovery [1]. Key-players in the intrinsic apoptosis pathway are the B-cell lymphoma protein 2 (Bcl-2) family of proteins. Despite the opposing pro- or anti-apoptotic functions of the various family members, they all exhibit a high degree of sequence and structural similarity in their “Bcl-2 homology regions”, in particular, the presence of a highly conserved hydrophobic ligand binding groove. Both pro-apoptotic and anti-apoptotic proteins within the Bcl-2 family have long been considered as targets for new anti-cancer drug development [2]. Here, we report the backbone chemical shift assignments for one of the anti-apoptotic proteins, Bfl-1 [3, 4], and NMR titration studies of its interactions with low-molecular weight molecules arising from fragment-based screening. References [1] A.D. Gossert, W. Jahnke, NMR in drug discovery: A practical guide to identification and validation of ligands interacting with biological macromolecules, Progress in Nuclear Magnetic Resonance Spectroscopy, 97 (2016) 82-125. [2] J. Huang, W. Fairbrother, J.C. Reed, Therapeutic targeting of Bcl-2 family for treatment of B-cell malignancies, Expert Review of Hematology, 8 (2015) 283-297. [3] E.Y. Lin, A. Orlofsky, M.S. Berger, M.B. Prystowsky, Characterization of A1, a Novel Hematopoietic-Specific Early-Response Gene with Sequence Similarity to Bcl-2, Journal of Immunology, 151 (1993) 1979-1988. [4] E. Ottina, D. Tischner, M.J. Herold, A. Villunger, A1/Bfl-1 in leukocyte development and cell death, Experimental Cell Research, 318 (2012) 1291-1303.

P31

Structural model of Mcl-1/Pyridoclax complex revealed by combining NMR, Docking Approaches and Molecular Dynamics.

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Apoptosis, or programmed cell death, plays a protective role against tumor formation and can be regulated by either the extrinsic or the intrinsic pathway. The latter one is regulated by Bcl-2 family proteins. [1] Among them, MCL-1 and Bcl-xL which are anti-apoptotic proteins implied in ovarian carcinoma proliferation and chemoresitance. Both proteins cooperate to protect cancer cells against apoptosis and their concomitant inhibition leads to apoptosis even in absence of chemotherapy. [2] An emerging option is the targeting of the anti-apoptotic members MCL-1 and Bcl-xL of Bcl-2 family. Indeed, this strategy was validated by the discovery of ABT-737 [3] whereas MCL-1 inhibition remains problematic. In this context, we have designed and synthesized foldamers [4],[5] that specifically inhibit MCL-1. Among them, Pyridoclax was designed to target the hydrophobic MCL-1 binding groove and we demonstrated, by SPR, that it could interact directly and selectively with MCL-1, and thus sensitize ovarian carcinoma cells to Bcl-xL targeting strategies. [6] A structural molecular-scale approach to investigate the interaction between MCL-1 and Pyridoclax was then conducted by NMR (hotspots, KD, koff) and showed that Pyridoclax can mimic the BH3 domain of pro-apoptotic proteins. NMR data were then combined with computational methods to identify the exact binding modes of Pyridoclax in the large hydrophobic MCL-1 groove and provided valuable information on the MCL-1:Pyridoclax complex that could be useful for the design of more active compounds that modulate the biological function of MCL-1.

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P32

Unraveling the dynamic processes in inhibitor-Muramyl ligase D complexes: towards new potent antibacterial agents

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The Muramyl ligase D (MurD) is an ATP-driven 47.7 kDa multi-domain intracellular bacterial enzyme, essential for the biosynthesis of the bacterial peptidoglycan. It represents an attractive target for the development of novel antibacterial agents. The intracellular steps of peptidoglycan synthesis have been greatly underestimated and only two such antibacterial agents are in clinical use by now. Several attempts to design effective Mur ligases inhibitors by mimicking substrates, products or tetrahedral intermediates are reported. However, the results are not satisfactory. The crystal structures for several types of these inhibitors in complex with MurD could not be determined. Moreover, the same number and type of binding interactions with MurD were observed in rigid crystal structures for D- and L-Glu sulfonamide type inhibitors, although they have significantly different inhibitory activities.

We are investigating the binding mode of MurD inhibitors with various molecular scaffolds in aqueous environment using the ligand-based and the protein-based NMR methods in combination with the extensive molecular dynamic (MD)simulations. For protein-based NMR studies we express and purify the 13C methyl selectively labelled, the deuterated 15N labelled and the deuterated 15N/13C methyl selectively labelled MurD enzyme from E. coli. Results reveal the complex dynamic processes in inhibitor-MurD complexes, which influence the inhibitor binding interactions and can be related to the differences in the inhibitory activities. The fast protein domain motions especially between the C- and N-terminal domains affect the conformation and flexibility of the bound ligands, the stability of the ligand-MurD interactions and the binding site adaptability. Indeed, the inhibitors that do not interact with the C-terminal domain can have comparable or even better inhibitory activities in comparison to the inhibitors that span from C- to N-terminal domain despite the reduced number of ligand-protein interactions. Our latest studies involve the characterization of MurD dynamics upon binding of enzyme substrates and inhibitors using the NMR spin relaxation experiments and the normal mode analysis based on MD trajectories. By analysing the distribution of the low frequency modes during the binding process we elucidate the impact of the protein internal dynamics on the change of the free energy of ligand binding.

Our studies provide upgraded knowledge for the rational structure-based design of novel more efficient MurD inhibitors. Moreover, the results confirm, that the proper treatment of dynamic effects can be crucial for the effectiveness of structure-based drug-design methods. The work was supported by the Slovenian Research Agency (Grant numbers P1-0010 and J1-8145) and EN-FIST Centre of Excellence.

P33

Structure And Allosteric Inhibition Of ERI3, A Nuclease Essential For Dengue Virus Replication

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Viruses that belong to the genus Flaviviridae include Dengue virus, West Nile virus, Zika virus, Japanese encephalitis virus, and yellow fever virus, amongst others. These viruses are responsible for widespread mortality throughout the world. The human host cell provides numerous proteins for flavivirus replication. Host proteins involved in viral replication are targets for antiviral therapy. One such factor is the protein encoded by the human ERI3 gene that belongs to the Enhanced RNAi (ERI) family of eukaryotic 3'-5' exonucleases. The Enhancer of RNAi (ERI) exoribonucleases form a distinct subfamily of DEDDh nucleases characterized by their ability to degrade structured RNA and DNA substrates and a strict conservation of functionally important active site residues. Although no function for ERI3 is known, human and mosquito ERI3 proteins have recently been implicated in Dengue virus replication and in accumulation of DENV-2 infectious particles by directly interacting with DENV-2 genomic RNA in the 3' untranslated region. We report crystal structures of ERI3 bound to rAMP and rCMP and Mg2+ at 1.8 Å resolution and describe its interaction with the DENV-2 RNA hairpin substrates using hybrid approaches that include NMR Spectroscopy and small-angle X-ray scattering. We used computational screening methodologies against several chemically diverse libraries combined with 19F-NMR fragment-based screening to identify compounds that selectively bind to an allosteric site on ERI3. Our structure-based lead compounds are being further optimized for the discovery of a selective inhibitor of ERI3 with possible clinical relevance in antiviral drug therapy.

P34

NMR as a key tool to develop small molecule binders of the RAL small GTPases

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The RAS subfamily of proteins are very well validated targets in cancer biology. The RAL (RAS-like) GTPase is structurally closely related to the RAS proteins. There are two isoforms RALA and RALB, both of which have been shown to play a role in human cancer. RAL is a downstream effector of RAS and therefore has the potential to offer an alternative way to disrupt RAS signalling. RAL signalling is achieved by exchange from the inactive GDP bound to GTP bound state which is mediated by guanine nucleotide exchange factors (GEFs). The activated RAL then signals by further interactions with effector proteins. Pre-clinical in vitro and in vivo genetic knockdown and deletion studies have demonstrated a clear role for RALA and B in tumour initiation, progression and metastasis. Although RAL mutations are uncommon, there is evidence of upregulation of active RAL in a number of different tumour types e.g. PDAC, metastatic bladder cancer, advanced prostate carcinoma. In this study we started with a fragment screen using NMR and SPR to discover new chemical entities that bind to RAL. The fragment starting points, which exhibited high millimolar affinities, have evolved and grown to deliver a set of molecules that exhibit low micromolar affinity for RAL. NMR has been a key method in the drug discovery process - CSP screening assays, Kd determination. The NMR data in combination with other biophysical techniques and biochemical assays has been a central driver to establish structure-activity relationships (SAR) to allow the medicinal chemistry to increase RAL affinity. This study demonstrates the impact NMR has made in a critical area of cancer drug discovery and how its interplay with crystallography, medicinal chemistry and biochemistry has developed promising compounds that aim to disrupt RAS signalling via targeting the RAL pathway.

P35

A Complete Pipeline for Enabling Efficient and Timely NMR Structural Biology on Challenging Pharmaceutical Targets in an Industrial Setting

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By now the power of structure-based drug design (SBDD) is so widely recognized that it has become essentially the de facto approach for target based, small molecule campaigns. NMR structural biology is indispensable for cases where crystallization fails, or where crystal contacts create artefactual binding sites. NMR can provide structural information at different levels of resolution, with a trade-off between the amount of information/ambiguity versus throughput. Combining NMR data (e.g. chemical shift perturbations and intermolecular NOEs) with docking approaches, provides invaluable structural information for medicinal chemistry efforts. However, NMR structural biology efforts on pharmaceutical targets are often hindered by a variety of challenges including: poor yields of recombinant protein, limited solubility or instability of the protein and long experimental and analysis time needed for NMR resonance assignment and structural information. ZoBio has been implementing and developing comprehensive strategies to enable efficient and timely NMR structural biology and has routinely obtained protein-ligand co-structures by combining sparse NOE data with data-driven docking. The impact of these strategies on enabling NMR structural biology on difficult targets will be highlighted with various examples.

P36

Effects of Excipients of Filgrastim Products on the Structure and Dynamics of the Drug Substance

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Filgrastim is the generic name for recombinant methionyl human granulocyte colony-stimulating factor. Filgrastim products are formulated as a low ionic strength solution containing cryoprotectant such as sorbitol, non-denaturing detergents, usually polysorbate-80, in a buffer at an unusually low pH of 4.0, or 5.0 in some case. It is well documented that the low pH stabilizes the protein fold and we have proposed the presence of a cation-p (pi) interaction as a major stabilizing interaction. In addition, there is a significant body of literature suggesting that interactions of excipient molecules with the API may provide benefits from a conformational stability aspect. In order to shed some light in this area, we have used NMR spectroscopy to probe the effects of the structure, via chemical shifts, and dynamics using relaxation measurements of filgrastim when subjected to various excipient conditions. The NMR results are analyzed in parallel with thermal unfolding studies using circular dichroism.

P37

NMR studies of liquid-liquid phase separation in antibody biopharmaceuticals

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Monoclonal antibodies (mAbs) and other biopharmaceutical proteins are the fastest growing sector of the pharmaceutical industry, representing increasingly important therapies in the treatment of a wide range of diseases. Administration of a high-concentration protein formulation (HCPF) (> 100 mg/ml mAb) with low total volume (< 1.5 ml) by the patient themselves is an attractive delivery strategy [1]. However, achieving such high protein concentrations during manufacturing can be difficult, whilst high concentrations may also lead to undesirable formulation characteristics, including aggregation, high viscosity, opalescence, and liquid-liquid phase separation (LLPS). During mAb LLPS, a medium-concentration solution separates into a low-concentration lean phase and a high-concentration dense phase, typically during refrigerated storage. LLPS is unacceptable in final biopharmaceutical formulations, and should be prevented through the use of excipients and optimisation of formulation. Conversely, LLPS may be a novel method to concentrate biopharmaceutical proteins and produce HCPFs by isolation of the dense phase.

In our work, we study mAb self-association and LLPS through NMR spectroscopy. Whilst isotopic labelling and multidimensional techniques are typically applied when studying large proteins (> 20 kDa) by NMR, these approaches are generally not feasible or practical in mAbs produced by mammalian cell culture on an industrial scale. However, as we previously showed, a surprisingly large amount of information can be ascertained about the behaviour and properties of 150 kDa mAbs and their formulations through 1H NMR using analysis of 1D spectra, transverse relaxation, and translational diffusion [2, 3]. Here, we further demonstrate possible applications of NMR in mAb biopharmaceuticals through the assessment of LLPS as a means of producing HCPFs. NMR reveals that mAb structure and behaviour is not irreversibly perturbed by LLPS, while a novel NMR method for assessment of mAb fragmentation is applied to assess the stability of HCPFs produced by LLPS. Ultimately, we demonstrate that LLPS can be used to produce high-concentration solutions of mAbs without compromising their structure and stability. We show that NMR spectroscopy is a valuable tool for characterisation of biopharmaceuticals even as large as mAbs.

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P38

NMR based drug design for Wnt signaling disease

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β-catenin is a key signaling protein which regulates cell signaling and gene transcription. Abnormal activation of β-catenin is linked to many cancers, particularly with colorectal cancer (CRC). RAS plays a critical roles in signal transduction from EGFR. The signal eventually reaches the nucleus and induces cell cycle and cell growth promotion. RAS is subjected to the regulation via the Wnt/β-catenin pathway. There have been many studies on β-catenin and RAS each, but their interaction has not been studied well since each of them has its own pathway. Here, we confirmed the binding of β-catenin and RAS through fluorescence experiment. To figure out the binding site of the β-catenin and RAS, we performed NMR titration experiments. We could identify the binding site by titration of β-catenin to RAS. NMR studies provide an insight on structure and interaction profiles of β-catenin and RAS. CRC generally occurs through the sequential genetic mutations of adenomatous polyposis coli (APC) and KRAS, which lead to abnormal activation of the Wnt/β-catenin and RAS/extracellular-signal-regulated kinase (ERK) pathways, respectively. When the Wnt/β-catenin signaling is negatively regulated, the RAS degradation arises together with the β-catenin degradation. The synergistic effect on tumor growth associated with mutations in both APC and KRAS is attributed to the stabilization of both β-catenin and oncogenic KRAS by APC loss. We screened a small-molecule library to identify compounds that destabilized both β-catenin and RAS proteins via inhibition of the Wnt/β-catenin pathway. The compound which is named KYA1797K efficiently destabilized β-catenin and RAS, and it reduced the proliferation of various CRC cells harboring APC and KRAS mutations. We confirmed the interaction between KYA1797K and regulators of the G-protein signaling (RGS) domain of axin using NMR titration experiments. KYA1797K enhanced formation of the β-catenin destruction complex and induced GSK3β activation, leading to phosphorylation of both β-catenin and KRAS at S33/S37/T41 and T144/T148, respectively. Phosphorylated β-catenin and KRAS were degraded via β-TrCP E3-linker-mediated proteasomal degradation. Our study provides a new small-molecule-based approach for treatment of CRC and other cancer types with activated both Wnt/β-catenin and RAS-ERK pathways via degradation of both β-catenin and RAS.

P39

Imatinib acts as an allosteric activator of Abl kinase

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Imatinib remains the first-line treatment for chronic myelogenous leukemia (CML) by targeting Bcr-Abl as a specific ATP-competitive inhibitor and its success has revolutionized targeted cancer therapy. Combining nuclear magnetic resonance (NMR) spectroscopy, x-ray crystallography, isothermal titration calorimetry (ITC) and biochemical assay, we show other than the ATP binding site, Imatinib binds specifically to a secondary, allosteric site in the myristate pocket of Abl with a sub-micromolar affinity and unexpectedly acts as an allosteric activator of Abl by preventing the αI-helix from forming a conformation which is required for assembly of Abl to its inhibited state. The findings suggest administration of high-dose of imatinib may result in poor inhibition of imatinib-resistant Abl mutants. Our studies highlight the benefit of combining imatinib with allosteric inhibitors to maximize the inhibitory effect on Bcr-Abl. We also describe an NMR-based method to distinguish myristate-site allosteric inhibitors from activators of Abl.

P40

NMR-based fragment screening to indentify new inhibitors of the chaperone HSP90

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The Heat Shock Protein 90 (Hsp90) is a well-studied yet poorly understood chaperone, essential for the viability of eukaryotic cells. Hsp90 targets partially folded clients and promote their interaction with diverse co-factors, allowing them to reach their stable, functionally active state. To perform this function, HSP90 interacts with many co-chaperones that modulate its activity by recruiting specific clients and by promoting distinct phases of its conformational cycle. Because of its role in the folding and activation of clients from crucial cellular pathway, Hsp90 is linked to numerous diseases such as cancers or neurodegenerative diseases. In human, two HSP90 isoforms co-exist in the cytoplasm, the beta isoform that is constitutively expressed and the alpha isoform, for which the expression is regulated by stress response elements and is thus overexpressed in pathology. The development of Hsp90 inhibitors has been pursued by pharmaceutical companies, with about fifty clinical trials started. However, no treatment has been approved due to strong side-effects on non-malignant cells, induced by disrupting the core chaperone cycle. We are working on the design of new Hsp90 inhibitors. Using an NMR-based fragment screening, we were able to identify nine new fragments that bind to the N-terminal domain of HSP90. Based on chemical shift perturbations, we determined that eight of them interact directly in the ATP-binding pocket, while one fragment also affects an areadistal to the ATP binding pocket. This effect might highlight an allosteric pathway between the ATP-binding site and another position on the NTD of HSP90. We are assessing effects of these compounds on ATPase activity and investigating the specificity of these inhibitors toward the two isoforms of HSP90, with the goal to develop isoform-specific inhibitors of HSP90.

P41

Expression and Purification of Isotopically Labeled Monoclonal Antibodies in Escherichia Coli for Heteronuclear Sequential Assignment by NMR

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Monoclonal antibodies (mAbs) are the fastest growing drug platform in the biopharmaceutical industry, and there is an increasing interest in the establishment of precise, yet practical methods, to assess mAb higher order structure (HOS), stability and dynamics. Two-dimensional (2D) NMR has emerged as a powerful tool for higher order structure (HOS) characterization and similarity assessment of mAbs at atomic resolution. To achieve the full potential of the NMR fingerprinting method, sequential assignment of the mAb spectra would be advantageous. However, NMR assignment of large proteins such as mAbs (150 kDa) typically require isotopic labeling, in particular perdeuteration, which can be expensive or in accessible when using mammalian cell lines as expression platforms. The most common and cost-efficient method to isotopically label proteins is to express them in Escherichia coli (E. coli) using minimal media composed of iso-

topically labeled components. Here we present a strategy for expression and purification of a mAb from an E. coli strain genetically engineered to form disulfide bonds in its cytoplasm using the NISTmAb sequence as a model. The resulting aglycosylated and unlabeled mAb, and the mAb singly or triply labeled with 13C, 15N, 2H are found to be well-folded and yield high quality heteronuclear NMR correlation data. Differences between mammalian-expressed and E. coli-expressed mAb were pin-pointed to the lack of glycosylation in the E.coli expressed protein. Isotopically labeled Fab and Fc fragment sequences from NISTmAb have been similarly expressed and purified. The HSQC subspectra of these Fab and Fc fragments overlay well with the intact mAb allowing heteronuclear backbone assignments made using these fragments to be mapped back on to the intact mAb.

P42

Solution NMR of Heptahelical Membrane Proteins

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We are interested in determining the structure and dynamics of G-protein coupled receptors (GPCRs) by solution NMR techniques, and to study their folding pathway. To this end we have further matured a thermostabilized version of the α1B-adrenergic receptor (α1B-AR), produced in Escherichia coli, using CHESS methodology [1] so that it is stable at 50°C for at least 3 weeks. I will show data of this GPCR incorporated in LMNG micelles, as well as in circular nanodiscs for both backbone as well as methyl groups, and discuss various approaches for obtaining structural and dynamical information. I will try to present a fair account of pros and cons for individual labeling methods and summarize performance of various NMR experiments on such systems. The particular variant of the α1B-AR is stabilized against the agonist prasozin – the apo form is much less stable. This particular mutants also binds the allosteric peptidic modulator ρ-Tia very well resulting in large change in the spectra. I will also show spectra of mutants in which the stabilizing mutations have been replaced by wild-type residues to access the effect of these replacements on protein stability. As a proof-of-concept my group has studied bacteriorhodopsin (bR) incorporated in nanodiscs. bR is produced as a fusion to mistic and refolded upon addition of retinal [2]. We have developed a strategy for obtaining extensive backbone assignments on uniformly labeled bR that uses a combination of TROSY-based triple-resonance NMR experiments, as well as biochemical and topological information. All this information is used as input for automatic assignment runs using the FLYA module of CYANA [3]. The aim of the study is to select for a set of experiments and to develop a strategy that works for such demanding cases. Individually amino-acid specifically labeled forms are prepared to validate those assignments.

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P43

A high-resolution description of the backbone dynamics of the β1-adrenergic receptor from NMR relaxation data

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G protein-coupled receptors (GPCRs) are physiologically important transmembrane signaling proteins that elicit intracellular responses upon binding of extracellular ligands. Recent breakthrough in crystallography has provided a wealth of static GPCR structures. However, dynamical information on the signaling process is scarce. We have recently shown that the GPCR response to various ligands can be followed from 1H-15N resonances at virtually any backbone site in a thermostabilized mutant of the turkey β1-adrenergic receptor (β1AR) [1]. Here we have obtained 15N relaxation rates on uniformly 15N/2H- and specifically 15N-valine-labeled β1AR, which provided global as well as local dynamical information. 15N R2 relaxation rates revealed significant conformational exchange in the micro- to millisecond time range throughout the receptor. We will discuss correlation between the observed dynamics, ligand binding and signal transmission.

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P44

Isotropic bicelles: new rim-forming agents, lipid phase transitions and application in the studies of single-pass membrane proteins by NMR in solution.

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Isotropic bicelles are a widely used membrane mimetic for solution NMR spectroscopy of membrane proteins. Bicelles are formed in the mixtures of lipids with surfactants and have a discoidal shape of particles. Despite the obvious power of bicelles in reproducing the various kinds of lipid environments, the vast majority of structural studies employ the single system, where the most convenient bilayer lipid - DMPC is mixed with the short-chain lipid DHPC. However, even for this system, the presence of the lipid bilayer in small bicelle particles was not confirmed. In the present study, we use NMR to investigate the behavior of various bicelle systems in order to establish the structure of particles and effects of the bicelle composition on the properties of transmembrane proteins. We developed an approach to verify the presence of lipid bilayer in the bicelle particles. The approach relies on the size on the lipid-to-detergent ratio dependence and detection of lipid phase transitions. The phase transitions could be clearly observed with the 31P NMR spectroscopy. Using the technique, we investigated many types of bicelles with different contents, varying both the lipid composition and the rim-forming agent. This study resulted in the bicelle parameters for an almost arbitrary composition of the lipid/detergent mixture. We show that isotropic bicelles, applicable for solution NMR spectroscopy, behave as predicted by the theoretical models, demonstrate the presence of lipid phase transitions and are likely to be bicelles rather than mixed micelles. We show that CHAPS is preferable as a bicelle rim-forming agent, and use of DH7PC should be avoided. According to our data, lipids with unsaturated fatty acids and ethanolamines are the unwanted components

of bicellar solutions and exclusively anionic lipids cannot be used to prepare isotropic bicelles, they should be taken in mixtures with zwitterionic ones. To avoid the denaturation of water-soluble domains, we suggested to use very mild Facade detergents as bicelle rim-forming agents and revealed that Facade-EM is the most prospective bicelle surfactant to study the membrane proteins with globular soluble domains. On the other hand, we demonstrate that change of the rim contents does not affect the structure of the transmembrane domain, but the dynamics and stability of the domain could be influenced substantially. Last, we developed an NMR-based approach to probe the effect of bilayer contents on the free energy of protein-protein interactions in bicelles. Using this technique, we investigated the dimerization of HER4 transmembrane segment in four different bilayers and showed that while the structure of the dimer of preserved, the free energy of the dimer is affected dramatically by the change in bilayer thickness. The work was supported by the Russian Science Foundation grant #14-14-00573.

P45

Molecular Mechanisms of Human GPCRs and Microbial Rhodopsins Uncovered by DNP-Enhanced SSNMR Spectroscopy

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Many membrane proteins are often important targets and/or research tools in drug discovery and life science. However, the high complexity and many intrinsic biochemical difficulties make these proteins challenging to study by conventional NMR spectroscopic methods. In this abstract, we would like to address the new opportunities in the investigations of the molecular mechanisms, in particular the functional regulations, of two major classes of seven transmembrane proteins, namely GPCRs and microbial rhodopsins, using dynamic nuclear polarization (DNP)-enhanced solid-state NMR (SSNMR) spectroscopy as the core technique. First we will demonstrate that DNP-enhanced SSNMR reveals the molecular mechanism of the subtype selectivity of peptide GPCRs, a long-last puzzle in the fields of biochemistry, pharmacology and drug discovery. We have focused on human bradykinin receptors (B1R and B2R) and have solved the atomic structures of the peptide ligand DAKD bound to B1R using nmol quantity of protein prepared from insect cell Sf9 expression system. High DNP enhancement of nearly 200 folds is essential for the success in detecting such low amount of protein-ligand complexes by SSNMR. A new approach integrating chemical-shift-based forward and backward conformation analysis has been developed for determining and for cross-validating the peptide structures. The integration of our NMR results with advanced molecular modeling and biochemical/pharmacology data provides a detailed picture that different structural factors and binding chemistry orchestrate in a complex fashion to cast the high peptide selectivity on these receptor subtypes. Second we will show that DNP supports a powerful SSNMR toolbox for investigating key functional aspects of microbial rhodopsins that are the most popular molecular tools in the field of optogenetics. In this abstract we will focus on the initial steps of photocycles of microbial rhodopsins, namely the light absorption and the retinal isomerization/K-state formation that are shared by all microbial rhodopsins despite their distinct functions and evolutionary pathways. We will showcase that by tailoring coherently the isotope labeling schemes and NMR methods, all residues of the retinal binding pocket in microbial rhodopsins such as in proteorhodopsins (PRs) can be probed. Furthermore, at cryogenic temperatures DNP-enhanced SSNMR permits the study of the key functional retinal moiety at the chemical resolution through CSA recoupling experiments. The NMR observations have been combined with QM/MM, MD and bioinformatics tools and the structural factors dictating the color tunability of microbial rhodopins have been uncovered. By further developing the heteronuclear NOE experiments of rotating methyl groups under DNP conditions, we have established new approaches for studying the structure and local dynamics of proteins. Based on these new methods an unexpected link between the structures, ligand dynamics and the initial modulations of the functional proton on the Schiff's base crossing different types of microbial rhodopsins has been discovered.

P46

Ligand modulation of sidechain dynamics in a wild-type human GPCR

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GPCRs regulate all aspects of human physiology, and biophysical studies have deepened our understanding of GPCR conformational regulation by different ligands. Yet there is no experimental evidence for how sidechain dynamics control allosteric transitions between GPCR conformations. To address this deficit, we generated samples of a wild-type GPCR (A2AR) that are deuterated apart from 1H/13C NMR probes at isoleucine $\delta 1$ methyl groups, which facilitated 1H/13C methyl TROSY NMR measurements with opposing ligands. Our data indicate that low [Na+] is required to allow large agonist-induced structural changes in A2AR, and that patterns of sidechain dynamics substantially differ between agonist (NECA) and inverse agonist (ZM241385) bound receptors, with the inverse agonist suppressing fast ps-ns timescale motions at the G protein binding site (1). In addition to A2AR, we have demonstrated the general feasibility of our approach by expressing and purifying other wild-type GPCRs labeled in the same manner from deuterated Pichia cultures (2). Our approach to GPCR NMR creates a framework for exploring how different regions of a receptor respond to different ligands or signaling proteins through modulation of fast ps-ns sidechain dynamics.

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Phosphorylation-induced conformation of β2-adrenoceptor related to arrestin recruitment revealed by NMR

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The C-terminal region of G protein-coupled receptors (GPCRs), stimulated by agonist binding, is phosphorylated by GPCR kinases, and the phosphorylated GPCRs bind to arrestin, leading to the cellular responses. To understand the mechanism underlying the formation of the phosphorylated GPCR–arrestin complex, we performed NMR analyses of the phosphorylated β2-adrenoceptor (β2AR) and the phosphorylated β2AR–β-arrestin 1 complex, in the lipid bilayers of nanodisc. Here we show that the phosphorylated C-terminal region adheres to either the intracellular side of the transmembrane region or lipids, and that the phosphorylation of the C-terminal region allosterically alters the conformation around M215 and M279, located on transemembrane helices 5 and 6, respectively. In addition, we found that the conformation induced by the phosphorylation is similar to that corresponding to the β-arrestin-bound state. The phosphorylation-induced structures revealed in this study propose a conserved structural motif of GPCRs that enables β-arrestin to recognize dozens of GPCRs.

P48

Formation of the beta-barrel assembly machinery complex in lipid bilayers as seen by solid-state NMR

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The beta-barrel assembly machinery (BAM) is a complex of five proteins (BamA–E), which catalyzes the insertion of beta-barrel proteins into the outer membrane of E.coli, an essential process for the viability of the cell. Thus far, a detailed understanding of the underlying insertion mechanism has been elusive but recent results suggest that local protein motion, in addition to the surrounding membrane environment, may be of critical relevance. We have devised a tailored high-sensitivity solid-state NMR approach to directly probe protein motion and the structural changes associated with BAM complex assembly in lipid bilayers. Our results reveal how essential BamA domains, such as the interface formed by the polypeptide transport associated domains, P4 and P5, become stabilized after complex formation and suggest that BamA beta-barrel opening and P5 reorientation is directly related to complex formation in membranes. Both the lateral gate, as well as P5, exhibit local dynamics, a property that could play an integral role in substrate recognition and insertion.

P49

Combinatorial Selective Labeling in Studies of Membrane Proteins: Mapping of Binding Interface of Spider Toxin with Isolated Voltage-Sensing Domain of Sodium Channel

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Voltage-gated Na+ (NaV) channels contain domains that have discrete functionalities. The central pore domain allows current flow and provides ion selectivity, whereas peripherally located four voltage-sensing domains (VSD-I/IV) are needed for voltage-dependent gating. Certain mutations trigger a leak current through VSDs leading to various diseases. For example, hypokalemic periodic paralysis (HypoPP) type 2 is caused by mutations in the S4 voltage-sensing segments of VSDs in the skeletal muscle channel NaV1.4. The gating modifier toxin Hm-3 (crab spider Heriaeus melloteei) inhibits leak currents through such mutant channels and represents useful hit for HypoPP therapy [1]. To study molecular basis of Hm-3 interaction with the VSD-I of NaV1.4 channel, we expressed the isolated domain (150 a.a., four transmembrane helices, S1-S4) in the cell-free system. Mixed micelles of zwitterionic detergents (DPC/LDAO 1:1) provided optimal conditions for NMR study. The limited stability of the VSD-I samples in this milieu (half-life time of 24 h at 45 °C) prevents usage of classical assignment approach based on the 3D triple-resonance (1H-13C-15N) experiments. Therefore we used combinatorial selective labeling (CSL) and extracted sequence-specific information from the 2D TROSY and HNCO spectra measured for several selectively 13C,15N-labeled samples. The price-optimal combinatorial selective labeling (CSL) scheme was calculated using new deterministic CombLabel algorithm. CSL provided straightforward assignment for 50% of VSD-I backbone resonances. That permitted to characterize the secondary structure and backbone dynamics of VSD-I in micellar environment. The tertiary structure of the domain was characterized by paramagnetic relaxation enhancement data. NMR data show that Hm-3 partitions into micelles through a hydrophobic cluster formed by aromatic residues and reveal complex formation with VSD-I through electrostatic and hydrophobic interactions with the S3b helix and the S3–S4 extracellular loop. Two different hydrophobic interfaces on the Hm-3 surface are responsible for the interactions with the micelle and VSD-I. The model of the Hm-3/VSD-I complex was built using protein-protein docking guided by NMR restrains. Our data identify VSD-I as a novel specific binding site for neurotoxins on sodium channels [1]. To best of our knowledge our report is the first NMR study of structural interactions between toxins and NaV channels. Work was funded by the Russian Science Foundation (#16-14-10338).

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P50

Deuteration and selective labeling of alanine methyl groups of β2-adrenergic receptor expressed in a baculovirus-insect cell expression system

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G protein-coupled receptors (GPCRs) exist in equilibrium between multiple conformations, and their populations and exchange rates determine their functions. However, analyses of the conformational dynamics of GPCRs in lipid bilayers are still challenging, because methods for observations of NMR signals of large proteins expressed in a baculovirus-insect cell expression system (BVES) are limited. Here, we report a method to incorporate methyl-13C1H3-labeled alanine with >45% efficiency in highly deuterated proteins expressed in BVES. Application of the method to the NMR observations of β2-adrenergic receptor in micelles and in nanodiscs revealed the ligand-induced conformational differences throughout the transmembrane region of the GPCR.

P51

Force from polyunsaturated lipids modulates gating of mechanosensitive channels

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Polyunsaturated fatty acids have pronounced effects on membrane proteins, as they modulate important physical properties of the lipid bilayer such as the lateral pressure profile. To understand the biophysical basis of this influence, we studied how polyunsaturated lipids modulate the gating behaviour of the mechanosensitive channels MscL and MscS. We found two distinct effects on the gating of these channels. Whilst MscL responds primarily to membrane thinning induced by polyunsaturated lipids, MscS is modulated by the change of lateral pressure caused by the unsaturation. To characterize the change of mechanical properties of these lipids, which are responsible for the observed modulation of the channel gating, we employed solid state 13C NMR on oriented membranes. Segmental order parameters for up to threefold polyunsaturated lipids were obtained from 13C-1H dipolar couplings measured using separated local field experiments. Using a dedicated geometrical model, we were able to determine the membrane thickness and lateral pressure profile of bilayers of polyunsaturated lipids, and correlate these membrane properties with the observed effect on mechanosensitive channels.

P52

Structural Mechanism of Antagonist Tolerance in GPCRs.

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G protein coupled receptors (GPCRs) play a major role in human health and disease, and subsequently account for an estimated 35% of all drug targets. Often, drugs that target GPCRs become less potent after repeated administration. This phenomenon is called tolerance. Understanding the mechanism of tolerance remains the focus of intense investigation. So far, studies have highlighted altered receptor concentration on the cell surface and reduced receptor occupancy by the drug as possible mechanisms. To understand how tolerance develops, we investigated AMD3100, the only FDA-approved antagonist of the chemokine receptor CXCR4. Tolerance to AMD3100 decreases the ability of the drug to release leukemic blasts from the bone marrow into the peripheral blood and to sensitize them to chemotherapy. After prolonged treatment with the drug, leukemic blasts increase CXCR4 on the cell surface and rehome to the bone marrow where they are protected from killing by chemotherapy. AMD3100 inhibits CXCR4 G protein signaling and β-arrestin1/2-dependent receptor endocytosis. Here, we show that inhibition of CXCR4 endocytosis is sufficient to increase the receptor concentration on the cell surface and to cause tolerance to AMD3100. At high concentrations, CXCR4 clusters, forming oligomeric assemblies. Intermolecular contacts within these assemblies allosterically change the structure of CXCR4 monomeric subunits and alter the binding site for AMD3100. This reduces affinity of the drug for the receptor, providing a novel mechanism for the development of antagonist tolerance.

P53

Paramagnetic NMR Finds the Collagen-Digesting Protease MT1-MMP to Bind Membranes by Opposite Tips of its β-Propeller

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Critical to migration of tumor cells and endothelial cells is the proteolytic attack of membrane type 1 matrix metalloproteinase (MT1-MMP) upon collagen, growth factors, and receptors at cell surfaces. Lipid bilayer interactions of the substrate-binding hemopexin-like (HPX) domain of MT1-MMP were investigated by paramagnetic NMR relaxation enhancements (PREs), fluorescence, and mutagenesis. The HPX domain binds bilayers by blades II and IV on opposite sides of its β-propeller fold. The EPGYPK sequence protruding from both blades inserts among phospholipid head groups in PRE-restrained molecular dynamics simulations. Basic side chains nearby in the interface are drawn to phosphoesters of the head groups. Bilayer binding to either blade II or IV exposes the CD44 binding site in blade I. Bilayer association with blade IV allows the collagen triple-helix to bind without obstruction. Indeed, vesicles enhance proteolysis of collagen triple-helical substrates by the ectodomain of MT1-MMP. Hypothesized side-by-side MT1-MMP homodimerization would allow binding of bilayers, collagen, CD44, and head-to-tail oligomerization. This work was supported by NIH grant R01 CA098799.

P54

Role of oligomerisation in CLIC-1 membrane insertion

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The Chloride Intracellular Channel (CLICs) proteins consist of a family of metamorphic proteins that exist in an equilibrium between a soluble and a membrane-bound state. The alteration of CLIC function has been involved in ischemia-reperfusion and different forms of cancer. CLIC1 has been directly linked with glioblastoma proliferative capacity. It can be found as a chloride channel or as a soluble reduced form with oxidoreductase activity. CLIC1 inhibitors block both its ion channel function and oxidoreductase activity. However, it is unknown what form or forms of the protein are relevant in the context of healthy and glioblastoma cells and how the equilibrium between them is affected in disease. In order to explore the mechanism of channel formation, we have studied CLIC1 membrane insertion, as well as its structure and oligomerisation states in solution. We have found that CLIC1 inserts in the membrane, but pH nor oxidation have any effect as previously reported. Our NMR data suggests that CLIC1 maintains the same structure in reducing and non-reducing conditions. However NMR, gel filtration and native MS indicate that CLIC1 exists in an equilibrium of monomers, dimers, tetramers and dodecamers in solution. Fittings of the X-ray structure to the SAXS envelop and NMR chemical shifts indicate that the non-covalent association of the dimers does occurs differently to the previously reported oxidised form. Taken together, our data suggests that CLIC1 exists as a mixture of oligomeric species in solution, and that oligomerisation occurs prior to the membrane insertion.

P55

An Ensemble Description of GPCR Allostery

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Thirty-four percent of current pharmaceuticals target G protein-coupled receptors (GPCRs), a class of membrane proteins that regulate diverse biological processes including sensory perception, hormonal regulation, and immune response. Structurally, GPCRs exist in a dynamic ensemble of conformations spanning inactive, intermediate, and active states capable of engaging G proteins. The adenosine A2A receptor (A2AR) is a prototypical GPCR and a drug target for the treatment of inflammation, cancer, diabetes, and Parkinson's disease. While X-ray crystal structures reveal only an inactive and an active conformation, nuclear magnetic resonance (NMR) spectroscopy show that detergent-reconstituted A2AR exhibits at least four functional states spanning inactive, intermediate, and active states capable of engaging G proteins. Though mechanistically poorly understood, the lipid bilayer plays an important role in GPCR regulation. Several GPCRs are known to require membrane cholesterol for function, including A2AR. Here, we employ fluorine (19F) NMR to characterize A2AR activity and drug-response in a synthetic lipid bilayer (nanodisc). We show that cholesterol is an allosteric effector of A2AR through modulation of its functional states. This knowledge furthers our understanding of GPCR allostery and protein-membrane interactions.

P56

Mistic - Interaction studies in native E. coli membranes using 400 and 800 MHz DNP

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Mistic is an α-helical self-inserting integral membrane protein of B. subtilis believed to trigger biofilm formation together with YugO, a K+-channel located just downstream in the mstX-yugO two-gene operon[1]. Due to its self-inserting properties Mistic attracted attention as a fusion tag to support production and incorporation of recombinant membrane proteins into lipid bilayers[2]. NMR studies revealed that Mistic interacts tightly with detergent[3]. However, it has an unusual polar surface with a net charge of -12 at pH 7 when solubilised with detergent. Consequently, Mistic seems to combine features of soluble as well as of typical integral membrane proteins[3]. Surprisingly, it was shown that polar interactions with the headgroup of anionic and zwitterionic detergents overcome the hydrophobic effect in stabilizing Mistic[4,5]. Nevertheless, the aliphatic chain length of detergents still is of importance as shown with non-ionic detergents[4,5].

To shed light on this unusual behavior we carry out 400 MHz and 800 MHz DNP solid state NMR experiments on isolated E. coli membranes containing selectively 13C-, 15N-labeled Mistic. We aim to look at the boundaries between loops and secondary structure regions to characterize the membrane topology of Mistic. Further, we hope to understand the importance of polar contacts in membrane binding. Several radicals are compared at 400 and 800 MHz (i.e, AMUpol shows an enhancement of about 80 and 8–10, respectively). We show the beneficial effect of the increased resolution at 800 MHz yielding surprisingly well-resolved 13C-15N correlations, allowing sequential assignment – which is in progress. First results demonstrate that Mistic is present in a soluble and a membrane bound state. When bound to membranes, Mistic prefers membranes with a high fraction of ionic POPG, supporting the importance of polar contacts in membranes. With our studies we aim to improve our understanding of how membrane proteins autonomously fold and confirm the structure of Mistic in an aqueous environment, in lipid bilayers, as well as its topology. We demonstrate that with DNP it is possible to study membrane proteins in their native environment.

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P57

NMR structural studies of human transmembrane proteins associated with human diseases

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The biological functions of human transmembrane proteins (hTMPs) are involved in a variety of processes, from basic life functions such as en-

ergy production to cell adhesion, communication and synaptic transmission. Also, since about 25-30% of the coded proteins of the human genome have been identified to encode hTMPs, they have become important targets for related clinical drugs. The starting point for understanding and studying the specific biochemical processes of hTMPs is to determine their three-dimensional structure. However, due to their inherent hydrophobicity, it is difficult to obtain a sufficient amount of protein, and because of the technical difficulties of extraction and purification, the number of membrane proteins with known structure is very small. Nonetheless, the study of the structure of these human transmembrane proteins has been extensively studied because it not only helps us understand our body systems, but also forms the foundation for new drugs development. We obtained sufficient amounts of proteins by overexpression in E. coli strains and various biophysical methods for structural studies using the NMR of the transmembrane domain of two hTMPs related to human disease. One is the transmembrane domain of amyloid precursor proteins (APP-TM) associated with Alzheimer's disease. There are several mechanisms that cause neurodegeneration, but what we are interested in is the formation of amyloid channels that cause side effects such as an abnormal increase in intracellular calcium concentration. In other words, APP-TM forms a Ca2+ permeable ion channel in the cell membrane, destroying calcium homeostasis in normal cells, and such amyloid channels are also found in other amyloidogenic disease-related proteins such as prions and α-synuclein. The other is Syndecan-4 (Syd4), which is involved in signal transduction and cancer progression. Syndecan-4 interacts with a variety of binding partners to increase wound healing, cell penetration and survival, activate adhesion, induce phosphorylation, and play an important role in cancer progression. Syd4 comprises large extracellular (ecto-), single transmembrane (TM) and short cytoplasmic (Cyto-) domains and acts as a diverse receptor when dimerized by highly conserved GXXXG motifs present in TM. The main process of action mechanism of Syd4 is the signal transmission through structural changes by ligand binding, so it is important to examine the three-dimensional structure in order to understand this process. High-purity protein samples obtained from optimized expression and purification processes have been characterized using various analytical techniques and are undergoing structural studies using solution/ solid-state NMR spectroscopy and computer modeling.

P58

Investigating Transport Mechanisms in TonB Dependent Transporter

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FhuA is an outer membrane protein of Escherichia coli, which belongs to TonB-dependent transporters (TBDTs) family. It primarily acts as a transporter of ferrichrome in order to provide supplement of iron in E.coli. However, it also can act as a transporter of colicin M, microcin 25, albomycin, and rifamycin CGP 4832. Furthermore, it serves as receptor for the phages T1, T5, 80, and UC-1. This makes it unique candidate for studying diverse functionality at molecular level. Alike all other TBDTs, FhuA structure is comprised of a beta barrel and a plug domain concealing the lumen from opening a passage. While transporting, a signal indicating transporter occupancy is transmitted across the outer membrane to energy-transducing and regulatory proteins such as TonB-ExbB-ExbD embedded in the cytoplasmic membrane. Crystal structures of FhuA bound to its substrates reveals closely related structures. They demonstrate that despite of substantial conformational changes upon substrate binding the plug domain seals the pore. However, mutational and deletions studies in these regions showed no alterations in its TonB dependency. Moreover, translocation of large molecules like colicin M (diameter of 3nm by 4nm) is not possible even the entire plug is removed. This indicates involvement of a unique structural, dynamic and allosteric mechanism for the translocation that is not yet revealed. Here we use solution NMR as the primary tool coupled with other bio-physical techniques to investigate the structural basis of this mechanism.

P59

Structural studies on dopamine 4 receptor with partner proteins

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Since GPCRs involve in key physiological and sensory processes in humans, those are prominent drug targets. Despite the fact that various GPCR structures have been identified, the key information about the unique ligand binding site possessed by each GPCR molecule are different, and there is a need for additional structural information in the field of drug development targeting GPCRs. Human dopamine receptor 4 (hD4R) is a member of G-protein coupled receptor (GPCR) A families, consisting of seven transmembrane domains and is related to neuronal and psychological condition of human such as addiction, bipolar disorder, schizophrenia and Parkinson's disease. Although numerous reports have addressed the biological properties on D4R and inactive structural information of D4R with antagonist, insights from detailed biophysical and structural investigations on D4R complex with interacting proteins or its active structure with agonist are mandatory. Therefore, we performed interaction studies of E3 ligase and arrestin-2 with hD4R by using NMR spectroscopy and found some specific interaction with them. In addition, we monitored the conformational change on active and inactive state of D4R interacting with agonist and antagonist by using hydrogen-deuterium exchange Mass Spectrometry (HDX-MS). These results provide information on the implication of complex with interacting ligands and proteins as well as on the conformational changes related to these interactions.

P60

Deuterated LMNG detergent for NMR studies of membrane proteins

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Examining the structure, dynamics, and interactions of membrane proteins by NMR is expedited by deuteration of the detergent or lipid that is most suitable for solubilizing a given protein. Maltose neopentyl glycol (MNG) detergents have lately become a mainstay of membrane protein studies and have been instrumental in the X-ray structure determination of a number of GPCRs. Here we report the synthesis of lauryl MNG (LMNG) deuterated at the decyl tails, designed to enhance the methyl region of protein NMR spectra. We compare the spectra of a membrane protein solubilized in the natural abundance and deuterated detergent.

P61

<sup>13</sub>C solid-state NMR signal assignment and structural stability of c-ring of F_oF₁-ATP synthase in lipid membranes

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F_{o1}-ATP synthase catalyzes ATP hydrolysis/synthesis coupled with a transmembrane H^+ translocation in membranes. The F_oc-subunit ring plays a major role in this reaction. We have developed an assignment strategy for solid-state ^{13}C NMR (ssNMR) signals of the F_oc-subunit ring of thermophilic *Bacillus* PS3 (TF_oc-ring, 72 residues), carrying one of the basic folds of membrane proteins. In a ssNMR spectrum of uniformly ^{13}C -labeled sample, the signal overlap has been a major bottleneck because most amino acid residues are hydrophobic. We propose a strategy for a full set of resonance assignments of membrane proteins using reverse-labeling of selective combinations of amino acids. To overcome signal overlapping, we developed a method designated as COmplementary Sequential assignment with MInimum Labeling Ensemble (COSMILE). According to this method, we generated three kinds of reverse-labeled samples to get rid of signal overlap. For an efficient sequential assignment, the stretches of three samples should be complementary. To assign the carbon signals sequentially, two-dimensional (2D) inter-residue C^{i+1}-C^i correlation and dipolar assisted rotational resonance (DARR) experiments were performed under magic-angle sample spinning (MAS). On the basis of inter- and intra-residue ^{13}C-^{13}C chemical shift correlations, 97% of C^α, 97% of C^β, and 92% of C' signals were directly assigned. On the basis of assignment, a hairpin fold of two helices with a central loop was predicted. The effects of saturated and unsaturated lipids on the TF F_oc-ring structure were examined. The DARR spectra are essentially similar to each other in saturated and unsaturated lipid membranes, suggesting that TF F_oc-rings have similar structures under the different environments. The spectrum of the sample in saturated lipid membranes showed better resolution and structural stability in the gel state. The unsaturated lipid-protein interaction seems stronger in terms of the effect on the structure.

P62

Novel insights into Munc18-SNARE interactions from NMR analyses

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Neuronal exocytosis is mediated by membrane-bridging complexes formed by the SNARE proteins: syntaxin-1, SNAP-25 and synaptobrevin. For efficient assembly, the SNAREs interact with additional proteins such as Munc18 and Munc13, but neither the nature of the intermediates nor the sequence of SNARE complex assembly is known. We focus on the cellular machinery that orchestrates assembly and disassembly of SNARE complexes, thereby tightly regulating the process of membrane fusion in neurotransmitter release. The precise functions of Munc18 have been difficult to define, as it seems to have both inhibitory and stimulatory roles in membrane fusion. Munc18 binds to syntaxin-1 folded into a self-inhibited closed conformation that involves intramolecular binding of its N-terminal Habc domain to its SNARE motif. The closed state of syntaxin-1 is inhibited from assembling it into the SNARE complex, and its opening is mediated by Munc13. In addition to locking syntaxin-1 in an inhibited form, Munc18 was recently shown to bind to synaptobrevin, supporting a model whereby Munc18 provides a template for the formation of the SNARE complex. Using heteronuclear NMR experiments and in vitro reconstitution assay of vesicle fusion, we are trying to elucidate how Munc18 controls SNARE complex assembly and neurotransmitter release. Our results suggest that the starting point for SNARE complex formation is the Munc18-syntaxin complex, as membrane fusion is abrogated in the absence of Munc18. Upon opening o syntaxin-1, which may occur concomitantly with binding of synaptobrevin to Munc18, the syntaxin-1 and synaptobrevin SNARE motifs can interact to initiate SNARE complex assembly. In our recent experiments we have investigated potential interactions of Munc18 with the Habc domain of syntaxin-1 in its open state. Our preliminary data suggest that Munc18 interacts with the Habc domain in a different mode than involving the closed conformation. Additionally, this new mode of interaction appears to be modulated by the presence of synaptobrevin, which could be a key feature of the sequence of events that lead to SNARE complex formation.

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Membrane Protein Structure Determination from Limited Paramagnetic NMR Data

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Membrane proteins (MPs) represent 30% of the human genome but only 3% of protein structures deposited in the Protein Data Bank (PDB) are MPs. This is problematic because more than half of drugs used on the market target some type of MP. Consequently, we lack a large chunk of MP structural information that, if available, would provide novel platforms for designing new drug candidates for implications in the clinic. Most protein structures available in the PDB have been determined by traditional methods such as X-ray crystallography or NMR. Although extraordinary progress has been made in the crystallization of MPs, generating high resolution three-dimensional MP crystals still remains challenging. This is largely due to the fact that MPs must be associated with a membrane mimetic such as detergent micelles. Furthermore, even if crystallization for a MP is achieved, the introduction of crystallization aides such as protein chaperones, stabilizing mutations or the presence of a non-native mimetic perturbs the overall biological relevance. NMR is a great alternative for MP structure determination but also has its limitations. Advancements to overcome this problem in the field have been substantial and include the 1) transverse relaxation optimized spectroscopy (TROSY) experiment and 2) perdeuteration during MP sample preparation. Even so, conventional NMR nuclear overhauser effect (NOE) restraints that are typically used to define protein structure are unattainable because helix-helix distances for MPs are typically >6 Å, which is outside the measurable NOE range. Here, we present an experimental strategy to overcome these challenges using limited paramagnetic NMR datasets. Simultaneous access to several long-range distance and orientational restraints (PCSs, RDCs and PREs) by NMR through a paramagnetic tagging strategy is used to drive structure prediction in ROSETTA. The Disulfide Bond Formation B (DsbB) E. Coli MP is used as a model system to demonstrate this experimental approach. DsbB plays an essential role in catalyzing the formation of disulfide bonds to ensure proper protein folding, function and stability. In order to generate limited NMR datasets for DsbB structure determination, paramagnetic lanthanide ion tags carrying an unpaired electron are attached to DsbB to give rise to orientation and distance restraints. These paramagnetic tags are attached to the protein at specific residue locations by using a copper catalyzed click chemistry reaction between the tag and an engineered

unnatural amino acid (UAA), p-Azido-L-phenylalanine (pAzF). Limited NMR datasets for different UAA-DsbB mutants are used to drive structure prediction in the ROSETTA biomolecular software package. This experimental and computational framework is also being used to probe structure perturbations of DsbB in different membrane mimetics.

P64

Toward the Structure of Synaptotagmin-1 Bound to Membrane Anchored SNARE Complex

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In neurons, calcium influx at the presynaptic terminal triggers neurotransmitter release within a few hundred microseconds. Synaptotagmin-1 is the calcium sensor responsible for this speed and precision of synchronous NT release. The mechanism of how Synaptotagmin-1 triggers membrane fusion after sensing calcium is poorly understood. Investigating the interaction of Synaptotagmin-1 with the membrane fusion machinery, particularly the SNARE complex, will provide important insights into the mechanism of calcium triggered NT release. Attempts to understand these interactions have resulted in multiple structures of Synaptotagmin-1 bound to SNARE Complex with distinct binding interfaces. Although these studies are instrumental in beginning to formulate models for calcium triggered neurotransmitter release, they only provide an incomplete picture and many important questions still remain unanswered. Studying the interaction between Synaptotagmin-1 and SNARE complex in a more physiological context, i.e between membranes is necessary to truly understand how synaptotagmin-1 binds to the SNAREs and together they trigger neurotransmitter release upon calcium influx. To study these interaction in a membrane environment, we are using a combination of nanodisc technology and lanthanide induced pseudo contact shifts based NMR methods.

P65

Dynamic properties of transmembrane helices as substrates of intramembrane proteases

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Intramembrane proteolysis is a conserved mechanism that regulates a variety of cellular processes ranging from transcription control to signaling. Intramembrane proteases affect a wide range of important biological functions, and are implicated in several severe diseases, including Alzheimer's disease. Cleavage of the C99 fragment of amyloid precursor protein (APP) at alternative positions in the TM helix by γ-secretase is considered as one of the triggers that lead to the development of Alzheimer's disease. As no specific consensus sequence has been detected for γ-secretase with more than 90 known substrates, dynamic parts within the TM helix are discussed as potential recognition sites. Cleavage is believed to be initiated at alternative sites that leads to release of the most abundant Aβ40 and the minor Aβ42 and Aβ38 peptides plus minor species. Numerous mutations within C99-TM domain are known to increase the Aβ42/40 ratio often leading to early onset Alzheimer's disease. Many of them appear to change conformational flexibility of substrate TM helices. Here we compare the TM helix of APP to that of two single point mutations by liquid state NMR spectroscopy, disrupting a double Gly37Gly38 motif in the center of C99-TM that has been postulated to control TM dynamics. We exchanged Gly38 against proline or leucine and measured the structural and dynamic properties by relaxation dispersion and H/D exchange. The investigated mutations had subtle effects on helix structure and dynamics but no impact on residues at the initial cleavage site. Exchanging glycine against leucine leads to a more stable helix whereas proline reduces helicity indicating that local changes translate into altered dynamics distributed among many residues. Subtle changes of H-bond flexibility at one site induce differences in the cooperative helix dynamics and, as a consequence, lead to altered presentation of the cleavage sites to the enzyme's active site, thus modifying position and/or efficiency of initial cleavage.

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P66

Structure and dynamics of the CheY response regulators from Rhodobacter sphaeroides

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The chemotaxis signalling network of E. coli. depends on autophosphorylation of a histidine protein kinase (HPK) in response to a signal from a sensor domain, with subsequent transfer of the phosphoryl group to an aspartate on response regulator (RR) proteins that bind to the flagellar motor and alter its rotation. CheY is a 14kDa single domain RR that is conserved across motile species. It is formed by 5 α-helices and 5 β-strands surrounding a conserved phosphoryl accepting aspartate residue, and once phosphorylated diffuses to the flagellar motor, binding to its FliM component to cause switching of rotational direction. The photosynthetic bacterium Rhodobacter sphaeroides has multiple chemosensory pathways formed by homologues of the E. coli chemosensory proteins. It has six CheY homologues with different effects on chemotaxis. Only CheY6 is able to stop the flagellar motor but either CheY3 or CheY4 are also required for chemotaxis.

NMR and computational methods have been used to answer questions about the structure, dynamics and function of two of these CheY's, CheY3 and CheY6. NOEs, chemical shifts and residual dipolar couplings are used to define the structures of CheY3 and CheY6 in their inactive and active states, where phosphorylation is mimicked using BeF3-. We have investigated fast timescale backbone dynamics using the {1H}-15N heteronuclear NOE and have used CPMG relaxation dispersion experiments to detect low populations of alternative conformations. CheY6 differs from the other R. sphaeroides CheYs and E. coli CheY by the insertion of a ten-residue loop before the C-terminal helix. We have deleted this loop region from CheY6 in order to determine, using in vivo and in vitro assays, if it plays a role in the unique function of CheY6 in R. sphaeroides.

P67

Structural basis of the signal transduction via transmembrane domain of type I receptors studied by high-resolution NMR

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Signal transduction by type I receptors, such as receptor tyrosine kinases (RTK) and cytokine receptors (utilizing Janus kinase), has been in the spotlight of scientific interest owing to the central role of these single-spanning membrane receptors in the regulation of development, cell motility, proliferation, differentiation, and apoptosis. The human epidermal growth factor receptor (EGFR) and growth hormone receptor (GHR) serve as excellent models of type I receptor to illustrate how ligand-induced conformational rearrangements and specific dimerization of extracellular domains lead to the allosteric activation of the cytoplasmic domains, resulting in signal propagation across the membrane. Dysregulated signaling from these receptors has been shown to play significant roles in promotion of number of human diseases, and inhibitors of these receptors have been among the most successful examples of targeted therapies to date. Many essential aspects of EGFR and GHR signal transduction at the molecular level have been elucidated lately. Nevertheless, there are several issues yet to be resolved, including the particular role of single-span helical transmembrane domain (TMD) and flexible juxtamembrane regions in the receptor activity switching in terms of an apparent loose coupling between structural rearrangements of the extracellular and intracellular regions. We experimentally determined the alternative dimeric conformations of the EGFR and GHR TMDs in different membrane-mimicking environments using high-resolution NMR spectroscopy combined with MD-relaxation in explicit lipid bilayer. Based on the available mutagenesis data, observed conformations correspond to the dormant and active states of both receptors. Fine adaptation of intermolecular polar and hydrophobic contacts that we found to accompany the different EGFR TMD dimerizations (observed in detergent micelles or in lipid bicelles) suggests that certain membrane properties can govern the TMD helix-helix packing and, thus, their alteration can trigger the receptor state. Whereas two distinct dimeric modes of GHR TMD (coexisting in micellar environment) revealed the functional role of juxtamembrane region rearrangements in alternation between protein-protein and protein-lipid interactions that can be initiated by ligand binding. Observed the TMD helix-helix packing diversity appears in favor of the lipid-mediated rotation-coupled activation mechanism, which implies that the sequence of structural rearrangements of EGFR and GHR domains is associated with perturbations of the lipid bilayer in the course of ligand-induced receptor activation, considering the receptor together with its lipid environment as a self-consistent signal transduction system. The work is supported by the Russian Science Foundation, grant #14-50-00131.

P68

ATP site ligands determine the assembly state of the Abelson kinase regulatory core via the activation loop conformation

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Abelson kinase (c-Abl) is a nonreceptor tyrosine kinase implicated in many cellular processes such as proliferation, division, survival, DNA repair and migration (Greuber et al., Nat. Rev. Cancer, 2013). The regulatory core of c-Abl consists of the SH3 and SH2 domains, followed by the kinase domain N- and C-lobes. This SH3-SH2-KD domain order is conserved among the Abl, Src, Csk, Brk, and Tec non-receptor tyrosine kinase families. Under physiological conditions, c-Abl is strongly down-regulated by the docking of the SH3 and SH2 domains to the kinase N- and C-lobes, referred to as the assembled state of the core. Abnormal chromosome translocation leads to the deregulated Bcr–Abl fusion protein and in consequence to Chronic Myeloid Leukemia, against which ATP-competitive and allosteric inhibitors have been developed. We have previously shown that the regulatory core of c-Abl adopts the assembled (inactive) or a highly mobile, disassembled (active) conformation depending on the binding of various ATP-site and allosteric inhibitors (Skora et al., Proc. Natl. Acad. Sci. USA, 2013). We have now established that the core assembly state strictly correlates with the conformation of the kinase activation loop induced by a total of 14 ATP site ligands, comprising all FDA-approved Bcr-Abl inhibiting drugs. The results show that the inactive A-loop conformation induced by type II inhibitors leads to the disassembly of the core, whereas ATP site ligands with active A-loop conformations preserve the assembled core. An analysis of all available Abl crystal structures reveals that the type II inhibitors induce a small but consistently observed push of the KD N-lobe via the A-loop and the P-loop towards the SH3 domain. A similar sized P-loop motion is expected during nucleotide binding and release, which would be impeded in the assembled state, in agreement with its strongly reduced kinase activity (Sonti et al., J. Am. Chem. Soc, 2018).

P69

Conformational sampling of the intrinsically disordered C-terminal tail of DERA is important for enzyme catalysis

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2-Deoxyribose-5-phosphate aldolase (DERA) catalyzes the reversible conversion of acetaldehyde and glyceraldehyde-3-phosphate into deoxyribose-5-phosphate. DERA is used as an industrial biocatalyst for synthesis of

drugs like statins and is a promising pharmaceutical target due to its involvement in nucleotide catabolism. Despite several biochemical studies suggesting the catalytic importance of C-terminal tyrosine residue found in several bacterial DERAs, the structural and functional basis of its participation in catalysis remains elusive because the electron density for the last 8 to 9 residues (i.e., the C-terminal tail) is absent in all available crystal structures. Using a combination of NMR spectroscopy and molecular dynamics simulations, we conclusively show that the rarely studied C-terminal tail of E. coli DERA (ecDERA) is intrinsically disordered and exists in equilibrium between open and catalytically-relevant closed states, where the C-terminal tyrosine (Y259) enters the active site even in absence of the substrate [1, 2]. Nuclear Overhauser effect distance restraints, obtained due to the presence of a substantial closed state population, were used to derive the solution-state structure of the ecDERA closed state. Real-time NMR hydrogen/deuterium exchange experiments reveal that Y259 is required for efficiency of the proton abstraction step of the catalytic reaction. Phosphate titration experiments show that besides the phosphate-binding residues located near the active site observed in the available crystal structures, ecDERA contains previously unknown auxiliary phosphate-binding residues on the C-terminal tail which could facilitate in orienting Y259 in an optimal position for catalysis. Thus, our data represent significant insights into the structural and mechanistic importance of the ecDERA C-terminal tail and illustrate the role of conformational sampling in enzyme catalysis.

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P70

The lantibiotic Nukacin ISK-1 exists in an equilibrium between active and inactive lipid-II binding states

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Nukacin ISK-1 is an antimicrobial lanthipeptide, which binds to lipid II, an essential intermediate in cell wall biosynthesis. Here, we determined the solution structure of nukacin ISK-1 and analyzed the interaction to lipid II. To our surprise, nukacin ISK-1 exists in two structural states in aqueous solution, with an interconversion rate on a time scale of seconds. The two structures are different in the relative orientations of the two lanthionine rings. Chemical shift perturbation induced by the titration of lipid II revealed that only one state was capable of binding to lipid II. On the molecular surface, a potential multiple hydrogen-bonding site formed by amino acid residues in the ring A region is adjacent to a hydrophobic surface formed by residues in the ring C region, and these sites interact with the pyrophosphate moiety and the undecaprenol part of the lipid II molecule, respectively.

P71

Modulation of chaperone activity by oligomerization

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Molecular chaperones alter the folding properties of cellular proteins via mechanisms that are not well understood. Dynamic properties of chaperones as well as relatively weak interaction with the substrate protein have impeded the elucidation of the mechanism in detail. Here we show the solution structures of Trigger Factor (TF) chaperone in the absence and presence of an unfolded substrate protein PhoA. The structures show that the unliganded TF forms a symetric100 kDa dimer in a head-to-tail orientation but binds to the substrate protein as a monomer. The monomeric TF binds the substrate protein using its multiple distinct substrate binding sites consisting of the hydrophobic amino acid residues, and keeps the substrate protein in an extended, unfolded state. On the other hand, dimerization of TF partially buries the substrate-binding sites, which is consistent with the fact that the monomeric TF has higher affinity for the unfolded protein. These observations seemingly imply that the dimeric form of TF is an inactive, resting state. However, the dimeric TF associates faster with substrate proteins and exhibits stronger anti-aggregation and holdase activity than the monomeric TF, as shown by the kinetic studies and the activity assays. The structural data show that the dimerization assembles the substrate-binding sites in the two subunits and forms a large contiguous surface inside the cavity, which accounts for the observed accelerated association with unfolded proteins. The results demonstrate how a molecular chaperone modulates its activity in consort with dynamic monomer-dimer switching, in order to provide distinct functional outcomes in the cell.

P72

The effect of arginine-382 on the autokinase activity of E. coli PhoR catalytic domain

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Protein phosphorylation is one of the most widely accepted regulatory mechanisms in signal transduction of biological processes. Two component systems mediated by phosphotransfer are the predominant signal transduction pathways in bacteria. A typical TCS includes a histidine kinase and a response regulator. Histidine kinase can be phosphorylated by ATP, then delivers the phosphoryl group to the downstream response regulator to regulate the cellular responses. The CA domain of histidine kinase participates in the phosphorylation process. It has an ATP-binding pocket sheltered by a loop called ATP lid. However, due to the high flexibility of the ATP lid, the phosphorylation mechanism of histidine kinase remains unclear. Here we report the effect of R382 residue on the autophosphorylation rate of PhoR from E. coli by utilizing mutagenesis and nuclear magnetic resonance (NMR) spectroscopy. PhoR belongs to HisKA family, which senses the concentration of the environmental inorganic phosphorus. The R382 site is located at the center of ATP lid of PhoR and is relatively conserved in the HisKA family. We mutated the R382 into amino acids with different charge properties, namely R382K, R382E and R382D. 31P NMR experiments were performed to record the reaction products of ATP and PhoR, where the ATP signals were converted into ADP and inorganic phosphorus. We have observed that the negative charged residue on site 382 slows down the reaction rate compared to the wild type protein. Isothermal titration calorimeter results showed that the binding affinity of ADP with PhoR plays an important role in the regulation of the autophosphorylation rate. These findings suggest that the charge of the ATP lid

might be an essential factor regulating the autokinase activity of HKs.

P73

Hydrophobin fungal amyloids from the opportunistic pathogen Aspergillus fumigatus

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Hydrophobins are fungal proteins characterised by a conserved amphipathic profile and an idiosyncratic pattern of eight cysteine residues involved in four disulphide bridges. Their functions are due to their remarkable physicochemical properties. Hydrophobins are secreted in a soluble form that self-assembles at hydrophobic/hydrophilic or air/water interfaces to form amphipathic layers showing the hallmarks of protein amyloids. Hydrophobin assemblies are thus functional amyloids used by fungi to breach the air/water barrier and develop aerial hyphae, to prevent water-logging, to cover aerial hyphae and spores rendering them hydrophobic thus facilitating aerial growth, spore dispersal and resistance to desiccation, to participate in the extracellular matrix or to form a protective layer during fruiting body development. Hydrophobins can also participate in host-fungi interactions. Aspergillus fumigatus is the most important airborne fungal pathogen, causing over 200 thousands deaths per year among immunocompromised people. Its spores, which are the infectious form of the mould, are covered by an amyloid-fibre layer with rodlet morphology formed by a hydrophobin called RodA. This rodlet coat renders the spores inert relative to the innate and adaptive human immune systems. We have solved the solution structure of RodA, studied its self-assembly in vitro, performed a mutational analysis to highlight the regions involved in the formation of the amyloid core of the rodlets, correlated the kinetics of rodlet formation in vitro with their rate of appearance on the spores and analysed the relationship between the structure of RodA and its immunological properties. We have also studied two close homologues of RodA, named RodB and RodC, which form functional amyloids on A. fumigatus conidial cell wall.

P74

Direct evidence of a low barrier hydrogen bond in the catalytic triad of a Serine protease

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Serine proteases are one of the largest groups of enzymes, found in both eukaryotes and prokaryotes, and are responsible for many different functions. The detailed information about the hydrogen-bonds in the catalytic triad (Asp…His…Ser) of these enzymes is of importance in order to fully understand the mechanism of action. The aspartate of the triad is hydrogen bonded to the histidine but the exact nature of this bond has been under discussion for some time. It is either a common short ionic hydrogen bond (SIHB) or a delocalized low barrier hydrogen bond (LBHB) were the hydrogen bond is shorter. So far, the evidence for LBHB in proteins have not been conclusive. Here we show clear NMR evidence that LBHB does exist in NS3, a serine protease from Dengue. The one bond coupling constant between the hydrogen and nitrogen was shown to be only 52Hz instead of the usual 90Hz. This together with a 1H chemical shift of 19.93 ppm is evidence that the hydrogen bond distance between His and Asp is shorter than for SIHB. Our result clearly shows the existence of LBHB and will help in understanding the mechanism of the catalytic triad in the important group of serine proteases.

P75

Structural insights into the mechanisms controlling the recruitment of REMORINs to membrane nanodomains

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The sub-compartimentalization of biological membranes in nanodomains is thought to orchestrate cell bioactivities in all organisms. Nevertheless, the mechanisms governing the organization of these nanodomains, enriched in specific lipid and protein components, are poorly understood and the functional relevance of protein spatiotemporal confinement remains to be fully demonstrated. Because of their unique lipid composition (e.g. phosphatidylinositolphosphates PIP and sterols), membrane nanodomains may act as dynamic signaling platforms recruiting immune response effectors. Among them, Remorin is a multi-domain protein known for its ability to decrease the cell-to-cell propagation of viruses. We show here that the C-terminal region is essential for Remorin to localize at the plasma membrane and fulfill its biological function. To decipher the molecular basis underlying the membrane association of Remorin, we apply solid-state NMR spectroscopy on liposomes mimicking the plasma membrane composition in the presence of Remorin C-terminal Anchor (REM-CA). Solid-state NMR is a unique technique to investigate the local structure of the peptide as well as lipid dynamics and membrane behavior upon interaction with the peptide. Our results suggest that the REM-CA penetrates partially inside the membrane and undergoes a conformational change in the presence of PI4P and sterols. While the role of REM-CA alone was then established, the structural and functional aspects associated with Remorin self-assembling properties remained unclear. Using a combination of different biophysical techniques including solid-state NMR, cryoEM and in vivo imaging, we showed that plasma membrane association of Remorins essentially depends on the formation of trimers formed by a short coiled-coil domain. We coupled solid-state NMR data to molecular modeling to propose a model for coiled-coil oligomers associated to nanodomains. Altogether, our results suggest that under the control of coiled-coil domain association, the preferential interaction with PI4P could be the driving force to address the Remorin to plasma membrane nanodomains and to perform its cellular functions.

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Structural studies on MazEF9 toxin-antitoxin system from Mycobacterium tuberculosis

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Toxin-antitoxin (TA) systems are stress-responsive genetic elements that govern apoptosis in bacteria. They are ubiquitous across microbial genomes and are unusually abundant in Mycobacterium tuberculosis. These TA systems contribute to its ability to persist in a drug-tolerant latent state in host cell granulomas. In Mtb, the MazEF TA system belongs to the Type II class, wherein, under favourable growth conditions, the protein antitoxin (MazE) binds to its cognate toxin (MazF) forming an inactive complex. Under conditions of stress, this complex dissociates thereby allowing the toxin to affect protein synthesis and consequently, bring about growth arrest.

In the case of MazEF TA systems in Mycobacterium tuberculosis, structures are known for MazF 1, 3, 4, 6 and 9. However, the structure of MazE4, determined in complex with MazF4, hitherto represents the sole structure of an antitoxin from this family of TA proteins. Structural data on the TA complexes has been scarce due to problems associated with poor solubility of the proteins and due to the propensity of these molecules to aggregate at concentrations necessary for structural studies.

Our interest lies in determining the structures of the MazF9 toxin in complex with its RNA substrate, the MazE9 antitoxin, and the MazEF9 complex, using nuclear magnetic resonance spectroscopy. Such a study would help decode the the poorly understood mechanism of transition between the catalytically active and the antitoxin bound inactive conformations of MazF toxins in Mtb in general, from a structural biology perspective. An important facet of MazF9 is the tRNA(Lys) and tRNA(Gln) specific endoribonuclease activity, which distinguishes it from all other MazF members. Structural elucidation of the tRNA substrate bound conformation of MazF9 will unravel its unique substrate choice.

Like in other TA systems, the MazE9 antitoxin protein is intrinsically disordered in the C-terminal region, which is expected to bind to and inactivate the toxin. This prompted the synthesis of the del(1-42) deletion mutant, to overcome issues associated with the large size of the complex. Large scale recombinant expression of MazF9, MazE9 and MazE9c (del 1-42 deletion mutant) has been achieved using a cytochrome b5 fusion tag. Preliminary biophysical characterization shows the full length MazE9 to exist in an intrinsically disordered form. Proton NMR spectra shows limited chemical shift dispersion in the amide and aliphatic regions, further corroborating the data from biophysical experiments. Low intensity resonance lines are observed close to 0.0 ppm, indicating the possibility of conformational exchange between the disordered and ordered forms. MazF9 on the other hand, shows several resonance lines at chemical shifts > 8.8 ppm and < 0.4 ppm, indicating a well structured protein. Isotopically enriched samples of MazE9 and MazF9 have been purified and data acquisition is underway. The results of these experiments will be presented.

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Regulation of the STAR protein Sam68 by post translational modifications

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The signal transduction and activation of RNA (STAR) proteins are a family of proteins that contain an RNA binding STAR domain. The best characterised member of the STAR family is Sam68, a predominantly nuclear protein that can also be found in the cytoplasm where it acts as an adapter protein in various signalling cascades. One of the main biological functions of Sam68 is its role in alternative splicing of specific target genes such as CD44, cyclin D1 and Bcl-X (Frisone et al, 2015).

The function of Sam68 in alternative splicing is regulated by post-translational modifications (PTMs) such as serine/threonine phosphorylation (Matter et al, 2002), tyrosine phosphorylation (Lukong et al, 2005), arginine methylation (Cote et al, 2003) and lysine acetylation (Babic et al, 2004). The precise mapping of these PTMs on Sam68 and how they affect Sam68 function mechanistically is currently unknown. We aim to study the PTMs of Sam68 using NMR by gaining spectra of Sam68 in various cellular extracts as well as in the presence of specific kinases. We have investigated the N-terminal intrinsically disordered region of Sam68 by NMR. We have showed that the addition of kinases induces phosphorylation of specific serine/threonine residues. Similar experiments will be done in nuclear and cytoplasmic extracts. The functional consequence of these phosphorylation events will be evaluated using splicing assays.

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P78

A structural and functional investigation of vaccinia virus envelope protein A26 and A27 complex by NMR and SPR spectroscopy

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Vaccinia virus (VV) is a prototype poxvirus that infects cells via two different entry pathways; endocytosis and virus/cell membrane fusion. Among VV envelope proteins, pH sensitive A26 (500 aa) plays a critical role that is able to suppress membrane fusion in virus entry pathway [JV W. Chang, 2010]. Previous studies showed that A26 is able to interact with A27 forming a complex via its C-terminal domain (1). And the A26-A27 complex is tethered to VV membrane protein A17 (2). As previously demonstrated, the A26-A27 complex could be partially or completely interrupted by N-terminal or C-terminal mutations (3). Although Cys441 and Cys442 of A26 are associated in the inter-molecular disulfide bonds formation with Cys71 and Cys72 of A27, respectively, however, A26 and A27 still form complex in the absence of disulfide bonds. Thus, it was suggested that the molecular interactions are more than disulfide bond (1). Till now, the molecular interactions between A26 and A27 remain largely elusive. In the current study, we carried out a structural and functional investigation of viral protein A26 (350-450 aa) and A27 (21-110 aa) complex by solution NMR spectroscopy and surface plasmon resonance (SPR), in order to uncover the molecular interactions and its underlying binding mechanism. In this communication, the structural and functional information determined from multi-dimensional NMR and SPR data are to be reported to elucidate its pH sensing property related to the viral protein complex formation. Our study shall help to better understand the structural and functional relationship of A26-A27 complex and to further advance our knowledge of VV entry at the molecular level.

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P79

Importance of Interaction Geometry of Aromatic Side chains Inside a Protein's Hydrophobic Core: A lesson from SUMO

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Inside a protein hydrophobic core, side chains of Aromatic amino acids are preferentially found to be involved in paired interactions(1). Statistical analyses of ring orientations in protein structures have indicated an inherent bias in the distribution of ring orientation to two distinct geometries, off-centred parallel displaced and face to edge arrangement and which among these two conformations are most preferred remained a topic of debate in the field(2-4). Despite the fact that these reports have highlighted the importance of ring orientation for a stable interaction, studies in context of a protein fold and function are limited. Interestingly, here we report that a highly conserved aromatic triad of three amino acids Phe-Tyr-Phe is a unique signature of SUMO (Small Ubiquitin Like Modifier) fold that distinguishes itself from other Ubiquitin Like homologous folds and its stability is substantially determined by this paired aromatic interaction. Moreover we also found that noncovalent SIM (SUMO Interaction Motif) binding interface of SUMO which plays a key role in SUMO-mediated protein-protein interaction networks in SUMO biology is critically dependent on the stereospecific T shape orientation of the Tyr-Phe aromatic moieties. Disruption of aromatic interaction by substitution with corresponding aliphatic residues of similar stable folds comes at the cost of protein's stability and function. Dynamics studies using solution NMR spectroscopy has shown that disruption of Tyr-Phe pair effect the packing and renders it more dynamic and flexible. Solution structure of the aromatic to aliphatic mutant shows a significant disruption of tertiary contacts compared to Wt fold. Impact of aromatic to aliphatic substitution on SUMOylation was also checked by both in vitro and in vivo SUMOylation assays where a significantly lesser fraction of substrate SUMOylation was observed compared to Wt protein. Altogether with stability and functional data, our results explain why the Phe Tyr Phe aromatic triad is 100% evolutionarily conserved from Arabidopsis to Human and shed light on the necessity of co-conservation of these aromatic residues.

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P80

Structural and Biophysical Characterisation of Mycobacterial MazEF6 TA Complex by Solution NMR

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M. tuberculosis possesses a large number of Toxin-Antitoxin (TA) systems that are known to confer multidrug tolerance, regulate biofilm formation and arrest cellular growth under conditions of stress inside the host cell. They provide the bacterium the ability to persist in a non-replicating state. The majority of the TA systems belong to one of MazEF, RelBE, ParDE, CcdAB, HigBA or VapBC families. Under conditions of normal growth, MazF (toxin) from Mtb are inactive, a state that is characterised by binding to the cognate antitoxin. Under conditions of stress, the toxin is released and is free to degrade cellular RNA which results in growth arrest. In the case of the Maz TA systems, structures are known for MazF3,4,6 and 9. The structure of MazE4 represents the sole antitoxin (determined in complex with MazF4) from this family of TA proteins. Structural analysis of homologous proteins from other bacterial species indicate that the C-terminal regions of the MazE (antitoxin) proteins are intrinsically disordered and helps the protein bind to the cognate toxin.The antitoxin dimerizes via its N-terminii. Structural data on the TA complexes has been scarce due to the problems associated with poor solubility of the proteins and due to the propensity of these molecules to aggregate at concentrations necessary for structural studies. Solving the structure of the toxin bound to the antitoxin can lead to the structural basis of inactivation of the toxin. While the mRNA bound form can give a detail account of its endoribonuclease activity. We have undertaken structural and biophysical studies of several of the TA proteins belonging to the Maz family. Initial attempts to obtain structural data on theMazE6, MazF6 and MazEF6 complex were unsuccessful. The MazE6 protein exhibited discernable temperaure dependence of chemical shifts of residues in the C-terminal region. A strategy was devised to study the structure and conformation of N-terminal deletion mutant (D1-46, MazE6c) of the antitoxin in the free and toxin bound forms. Isotopically enriched samples of MazE6c were prepared. Near complete sequence specific assignments for backbone 1H, 13C and 15N nuclei of the protein in the free form have been obtained from triple resonance experiments. Analysis of secondary chemical shifts and {1H}-15N heteronuclear NOE data indicate that MazE6c is structurally disordered. Superposition of spectra of MazE6 and MazE6c show that the C-terminal region is disordered in the full length protein too. Preparation of samples of the MazF6-MazE6c complex are underway. Isotope edited NOESY experiments, chemical shift perturbation, saturation transfer and RDC data will be used to calculate the structure of the MazEcF6 complex. The results of these studies will be presented.

P81

The chaperonosome: structure of a histone chaperone-bound histone octamer

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A fundamental question in biology is how chromatin is opened up to allow

access to the genomic DNA for all the protein machineries that control DNA replication, transcription, and repair. Histone chaperones are essential mediators of this process by regulating the assembly and disassembly of the nucleosome, the basic unit of chromatin (1). Here, we focus on the histone chaperone mechanism of APLF, a chaperone that is an integral part of the non-homologous end-joining (NHEJ) machinery, the cell's emergency system to repair DNA double-strand breaks (2).

We recently showed in an NMR study that the acidic domain of APLF (APLFAD) is intrinsically disordered, yet binds the core histone complexes H2A-H2B and H3-H4 specifically and with high affinity relying on two conserved aromatic "anchor" residues (3). Here, we show that APLFAD can bind simultaneously to both histone complexes, forming a 120 kDa complex. Using a divide-and-conquer strategy, we mapped the binding interfaces in this complex by NMR. APLFAD has distinct binding sites for H2A-H2B and H3-H4, each harbouring a double-aromatic-anchor motif to specifically engage the histone complex. Functionally, APLFAD covers most of the histones' DNA-interaction surface. In a chaperone assay, APLFAD prevents unspecific histone-DNA interactions and promotes nucleosome formation. This activity may be facilitated by an additional secondary binding mode that we mapped by NMR between APLFAD and histones and which may be involved in the handoff of histones to DNA or other chaperones.

Based on our NMR data, complemented with analytical gel filtration, native and crosslinking mass-spectrometry, SAXS and mutational analysis, we discuss a structural model of histone chaperone APLF bound to the histone octamer. This model emphasizes how hydrophobic residues, well-placed in an otherwise low-complexity protein sequence, can provide specificity for the histone-chaperone interaction, ultimately allowing precise control over the histone octamer and the nucleosome. Hence, we call this histone chaperoning complex the "chaperonosome".

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P82

Expression optimization of auto-induced and isotope-labelled cytochrome b5 from the parasitic protist Giardia intestinalis for one-dimensional and multidimensional NMR experiments

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The protozoan parasite Giardia intestinalis does not synthesize heme and lacks many heme proteins common to eukaryotes, yet it expresses four members of the cytochrome b5 family of small electron transfer proteins. Giardia cytochromes b5 have comparatively low reduction potentials, and each has a distinct subcellular location. These observations are consistent with structural features and biological roles that differ from those of the more thoroughly-studied mammalian isotypes. We are especially interested in characterization of Giardia cytochrome b5 isotype III (gCYTB5-III), which, unusually for a member of this protein class, localizes exclusively to the cell nucleus. Heme proteins are especially interesting targets for NMR studies. In addition to multidimensional NMR experiments, 1H-1D NMR can provide useful information on the local heme environment owing to the significant effects on the chemical shifts of nearby protons caused by the strong aromatic ring current of the porphyrin and the paramagnetism of the iron in the ferric state. To obtain recombinant, heme-replete gCYTB5-III for NMR experiments, we optimized expression with a lactose-driven auto-induction system as an alternative to the more conventional induction of expression with isopropylthiogalactoside (IPTG). Auto-induction mitigates user intervention and can lead to higher protein yields; significant for our purposes, it may also lead to higher expression of the heme-replete holoprotein over heme-deficient apoprotein. To establish the general utility of auto-induction expression for heme proteins and for comparison purposes we also studied recombinant bovine microsomal cytochrome b5, which has been well-characterized structurally and by NMR. Both cytochromes were expressed in E. coli strain BL21; optimal expression in unlabeled rich media (Terrific broth) yielded 160 mg purified protein per L culture, while the yield from 15N-labelled minimal media (Studier's N-5052) was fourfold lower. 15N-labelling efficiency in the latter was confirmed by mass spectrometry. The 1H-1D spectrum of bovine microsomal cytochrome b5 compared well to that reported previously, while that of gCYTB5-III indicated significant differences in the local environment about the heme, despite a similar coordination environment consisting of a pair of axial histidine ligands. 1H-15N HSQC experiments on these proteins are in progress. This research extends auto-induction and isotope-labelling methods to heme proteins, and helps reveal the structural features of the enigmatic gCYTB5s.

P83

An Atomistic View of Abl kinase regulation

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Despite the success in treating chronic myelogenous leukemia(CML) by targeting Bcr-Abl with the small-molecule inhibitor Gleevec®, a common problem is the development of drug resistance in many patients. The underlying mechanism of how these mutants exert their allosteric effect cannot be rationalized on the basis of existing structural data. We used NMR spectroscopy to show how structural elements within the Abl regulatory module (RM) create a multilayered allosteric mechanism allowing Abl kinase to function as a finely tuned switch. We dissected the structure and energetics of the regulatory mechanism to precisely measure the effects of various activating or inhibiting stimuli on Abl kinase activity. We find that in vitro Abl kinase activity is correlated to the relative population of the activating/extended state as measured by NMR. This provides a powerful approach to classify the conformational state of Abl in solution. This information is critical to understanding the allosteric network in conjunction with structure and dynamics is responsible for kinase regulation. This provides a potentially fruitful avenue to design allosteric inhibitors that are more specific and less prone to resistance development. The data provide a mechanistic basis for the development of drug resistance and reveal a previously unknown activator region within Abl. Our findings show that drug-resistance mutations in the Abl RM exert their allosteric effect by promoting the activated/extended state of Abl and not by decreasing the drug affinity for the kinase.

P84

Direct detection of N-H[...]O=C H-bonds in a 13C- and 15N-labelled cyclic lipodepsipeptide and the investigation of its self-assembly

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Cyclic lipodepsipeptides (CLPs) are secondary metabolites typically produced by soil-dwelling or plant-associated bacteria belonging to the genera of Pseudomonas or Bacillus. The denomination of this biomolecule family reflects the primary structure of the included compounds as they are built of a fatty acid moiety linked to the N-terminus of a peptide chain; which is cyclized by an ester (or 'depsi') bond formation between its C-terminus and an OH group bearing amino acid side chain of typically a Thr or a Ser. CLPs resolve multifarious tasks for their bacterial producers: they facilitate bacterial motility, modulate biofilm formation and may also take role in stimulating the immune system of plants what has already been exploited in the field of crop protection. Moreover, in vitro testing has revealed activity against a wide range of microbials (e.g. fungi, viruses, bacteria); and actually, the CLP daptomycin is one of the only two antibiotics that reached clinical applicability in the past 30 years. To promote the development of novel CLP-like chemicals with optimized biological function(s), our group targets the conformational assessment of natural and synthetic CLPs using solution state NMR spectroscopy as main experimental toolbox. Serving the pioneering example for an isotopically labelled compound within this metabolite family, we recently produced the 13C- and 15N-enriched version of a CLP called viscosinamide (VA). Then, modified HNCO experiments were conducted to refine the current knowledge on the peptide's solution state backbone conformation regarding the geometry of intramolecular N–H[...]O=C -type H-bonds. In agreement with the outcome of molecular dynamic simulation studies, the structure-stabilizing H-bond network of VA is fully identical for its free monomeric state and for its coaggregate with a cell membrane mimicking DPC micelle. Notably, the 3D structure of viscosinamide – and of the clear majority of natural CLPs – is folded in a fashion (i.e. adopting a left-handed α-helix capped by a loop) that infers the separation of the hydrophobic and hydrophilic amino acid side chains and thus engenders amphipathic character. In low polarity solvent therefore, the monomeric lipopeptide molecules exist in fast exchanging equilibrium with their tubular-shaped self-associations. The population ratio of the two states can be influenced by changing the peptide concentration and/or the solvent polarity. By tracking the chemical shift evolution of the backbone amide atoms in parallel, the intermolecular contacts in the assembly of VA could be disclosed; while complementary diffusion spectroscopy measurements gave additional insights into the steps of the self-association procedure. Since a possible explanation behind the lytic effect of CLPs (and other antimicrobial peptides) is the creation of likewise 'pores' in the targeted cell membrane, we believe that the presented results mean important elements for their structure/function/mode of action relationship puzzle.

P85

Mechanisms of chitosan recognition by chitosan-related proteins

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Chitosanases are enzymes catalyzing hydrolytic cleavage of the beta-1,4 glycosidic linkages of chitosan, a cationic polysaccharide consisting of glucosamine residues. Among the enzymes, some chitosanases have multimodular structures consisting of a catalytic module and chitosan-binding modules. These enzymes may find important industrial application in utilization of the enormous chitin/chitosan biomass. However, the mechanism of chitosan binding and degradation by chitosanases is not fully understood as compared with other polysaccharide degrading enzymes, such as cellulases or chitinases. Here, we present the chitosan-binding mode of chitosan-related proteins, chitosan-binding modules from Paenibacillus IK-5 (DD1 and DD2) and a chitosanase from Streptomyces N174 (CsnN174), and discuss their complicated binding and recognition mechanisms. Although amino acid sequences of DD1 and DD2 are highly homologous to each other, NMR and ITC experiments showed that these two modules have different affinity toward chitosan oligosaccharides. Structural analysis by NMR spectroscopy and crystallography revealed an important amino acid, which discriminates the binding affinities between DD1 and DD2. The results were confirmed by mutational studies of these chitosan-binding modules. CsnN174 is a single-modular protein with a long substrate binding cleft. To investigate the oligosaccharide binding mechanism, chitosan hexamer titrations by ITC and NMR were conducted using CsnN174. The experimental data obtained could not be interpreted by simple two-state binding mechanism. The NMR line shape analysis of chemical shift perturbation data revealed the three-state mechanism for this enzyme; that is, CsnN174 initially binds substrate, and subsequently undergoes the conformational change.

P86

Structural study of human neuromodulators Lypd6 and Lypd6b

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Several endogenous ligands of nicotinic acetylcholine receptors (nAChRs) belonging to the Ly-6/uPAR family were discovered in higher animals. These proteins share structural homology with 'three-finger' snake α-neurotoxins, specific inhibitors of nAChRs. Some of these proteins (e.g. Lynx1, Lypd6) are membrane-tethered via GPI-anchor and co-localize with nAChRs modulating their functions in the brain. Others (SLURPs) are secreted and act as autocrine/paracrine hormones in epithelium. Lypd6 and Lypd6b are expressed in the brain and unlike to other human Ly6/uPAR proteins have additional long N- and C-terminal sequences flanking the 'three-finger' LU-domain. C-terminal sequences contain sites for GPI-anchor attachment. Lypd6 increases the amplitude of nicotine-induced calcium currents in mouse trigeminal ganglion neurons. Lypd6 of D. rerio fish is involved in the regulation of Wnt/β-cathenin signaling pathway, and the knockdown of Lypd6 leads to the impairment of embryonic development. Expression of Lypd6b in X. laevis oocytes increases the sensitivity of nAChRs to acetylcholine and their desensitization rate. In the present work the water-soluble LU-domains of human Lypd6 and Lypd6b have been expressed in E. coli cytoplasmic inclusion bodies with subsequent solubilization and refolding, as well as LU-domain of Lypd6 with the additional N-terminal sequence. Analysis of recombinant N-Lypd6, Lypd6 and Lypd6b by NMR revealed that N-Lypd6 is likely to be unfolded. Spatial structure and intermolecular dynamics of Lypd6 and Lypd6b were studied using 13C,15N-labeled proteins. According to obtained NMR data, the proteins adopts typical 'three-finger' fold and possess three loops (I, II, III) protruding from the β-structural core ('head'). In contrast to other Ly6/uPAR proteins, the Lypd6 and Lypd6b contain only one β-sheet formed by five strands and involving residues form all three loops. The loops I and III are stabilized by additional disulfide bonds and accommodate two 'unexpected' α-helical elements. C-terminal regions (87-95) of Lypd6 and Lypd6b demonstrate conformational heterogeneity in solution possibly connected with cis-trans isomerization of Leu85-Pro86 bond going with characteristic time 0.1 s. The sites of exchange motions were also observed in the β-strands of the molecules. 15N-relaxation data revealed significant ps-ns mobility in the unstructured C-terminal region of both proteins and in the Lypd6b loop II. The overall rotation correlation time determined for Lypd6 and Lypd6b (5.5 ns at 30 °C) confirmed monomeric state of proteins in solution. Obtained spatial structure of Lypd6 permitted to model its interactions with the $\alpha4\beta2$ neuronal nAChR. Protein-protein docking and molecular dynamics revealed tight contacts between the loop I of Lypd6 and the entrance to the agonist binding site that is located between the $\alpha4$ and $\beta2$ subunits of the receptor. The work was supported by

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P87

Molecular mechanisms of BIP regulations

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The endoplasmic reticulum (ER) is an essential organelle in eukaryotic cells responsible for folding and maturation of the majority of secreted and membrane proteins. Binding Immunoglobulin Protein (BIP), the only Hsp70 chaperone in the ER lumen, is a key player of the ER protein quality control system. It's main functions include assisting folding and maturation for newly synthesized proteins as well as the regulation of the unfolded stress response (UPR) network. Majority of BIP functions, as other Hsp70s, rely on the ability to cycle between several functionally and structurally distinct conformations. However, little is known of specific features of the BiP conformational landscape that tune BiP to its unique tasks and ER environment. We are using solution NMR (including chemical shift perturbation analysis and relaxation dispersion measurements) and a wide range of biophysical and biochemical techniques as well as molecular dynamic simulations to explore how functional conformations of BIP are regulated by posttranslational modifications, Ca2+ binding, and interactions with other components of the ER protein quality control network. Here we present the NMR and ITC characterisation of the BiP chaperone cycle that revealed surprising mechanisms of Hsp70s activity regulation: BiP, as every Hsp70 chaperone consist of two domains –Substrate Binding Domain (SBD) and Nucleotide Binding Domain (NBD). There are two key functional conformations of BIP: ATP bound domain-docked, that upon substrate binding and ATP hydrolysis converts into ADP-bound with domains spatially separated. Our findings suggest that different members of the Hsp70 family apparently fine-tune their function evolutionally, posttransnationally or by exploitation of the unique environmental factors by adjustments of their conformational landscape rather than by altering chaperone structure. The conformational heterogeneity of ATP-bound BiP enables gradual post-translational regulation of the BiP chaperone cycle and its chaperone activity by subtle local perturbations of the SBD allosteric 'hotspots'. Particularly, BiP inactivation by AMPylation of its SBD relies on a redistribution of the BiP conformational ensemble and stabilization its domain-docked conformation in presence of ADP and ATP, but preserves Hsp70 inter-domain allostery and BiP structure. Similarly, such regulation may be also achieved by specific environmental factors. In case of BIP, Ca2+ ions that are abundant inside the ER lumen are known to bind into nucleotide binding site, substituting Mg2+. Our analysis show that BIP inactivation by Ca2+ relies on stabilization of a domain-undocked conformation and significant alteration in nucleotide binding propensities that lead to trapping of ADP-bound conformation. While mounting evidence suggests that impaired BiP activity is linked to neurodegenerative diseases, diabetes, cardiovascular diseases, cancer progression and anticancer drug resistance, these tunable properties of the BiP conformational landscape are particularly interesting because they potentially provide new opportunities to develop BiP-specific allosteric drugs.

P88

NMR Studies of Structure and Function of AIPL1 FKBP Domain

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Aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1) is a specialized chaperone of phosphodiesterase 6 (PDE6), a key effector enzyme in the phototransduction cascade. AIPL1 is composed of a N-terminal FKBP-like domain and a C-terminal tetratricopeptide repeat (TPR) domain. AIPL1 FKBP domain binds the farnesyl moiety of PDE6, while the TPR domain binds to chaperone Hsp90. Mutations in AIPL1, including many missense mutations in the FKBP domain, have been associated with Leber congenital amaurosis (LCA), an early-onset inherited retinopathy that causes severe blindness in children.

The AIPL1 FKBP domain shares a high sequence homology with the FKBP domain of aryl hydrocarbon receptor-interacting protein (AIP). Both AIP FKBP and AIPL1 FKBP do not bind FK506, lack PPIase activity, and have a long insert sequence (57 aa) replacing the hairpin loop of classical FKBPs. However, AIPLI FKBP binds isoprenyl moieties, but AIP FKBP does not. Moreover, AIPL1 is expressed selectively in the retina and the pineal gland. But, AIP is ubiquitously expressed and acts as a co-chaperone with Hsp90 in the maturation of the aryl hydrocarbon receptor and other nuclear receptors.

Here we report the NMR studies and resonance assignments of the N-terminal FKBP domain of AIPL1 in apo and in complexes with isoprenyl ligands. These results revealed the structure and dynamics of AIPL1 FKBP and the mechanism of isoprenyl binding.

P89

Conformational sampling of arginine: a view from long-range scalar couplings

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Amino acid side chains are fundamental to all aspects of the biology and pathology of proteins. They play critical roles in processes as diverse as: folding, catalysis, binding and allosteric regulation. The guanidinium group in arginine allows it to participate in a wide range of chemical interactions and arginine is often found at binding interfaces, particularly in interactions with nucleic acids. The position of this guanidinium group is dictated by the conformation of the aliphatic chain it terminates. Side chains are generally dynamic and it has become clear that function can be intrinsically linked to motion as well as structure. Despite its importance, methods to probe the behaviour of arginine, and other side chains lacking methyl groups, are limited.

Scalar coupling constants across dihedral angles provide a wealth of information on both the conformation and dynamics around those angles[1,2]. Determining these coupling constants along the arginine side chain allows full description of its behaviour in solution and, consequently, the position and spatial sampling of the guanidinium group. We have adapted and developed a range of both proton and carbon excitation/detection pulse sequences to measure these constants across all four arginine chi-angles. These methods have been used to probe the native conformational behaviour of arginine residues in the dynamic, medium-sized (20kDa) protein T4 lysosyme L99A at both 25°C and 5°C.

The results also highlight correlations between dihedral angle sampling and chemical shift in arginine similar to those observed in aliphatic side chains[3,4,5]. Whilst this relationship has yet to be fully explored, it is a promising start for the development of methods to obtain similar structural and dynamic information in systems not suited to extended multidimensional J-coupling experiments, such as large proteins or minor state conformations.

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P90

Structural and positional studies of the antimicrobial peptide brevinin-1BYa and its analogue

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Brevinin-1BYa (FLPILASLAAKFGPKLFCLVTKKC), first isolated from skin secretions of the foothill yellow-legged frog *Rana boylii*, is particularly effective against *C. albicans*, and is also active against *E. coli* and *S. aureus*. The structures of brevinin-1BYa and its less-potent analogue [C18S,C24S]brevinin-1BYa were investigated in various solution and membrane- mimicking environments by 1H-NMR spectroscopy and molecular modelling. Brevinin-1BYa possesses two cysteine residues, one at the C-terminal end of the peptide and the other 6 residues prior to it, joined by a disulphide bridge. The peptide does not appear to possess a secondary structure in aqueous solution. In a 33% 2,2,2-trifluoroethanol (TFE-d_3)-H_2O solvent mixture, as well as in membrane-mimicking sodium dodecyl sulphate micelles, and dodecylphosphocholine micelles, however, the structure is characterised by a helix-hinge-helix motif, with a hinge located at the Gly13/Pro14 residues, and the two α-helices extending from Ile^4 to Lys^{11} and from Lys^{15} to Thr^{21}. Positional studies of both the native peptide and its serine analogue in sodium dodecyl sulphate micelles using 5-doxyl labelled stearic acid and manganese chloride paramagnetic probes show that the N-terminal helical segment of the peptide lies parallel to the micellar surface, with the residues of the hydrophobic side of the amphipathic helix facing towards the micelle core and the hydrophilic residues pointing outwards, with similar results obtained for the C-terminal segment of the native peptide.

P91

Structural studies of the antimicrobial peptide maximin-3

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Maximin-3 (GIGGKILSGLKTALKGAAKELASTYLH) is a 27-residue long cationic antimicrobial peptide found in the skin secretion and brain of the Chinese red belly toad *Bombina maxima*. The peptide is interesting as it possesses anti-HIV activity, not found in the other maximin peptides, in addition to its antimicrobial, antitumor, and spermicidal activities. Its conformation was investigated in a 50/50% 2,2,2-trifluoroethanol (TFE-d_3)/water mixture using two-dimensional NMR spectroscopy. Maximin-3 was found to adopt an α-helical structure from residue Ile^2 to Ala^{22}, with a break around Lys^{15} and Gly^{16}, and a coiled structure extending from Ser^{23} to the C-terminus. The peptide has a well-defined separation between polar and hydrophobic residues, therefore it is amphipathic. The interactions with sodium dodecyl sulfate (SDS) micelles, a widely-used bacterial membrane-mimicking environment, are then modelled employing molecular dynamics simulations. The peptide maintains an α-helical conformation, occasionally displaying a flexibility around the Gly^9/Leu^{10} and Gly^{16}/Ala^{17} regions. It is found to preferentially adopt a position parallel to the micellar surface, with the C-terminal segment from Ser^{23}-His^{27} protruding into the aqueous solvent. The hydrophobic face of the amphipathic helix is in direct contact with the micelle core, while the polar residues face the aqueous solvent and form electrostatic interactions with the detergent's polar sulphate headgroups.

P92

Designed domain swapping in proteins

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Domain swapping is the process by which dimers or higher order oligomers are generated through the exchange of structural elements between identical monomeric proteins. Although engineered domain swapping is a compelling strategy for protein engineering and assembly, its application has been limited due to the lack of simple and reliable design approaches. Here, we demonstrate that the five-residue 'cystatin motif' from the domain-swapping protein Stefin B, when engineered into a surface loop between beta-strands of the non-domain-swapping proteins, single-chain monellin (MNEI) and human ubiquitin (UBQ), prevents the loop from folding back upon itself, and drives domain swapping. Engineering the motif independently into three distinct surface loops of MNEI, connecting different beta-strands, generated topologically distinct swapped dimers, which was confirmed by high resolution structural studies. We also show that engineering the motif simultaneously into two loops of MNEI induces double domain swapping. Finally, engineering the motif into one of the β-hairpin motifs of a sso7d variant resulted in dimerization, while its introduction into Ubiquitin resulted in the formation of higher-order oligomers. Thus, introduction of the cystatin motif can be used as a general mutational approach for engineering domain swapping in diverse beta-hairpin proteins.

P93

Revealing binding secrets of mussel-mimetic peptide on nanomaterial surface: From NMR as a powerful tool for detection

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Bio-inspired mussel adhesive proteins (MAP's) can modify their primary structures and its functionality to achieve strong binding and recognize inorganic material surfaces.[1] One of the key chemical components behind the strong mussel adhesion is L-DOPA (3, 4-dihydroxy L-phenylalanine).[2] Furthermore, neighboring residues contribute considerably to the binding mechanism. However, the molecular basis of peptide interactions toward inorganic surfaces is not yet entirely understood. A thorough understanding of alterations in the primary peptide structure and its interaction with the inorganic substrate will help novel de-

sign biosensors and sophisticated bio-inspired materials. To investigate these changes in the primary peptide structure and the type of interactions between the peptide and inorganic substrates. We have identified enzymatically activated 12-mer mussel-inspired peptides, which specifically bind to Alumina substrates.[3] In the present contribution, we report a solution NMR spectroscopy study revealing insights into interactions of mussel peptides, showing sequence-specific and strong adhesion onto Al2O3 nanoparticle (NP) surfaces. Nuclear Overhauser Effect (NOE) based ligand-receptor screening methods such as Saturation Transfer Difference (STD), and NOESY experiments were applied to understand the orientation and surface-bound structure of the peptide on the nanomaterial surface.[4] The orientation of the peptide on the surface was determined by transferring magnetization from nanoparticle surface to the nearby proton of the peptide.[5] In-depth insights of the aqueous peptide-Al2O3 interfaces binding via either entropically or enthalpically driven mechanisms have been revealed by using molecular dynamic simulations.[6,7]

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P94

TAp63α is activated via timed multistep phosphorylation and a "kinetic fuse"

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p63 is a member of the p53 family of transcription factors. p53 has been termed the "guardian of the genome" and is widely regarded as one of the most important cancer suppressors. p63 has a dual set of roles, depending on the isoform of the protein. Two different promoters either give rise to isoforms containing the transactivation domain (TAp63) or lacking it (ΔNp63). Additionally several splice variants determine the C-terminal specific isoform (α, β, γ, δ and ε). The most abundant isoforms are ΔNp63α and TAp63α. ΔNp63 is present in skin stem cell tissue and plays a role in the regulation of differentiation into stratified epithelium. TAp63α on the other hand is critical for maintaining the genomic integrity within mammalian oocytes. Within the cells TAp63α is expressed in high quantities, but remains totally inert. In contrast to all other members of the p53 family it is inherently locked in a dimeric state by a charge charge interaction network. This network obscures the transactivation domain of the protein, thus making transactivation impossible. Additionally the DNA binding domains are locked in a sterically unfavorable conformation, massively lowering the DNA binding affinity. Nevertheless TAp63α behaves much like p53, and therefore DNA double strand breaks, either chemically induced or via ionizing radiation, lead to activation of the protein. Activation subsequently leads to tetramerization, DNA binding and transactivation on pro-apoptotic promoters. TAp63α is activated by a series of phosphorylations, by a set of two different kinases. An initial phosphorylation at S582 by the checkpoint kinase 2 (Chk2) functions as a priming site for four additional phosphorylations by Casein Kinase I (CKI) within a serine/threonine rich patch. CKI is reported as a processive kinase, phosphorylating preferentially residues in a +3 position to an already phosphorylated serine/threonine. The serine/threonine rich region in TAp63α has a sequence which should enable processivity, as each phosphorylation of CKI creates another substrate the kinase itself. By fast 1D- and 2D-NMR spectroscopy we were able to determine the kinetics of phosphorylation of the peptide sequence. In contrast to the anticipated result we see a stalling of phosphorylation kinetics after two phosphorylations by CKI. Phosphorylations three and four are an order of magnitude slower than the initial two phosphorylations. This result correlates well with the in vivo and in vitro observation that at least three phosphorylations by CKI are needed to disrupt the auto inhibitory complex, therefore indicating that a kinetic fuse is in place to avoid premature activation of TAp63α in case of repairable DNA damage.

P95

Effects of glycosylation on protein structure, function and dynamics

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Even though more than half of all human proteins are glycosylated, the functional characteristics of this important posttranslational modification is so far poorly understood and structural information is rare. One obstacle to investigate these proteins is due to the fact that they can most often not easily be overexpressed in Escherichia coli. Obtaining the correctly modified protein requires more complicated large-scale recombinant eukaryotic expression systems (such as insect cells). Furthermore, the size, flexibility and heterogeneity of these proteins are a challenge for the analysis and structure determination.

By using NMR spectroscopy, we aim to delineate the changes in the protein structure and dynamics upon glycosylation, also when the target protein is unstructured or when multiple conformations are present. This requires the development of specific isotope labeling protocols for the recombinant expression of glycosylated proteins in eukaryotic hosts. The initial focus is on the C-mannosylation of tryptophan residues in TSR1, the thrombospondin type 1 repeat from the netrin receptor UNC-5. The addition of a hydrophilic mannose undoubtedly affects the localization and characteristics of the tryptophan thereby modulating the protein properties. This modification possibly plays a key role in the function of the protein. It has been suggested that ligand binding, receptor internalization, proper folding, export, signal transduction and cell migration are affected by C-mannosylation. Subsequently, our primary research can be expanded to study other glycosylated protein systems.

P96

Allosteric Regulation of Catalysis Through Substrate Inhibition of Fluoroacetate Dehalogenase

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While enzymes have evolved to be both specific and efficient catalysts, as many as 20% exhibit inhibition with excess substrate. Here, X-ray crystallography and fluorine (19F) nuclear magnetic resonance (NMR) studies of the homodimeric enzyme, fluoroacetate dehalogenase (FAcD), reveal that the inhibitory effects that ensue at higher substrate concentrations arise from a second substrate binding site which lies along the binding pathway

of the occupied protomer. 19F NMR reveals that binding of a second substrate leads to both a reduction in the catalytic rate and attenuation of key side chain dynamics in the vicinity of the active site. Second-site binding occurs at a critical site along an allosteric pathway believed to be involved in catalysis and connecting the substrate-bound and empty protomers. This is shown to reduce conformational degrees of freedom, dampen interprotomer allosteric coupling, and reduce catalysis. These results underline the role of cooperative processes and dynamics in allostery and catalysis.

P97

Investigating chemical probes for protein dynamics using 19F NMR

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19F NMR is regularly used in the study of protein topology and conformational dynamics. Conjugation of this reporter to thiols allows for the study of local environmental changes in the protein and under certain conditions, identification of unique protein conformational states. Here, we report on studies for two types of fluorinated tautomers; pyridones and beta di-ketones as potential thiol specific tags for the study of protein conformations and dynamics. The 19F compounds were tested in vitro for sensitivity to changes in solvent polarity, pH and temperature. A polarity series was prepared for both types of tautomers using mixtures of methanol:water ranging from relatively non-polar (19:1) to polar (0:1). The 19F NMR spectra were compared to the currently used thiol specific tag, 2-bromo-N-(4(trifluoromethyl)phenyl)acetamide (BTFMA), and the commonly used tag, 3-bromo-1,1,1-trifluoro-propan-2-one (BTFA). 5-fluoro-2-hydroxypyridine (5-MFHP) demonstrated the greatest range of chemical shift as a function of solvent polarity, at 2.56 ppm. This is ideal for smaller peptides which can provide structure and conformational elucidations without significant line-broadening that is typically associated with larger proteins. 6-trifluoromethyl-2-hydroxypyridine (6-TFMHP) displayed the greatest chemical shift range of 1.61 ppm among the trifluoromethyl tags which are far more sensitive than BTFMA, which was 1.03 ppm under the same conditions and is preferable for larger and more complex macromolecules due to the higher signal intensity obtained from the CF3 group compared to CF on 5-MFHP. The beta di-ketones, while providing more information via various peaks, also yields a more complex spectra that would make deconvoluting the protein peaks more difficult.

P98

Preparation of residue- and site-selectively protonated large proteins using cellular and cell-free E. coli expression systems – Revisiting direct 1H-NMR spectroscopy

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The observation of 1H-NMR signals in residue- and site-selectively protonated, and otherwise fully deuterated, proteins was originally reported a half century ago, as the first successful isotope-aided method for investigating protein structures in solution. However, due to the limited spectral resolution, the one-dimensional 1H-NMR approach for selectively protonated proteins (originally called “selectively deuterated proteins”) was not widely used and completely overshadowed by multidimensional multinuclear spectroscopy in the 1980-90s. During our continuing endeavors to further optimize the stereo-array isotope labeling (SAIL) method for extraordinarily large proteins and protein complexes, we have recently revisited this traditional approach. We are convinced that one-dimensional and possibly other low-dimensional 1H-direct observation NMR spectroscopy should be revitalized in the coming era, featuring ultrahigh-field spectrometers, for observing the isolated 1H-12C pairs in quite large proteins (Kainosho, M. et al. J Biomol NMR (2018). https://doi.org/10.1007/s10858-018-0198-x). In this presentation, we especially focus on the practical protocols for preparing selectively protonated proteins, using conventional E. coli cellular and cell-free expression systems. Some of our latest NMR results will also be presented.

P99

Structural determinants for the dual function of Hsp33 as a holding chaperone and as an unfoldase/aggregase chaperone

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Various activities of molecular chaperones contribute to ensuring cellular proteostasis and to responding to cellular stressors. The prokaryotic molecular chaperone Hsp33 was originally identified as a redox-regulated holdase (holding chaperone) that binds to the folding intermediates of client proteins to prevent their ultimate, irreversible denaturation. In addition, it has been suggested that Hsp33 is also involved in a chaperone network that regulates the stability of elongation factor Tu (EF-Tu) in cells. The holding activity of Hsp33 is post-translationally achieved by dual stresses of heat and oxidation, through an oxidation-induced unfolding of the C-terminal redox-switch domain (RSD). Our NMR investigations suggested that a dynamic interaction between the interdomain linker stretch and the N-terminal stretch in the reduced state serves as a heat-sensing module, while the RSD functions as a redox-sensing module. In particular, the former dynamic interaction, rather than the latter RSD fold, could be identified as the structural determinant for the activation process of Hsp33. Meanwhile, the reduced Hsp33, but not its oxidized forms, was relevant for direct molecular interaction with EF-Tu. This interaction was critically mediated by the folded RSD of the reduced Hsp33 and the guanine nucleotide-binding domain of EF-Tu. Furthermore, the bound Hsp33 catalyzed the aggregation of EF-Tu via evoking its aberrant folding, which generated susceptibility to Lon protease. These results suggest that the RSD fold is the structural determinant for the own function of reduced Hsp33. Collectively, Hsp33 can be appreciated as a unique molecular chaperone that displays a dual function as an oxidation-dependent holding chaperone and as an oxidation-independent unfoldase. In addition, our findings constitute the first example of an aggregase activity displayed by a molecular chaperone, which is potentially involved in protein turnover and/or the response to thermal stressors via impairing the EF-Tu functionality.

P100

Biophysical characterisation of Aspergillus fumigatus conidial functional amyloids

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Aspergillus fumigatus is the major fungal airborne pathogen and is at the origin of over 200,000 deaths worldwide per year. This fungus secretes proteins from the hydrophobin family that spontaneously self-assemble at hydrophobic/hydrophilic or air/water interfaces to form amphiphilic layers that show the hallmarks of amyloid fibres, such as those associated to Alzheimer's disease. These functional amyloids are used by fungi for different purposes such as breaching the air/water barrier, coating spores or mediating host-fungi interactions 1.

The spores (conidia) of A. fumigatus are covered by an outer layer with rodlet morphology consisting of amyloid fibres made up of the RodA hydrophobin. This rodlet layer renders the spores inert relative to the immune system1. In addition to RodA, two other hydrophobins, named RodB and RodC, are present in the conidial cell wall. Both are close homologues of RodA (50% identity) and, as shown herein, can form amyloids in vitro.

We are characterising the structure of the soluble form and the mechanism of self-assembly of RodB and RodC following a similar strategy to the one used for RodA to get a better understanding of A. fumigatus conidial functional amyloids. For RodC, we have analysed the effect of single point mutations on the kinetics of amyloid formation to pinpoint the residues that are important for self-assembly and that might be involved in the cross-β core of the amyloid fibres. We have also used CD and FTIR to understand the secondary structure modifications that occur upon auto-association and AFM to observe the structures formed by RodA, RodB and RodC.

Our results indicate that (i) the three proteins undergo significant conformational changes with a gain of ß-sheet structures upon amyloid formation and that (ii) the monomers are surface active and behave differently when exposed to varying interfaces.

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2 Aimanianda V et al. (2009) Surface hydrophobin prevents immune recognition of airborne fungal spores. Nature 460: 1117–1121.

P101

NMR studies on the oxidation of IL-33

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Interleukin-33 (IL-33) is a member of alarmin cytokine from IL-1 family. IL-33 is secreted from the nucleus of endothelial and epithelial cells to the extracellular space during tissue damage. Extracellular IL-33 binds strongly to the cell-surface receptor ST2/IL1RL1. Oxidation and the formation of two disulfide bridges of cysteine residues can terminate IL-33 activity at its receptor ST2. In previous study, it has been suggested that C208-C259, and C227-C232 formed two interchain disulfide bonds (Cohen, E. S. et al. Nat Commun 6, 8327). Despite these observations, the mechanisms of oxidation and inactivation of IL-33 remain elusive. The purpose of this study was to decipher oxidation states of IL-33 using NMR and the other biochemical methods. Each 4 cysteine residues of IL-33 has been replaced with alanine to evaluate the importance of cysteine during the oxidation. Oxidation of the series of IL-33 has been monitored by non-reducing gel electrophoresis. Addition of DMEM media to the IL-33 wildtype, C208A, and C227A caused the oxidation. However, cysteine 259 mutation has blocked the oxidation. It is suggested that C259 is critical for IL-33 oxidation, which in turn termination of IL-33 cytokine activity. Furthermore, using NMR spectroscopy, we have investigated the conformational changes upon oxidation of IL-33.

P102

NMR Structure-activity studies of antimicrobial peptides with enhanced activity

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Many antimicrobial peptides (AMPs) found in the last century have been studied to be commercial drugs, but there have been few successful cases. However, due to their advantages such as different mechanisms from conventional antibiotics and lack of side effects, there is still the potential for being used as candidate of good antimicrobial agent. It is also indispensable to discover new compounds to counteract the existing antibiotic resistant bacteria in the world Lactophoricin (LPcin), a cationic amphipathic peptide consists of 23-mer peptide from bovine milk, was currently utilized as the framework to design the novel AMP analogs and study the correlation between structure-activity of AMPs. 11 LPcin analog peptides were designed to improve the antimicrobial activity, using conservative sequence modification and designed additional 9 analog peptides based on LPcin-YK3 which had showed the best antibacterial activity among 11 LPcin analog peptides. Antimicrobial activity of designed novel AMP analogs was confirmed by bacterial killing assays and growth inhibition assays for bacteria and fungi, and the stability was confirmed by hemolysis assay and cytotoxicity assay for mouse and human eukaryotic cells. In order to define the correlation between antimicrobial activity and structure of AMPs we conducted structural studies of novel AMPs using various spectroscopic methods as well as NMR. Analog peptides were obtained with high yield and high purity by optimizing expression using E. coli and purification using many biophysical techniques. Structures of analog peptides in membrane environments are studied using solid-state NMR to identify their 3D structures and topologies.

P103

Structure and Dynamics Analysis of Yeast Ubiquitin Hydrolase 1 (YUH1) Using Paramagnetic NMR Spectroscopy

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Ubiquitination and deubiquitination of proteins are involved in numerous cellular processes such as proteolysis, signal transduction, DNA repair, etc. Yeast Ubiquitin Hydrolase 1 (YUH1), which is composed of 236 residues (26.4 kDa), is a deubiquitinating enzyme, capable of hydrolyzing ubiqui-

tin adducts for homeostasis of free ubiquitin levels in the cell. However, the molecular mechanism of its specific substrate recognition and hydrolysis is not fully understood because the three-dimensional (3D) structure of monomeric YUH1 in solution has not been solved yet whilst the crystal structure of its complex with the tight-binding inhibitor ubiquitin aldehyde (Ubal) has been reported previously. Hence, we addressed the protein structure determination and dynamics analysis of monomeric YUH1 to elucidate the structural basis of this specificity with the ubiquitin and dynamical processes. First, we performed 3D triple-resonance NMR experiments for backbone and side-chain resonance assignments. Approximately 98% of the backbone and 75% of the side-chain resonances were assigned. 2322 NOE-derived distance restraints, including 471 long-range ones, were obtained from 3D 15N- and 13C-separated NOESYs, and 13C/13C-separated NOESY with selective 1H/13C-labeling of the methyl groups. The 3D structure of YUH1 was calculated with the program CYANA using automated NOE assignment. Since the conformers obtained exclusively from NOE-derived distances and dihedral angle restraints were not well converged, we further measured pseudo-contact shifts (PCSs) and paramagnetic relaxation enhancements (PREs) to collect long-range structural information. MTSL and 4PhSO2-PyMTA paramagnetic probes for PRE and PCS, respectively, were incorporated at four separate sites (N5C, S104C, N140C, N225C). 4PhSO2-PyMTA with a short linker allowed us to obtain a sufficient number of relatively large PCS values by suppressing tag flexibility. Besides, we analyzed the dynamics of YUH1 by T1/T2 relaxation and heteronuclear NOE experiments. The final structure ensemble of monomeric YUH1 is well converged with a backbone RMSD of 0.81 Å to the mean coordinates and similar to the crystal structure of the complex with Ubal (backbone RMSD 2.49 Å), except for the region in ubiquitin recognition and hydrolysis. Contrary to the rigid structure of this active site in the YUH1 complex, our structural and relaxation analysis demonstrate that the same region of the monomer is structurally significantly different and considerably flexible with internal motion on a timescale of approximately 0.8 ns. This large structural and dynamical differences may be attributed to the interaction of Ubal or the difference between crystal and solution states. Meanwhile, it is believed that this active site, which is composed of a unique 20 residue loop and the N-terminus of YUH1, functions to restrict or filter the size of the substrate attached to ubiquitin and regulates the catalytic activity. Thus, the significant structural and dynamical changes presumably contribute to the specificity and efficiency of deubiquitination.

P104

High resolution protein 3D structure determination in living eukaryotic cells

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The interior of eukaryotic cells is different from that of prokaryote, which is comparted by a nucleus and other organelles as well as the meshwork of the cytoskeleton. Although in-cell NMR studies in eukaryotic cells have become possible, high-resolution protein structures have been determined only in E. coli cells. In order to elucidate the subtle difference between in vitro and in-cell structures, it remains necessary to achieve the de novo 3D protein structure determination from a sufficient number of NOE-derived distance restraints between side-chains. Here we show the first de novo protein structure determinations in living eukaryotic cells provided by the sf9 cell/baculovirus system, exclusively based on information from NMR spectra. As model systems, three small- and two medium-sized proteins were selected; protein GB1 (7 kDa), the T. thermophilus HB8 TTHA1718 (7 kDa), human ubiquitin with three alanine mutations, (8 kDa; Ub3A), rat calmodulin (17 kDa; CaM), and C-terminally truncated human HRas (19kDa; HRas). For GB1 in sf9 cells, we could obtain high quality 3D triple-resonance NMR spectra even for side-chain resonance assignments. We could assign approximately 98% of the backbone resonances of GB1 in sf9 cells unambiguously. Structures were first calculated with the program CYANA. The resulting structures were further refined with the assistance of Bayesian inference. 1900 conformers collected from the trajectory of GB1 are well defined with an average backbone RMSD of 0.51 Å to the mean coordinates. The structure demonstrated that, in sf9 cells, the relative position of the a-helix is tilted significantly in a direction away from the b-sheet. The structure determinations of Ub3A and TTHA1718 were also performed from 3D NOESY spectra in sf9 cells. The resulting structure ensemble of Ub3A with 4400 conformers is well-defined with an average backbone RMSD of 0.39 Å to the mean coordinates. The backbone RMSD between its mean structure and the structure in diluted solution is 1.31 Å. Backbone RMSDs of TTH1718 (residues 1-8,19-66) with 1000 conformers to the mean coordinate and the structure in diluted solution are 0.88 and 1.27 Å, respectively. For CaM and HRas, we prepared samples with methyl- and aromatic-selective 1H/13C- labeling in sf9 cells, since these proteins exhibited heavily overlapped NMR spectra when labeled uniformly. In both cases, well resolved 3D 13C-separated NOESY spectra were acquired. Comparison of in-cell NMR spectra of CaM with corresponding spectra in diluted solution suggested that CaM in sf9 cells exists in a conformational state similar to Mg2+-bound CaM. HRas in sf9 cells is suggested to be in the 'inactive' GDP-bound state, which is reasonable considering that the C-terminal truncation disables the GDP to GTP exchange at the cell membrane, while the bound GTP is hydrolyzed by its intrinsic GTPase activity during the sample preparation.

P105

The F141L mutation in human calmodulin disrupts the structure and leads to misregulation of a voltage-gated cardiac calcium channel

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Calmodulin (CaM) is the universal calcium sensor and mediator of calcium-dependent regulation of cellular activity. To date, more than 300 interaction partners of CaM are known, and CaM is one of the genetically most conserved proteins in vertebrates. However, a few CaM mutations have been identified in humans with cardiac arrhythmia syndromes. One of those mutations, F141L, adversely influences the calcium-dependent regulation of the cardiac voltage-gated calcium channel (Cav1.2) resulting in Long-QT syndrome. Here we show that the F141L mutation leads to severe structural changes in CaM that can explain the altered interaction with Cav1.2. Cav1.2 allows for influx of calcium ions from the extracellular space to the intracellular space in cardiac myocytes. In healthy individuals, wild-type CaM inactivates Cav1.2 when a certain calcium concentration is reached, thus allowing for repolarization. Upon increasing calcium concentration, CaM binds calcium and the resulting structural change is transmitted to the ion channel which is inactivated. The F141L mutation disrupts this calcium-dependent inactivation, thus leaving the ion channel open for too long causing an arrhythmia known as LQT syndrome. Here we show, that the mutation interferes with the hydrophobic packing of the C-lobe and completely disrupts the structure of the C-lobe in the absence of calcium. In the presence of calcium the C-lobe folds to a structure similar than the wild-type. This form is then binding to the IQ domain of Cav1.2 in a similar fashion than the wild-type. As a consequence of those structural changes, the affinity of CaM F141L for the Cav1.2 is too high in the low-calcium resting state, but too low in the calcium-loaded state causing misregulation of Cav1.2.

P106

Solution structure and dynamics of anti-CRIPSR protein AcrIIA4, Cas9 inhibitor probed by NMR spectroscopy

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The CRISPR-Cas system is a bacterial immune system against invading phages and foreign plasmids. An RNA-guided endonuclease Cas9 cleaves DNA targets of phages in the type II CRISPR-Cas system. Anti-CRISPR (Acr) proteins are phage proteins that act against host bacterial immunity by inactivating the CRISPR-Cas system. Here we report the solution structure and dynamics of AcrIIA4 from Listeria monocytogenes prophage, which also inhibits Streptococcus pyogenes Cas9 (SpyCas9) used for genome engineering technologies. AcrIIA4 has a compact monomeric abbbaa fold comprising three a helices, three antiparallel b strands, and a short 3.10 helix. Distinct backbone dynamics of AcrIIA4 in fast and slow timescales at loop regions implicates that the conformational plasticity at the binding interfaces contributes to the recognition for SpyCas9. AcrIIA4 binds to apo-SpyCas9 with KD 4.8 uM and to single guide RNA (sgRNA)-bound SpyCas9 with KD 0.6 nM. Thus, the binary complex of AcrIIA4 and SpyCas9 can associate with sgRNA to form a tight ternary complex, avoiding competition with the DNA substrates for SpyCas9 binding. We postulate AcrIIA4 initially forms a weak surveillance complex with Cas9, which turns into a tight inhibitor complex in the presence of mature guide RNA, effectively disabling the Cas9 nuclease activity.

P107

Structure in solution of fibronectin type III-domain 14 reveals its weak synergistic heparin binding site

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Fibronectin is a large multi-domain protein of the extracellular matrix that harbors two heparin binding sites, Hep-I and Hep-II, which shown to support heparin dependent adhesion of melanoma and neuroblastoma cells. The stronger heparin/HS binding site on fibronectin, Hep-II, spans across fibronectin type III-domains 12 to 14 (FN 12-14). Previous site-directed mutagenesis and NMR chemical shift perturbation studies as well as crystallographic structural analysis all agree in that the main heparin binding site is located on the surface of fibronectin type III domain 13. However the so-called 'synergy site' for heparin binding located on fibronectin type III domain 14 still remains elusive since no actual binding sites could be identified to date. Using multidimensional nuclear magnetic resonance (NMR) spectroscopy and isothermal titration calorimetry (ITC), we show here that heparin is able to bind to a cationic 'cradle' of fibronectin type III-domain 14 (FN14) formed by the PRARI sequence, which is involved in the integrin $\alpha 4\beta 1$ interaction, and to the flexible loop comprising residues KNNQKSE between the last two beta sheets of FN14. ITC data reveal that the individual domain FN14 binds to the sulphated sugars Dp (disaccharides degree of polymerization) 8 and Reviparin with similar affinities as the individual domain FN13 that contains the Hep-II site. Noteworthy, by introducing the last beta strand of FN13 and the linker region between type III-domains 13 and 14 preceding the FN14, the perturbation of NMR chemical shifts by heparin is significantly reduced, especially at the PRARI site. This indicates that the Hep-II binding site of fibronectin is mainly located on FN13 and the synergic binding site on FN14 only involves the KNNQKSE sequence with much lower affinity.

P108

Solution structure of RING domain of Doa10 and its interaction with E2 enzyme, Ubc6

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There are more than 15 E3 ligases which are involved in ER-associated degradation (ERAD) in mammalian cells, whereas only two E3s, Doa10 and Hrd1, have been identified in yeast. Hrd1 mediates proteasomal degradation of membrane and luminal proteins in ERAD, while a wide variety of proteins including cytoplasmic and membrane proteins of ER and nucleus are specified as substrates by Doa10. Doa10 is a membrane protein which has 14 transmembrane helices and N-terminal C4HC3 type RING domain (RING-CH) in cytosolic face .RING type E3 ligase is one of the two E3 superfamilies. E3 RING ligases typically situate the substrate and the E2/Ub complex by binding to both of them, enabling the direct transfer of Ub to substrate from E2. It is widely accepted that the function of RING E3 in proteasomal degradation pathway is mediated by its small bivalent zinc coordinating domain termed RING. The characteristic primary sequence of RING domains is cysteine rich and can be described as Cys-X2-Cys-X9-39-Cys-X1-3-His-X2-3-Cys-X2-Cys-X4-48-Cys-X2-Cys. Coordinating the two zinc ions confers a compact stable fold which commonly exhibits $\alpha\beta$ structure in cross brace manner on RING domain. Doa10 needs two E2 enzymes, Ubc6 and Ubc7, for its inherent activity in vivo. Here, we determined the solution structure of RING domain of Doa10 by NMR spectroscopy and elucidated its direct interaction with Ubc6. Also we present that Ubc6 can be degraded by Doa10 mediated ubiquitination, which means that the Ubc6 acts as both an E2 enzyme and a substrate of Doa10.

P109

The structure and binding mode of the MIA protein as a blueprint for non-cytosolic SH3-domains

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The Melanoma Activity Inhibitory (MIA) protein belongs to a family of four (MIA, OTOR/FDP, TANGO1, and TALI) non-cytosolic proteins or domains that adopt a SH3-like fold. Whereas MIA and FDP are small, secreted proteins with a SH3-like fold, TANGO1 and TALI are large transmembrane proteins located at the exit sites of the endoplasmic reticulum (ER) that contain an N-terminal SH3-like domain in the ER lumen. Despite highly conserved sequence patterns within the SH3-like domain of these proteins, they appear to be responsible for different tasks. While MIA is highly expressed in melanoma cells and cartilage, FDP is found exclusively in cartilage tissue of the ear. TALI and especially TANGO1 are major players in the export of huge cargo like collagens by forming megacarriers. Of all family members, only MIA has been investigated on a structural level. Here we present the interaction between MIA and fibronectin type III modules as well as disruption of this complex by small molecular ligands via NMR spectroscopy and molecular docking. Our data might serve as a blueprint for the modus operandi of these non-cytosolic SH3-domains. Uncovering the molecular basis of this interaction might lead to deeper understanding of important cellular mechanisms such as

malignant melanoma treatment.

P110

Backbone assignment of the 58 kDa B-subunit of the cholera toxin using 15N and 13C detection

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Cholera is an acute, diarrheal illness caused by infection of the intestine by the bacterium Vibrio cholerae. An estimated 2.9 million cases and 95,000 deaths occur each year around the world. The crystal structure of the highly homologous heat-labile enterotoxin (LT) and the cholera toxin are known. The cholera toxin is an oligomeric complex made up of six protein subunits: one copy of the enzymatic A subunit and five copies of the receptor binding B subunit (CTB). Cholera toxin has been suggested to bind to cells in the human intestine via the GM1 ganglioside. We decided to use NMR as a tool to study the protein-ligand interaction, and as a prerequisite, the backbone and preferably also the side chain assignment is required. The backbone assignment of the perdeuterated receptor binding subunit from LT was recently reported. CTB is readily overexpressed in V. cholera but is inherently difficult to deuterate. Instead we used a series of 13C and 15N detected 2D and 3D experiments (2D NH, 2D NCA, 2D NCO, 3D HCACO) to obtain essentially complete sequential assignment of the pentameric 58 kDa CTB. Side chain assignments were obtained from 1H-detected 3D HCCH and 13C-NOESY and 15N-NOESY experiments. Chemical shift perturbation on ligand binding indicate an allosteric mechanism in accordance with previously reported ligand binding cooperativity.

P111

Structure and dynamics of the phosphate-sensing SPX domain

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Several essential cellular processes including DNA and ATP synthesis require intracellular inorganic phosphate (Pi). On particular, for the maintenance of the thermodynamic potential of the ATP/ADP equilibrium, a tight regulation of cytosolic Pi levels is essential. This regulation of Pi homeostasis comprises Pi importers and exporters as well as proteins involved in storing and depleting Pi. Many of these proteins contain an N-terminal SPX domain, which has been suggested to serve as a Pi sensor. In plants, mutations in certain SPX-containing proteins cause changes in Pi transport and Pi starvation signaling. The crystal structures of several SPX proteins are known, showing that SPX comprises six alpha-helices arranged in a bundle (1). Understanding structure and dynamics of SPX in solution, as well as SPX interactions will help to better understand the mechanism of Pi regulation.

Here, we show an initial characterization of the SPX domain of yeast inorganic polyphosphate polymerase vacuolar transporter chaperone 2 (VTC2). This protein acts probably as a regulatory subunit of the VTC complex, which preforms the polyphosphate synthesis in the vacuole. The SPX domain boundaries in solution were redefined by solution NMR spectroscopy, leading to the discovery of an additional alpha-helix. The dynamics of the protein were quantified in presence and absence of two ligands – phytic acid and diphosphoinositol phosphate analogue 5 (PCP-InsP5). Both binding events increase the protein dynamics in both termini. Additionally, the presence of 5 PCP-InsP5 revealed reduced protein dynamics in the loop between helix 2 and helix 3 and their helix edges, as well as in helix 6, indicating ligand specificities regarding protein dynamics. The data suggest that ligand binding induces changes in protein dynamics of SPX domain, which might be responsible for the regulative effect on VTC complex function.

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P112

NMR characterization of Adenomatous Polyposis Coli protein: Resonance Assignment and Interactions with Binding Partners

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Colorectal cancer is the 2nd leading cause of cancer morbidity and mortality worldwide. The 5-year survival rate ranges from 93% for early stage to 8% for advanced stage. Although a complex interplay of genetic and epigenetic factors control the broad phenotypic spectrum of familial and sporadic colon cancers, the tumor suppressor adenomatous polyposis coli (APC) is considered the single “gatekeeper gene” for this disease. Mutations inactivating APC are found in 80% of all human colon tumors. The APC gene spans 58 kb with a 15-exon coding region of 8529 bp. Full length canonical APC protein consists of 2843 amino acids (310 kDa). This large scaffolding protein consists of at least eight functional subdomains. One well-established role of APC as a tumor suppressor is to negatively regulate the WNT signal transduction pathway via the formation of a “destruction complex” that promotes ubiquitination and subsequent proteasomal degradation of the transcription factor beta-catenin. APC contains several binding sites for beta-catenin. There are four so-called “15 amino acid repeats” (spanning aa 1020-1169) and seven so-called “20 amino acid repeats” (spanning aa 1262-2033). The former are sufficient for binding, whereas the latter are required for the destruction of beta-catenin. Nearly all APC mutations found in patients with colon cancer result in the expression of a truncated APC protein, typically including only the N-terminal 25-50% of the full-length protein. The 15-aa repeats are nearly always retained in truncation mutations, indicating that this central region imparts some selective advantage to cancer cells. No structural or spectroscopic characterization of this region has been published, although structural prediction software predicts a high probability of disorder, interspersed with small regions of predicted structure. Establishing whether this central domain of APC is a fully intrinsically disordered protein or possesses regions of stability will shape our understanding of the function of this critical protein-binding region. Solution NMR is an attractive method to probe the structure and dynamics of the central region of APC, assess the binding kinetics and stoichiometry of the interaction with binding partners and uncover the specific residues that interact noncovalently as a prelude to the development of molecular probe agonist/antagonists. The initial steps of this research project are described in this poster. Specifically, this poster describes the screening of different constructs, condition optimization for NMR spectroscopy, expression and purification of the protein in minimal media, backbone resonance assignments using triple-resonance experiments collected using Non-Uniform Sampling and characterization of the protein-protein interactions between APC and binding partners. The long-term goal of this project is to leverage the structural and protein-partner analysis of the central domain of tumor suppressor APC to inform future development of novel therapeutic strategies to address colorectal cancer.

P113

Structural studies of human lecithin retinol acyltransferase by NMR

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The lecithin retinol acyltransferase (LRAT) is a critical enzyme in the human visual cycle. It transfers the acyl group from the sn-1 position of phosphatidylcholine to all-trans retinol, producing all-trans retinyl esters. Several mutations in LRAT lead to the loss of vision. In order to understand how these mutations lead to loss of vision, we used NMR to study the kinetics and structural properties of a truncated form of wild-type LRAT (tLRAT), as well as several mutants that lead to the loss of vision. The NMR data presents an unexpected structure that is significantly different from published theoretical models. Moreover, the disease-causing mutants appear to have a 3D structure that is similar to the wild-type form.

P114

Structure-function studies of the H. polygyrus TGF-beta mimic TGM

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The mouse parasite Heligmosomoides polygyrus evades host immune responses by secreting a protein known as HpTGM that binds directly to the TGF-receptors. This activates the TGF-beta pathway and dramatically increases the population of immunosuppressive Fox3p+ Tregs. HpTGM, which is comprised of five homologous domains (D1-D5), each with approximately 90 amino acids and two characteristic disulfides, belongs to the complement control protein (CCP) family. The CCP family has no homology to the TGF-beta family and thus HpTGM is a structurally distinct evolved TGF-beta mimic. To better understand how HpTGM recognizes and assembles TbRI and TbRII into a signaling complex, binding studies with the different domains of HpTGM and the TGF-beta receptors were performed using NMR and ITC and correlated with results from cell-based TGF-beta reporter assays. These showed that TGM D1 and D2 cooperate to bind TbRI with the same high affinity as full-length HpTGM, while TGM D3 alone binds TbRII. Through NMR-based structural analysis, TGM-D3 was shown to adopt the same four-stranded antiparallel b-sheet characteristic of CCP family proteins, but differs in that it formed a continuous half b-barrel rather than two unconnected antiparallel b-strands. Through NMR, we further showed that TbRII uses the same exposed b-strand to bind TGM-D3 NMR as it does to bind TGF-bs, consistent with TGM-D3 competing against TGF-beta for binding to TbRII. TGM D1-D2 did not compete against TGF-beta in luciferase reporter assays, indicating that TbRI appears to use a different surface to contact TGM D1-D2 and TGF-beta. The emerging picture from these studies is that TGM appears to mimic TGF-beta in two important ways - first by binding TbRI and TbRII in close spatial proximity - second by binding TbRII through a single chain, but by binding and gaining very high affinity for TbRI by contacting it through multiple chains.

P115

Characterization a MazEF toxin-antitoxin pair from Mycobacterium tuberculosis in light of its virtue for population-wide survival

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As most of bacteria and archaea, Mycobacterium tuberculosis (the causative agent for tuberculosis in human) possesses toxin-antitoxin (TA) systems in its chromosome with a significant abundance. The typical components of TA system are ribonucleolytic toxin and its neutralizing antitoxin. A type of TA system called MazEF system is involved in quorum sensing (QS), a population controlling mechanism of unicellular organisms. A five amino acids long peptide generated during ribosome stalling triggered by activated MazF is one type of quorum sensing molecules. Once it is generated, the peptide interacts with MazF and elevates the toxicity of MazF. Due to its secretory nature and bacterial killing property, the peptide was termed extracellular death factor (EDF). EDF enables communications in a group of bacteria and the communications allow the maintenance of population density. The population controlling ability is strikingly important for a bacterial community in order to survive hostile conditions such as nutritional stress, low oxygen pressure and immune responses for a long period. The long-lived nature of M. tuberculosis is attributed to its intricate population-wide regulation as well as resistances gained in an individual cell level. This study presents the structure of free M. tuberculosis (strain H37Rv) MazF4 and MazF4 in complex with its cognate antitoxin MazE4. In conjunction with the structural studies, modulation of MazF4 endoribonuclease activity by MazE4 and an EDF homolog from M. tuberculosis was assessed via in vitro RNA cleavage assay.

P116

Integrative structural biology: how NMR can gain leverage from Cryo-EM Introducing the new Thermo Scientific Krios G3i and Glacios CryoTEMs

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In 2017 R. Henderson, J.Frank and J Dubochet have been awarded the Nobel prize in Chemistry for having pioneered cyo electron microscopy (Cryo-EM) and Single Particle Analysis (SPA). During the last few years Cryo-EM and SPA have grown from techniques able to produce low-resolution structures of protein complexes (aka blobology) to tools capable of achieving atomic and quasi-atomic resolution for complexes that nobody could solve with any other technique. This incredible leap forward has made possible by introduction and adoption of new tools, in particular direct electron detectors (DED), ultra-stable cryo-microscope, such as Titan Krios, and the adoption of new SW for automatic data collection and processing. Cryo-EM benefit of specific advantages, respect to other structural biology techniques such as NMR and X-ray diffraction: • Crystallization or isotopic labelling is not needed. • 0.5-1mg/mL of sample is required. • Different functional conformation of a complex may be sorted out. Cryo-EM has proved to be a very useful technique to be integrated with X-ray and NMR for structure-based drug design (1,2,3). In particular Cryo-EM is able to study structures of complex that are too big and too heterogeneous to be analyzed by other means. While NMR is especially suited to analyze the dynamic of small protein's domain (4). The combination of the two technology brings much more than the sum of its part. So it is no surprise that many structural biology groups all over the world are seeking access to this technology in order to find answers to their most

relevant biological questions. Nevertheless most new comers to the field are struggling to overcome the adoption barrier that this technique may pose in term of: sample preparation and screening, automatic data acquisition and progressive users training. In this presentation we will see how the fast pace of cryo-EM growth is going to change the structural biology landscape for the best. In particular we will discuss the • Glacios™ Cryo-TEM: A 200kV X-FEG autoloader-provided system capable of automatic screening of multiple grids and reduced footprint. • The new KriosTM G3i: The latest Krios version with improved automation, increased cryo-performance and higher throughput. References: 1: Cui T, Mowrey D, Bondarenko V, Tillman T, Ma D, Landrum E, Perez-Aguilar JM, He J, Wang W, Saven JG, Eckenhoff RG, Tang P, Xu Y Biochim Biophys Acta. 2012

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Integrative structural biology of the calcium dependent type 2 secretion pseudopilus

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Gram-negative bacteria use trans-envelope nano-machines to secrete proteins or complexes essential for nutrient acquisition, adaptation and virulence. The type 2 secretion system (T2SS) assembles membrane anchored fibers called pseudopili that promote secretion of specific and folded proteins from the periplasm to the extracellular medium1. Pseudopili are polymers of protein subunits assembled in helical fibers similarly to type 4 pili and flagella. Medical, environmental and fundamental relevance of these molecular machines can explain the growing interest in their study. However, despite considerable advances in genetic, biochemical and structural analysis, the molecular mechanism of fibre assembly remains unclear. In this work, we determined the structure of the T2SS pseudopilus from Klebsiella by combining the NMR structure of its subunit PulG in calcium bound state with the 5 Å resolution cryo-electron microscopy (cryoEM) reconstruction of assembled fibers2. The structural data together with mutagenesis and functional assays revealed the key role of calcium on the folding and the stability of PulG as well as the integrity of pseudopili. Our results suggest wider significance of the regulatory role of calcium in the pseudopilus length control, essential for protein secretion.

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P118

Insights into the folding of a liver-fluke derived granulin

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Granulins are a family of growth factors involved in cell proliferation. The liver-fluke granulin, Ov-GRN-1, isolated from a carcinogenic liver fluke Opisthorchis viverrine, can significantly accelerate wound repair in vivo and in vitro. However, Ov-GRN-1 is difficult to express in high yields making it unlikely to be a valuable drug lead. We have recently shown that a truncated analogue (Ov-GRN12-35_3s) can also promote wound healing in a mouse model. NMR analysis of this analogue indicates the presence of multiple conformations, most likely as a result of proline cis/trans isomerisation. To further investigate whether the proline residues are involved in adopting multiple confirmations we have synthesised a set of analogues involving mutation of the proline residues. We have shown that the proline residues have a significant influence on the structure, activity and folding of Ov-GRN12-35_3s. Furthermore, it appears the N-terminal region is critical for the cell proliferation activity. These results provide insight into improving the oxidative folding yield and bioactivity of Ov-GRN12-35_3s, and might facilitate the development of a novel wound healing agent.

P119

NMR elucidation of monomer–dimer transition and conformational heterogeneity in histone-like DNA binding protein of Helicobacter pylori and its interaction with human SUMO-1

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Major global healthcare issues in the 21st century are due to infections caused by drug resistant bacteria including Helicobacter pylori. H. pylori colonizes under harsh acidic/oxidative stress conditions of human gastrointestinal tract and can survive there for infinitely longer durations of host life. The bacterium expresses several harbinger proteins to facilitate its persistent colonization under such conditions. One such protein in H. pylori is histone-like DNA binding protein (Hup), which has been recognized as a novel drug target owing to its capabilities to perform both architectural and regulatory cellular functions. Legitimately, if the binding of Hup to DNA is suppressed, it will directly impact on the survival of the bacterium, thus making Hup a potential therapeutic target for developing new anti-H. pylori agents. However, to inhibit the binding of Hup to DNA, it is necessary to gain detailed insights into the molecular and structural basis of Hup-dimerization and its binding mechanism to DNA. As a first step in this direction, we achieved the NMR assignments and structural features of Hup at pH 6.0. The study revealed the occurrence of dynamic equilibrium between its monomer and dimer conformations. The dynamic equilibrium was found to shifting towards dimer both at low temperature and low pH; whereas DNA binding studies evidenced that the protein binds to DNA in its dimeric form. These preliminary investigations correlate very well with the diverse functionality of protein and will form the basis for future studies aiming to develop novel anti-H. pylori agents employing structure-based-rational drug discovery approach. Moreover, infection with H.pylori represents a key factor in the etiology of various gastrointestinal diseases including gastric cancer. Disease outcome is the result of complex inter-

play between the host and pathogen which can be explained in part by interaction of human and bacterial proteins (secreted in-vitro). These secreted proteins contribute to gastric inflammation and epithelial damage; thus aid bacterial invasion in host tissue, and may also interact with host proteins, conspirating a mechanism against host immune system. As Hup is also secreted in vitro by H. pylori, thus it may have its role in disease pathogenesis. This is possible only if Hup interact with some host's proteins like SUMOs which participate in various innate immune pathways and thus promote an efficient immune response to combat pathogenic infections. Sequence analysis of Hup revealed the presence of two closely similar SIMs. Additionally, SUMO proteins epitomize negatively charged surface which confers them the ability to bind to DNA/RNA binding proteins. Based on the presence of both SIMs and negatively charged surface, it is legitimate to consider that SUMO proteins would bind to Hup. The present study has established this interaction for the first time making composite use of NMR and molecular docking methods.

P120

Hyperpolarized water to visualize disordered, well-folded and lowly-populated states in proteins

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A number of high-field dynamic nuclear polarization (DNP) methods were developed in recent years to improve the sensitivity of solution NMR; foremost among these the dissolution DNP (dDNP) approach.[1] Ex-situ dDNP of hyperpolarized water ("HyperW") holds great potential in protein NMR studies: when injecting this solvent into an NMR tube containing a protein that can readily exchange its amide protons, amide-based hyperpolarized protein NMR studies become feasible. [2],[3] 2D 1H-15N correlations are collected with high sensitivity due to rapid exchange which transfers magnetization from hyperpolarized water to the amide groups, and the long-lived (≈1-2 min) signal enhancements that can be achieved thanks to the long relaxation times T1 of water protons. HyperW can be particularly useful for studies of intrinsically disordered proteins or domains, which exhibit fast amide exchange.[4]-[6] With optimizations and resolution improvements[6] of enhanced 2D protein NMR spectra at hand, one can use the HyperW method in the structure and dynamics study of a variety of proteins. So far these have mainly focused on intrinsically disordered peptides and proteins.[2]-[6] Here, we extend HyperW and compare its applicability to three types of proteins: PhoA – a fully unfolded protein fragment; barstar - a folded protein; and a drkN-SH3 domain which exists in equilibrium between a folded and an unfolded state. For the unstructured PhoA fragment, the sensitivity enhancements were very high, thanks to the fast amide exchange experienced by residues throughout the sequence. For the barstar protein an "exchange filter" behavior was observed, whereby higher enhancements were measured for surface exposed yet fully folded residues. For the drkN-SH3 domain, HyperW HMQC managed to bring the lowly-populated, usually invisible folded state to light with the aid of a 3-site exchange magnetization transfer. This demonstrates the many strengths of the HyperW method: it can serve for sensitizing the NMR of disordered proteins; it can also be applied to study fully folded proteins and probe the latter's solvent accessibility; and it can be used to visualize what are normally "invisible" protein states. Acknowledgements: Financial support from the Israel Science Foundation Grant 795/13, the Program of the Planning and Budgeting Committee from the Israel Science Foundation (iCORE) Project 1775/12, the Kimmel Institute of Magnetic Resonance (Weizmann Institute), EU'S Instruct-ERIC, and the Perlman Family Foundation, are gratefully acknowledged. 1. Ardenkjær-Larsen, J. H., et al., PNAS, 2003 2. Harris, T., et al., J. Phys. Chem. B, 2014 3. Chappuis, Q., et al., J. Phys. Chem. Lett., 2015 4. Olsen, G., et al., J. Magn. Reson., 2016 5. Kurzbach, D., et al., Angew. Chem. Int. Ed., 2017 6. Szekely, O., et al., Anal. Chem., 2018

P121

Delineating the differential structural-stability and dynamic features of Interleukin-8 orthologs using NMR

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Chemokines are 8-10 kDa disulphide bridged proteins that provide regulatory cues during leukocyte trafficking. Neutrophil activating chemokines (NACs) are subgroup of chemokines that are actively involved in the recruitment of neutrophils during various exalted inflammatory events at the site of infection. This process also includes interaction of chemokines with cell surface glycosaminoglycans (GAGs), and G-protein coupled receptor of neutrophil cells. Structural stability and activity of each protein is solely dependent on its amino acid sequence. In order to unravel the species specific structure-stability features, recombinant human (hIL8) and canine IL8 (cIL8) orthologous proteins were overexpressed and purified. Backbone resonance assignment of both proteins has been obtained using triple resonance NMR experiments. Both the proteins exist as dimer at pH 7 as evidenced by diffusion NMR and 3D-NOESY spectroscopy. The NMR based structure calculation shows that cIL8 has smaller c-terminal α-helix and β3 strand then hIL8. Hydrogen-deuterium exchange analysis for exchangeable backbone protons evidenced that hIL8 molecule is higher stable then cIL8 molecule. NMR based temperature dependent amide proton chemical shift measurements and relaxation studies differentiated the dynamic differences between the orthologous IL8 proteins. Furthermore, heparin binding studies suggested that hIL8 binds stronger to heparin as compared to cIL8. Considering IL8 as the most conserved and functionally significant chemokine for neutrophil trafficking, the comparative analysis of the IL8 orthologs provided mechanistic insights into the evolution-structure-function relationship of NAC family chemokines.

P122

Visualisation of the invisible state of human lysozyme by solution NMR

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It is becoming increasingly clear that transiently and sparsely populated high free energy states of protein are key intermediates in a wide range of biological and chemical processes. These have been recalcitrant and invisible to most biophysical techniques through their heterogeneous nature and low populations. Here we consider human lysozyme, a globular protein that populates molten-globule like excited protein conformers, which are prone to self-assemble into amyloid fibrils that trigger detrimental non-neuropathic systemic amyloidosis in patients. While an understanding is emerging of how these partially unfolded lysozyme molecules occur during the aggregation pathway, an essential understanding of the structure and dynamic aspects of the process is still lacking.

We have undertaken a very detailed investigation of the partially unfolded intermediate state of lysozyme by monitoring the global unfolding process

at low pH by CEST and RD NMR. 15N CEST profiles at several magnetic fields and temperatures clearly demonstrate the exchange between the native and unfolded states with the population of the latter increasing with temperature. Remarkably, an additional state is also revealed in CEST profiles, which is distinctive from both native and unfolded state. This is likely to be the partially unfolded intermediate state that is in exchange with the unfolded state and self-assembles into amyloid fibrils. Global fitting of all the 15N CEST and CPMG data results in robust kinetic and thermodynamic parameters for the three exchanging states, in particular, characterising the intermediate state, which is only populated up to 0.5 % and has been invisible to most biophysical techniques, even NMR.

Applying CEST on a range of nuclei (15N/1H/13C) has allowed us to significantly extend our mechanistic understanding: 1H CEST on amides not only cross-validates the same exchange parameters from 15N CEST, but provides the 1H chemical shifts of the intermediate state. Also, 13C/1H CEST on the side-chains, both Ca/Ha and methyl groups, have enabled the measurement of the chemical shifts of the invisible unfolded state. The 1H/13C/15N chemical shifts from CEST have been utilised in restrained molecular dynamics simulations to obtain early structures on the invisible intermediate state as well as the unfolded state. Such structures, together with the NMR dynamics of the excited lysozyme conformers are providing the deepest available glimpses towards the targeting of lysozyme amyloidosis.

P123

Structural and dynamical characterization of the plasticity of PDZ

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The human serine protease HtrA2/Omi is a member of the HtrA (high-temperature requirement) proteins located in mitochondria. The enzyme is involved in the regulation of homeostasis, caspase-dependent and –independent apoptosis as well as in pathogenesis. The proteolytic activation of the protease occurs by ligand binding to its C-terminal PDZ domain. Upon ligand binding, PDZ releases the protease domain leading to activation. We have been investigated the PDZ domain of HtrA2/Omi to gain better insights on its structure, dynamics and ligand binding properties using NMR spectroscopy. We assigned 72% of the NMR backbone resonances of the apo form of PDZ. Furthermore we show that PDZ in its apo state forms monomers, whereas PDZ bound to a canonical peptide forms dimers. These findings are supported by the analysis of internal obtained NMR line width, size exclusion chromatography and crystal structure. Moreover, we obtained a crystal structure of apo PDZ showing that helix $\alpha 1$, which is mainly involved in the interaction with the protease domain, is affected by ligand binding With these investigations, we provide a structural and dynamical characterization of the plasticity of PDZ and its changes upon ligand binding that will lead to a better understanding of the HtrA2 activation mechanism.

P124

Intramembrane cleavage of the Alzheimer linked-protein TREM2 is regulated by its intrinsic dynamics

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TREM2 (Triggering factor expressed on myeloid cells 2) is a type I single-pass transmembrane protein which has been linked with an impaired immune reaction in the brain and therefore associated with an increased risk for several neurodegenerative disorders like Alzheimer's or Parkinson's disease. TREM2 undergoes regulated intramembrane proteolysis (RIP) which includes shedding of the extracellular domain (ECD) into the cerebrospinal fluid followed by intramembrane cleavage of the remaining membrane anchored C-terminal fragment (CTF) by γ-secretase. While a structure of the ECD is available, structural information about the transmembrane domain (TMD) are still missing.

Using solution state NMR with isotope-labelled TREM2-TMD we could determine the structure of the wild-type (wt) TREM2-TMD in DPC micelles and investigated its intrinsic dynamics with NMR relaxation experiments. The presented structure and dynamical data suggest that TREM2-TMD contains a highly flexible kink region located in its C-terminal part. Of special interest, in the TMD a positively charged lysine residue at position 186 is directly located in the otherwise hydrophobic transmembrane region. Disease-related K186N variant was found to accumulate at the cell surface without being able to engage in downstream signalling. K186 seems to be crucial for binding to DAP12 since the interaction can be completely abolished by this mutation. Interestingly, the structure of wt TREM2-TMD in complex with DAP12-TMD resembles the structure of the K186A variant. Consequently, the K186A variant leads to a severe loss in the intrinsic dynamics as compared to the wt TMD. In summary, we here provide novel structural and dynamical features which might be involved in the process of intramembrane cleavage.

P125

Pseudocontact shifts of backbone amide protons generated by double-histidine Co2+-binding motif at multiple sites for protein structure determination

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Site-specific attachment of a paramagnetic cobalt ion to a protein generates pseudocontact shifts (PCS) in the NMR spectra of the protein. The PCSs contain accurate information about the location of nuclear spins relative to the coordinate frame of the $\Delta\chi$ tensor, provided that the metal ion is rigidly attached to the protein. The double-histidine motif achieves this for a Co2+ ion better than established lanthanide tags. Labeling of the protein with cobalt at four different sites may be sufficient to determine the 3D structure of the protein from the PCSs of backbone amide protons only, using the software GPS-Rosetta.

P126

Methyl-selective 13C incorporation using alpha-ketoisovalerate for the yeast Pichia pastoris expression system

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Methyl groups in proteins are sensitive detection probes for NMR observation of high molecular weight proteins, and thus, selective isotope labeling of methyl groups is indispensable for methyl-detected NMR approach. A robust method to incorporate 13C into methyl groups has been developed for typical methyl-containing amino acids, Ile, Leu, and Val using E. coli recombinant protein expression systems, in which the metabolic precursors are supplemented in the culture media to achieve the methyl-selective 13C incorporation. The methylotrophic yeast Pichia pastoris (P. pastoris) is the most widely used yeast to express recombinant proteins, and various heterologous proteins including human membrane proteins could be expressed by P. pastoris. However, this expression system was not optimized for methyl-specific isotope labeling, especially for Val/Leu-methyl specific isotope incorporation. By exploring various culture conditions, we previously found that lowering the pH of the culture medium facilitates the cellular uptake of the Val/Leu precursor, alpha-ketoisovalerate (KIV). This approach was further developed and could be applied to different phenotypes (Mut+ and MutS) of P. pastoris strains, and the labeling efficiency of Val residues reached 74%, which was much higher than the reported values for yeast expression systems using the labeled KIV. Moreover, we modified the protocol to produce the labeled protein at biologically natural pH conditions with twofold higher yield than the original protocol. This protocol was expanded for the production of the Val-methyl-selective 13C-labeled protein, which is useful to simplify NMR spectra of larger proteins.

P127

NMR Crystallography in Tryptophan Synthase: Proton Positions, Stable Intermediates, and Transition States

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NMR-assisted crystallography – the synergistic combination of solid-state NMR, X-ray crystallography, and first-principles computational chemistry – holds remarkable promise for mechanistic enzymology; by providing atomic-resolution characterization of stable intermediates in the enzyme active site – including hydrogen atom locations and tautomeric equilibria – it offers insight into structure, dynamics, and function. Here, we make use of this combined approach to characterize the aminoacrylate intermediate in tryptophan synthase, a defining species for pyridoxal-5'-phosphate-dependent enzymes on the β-elimination and replacement pathway. By uniquely identifying the protonation states of ionizable sites on the cofactor, substrates, and catalytic side chains, as well as the location and orientation of structural waters in the active site, a remarkably clear picture of structure and reactivity emerges. Most incredibly, this intermediate appears to be mere tenths of angstroms away from the preceding transition state in which the β-hydroxyl of the serine substrate is lost. The position and orientation of the structural water immediately adjacent to the substrate β-carbon suggests not only the fate of that hydroxyl group, but also the pathway back to the transition state and the identity of the active site acid-base catalytic residue. Enabling this analysis is the ability to measure active-site isotropic and anisotropic NMR chemical shifts under conditions of active catalysis, and the development of fully quantum mechanical computational models of the enzyme active site that allow the accurate prediction of NMR spectral parameters.

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P128

A Stabilizing Role of Side-Chain to Main-Chain Hydrogen Bonds in PolyQ Helices

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Poly-glutamine (polyQ) tract expansions have been linked to nine human neurodegenerative diseases. The conservation of such sequences points to a relevant role, which is suggested to involve their organization into structural elements depending on their protein sequence context1,2,3. For the particular case of the androgen receptor (AR) we recently reported that the Leu-rich segment N-terminal to the polyQ tract acts as a helical N-capping sequence that propagates helicity into the tract itself1. Based on that, we have collected CD, NMR and molecular simulations data on a set of recombinant, isotopically enriched peptides representing increasingly longer AR polyQ tracts up to the lengths found in the average human population (16-25 Gln residues, depending on ethnicity)4. In agreement with helix-coil transition theory, experimental data shows that the helicity of the sequence positively correlates with the tract length, and that a rotameric selection affects the sidechains of the initial glutamine residues in the tract upon helicity gain. In turn, simulations unveil that helix stabilization is achieved through hydrogen bonds involving both the backbone and glutamine sidechains, resulting in a non-canonical helical arrangement. Proteome analysis shows that especially leucine and, to a lower extent, other helical, hydrophobic residue types are highly enriched in adjacent sequences N-terminal to polyQ tracts. We propose that these generate a hydrophobic shielding for Gln side-chain-involving hydrogen bonds, providing energetic stabilization by avoiding exchange with water5. CD data acquired on sequence mutants strategically designed to evaluate these observations experimentally validate the helix-stabilizing role of these hydrogen bonds and the relevance of hydrophobic shielding. This supports a structural role for the polyQ tract, as a minimum number of glutamine residues are required to stabilize the helicity while further growth of the tract is detrimental because of increased aggregation rates6,7.

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P129

Direct observation of protein monovalent cation binding sites by NMR.

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Monovalent cations are important components of many biological macromolecular assemblies, but are typically challenging to study by NMR. Through our studies of Kbp, a potential regulator of cellular homeostasis in E. coli whose structure is K+ dependent, we have explored strategies to discover and characterise the monovalent cation binding site. Potassium is key to cellular homeostasis. E. coli possesses several specific K+ influx and efflux systems that maintain the intracellular K+ concentration over a broad range of external pH, osmolarity and K+ concentrations. Although regulatory proteins and sensor domains have been identified for a several K+ ion transport systems, the exact mechanism by which K+ concentration is sensed in the cell and therefore how these systems are regulated remains unknown. Expression of the cytoplasmic protein Kbp is strongly upregulated in response to osmotic stress. We discovered that Kbp is an unprecedented, highly selective, soluble K+ binding protein and determined its K+-bound structure (1). NMR, CD and ITC studies of Kbp show that K+ binding induces a conformational change in Kbp that orders the protein structure that cannot be brought about by even high concentrations of Na+. We have used a range of techniques to explore the conformational repertoire of Kbp and identify the ion binding site. Our latest results include progress towards an NMR method for the direct observation of the interaction of proteins with monovalent cations backed by quantum mechanical calculations. (1) Ashraf et al (2016) Structure 24:741-749.

P130

Solution structral analysis of novel small heat shock protein Orf7.5 from Synechococcus elongatus PCC 7942.

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In cyanobacterium Synechococcus elongatus PCC 7942, a novel heat shock gene, orf7.5, which encodes the small acidic polypeptide (63 aa), was cloned. Northern blot analysis revealed that the expression level of orf7.5 is transiently increased in response to the heat shock stress. To reveal the role of orf7.5 in thermal stress adaptation, we constructed a stable mutant in which orf7.5 was disrupted. This mutant shows the remarkable inhibition of growth at 45 degrees C and a decrease in the basal and acquired thermo-tolerances at 50 degrees C. Furthermore, accumulation of the groESL transcript in this mutant was strongly reduced. Characters of this mutant were complemented to wild type phenotype with the DNA fragment coding normal Orf7.5, not mutated Orf7.5. From these results, we concluded that the Orf7.5 controls the expression of the groESL operon, and the relationships between structure and function of Orf7.5 must be elucidated. Orf7.5 has the small molecular weight and is classified in the category of small heat shocl protein (sHSP). However, the amino acid composition of Orf7.5 is different from other already known sHSPs, and crystalline domain, which is found in usual sHSPs, is not found in Orf7.5. This feature strongly suggests that Orf7.5 has unique structure and function which are not found in other sHSPs. To clear the physicochemical and biochemical properties of Orf7.5, we have constructed the overexpression system of Orf7.5 with the use of transformed E.coli. Gene fragment coding Orf7.5 was amplified and inserted into the NdeI/XhoI sites of pCold PROS2 vector and transferred into the BL21(DE3)/pLysS. Overexpressed PROS2 tagged protein was purified with Ni2+-affinity and size exclusion chromatography. Purified tagged proteins were digested with Factor Xa and purified Orf7.5 was obtained with size exclusion chromatography. On the physicochemical properties of purified Orf7.5, we found follow. 1) With the TOF-MAS analysis and cross-linking experiments with SDS-PAGE shows that Orf7.5 mainly presents as monomer in solution state. 2) Secondary structural change monitored with UV-CD analysis shows that Orf7.5 is quite stable below 50 degrees C. 3) Almost the same heat stability is observed with the 1H-15N NMR spectra measured at different temperature. On biochemical properties of purified Orf7.5, we found follows. 4) For maltose dehydrogenase and citrate synthase as model proteins, in the presence of Orf7.5, aggregation formation induced by heat denaturation is significantly inhibited. In consideration of these characters for Orf7.5, we further analyze the solution structure, with heteronuclear multidimensional NMR spectroscopy using 13C, 15N doubly labelled Orf7.5.

P131

Temperature-Dependent Protein Fold Switching

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The occurrence of metamorphic proteins that have the capacity to switch between completely different folds presents a challenge to the classical view that a protein's amino acid sequence encodes a unique 3-dimensional native structure. These include naturally occurring proteins that can undergo large-scale changes in their fold topologies under physiological conditions in response to environmental triggers, such as pH, ligand, and redox state, thereby expanding their functional capacities. In addition to natural examples, the engineered fold switch system of GA (3-α) and GB (4β+α) has demonstrated that these different folds can be connected through short mutational paths. In more recent studies, a new model system of fold switching was engineered, in which the 3-α GA fold was co-evolved to 100% sequence identity with the α/β-plait fold of the ribosomal protein S6. The 56 amino acid GAS6 sequence independently folds into a 3-α helical bundle and adopts the α/β-plait fold topology when embedded in the larger 95 amino acid sequence of S6GA. Thus, the S6GA engineered construct signified that a single amino acid sequence can code for more than one fold. Here, I present the mechanism of temperature dependent fold inter-conversion between the α/β-plait and the latent 3-α fold in this novel "fold-within-a-fold" system. To perturb the α/β-plait structure of S6GA and shift the equilibrium towards the alternative 3-α state, single amino acid mutations of residues that specifically stabilize the α/β-plait were investigated. Subtle mutations such as valine to threonine or isoleucine, particularly in the C-terminus of the S6GA sequence, lead to mutant proteins that simultaneously populate the α/β-plait and 3-α conformations with varying proportions. 2D 15N HSQC NMR analysis showed that these mutants exhibit temperature dependent fold inter-conversion within a narrow temperature range, 5 – 37 °C, in which the 3-α state is predominantly populated at lower temperature and the equilibrium gradually shifts to the α/β-plait state as the temperature is raised to 37 °C. A number of NMR spectroscopy techniques are used to analyze the structure, dynamics, energetics, and kinetics of the fold switch as a function of temperature. This investigation demonstrates the role of disordered ends in the ability of a protein to switch between different conformations. Results from this study also provide insights into the likelihood of new folds evolving from existing 3D structures through mutations and environmental triggers such as temperature that stabilize alternate folds and functions.

P132

Solid-state NMR Investigation on the Structure of Histones and DNA in the Nucleosome

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Understanding the structure of chromatin is indispensable as it provides fundamental atomic information to delineate the roles of chromatin in cellular processes like DNA replication, transcription and repair. The nucleosome core particle (NCP) is the fundamental unit of eukaryotic chromatin composed of four core histone proteins organizing 147 bp of DNA. We have performed dipolar coupling and J-coupling based solid-state NMR (SSNMR) experiments to elucidate the structure of human histone H4 (hH4) and DNA in NCPs reconstituted with different DNA sequences and a nucleosome array. The currently presented results indicate that hH4 tails adopt similar structures in the NCP and the nucleosome array reconstituted with 145 bp Widom '601' high affinity positioning DNA sequence. Furthermore, the structure of hH4 is elucidated for telomeric NCP, revealing that the well-structured globular regions of hH4 in the telomeric NCP have the same conformation as in the Widom '601' NCP and exhibit relatively higher mobility in several regions. In particular, one of the hH4 tails in the telomeric NCP show restricted flexibility and conformational heterogeneity, likely due to the interactions with other regions of the NCP. In addition, we also present preliminary SSNMR characterization of DNA in the NCP

P133

NMR Structural Studies of a Novel Staphylococcal Inhibitor of Myeloperoxidase

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The bacterium Staphylococcus aureus produces an array of anti-inflammatory molecules that prevent the innate immune system from recognizing it as a pathogen and clearing it from the host. In the acute phase of inflammation, our immune system relies on neutrophils to clear invading bacteria. Recently, novel classes of secreted proteins from S. aureus, including the Extracellular Adherence Protein (EAP) family and the Staphylococcal Peroxidase Inhibitor (SPIN) have been identified as highly selective inhibitors acting on Neutrophil Serine Proteases (NSPs) and myeloperoxidase (MPO) respectively.

SPIN is a protein found only in Staphylococci, with no sequence homology to any known proteins. Solution NMR structural studies of SPIN are therefore expected to provide a deeper understanding of its interaction with MPO. In this study, we report the backbone and side-chain 1H, 15N, and 13C resonance assignments of SPIN. Furthermore, using the chemical shifts of these resonances, we predicted the secondary structure of SPIN in solution via the TALOS-N server. The assignment data has been deposited in the BMRB data bank under accession number 27069.

P134

NMR-based structural biology of the interaction between c-Myc and Miz-1: Towards the rational development of a new generation of c-Myc inhibitors

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c-Myc is a b-HLH-LZ transcription factor involved in the tumorigenesis of the majority of cancers. c-Myc heterodimerizes with another b-HLH-LZ transcription factor called Max to bind DNA and activate the transcription of pro-oncogenic genes. Hence, the discovery of small molecules that could prevent this step by binding to the b-HLH-LZ of c-Myc (dubbed c-Myc°) to block the heterodimerization and DNA binding is actively being pursued. Despite being a prominent target to treat cancer, no such effective inhibitors have entered the clinic yet or have been found to be promising leads. In fact, c-Myc is an extremely difficult target and is even considered undruggable. Such a designation is attributed to its intrinsically disordered nature and the absence of (a) stable, well-defined and druggable binding site(s) through rationale medicinal chemistry. Recently, the stabilization of Miz-1 (Myc Interacting Zinc Finger protein 1) from degradation was shown to repress c-Myc transcriptional activities in cancer cells. In this context, we sought to characterize the interaction between c-Myc° and a region of Miz-1 (dubbed Mid2: a.a. 641-715) previously identified to interact with c-Myc°. In this poster, we show that the Mid2 and the Max b-HLH-LZ compete to form complexes with c-Myc°. Our results also demonstrate the formation of thermodynamically stable complex in the slow exchange regime between the c-Myc° and Mid2. Moreover, we note from 1H-15N HSQC spectra that both components undergo drastic conformational changes upon association. As discussed, the conformations of c-Myc° and the Mid2 in the complex possess structural knowledge to accelerate the discovery of a new generation of c-Myc inhibitors. Indeed, on the one hand, the 3D structure of the Mid2-bound c-Myc° might reveal a well-defined binding pocket large enough for the design of new molecules capable of targeting this state. On the other hand, the 3D structure of the c-Myc°-bound Mid2 might possess the chemical and structural information necessary to initiate the rationale design of promising and new inhibitors.

P135

Two distinct mechanisms of transcriptional regulation by the redox sensor YodB

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For bacteria, cysteine thiol groups in proteins are commonly used as thiol-based switches for redox sensing to activate specific detoxification pathways and restore the redox balance. Among the known thiol-based regulatory systems, the MarR/DUF24 family regulators have been reported to sense and respond to reactive electrophilic species, including diamide, quinones, and aldehydes, with high specificity. Here, we report that the prototypical regulator YodB of the MarR/DUF24 family from Bacillus subtilis uses two distinct pathways to regulate transcription in response to two reactive electrophilic species (diamide or methyl-p-benzoquinone), as revealed by X-ray crystallography, NMR spectroscopy, and biochemical experiments. Diamide induces structural changes in the YodB dimer by promoting the formation of disulfide bonds, whereas methyl-p-benzoquinone allows the YodB dimer to be dissociated from DNA, with little effect on the YodB dimer. The results indicate that B. subtilis may discriminate toxic quinones, such as methyl-p-benzoquinone, from diamide to ef-

ficiently manage multiple oxidative signals. These results also provide evidence that different thiol-reactive compounds induce dissimilar conformational changes in the regulator to trigger the separate regulation of target DNA. This specific control of YodB is dependent upon the type of thiol-reactive compound present, is linked to its direct transcriptional activity, and is important for the survival of B. subtilis. This study of B. subtilis YodB also provides a structural basis for the relationship that exists between the ligand-induced conformational changes adopted by the protein and its functional switch.

P136

A structural characterization of RSV interferon antagonist NS1

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Respiratory syncytial virus (RSV) is the main agent responsible for acute lower respiratory tract infections in infants, immunosuppressed and elderly worldwide. RSV has evolved a unique strategy to evade the host immune response, by encoding two nonstructural proteins, NS1 and NS2, which are the first genes transcribed and the most abundant RSV proteins upon infection. No homologs are known. NS1 and NS2 are multifunctional proteins, which have been shown to antagonize type I and III interferons (IFN) by downregulating IFN synthesis and signaling. Intriguing aspects of these proteins are their propensity to form multimers and amyloid-like behavior, since they bind thioflavine T. A crystal structure of NS1 was recently published. Here we propose to address the structure of RSV NS1 in solution, either as a monomer or as an oligomer. We optimized its production as a recombinant protein in E. coli and its subsequent purification. NS1 contains very reactive cysteines, the oxidation state of which needs to be tightly controlled. We first studied its concentration dependent association by size exclusion chromatography and dynamic light scattering. We then evaluated the potential of NMR as a method to get structural insight into NS1. We show preliminary data obtained by solid state NMR on an aggregated form of NS1 and by solution NMR on its monomeric form. Due to its propensity to form oligomers, we investigated the influence of physico-chemical parameters like pH, temperature and pressure on the stability of a monomeric species in solution.

P137

Structure and interaction studies on INI1 and HIV-1 integrase

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Integrase interactor1 (INI1) is a core subunit of the ATP-dependent chromatin-remodeling complex, SWI/SNF. It interacts with the human immunodeficiency virus type 1 (HIV-1) integrase, which plays a role as one of the host factors that regulates HIV-1 propagation and integration. INI1 consists of three regions; DNA binding, two direct imperfect repeats (Rpt1 and Rpt2) and coiled-coil domain. The human immunodeficiency virus (HIV-1) integrase (IN) is a key protein for infection to human. Repeat domain 1 (Rpt1) of INI1 specifically binds with HIV-1 IN. HIV-1 IN consists of three domain, N-terminus (NTD), catalytic core domain (CCD) and C-terminus (CTD). We have determined solution structure of the INI1-Rpt1 by using NMR spectrocopy. The resonance assignments of the Rpt1 domain were completed using a combination of HNCACB, CBCA(CO)NH, HNCO, HNHA, (H)CCC(CO)NH, H(CCCO)NH, and HCCH-TOCSY data. NMR data provided that INI1-Rpt1 contains of two α-helices and two β-sheets. Fluorescence assay and NMR titration provide a detailed inter-molecular interaction between INI1-Rpt1 and HIV-1 IN. Our results provide functional insight of INI1 and HIV-1 IN interaction during HIV-1 infection.

P138

Metal binding properties of the cysteine-rich biomarker Hepcidin-25

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Hepcidin regulates iron homeostasis in response to inflammation, erythropoietic demand, and iron stores (reference 1). The native state of hepcidin-25 is an attractive target for the development of a reliable analytical tool that can quantify the hepcidin concentration in biological samples and reveal iron metabolic disorders (reference 2). Therefore, a selective immunoassay would have to discriminate between different types of hepcidin and quantify only hepcidin-25's concentration. The peptide contains well defined β-sheets and a β-hairpin loop stabilized by four disulfide bonds (reference 3). Recently, it was shown that hepcidin-25 contains an ATCUN motif at its N-terminus. This motif is known to have high affinity towards Cu2+ and Ni2+ (reference 4). Here, we present an optimized procedure for preparing natively folded hepcidin-25 (2.80 kDa) and structural analysis of metal binding to hepcidin-25. Hepcidin was expressed as a His6-SUMO-hepcidin-25 fusion protein (16.20 kDa) in Escherichia coli, Origami B strains, and purified as a soluble recombinant protein in three steps. After purification based on the nickel affinity chromatography, the purified His6-SUMO-hepcidin-25 fusion protein was cleaved by the SUMO-specific ULP1 protease. The liberated hepcidin-25 was further purified on a HiLoad 16/600 Superdex 30 column and folded in the last step of purification in the presence of glutathione. The presence of natively folded hepcidin-25 after RP-HPLC was confirmed by ESI-MS and NMR spectroscopy. Based on published chemical shifts (reference 3), we achieved a nearly complete assignment of the labeled and unlabeled hepcidin-25 at pH=3 at room temperature as well as at 325 K in order to minimize line broadening caused by intermediate dynamics of the loop region. Comparison of 1H chemical shifts and TOCSY spectra at pH=7 in the presence and absence of Ni2+ demonstrates that the metal binds at the N-terminus of hepcidin-25. Coordinating Ni2+ causes chemical shift perturbations as a consequence of changes in the electronic structure and the most strongly affected amino acids are those of the ATCUN motif, specifically aspartic acid [Asp-1], threonine [Thr-2] and histidine [His-3]. Chemical shift changes due to metal complexation decrease further away from the metal binding site.

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Interaction of amyloid precursor protein transmembrane fragments with sterol and conformational variability of its wild-type and "australian" mutant forms

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Despite some progress in study of the molecular mechanisms of Alzheimer's disease (AD) pathogenesis initial steps of this pathogenesis are still unknown. Amyloidogenic Aβ-peptides forming plaques in the brain are products of consecutive intramembrane cleavage of Amyloid Precursor Protein (APP). More than half of familial mutations of APP predisposing to AD development occur in its transmembrane domain and juxtamembrane segment including metal-binding domain and it is thought that they affect stuctural-dynamical properties, dimerization and proteolysis of APP in the membrane. At the same time cholesterol interactions with the amyloid precursor protein (APP) have been rather extensively explored, and several interaction sites characterized and Alzheimer's disease pathogenesis is known to be closely correlated with the membrane concentrations of various lipid species, the dependencies on cholesterol content being especially frequently discussed. .

Australian (APP L723P) mutation is identified to be associated for developing autosomal-dominant, early onset Alzheimer's disease. The 13C/15N-isotope labeled APP fragments including mutant forms were produced using high-performance systems of bacterial or cell-free expression and solubilized in different membrane mimetics. We measured increased α-spiral flexibilixy of N-terminal region of L723P mutant compared to wild-type peptide and showed that unlike wild-type fragment, containing full-length APP transmembrane domain, L723P "Australian" mutant gradually converts from α-helical to β conformation and this process accompanied by high molecular weight aggregates formation. For investigation of interactioin if APP tm site with cholesterol we used a spin-labeled cholesterol analogue to probe with the aid of high-resolution NMR the cholesterol interaction sites of the APP transmembrane domain in two different membrane-mimicking systems having characteristic traits of more ordered and perturbed lipid environments (bicelles and micelles, respectively). In bicelles, the sterol molecule proved to interact with the peptide in a manner similar to previously described cholesterol interactions, near the amphiphilic juxtamembrane region and the N-terminal part of the transmembrane helix. Transition into the micellar environment was accompanied by appearance of another interaction site Thereby mutants of APP transmembrane fragments are shown to be promising objects for determination of molecular mechanisms of amyloidogenesis and identifying structural and functional determinants of APP which is necessary for understanding of AD pathogenesis, despite that observed modulation of the effects of sterol interactions by the overall state of the lipid environment suggests that the role of lipids in the AD pathogenesis cannot be fully understood without taking into consideration the entire lipid subsystem of the membrane rather than considering the effects of individual lipid species in isolation.

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Native and Intermediate conformations of the three HU dimers of E.coli

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HU is an essential Nucleoid Associated Protein for the bacteria. In enterobacteria, HU is encoded by two genes (hupA and hupB), leading to the formation of two homodimers (HUα2 and HUβ2) and a heterodimer (HU$\alpha\beta$). DSC experiments showed that those three dimers melt through a two step mechanism [1]. The first step, at Tm1, is a transition from a native dimeric conformation (N2) to an intermediate dimeric conformation (I2). The second step is a transition to a denatured monomeric form.

The N2 conformations of HU proteins are well characterized. Each chain consists in an N-terminus helix-turn-helix (HTH) motif followed by a three-stranded antiparallel beta-sheet flanked by a small C-terminus helix. The two chains intertwine to form the dimer, which can be seen as a rather rigid core, containing a hydrophobic pocket, and two moving harms. Preliminary NMR data on HUβ2 combined with high-temperature molecular dynamics simulations allowed us to obtain the first model of the intermediate state at the atomic level. I2 exhibits a larger hydrophobic pocket compared to the N2 conformation and a loss in helicity [2].

The N2/I2 conformations are in slow exchange on the NMR timescale. It is a reversible and strongly temperature-dependent process. We undertook a structural and dynamical study of each conformation of the three dimers in function of the temperature.

Populations ratios were determined by NMR for each dimer at a large range of temperature. The transition temperatures are 311 K, 288 K and 312 K for HUα2, HUβ2 and HU$\alpha\beta$, respectively. We decided to work on the intermediate at T$\approx$Tm1 where we have a 50/50 ratio.

To assign unambiguously the residues in both native and intermediate conformations, we first obtained the structures of the three dimers in their N2 form at T<Tm1-15 K, and followed the evolution of the chemical shifts in function of the temperature.

The homodimers are fully assigned in both their N2 and I2 forms. The backbone chemical shift differences between the two forms indicate that the two conformations present 37% of differences mainly located in the HTH domain and in the last helix. This is due to partial or total unfolding of the helices in the I2 conformation. Moreover, some inter-chains contacts (long range NOESY cross-peaks) present in N2 are lost in I2. Combination of the NMR data obtained so far and coarse-grained modelling calculations allowed us to propose a new model of the I2 conformation: an "open" conformation of the dimer, in which the HTH domain of each chain is partially unfolded and not in contact, the β-sheet domain is conserved and the C-terminal helix is unfolded.

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Solution structure of Helicobacter pylori HypA from 4D NMR experiments

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Helicobacter pylori HypA (HpHypA) is Ni metallochaperone necessary for [Ni,Fe]-hydrogenase maturation and for the insertion of Ni(II) in the active site of urease. HpHypA contains a tight-binding structural Zn(II) site as well as a unique lower affinity Ni(II) binding site. The resonance assignment and solution structure of apo- HpHypA, containing Zn(II) but devoid of Ni(II), was determined using 2D, 3D and 4D NMR data.

Distance restraint were extracted from 4D NOESY experiments: 4D [1H,1H]-NOESY experiments recorded in the 15N,15N-resolved, 13Cali,15N-resolved, 13Cali,13Cali-resolved, 13Cali,13Caro-resolved and 13Caro,13Caro-resolved varieties were recorded using a Bruker AVANCE 800 MHz spectrometer equipped with 5 mm TCI-HCN z-gradient cryo-probes. All 4D experiments utilized sparse sampling of indirectly sampled dimensions and were processed using the Signal Separation Algorithm, implemented in the cleaner4d software [1]. The spectra were inspected and manually peak-picked using the UCSF Sparky software [2], and the sidechain resonances were manually assigned using the sequence-specific backbone assignment.

The structure was determined with an approach (named YARIA) that combines the iterative protocol for NOE assignment implemented in ARIA software [3] with the YASARA molecular dynamics software [4].

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Tuning protein stability with ionic liquids: investigation of destabilizing vs stabilizing effects

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In a cell, small hydrophilic molecules, such as metabolites and osmolytes, can reach nearly molar concentration. These small molecules generally protect cells against stress[1] reinforcing the idea that background interactions, originating from cytosolic small molecules, cannot be disregarded as modulators of protein stability. In this context electrostatic forces play a pivotal role[2]. Salt effects on proteins are well known[3] and the cytoplasmic milieu has a Coulombic character due to its high content in electrolytes and ionic metabolites[4]. Intuitively, it would be expected that individual charged molecules in the cell millieu should interact weakly (un)specifically with proteins (ion-protein interactions) or as ion-pairs in protein interactions. Our work points to this fact where nonspecific direct interactions of small organic ions are able to regulate protein stability in solution[6]. Using NMR spectroscopy and calorimetry, combined with MD simulations we have elucidated how aqueous solutions of ionic liquids (ILs are "green" solvents consisting of molten salts of complex ions) can act as small charged molecular crowding agents to modulate stability and dynamics in a small alpha-helical protein, Im7. Our studies have confirmed that direct preferential binding of anions and cations to the protein surface in combination with ion hydration are key to understand protein destabilisation[5]. Furthermore, we found that ion topology is determinant for stability as the interactions with the protein are strongly dependent on the balance between electrostatic and hydrophobicity and anion/cation combination[6]. As a result, the correct combination of cation and anion can lead to a destabilising or stabilising IL. In this context, we have used a globular and highly-stable protein GB1 and src-SH3 protein domain with a ΔGU aprox =0 in aqueous solution to study the (de)stabilising mechanism of ILs using a designed biocompatible IL, choline glutamate ([Ch][Glu]) - stabiliser - and 1-butyl-3-methyllimidazolium dicyanimide ([BMIM][dca]) - destabiliser. In this communication, through the combination of 15N relaxation and proton-deuterium exchange NMR techniques combined with fluorescence and calorimetric studies, we will present a mechanism for protein (de)stabilisation promoted by ILs. Our work will contribute to a further understanding of the basis of protein folding towards stability. [1]D. Harries, J. Rösgen, Methods Cell Biol. 2008, 84, 679–735. [2]A. C. Miklos, M. Sarkar, Y. Wang, G. J. Pielak, J. Am. Chem. Soc. 2011, 133, 7116–20. [3]K. Hamaguchi, E. P. Geiduschek, J. Am. Chem. Soc. 1962, 84, 1329–1338. [4]J. J. Spitzer, B. Poolman, Trends Biochem. Sci. 2005, 30, 536–541. [5] A. M. Figueiredo, J. Sardinha, G. R. Moore, E. J. Cabrita, Phys. Chem. Chem. Phys., 2013, 15, 19632-43. [6] M. Silva, A. M. Figueiredo, E. J. Cabrita, Phys. Chem. Chem. Phys., 2014, 16, 23394-403.

P143

Chemical synthesis and natural abundance NMR: An efficient tandem to unravel 3D structures of Disulfide Rich Peptides & Miniproteins

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Disulfide rich peptides (DRP) are ubiquitous natural compounds composed of 10 to 100 residues and characterized by evolutionary-conserved cysteines reticulated by a specific bonding pattern. These remarkably stable molecules are active in adverse environments, exhibiting incredibly diverse host-defense or predation-related functions. DRPs are potent and selective binders of various targets, making them attractive compounds as pharmacological/agronomical tools. To undertake the structural studies of these natural compounds when the recombinant expression fails, we turn our attention to extracted or chemically synthesized DPRs, which are typically obtained without isotopic labelling. Our structural work is thus based on 1H NMR combined with natural abundance 13C and 15N 2D spectra (700MHz spectrometer with cryoprobe).

State-of-the art solid phase peptide synthesis was used for the total synthesis of medium size DRPs (40-50 residues) and combined to native chemical ligation (NCL) using our recent methodology based on a N-(2-hydroxy-5-nitrobenzyl)-Cys (N-Hnb-Cys) for longer DRPs [1]. The reduced forms were oxidatively folded under thermodynamic control to achieve the natural disulfide connectivity.

We illustrate our medium-throughput NMR protocol with several examples of medium-size DRPs [2-7]: i) antimicrobial peptides that play major roles in innate immunity and ii) toxins of diverse origins. To challenge our methodology, we recently undertook the structural studies of two particularly intriguing double-domain antimicrobial DRPs: i) AvBD11 (82 residues) is one of the major component of vitelline membrane of hen eggs [8], and was directly extracted from eggs. Its atypical structure, composed of two β-defensin motifs, had never been described before [9]; ii) Cg-BigDef-1 (93 residues) [10] from the oyster Crassostrea gigas contains an N-terminal hydrophobic domain and a C-terminal β-defensin motif.

Our standard set of experiments and our calculation protocol are usable for a large variety of folds (defensins, cystein-stabilized-α-helix-β-sheet motifs, inhibitory cystine knots) regardless of whether the peptide is extracted or synthesized. Remarkably, the 3D structure determinations of unlabeled multi-domain DRPs were made possible by exploiting the structural data of each domain, synthesized independently.

1. Terrier et al. 2016; 2. Jouvensal et al. 2003 ; 3. Da Silva et al. 2010 ; 4. Derache et al. 2012; 5. Meindre et al. 2014 ; 6. Bailleul et al. 2016; 7. Zoukimian et al. Submitted; 8. Hervé-Grepinet et al. 2010; 9. Guyot et al. In prep.; 10. Rosa et al. 2011

P144

Structural differences of mule deer prion protein provides insight into chronic wasting disease

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Chronic wasting disease (CWD) is the most efficiently transmitted prion disease of free-ranging wildlife, including elk, mule deer, white-tailed deer, red deer, reindeer and moose [1]. It is known that prion proteins selectively infect some mammalian species rather than others. Various species barriers were identified, which are impacted by the existence of different prion strains. For instance, in deer and elk the presence of conformational PrPSc strains was confirmed based on different disease progression in different species[2]. In this study, we highlight the importance of PrP structure in prion susceptibility. Specifically how a polymorphism in position 226, in which elk PrP contains glutamate and deer PrP contains glutamine, might influence prion susceptibility and pathogenesis. We have determined the high-resolution structure of the cellular form of mule deer prion protein (mdPrP), residues 94-233. We compare the structure to previously published cervids PrPs structures of elk PrP (ePrP) and white-tailed deer PrP (wtdPrP). Although the overall structures of the mammalian prion proteins are similar, we observe several local structural variations in the examined structures. The most remarkable differences are located in the α2-α3 loop with a different rearrangement of residues together with the hydrogen-bond formation that creates a tighter packing of the α1 helix and α2-α3 loop in mdPrP structure in comparison to ePrP and wtdPrP. Additionally, in mdPrP hydrophobic interactions of amino acid residues at the end of α3 helix and β2-α2 loop lead to a lower solvent accessibility of Ser225 and Gln226 which are related to the CWD transmissibility. We found that a single amino acid variation can alter the PrP structure and this can have important consequences on the different pathogenesis of CWD prions. The recent occurrence of CWD in Europe is most probably related to cervids developed genetic or spontaneous diseases which spread horizontally to other cervids [3] and there is still lack of information how this disease transmit. Our study is outstandingly important for better understanding the underlining mechanism of CWD prion transmission.

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P145

Conformational response of the Hsp90 N-terminal domain upon nucleotide binding and isoform-specific structural features

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The Hsp90 chaperone plays an essential role in the folding and stabilization of a structurally and functionally diverse set of client proteins, many of them involved in signal transduction. Hsp90 is a homodimer composed of an N-terminal ATP binding domain, a middle domain (MD) which mediates client interactions, and a C-terminal dimerization domain. ATP binding relocates several elements on the NTD and MD, creating transient contacts between the NTDs that result in an open to closed conformation change, essential for the chaperone cycle. Several factors, such as post-translational modifications, co-chaperones or clients, are able to target certain intermediate states of Hsp90 by affecting switch points involved in communication pathways. However, the mechanisms underlying intra- and interdomain allosteric communication of Hsp90 remain unknown. Here, we investigate the conformational response of the NTD of yeast Hsp90 to adenosine nucleotides and aim to identify key residues involved in allosteric pathways. For this purpose, we analyzed the structural rearrangements of the NTD upon binding to different nucleotide analogues by NMR. Our results indicate that ATP and ADP cause different repositioning of the ATP lid and the surrounding helixes, which transmitted to a distant strand involved in secondary dimerization. These structural effects are strongly dependent on the phosphate bond between beta and gamma groups, since the non-hydrolysable analog AMPPNP leads to rearrangements that resemble the ADP-bound form. Interestingly, comparison of the NTDs of the two isoforms of yeast Hsp90 reveals alterations in some of these regions, which correlates well with the differences in their ATPase activity and in vivo sensitivity towards the inhibitor radicicol.

P146

Exploring the mechanisms of activation and ubiquitination of parkin

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Parkinson's Disease (PD) is the second most common neurodegenerative disease that affects approximately 1% of the population worldwide. It is believed that PD is caused by mitochondrial dysfunction, which leads to the loss of dopaminergic neurons in the substantia nigra. PD is primarily sporadic, however a number of mutations have been linked to the disease, which can have either environmental causes or heritable causes. Heritable mutations in both PARK2 and PARK6 give rise to the early onset or autosomal recessive juvenile parkinsonism (ARJP) forms of PD, which account for 10% of the PD cases. PARK2 encodes the 53 kDa protein parkin, which is an E3-ubiqutin ligase. Parkin's mechanisms of ubiquitin conjugation and ligation are poorly understood, but involve at least two phosphorylation steps to activate ubiquitination activity. Using NMR and Mass Spectrometry, we have characterized various activation states of parkin. NMR methyl assignment of the C-terminus of parkin was performed to aid in the identification of the dynamic regions of interest. HDX and chemical shift perturbation experiments show that two distinct domain rearrangements occur during parkin activation. These conformational changes lead to a short-lived arrangement between parkin and its E2-ubiquitin conjugate needed to transfer ubiquitin. Through characterizing parkin's dynamic nature, we aim to expand current understanding of PD pathogenesis.

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Structural insights into the di-nucleotide formation of the primase ORF904 from the archaeal plasmid pRN1

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DNA replication is a crucial stage for the living organisms. DNA polymerase can only initiate the synthesis of a DNA strand with the help of an initial RNA or DNA primer, which is synthesized by primase. The replication protein ORF904 from the archaeal plasmid pRN1 is multifunctional with ATPase, Primase and DNA polymerase activity. It consists of at least two conserved domains. The novel N-terminal domain contains a catalytic prim/pol domain tethered to a novel helix bundle domain. And its primase activity is highly sequence specific requiring a GTG motif in the template DNA.

We investigated the NMR structure of the helix bundle domain (HBD) of the primase ORF904 in complex with the ssDNA template C+2T+1GTGCTCA and NTPs. In the present of ATP and ssDNA template, HBD can specifically bind GTP or dGTP. And there is a large conformational change of HBD after binding to GTP or dGTP. We try to determine the quaternary complex structure of HBD, ssDNA template, ATP and dGTP. This complex structure may help us to characterize the molecular steps required for the first two NTPs binding and di-nucleotide formation.

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Structural and Dynamics Studies of Mycobacterium tuberculosis FKBP12 protein: Screening and Development of Novel Active Compounds

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Mycobacterium tuberculosis is the causative microorganism of the tuberculosis disease. In 2016, World Health Organization (WHO) reported 6.3 million new tuberculosis cases worldwide. Such disease still is a significant cause of morbidity and mortality, mainly in low-income and middle-income countries. Although many efforts had been done over the past years, WHO estimates that 490 000 new cases of multidrug-resistant M. tuberculosis has emerged in 2016 and this large number arise due to abandonment of the long duration treatment against active tuberculosis (at least 6 months for new cases), in which four different antibiotics are used. Treatment in such cases of multi-resistant strains has still low success rates. In this way, stronger efforts in the research of new and more effective tuberculosis drugs and targets are essential. The peptidyl-prolyl cis-trans isomerase enzyme (PPIase) FKBP12 was chosen as a target for these studies. Although FKBP12 human ortholog is already used as a target for compounds, this protein is also present in different causative microorganisms of diseases. The PPIases of microorganisms are known and validated

as biological targets, and differ by about 40% in primary sequence compared to human ortholog. Putative FKBP12 from Mycobacterium tuberculosis (MtFKBP12) was overexpressed in E. coli BL21(DE3), purified by nickel affinity chromatography and, subsequently, had its His-tag cleaved by TEV protease in 5:1 molar ratio per 16h at 4°C. The second HisTrap step was performed in order to obtain MtFKPB12 with high purity and homogeneity. To observe the oligomeric state of MtFKPB12 a size-exclusion chromatography using a Superdex-75 was performed. All steps were monitored by SDS-PAGE 15%. 15N/13C labeled MtFKBP12 was fully assigned using double and triple resonance NMR experiments. The structure was solved using distance restraints, obtained from 15N- and 13C NOESY-HSQC spectra, dihedral restrainst obtained from TALOS N and Residual Dipolar Couplings (RDC). To investigate the backbone dynamics of the MtFKBP12 protein, we measured the relaxation parameters: 15N R1 and R2 relaxation experiments and 1H-15N heteronuclear NOE. The relaxation data show little significant variation in the values of 15N R1, which are mostly in the range of 1.6-1.8 s-1 (<R1> = 1.69 s -1). For the values of R2, most of the residues present values between 7 and 9 s-1 (<R2> = 8.05 s-1). Most heteronuclear 1H-15N NOE values are in the range of 0.7 to 0.83 s-1 (mean NOE = 0.714 s-1). The major significant variations in the N-terminus and loops between $\beta 1$ and $\beta 2$ and between π and $\beta 5$ strands. For binding studies, we used the tetrapeptide ALPF . Perspectives include the identification of low affinity ligands that could subsequently become leading compounds for drug production.

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Determination of the solution structure of a potentially inflammation-inhibiting peptide (VIPER) derived from a poxviral immune evasion protein (A46)

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Toll-like receptors (TLRs) play a role in viral detection leading to cytokine and IFN induction, and as such they are targeted by viruses for immune evasion. The poxviral protein A46 has been identified to inhibit TLR signalling by interacting with TIR domain-containing proteins of the receptor complex to collectively inhibit all TLR adaptor proteins that positively regulate transcription-factor activation [1]. One 11 aa peptide (KYSFKLILAEY) termed VIPER (Viral Inhibitory Peptide of TLR4) was reported to retain the inhibitory properties of full length A46 against TLR4 signalling. A 9R homo-polymer delivery sequence at the C-terminus provided delivery of the peptide into cells. 9R-VIPER showed its efficacy in a TLR4-dependent flu induced lung damage model [2]. In this study, structural comparisons are presented between 9R-VIPER, which is active in preventing TLR4-dependent cytokine induction in cell culture, and a mutant that exhibited loss of function (9R-VIPER L6AE10A), through solution NMR spectroscopy. Assignments of the resonances were accomplished 1H-15N HSQC, 1H-13C HSQC, TOCSY and NOESY at Agilent 800 MHz NMR. Chemical shift assignment and Nuclear Overhauser Effects (NOE) results were used to probe peptide conformation and generate 3D structures. Finally, the 3D structure of the VIPER region in the vaccinia virus A46 protein (residues 88-98) (PDB ID code: 4M0S) [3] is shown and subsequently superimposed with the VIPER and VIPER mutant peptide structure ensembles. We find that despite a relatively minor sequence difference, the loss of hydrophobicity, negative electrostatic interactions as well as structure changes result in subtle but potentially significant differences in the region of the peptide proposed to interface with TLR4.

P150

Amphipathic helical proteins and their association with phospholipid membranes

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Our lab is interested in the interactions of proteins and peptides with the membrane of mammalian cells, particularly in the area of cancer and pathogen cell death. To this end, we employ NMR, along with other structural techniques such as atomic force microscopy, circular dichroism and size exclusion chromatography, to gain a better insight into the processes that govern protein folding and misfolding. In the treatment of trypanosomiasis, a neglected tropical disease, determining the structure of BMAP-27, a trypanocidal bovine peptide and the nature of its interaction with the membrane is one of our interests. We have also been working on HAMLET (Human Alpha-lactalbumin Made Lethal to Tumour cells), which is a protein-fatty acid complex that remarkably selectively kills tumour cells while leaving healthy, differentiated cells intact. In both cases, the large presence of Lys residues in the α-helical regions has suggested that there may be a common way of penetrating the phospholipid bilayer membrane. The similarity of the alpha α-synuclein model in terms of its many Lys side chains (Fusco et al, 2016) appears to be relevant. A common means of associating with and subsequently penetrating the phospholipid bilayer, whereby Lys residues have electrostatic interactions with the charged groups of the membranes' lipids and more hydrophobic regions of the protein are inserted into the membrane, not only has profound implications for the design of novel trypanocidal and tumorcidal peptides but also our understanding of protein-membrane interactions. Our lab investigates the structure of such proteins through homo- and hetro-nuclear NMR and through additional techniques such as circular dichroism in order to gain a holistic understanding of this crucial process. Reference: Fusco G et al, Sci Reports 2016, 6:27125

P151

Using NMR and specific labeling to delineate the mode of binding for di-Ubiquitins

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The ubiquitin system regulates a large number of cellular processes in eukaryotic species such as protein degradation and signal transduction. It is essential for maintaining cellular homeostasis. Ubiquitin is ligated to substrate proteins as monomers and linear or branched chains and the topology of ubiquitin modifications are proposed to regulate substrate. Deubiquitinases are responsible for the cleavage of these ubiquitin chains from substrate enzymes. As ubiquitination directs a variety of substrate fates including proteasomal degradation, the different possible ubiquitin linkages and resulting topology allow for the differentiation between the multiple biochemical consequences. By engineering di-ubiquitin chains containing differential proximal and distal isotopic labels and measuring substrate

binding via NMR, we could show how the units in di-Ubiquitin interact with their cognate DUB. In two examples, the deubiquitinases (DUBs) USP7 and ZUFSP were investigated. In the case of Ubiquitin Specific Protease 7, we could clearly demonstrate that the driver for specific recognition and cleavage of K48 linked poly-Ubiquitin is the interaction of an acidic patch in USP7, consisting of the side chains of residues D305 and E308, with a free lysine side chain in the distal unit. This preferential binding significantly protracted the depolymerization kinetics of Lys-48-linked ubiquitin chains relative to Lys-63-linked chains. In ZUFSP, our studies revealed that full-length ZUFSP interacted with both Ub moieties of labeled K63-linked and K48-linked di-Ub. The catalytic domain alone showed no appreciable interaction with K48- or K63-linked di- Ub, or with mono-Ub. This explained the biochemical finding that K48-linked chains and, to a lesser extent, linear or mono-Ub inhibited processing of K63- linked Ub4-Rh110 by ZUFSP. In summary, amino acid specific labeling and enzymatic conjugation of ubiquitin suggests multiple opportunities for making polyubiquitin and may be a strategy more broadly applicable to finding the specific interactions driving linkage recognition.

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Structural investigation of riboswitch folding important for regulation of bacterial gene expression.

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Riboswitch is a structural RNA motif that is located at the 5'- end of mRNA, regulates protein expression upon binding small molecules. Riboswitches are composed of the aptamer domain and the expression platform. The aptamer domain specifically binds a ligand and the expression platform is responsible for the gene expression through its ability to switch the conformation between two different secondary structures in response to ligand binding. We have studied the folding mechanism of cyclic-di-GMP (bis-(3'-5')-cyclic dimeric guanosine monophosphate,) riboswitch. Cyclic-di-GMP is widely used by bacteria to regulate processes ranging from biofilm formation to the expression of virulence genes. Cyclic-di-GMP riboswitch is the first known example of a gene-regulatory RNA that binds a second messenger, and it is present in pathogens such as Clostridium difficile, Vibrio cholerae and Bacillus anthracis. We have investigated the structural mechanism of ligand binding of c-di-GMP riboswitch using NMR spectroscopy. The proton and carbon resonances of the riboswitch were assigned, and the secondary structure was determined by NMR. We will present the conformational changes of riboswitch RNA upon addition of metal ions and c-di-GMP to the riboswitch. Investigation the tertiary structure and ligand recognition mechanism of the riboswitch is essential to understand the gene regulation mechanism of the riboswitch. Our results will provide an insight into the new design of RNA targeting antibiotics.

P153

Proton-detected solid-state NMR detects the architecture of RNA in microcrystals

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RNAs play critical roles in various biological processes. However, RNA structure determination remains a challenging topic. In this presentation, we will report a proton-detected magic-angle-spinning solid-state NMR (MAS SSNMR) strategy to characterize the structure of the dimeric RNA in crystalline. A 23-mer RNA found in human immunodeficiency virus type-1 (HIV-1), which forms homodimers in crystals, was used as the model system to evaluate the strategy. The 1H-direct detected 1H-15N correlation spectra are acquired on fully protonated RNA at a MAS frequency of 40 kHz. The experiments with 15N-15N proton assisted recoupling (PAR) yield information about the inter-nucleotide N-H...N hydrogen bonds within the Watson-Crick base pairs, resulting in a similar spectral pattern of the hNN COSY that widely used in solution NMR. A comparison of the spectral patterns of the uniformly labelled sample and those of the isotopically diluted sample with naturally abundant RNA confirms the kissing-loop structure of the dimeric RNA. This strategy will contribute to advances in the structure determination of micro-crystallized RNA.

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P154

Investigation of DNA G-Quadruplex Structural Dynamics using Real-Time NMR-spectroscopy

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The complex energy landscapes of DNA G-quadruplexes cause a broad structural heterogeneity and numerous polymorphous conformations. The involved possible folding pathways are commonly described with kinetic partitioning that can lead to stable on- and off-pathway intermediates. In thermodynamic equilibrium, this results in an ensemble of distinct folded conformations that can interconvert throughout a readily folded state.(1) Especially G quadruplex forming elements from promoter regions of several oncogenes give potentially rise to inherent conformational dynamics. A distinct feature is the existence of (n+1)-G registers found frequently in G-quadruplex sequences next to the TSS. This leads to the possibility of a dynamic exchange of different n-layered conformations in the folded state, incorporating different Guanosine-residuals into the G-quadruplex core structure. By gaining insight into those refolding processes, we can obtain vital information on the overall energy landscape and contribute to a better understanding of the regulatory role of promoter G quadruplexes.(2)

Herein we present a novel strategy to transiently stabilize single G-quadruplex conformations by using photolabile-caged nucleotides.(3) We introduced a NPE protection group at the O6-position of deoxyguanosine to efficiently prevent Hoogsteen interactions and utilized this approach successfully for a transient conformational selection of different parallel and hybrid folded G quadruplexes associated with significant biological function (cMYC, hTERT). Photolysis with a visible light laser within the NMR tube enables nearly dead-time-free real-time NMR measurements with atomic resolution. We observed the re-equilibration of the trapped conformations to the native conformational ensemble on a relatively slow timescale. In combination with K+ induced observation of the folding into the ensemble of all respective G-register isomers, we gained mechanistic insight into inherent conformational dynamics. Careful analysis of the kinetics and the involved activation energies revealed distinct refolding dynamics subsequent to initial folding. To the best of our knowledge, this study reports the first direct observation of these G-quadruplex structural dynamics under physiological conditions and marks the impact to biological function and drug design.

Acknowledgment: This work was supported by the State of Hesse (BMRZ) and by the DFG (GRK 1986 — Complex Light Control).

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P155

Observation of imino proton signals of DNA and RNA introduced inside the living human cells by using in-cell NMR spectroscopy

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Intracellular environment is extremely crowded with large and small molecules and highly dense. It has long been suggested that the properties of proteins and nucleic acids inside the living cells are different from those in in vitro conditions; these properties include structure, structural stability, dynamics, intermolecular interaction, etc. There are increasing reports about the above-mentioned properties for nucleic acids under molecular crowding conditions using crowding cosolutes. They demonstrated that some short oligo nucleic acids that form canonical double helical structures, comprising Watson-Crick base pairs, de-stabilize under in vitro conditions supplemented with molecular crowders such as ethylene glycol [1]. Additionally, the stabilities of the structures were shown to be dependent on the kinds of cosolutes used. Cells contain various kinds and enormous number of molecules, therefore, it would be fruitful to investigate the structures and structural stabilities of DNAs and RNAs inside the living human cells. For this purpose, in-cell NMR spectroscopy is an attractive and robust methodology. So far, however, in-cell NMR spectroscopy has been applied mostly to proteins. There are only a few reports on nucleic acids. Notably, the reported in-cell NMR studies on nucleic acids were performed almost entirely by using Xenopus laevis oocytes. Here, we succeeded in observing the in-cell NMR signals of nucleic acids inside the living human cells [2]. We used DNAs and RNAs, which form hairpin structures in in vitro. They were introduced into HeLa cells by a method utilizing bacterial toxin streptolysin O, which enables to form and reseal the pores on the cell surface. Confocal fluorescence microscopy showed that these DNAs and RNAs locate mainly inside the nuclei. The observed signals directly suggested the formation of the hairpin structures inside the living human cells.

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P156

Probing the NMR solution structure of the CPEB3 ribozyme.

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In this project the study of the NMR solution structure and folding mechanism of the cytoplasmic polyadenylation element binding protein 3 (CPEB3) ribozyme is presented. Ribozymes are naturally occurring catalytic active RNA molecules. The CPEB3 ribozyme is until now the only confirmed small ribozyme in mammals but its role remains elusive.1 Since RNA functions are directly linked to the structure structural studies are essential for understanding the ribozyme function. The CPEB3 is one of the Hepatitus Delta Virus (HDV)-like self-cleaving ribozymes.2 We suggest that the self-cleavage mechanism of the CPEB3 ribozyme is similar to the known HDV cleavage activity. The cleavage reaction of the HDV occurs with Mg2+ facilitated acid-base catalysis in which a conserved cytosine directly participates. The conserved cytosine C75 is within the catalytic core and has a perturbed pKa value.3 In analogy to C75 of HDV, the CPEB3 ribozyme contains also a conserved cytosine C57 at the same position. Substitution of this cytosine with any other nucleotide prevents the ribozyme cleavage reaction.2 Therefore we want to determine the pKa value of C57 in the absence and in the presence of Mg2+. However the first pH-titrations with the chimp CPEB3 construct did not show any elevated pKa value, independently of the Mg2+ concentration applied. Another goal of this work is to elucidate the NMR solution structure of the CPEB3 ribozyme. As the CPEB3 ribozyme has 67 nucleotides, it is challenging to assign the structure due to the large resonance overlap. Therefore we use various truncated constructs, 15N and 13C labeling schemes, and a number of multinuclear and multidimensional NMR experiments. Working at the limit of NMR we could unambiguously assign a large fraction of resonances. Further, we introduce 5-Fluoro-uridine into the CPEB3 RNA using in vitro transcription and apply 19F-NMR spectroscopy to support and facilitate the assignment. This 19F labeling technique allows us to follow the 15 uracil resonances upon folding into its active state in the presence of Mg2+. Five of these uraciles are located in the catalytic core of the ribozyme. These results are the basis to understand the individual steps of the ribozyme function on a structural level and to enlighten the biological role of the CPEB3 ribozyme.

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P157

Nucleic Acid Resonance Assignment in a Single Shot

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The general assignment strategy for RNA starts on separated imino proton resonances. The HNN experiment ensures information about the base

pair architecture. In the next step, aromatic resonances are assigned for the base paired nucleotides using a CC-TOCSY transfer. Sugar resonances can be obtained from HCN, HCCH-TOCSY and HCCH-COSY experiments in combination with HCP and HCP-TOCSY. Assignments are completed by recording NOESY spectra on RNA samples in H2O and D2O solution [1]. Here we present an exclusively scalar-coupling based UTOPIA ("unified time-optimized interleaved acquisition") [2] approach to the resonance assignment of 13C/15N labeled RNA molecules. It involves interleaved recording of four 3D experiments that detect either of 1H, 13C, 15N and 31P nuclei using four receivers. The combined information provides sequence-specific assignments of guanosine and uridine imino groups and their hydrogen-bonded acceptor 15N nuclei in the corresponding bases of cytidine and adenosine, respectively. This is achieved by correlating backbone 31P chemical shifts with ribose resonances in the 3' and 5' directions. Interconnection points between the different experiments are the anomeric (C1') and the aromatic base (C6/C8) carbons that are finally linked to the imino 1H and 15N resonances with the help of a 13C TOCSY based intra-base correlation sequence. Finally, hydrogen bond interactions of the latter are monitored via a 15N-15N COSY-type transfer. Since only a single recovery delay is needed, appreciable time savings can in principle be achieved. However, although different nuclei are detected during each acquisition period, the implementation of individual pulse sequences and their sequential order requires careful optimization to prevent the destruction of polarization that is needed in subsequent experiments. For sensitivity reasons, three of the four experiments start with 1H magnetization Therefore, bandselective excitation of different spectral regions is employed, thus avoiding a saturation of unused proton spins. The method is demonstrated with a uniformly 13C/15N labeled 21-nucleotide RNA hairpin.

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P158

Segmental one-strand isotope-labeling of nucleosomal DNA for NMR interaction studies

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The nucleosomal DNA is a crucial contributor to interactions of the nucleosome with other proteins, including nucleosome remodelers and epigenetic readers. However, the limited number of building blocks, low chemical shift dispersion and the rigidity of the double helix lead to extensive signal overlap and peak broadening in NMR experiments, which makes it difficult to observe in interaction studies. To fill this potential information gap, we developed a method for isotope-labeling of six thymine residues in one strand and one segment of the nucleosomal 601 sequence, between Super Helix Locations (SHL) -7 and -6. The presence of methyl groups in thymine allows for enhanced NMR signal intensity, which enabled us to observe them in regular 1H13C HMQC experiments. We used an unlabeled 15 bp DNA duplex of the same sequence to assign all signals in a NOESY sequential walk and were able to transfer the assignments to the methyl signals of the labeled 30 bp nucleosomal DNA fragment. We show that these six methyl signals can also be observed in the full-length 100 kDa DNA601. In future experiments, we will use these signals in combination with paramagnetic tags on histones or interacting proteins to probe the binding of readers and remodelers to the nucleosomal DNA.

P159

High-Resolution Structure of a DNA-RNA Hybrid G-Quadruplex Reveals Interactions That Favor RNA Parallel Topologies

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Among the various alternative DNA structures, G-quadruplexes (G4) exhibit a remarkable variety of topologies with parallel, antiparallel or hybrid-type folds. In contrast, RNA quadruplexes seem to be mainly restricted to parallel G4 topologies. In this study, a DNA G-quadruplex adopting a (3+1)-hybrid structure was modified at its outer 5'-tetrad by substituting anti-favoring riboguanosine nucleosides (rG) for appropriate syn residues. 2D NOE as well as 1H-13C HSQC spectra allowed for the complete assignment of rG-modified G4s and close spectral similarities to the native structure confirmed the conservation of the global quadruplex fold. However, rG incorporation induced conformational rearrangements to yield a quadruplex featuring a 5'-tetrad with reversed polarity using C8 carbon chemical shift changes as a convenient probe for the glycosidic conformation.

A high-resolution structure of a disubstituted quadruplex variant with a reversed 5'-tetrad reveals a non-conventional C8-H8···O2' hydrogen bond within a medium groove between the 2'-OH of an rG residue with a C2'-endo sugar pucker and H8 of a 3'-neighboring anti-G residue. Direct NMR experimental evidence for this interaction was also obtained by changes in the corresponding C8-H8 coupling constant. Interestingly, a C3'-endo sugar conformation for another guanine ribonucleotide relocates its 2'-OH substituent from the quadruplex narrow groove into a medium groove, precluding its participation in a corresponding hydrogen bond. Both the formation of favorable CHO hydrogen bridges between G residues in anti conformation and unfavorable interactions of the 2'-hydroxyl group in a narrow groove may constitute major contributors to the poorly understood prevalence of parallel RNA topologies.

P160

NMR as a versatile tool in G4-quadruplex folding and dynamics

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DNA can adopt a variety of secondary structures, notoriously guanine-rich sequences fold into quadruplex structures consisting of G-tetrads instead of base pairs. The four guanine bases in these tetrads are connected via Hoogsteen hydrogen bonds. These G4-quadruplexes show great diversity in strand orientation and base configuration depending on sequence and environmental conditions. Furthermore they have been proven to be of relevance in both gene regulation and oncogenesis. NMR spectroscopy proves to be the perfect tool to study these biologically relevant structures. Using various NMR techniques we can not only illuminate quadruplex topologies but also assess ligand binding to G4-structures. Furthermore time and temperature resolved measurements allow for studies of G4-dynamics and folding enthalpies. Based on the well-known c-MYC sequence from the promotor region of the NHE III element, a comprehensive exploration of quadruplex folding parameters and dynamics is presented.

P161

RNA screening using 19F-NMR

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The role of fragment based screening (FBS) using NMR is well established in the case of proteins. For RNA, the FBS is less commonly used for finding new ligands. Current advancement in lab-automation (sample preparation robots) together with advanced NMR instrumentation and software allows for fast automated screening data acquisition and analysis. A new generation of fragment library (Diamond-SGC-iNEXT (DSI) Poised Library) consisting of 769 fragments which has the advantage of streamlining easy follow-up chemistry was utilized within this study. 106 fragments contain fluorine, which can be screened using 19F-NMR. As a first target test system for screening, we chose the second-messenger binding riboswitches "X", "Y", and "Z", which bind c-GAMP, c-diGMP and ZMP, respectively and thereby modulate transcriptional or translational efficiency. These riboswitches play a critical role in bacterial cellular metabolism[1]. They do not occur in eukaryotes and therefore represent excellent targets for drug development. Herein, we investigate the binding of three RNA constructs[2–4] from the above riboswitches against a library of 106 fluorine containing fragments. Our experimental design allows for screening of all fragments on a specific RNA sample in as little as 4 days, including RNA preparation. Utilizing 19F-1D and 19F-CPMG experiments, binding or non-binding is confirmed by chemical shift perturbation and modulation of the relaxation behavior. Binding fragments for the three riboswitch RNAs could be identified leading the way for further investigation and potential drug design.

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P162

Secondary structure probing of the Hatchet ribozyme by site-specific 15N-labeling and solution NMR-spectroscopy

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Recently Ronald Breaker et al. [1] discovered four new classes of catalytic active ribozymes using bioinformatics search strategy. For some of these novel ribozymes high resolution structures are available, but for the hatchet ribozyme structural information is rare limited to a secondary structure proposal. By using solid phase synthesis and site specific 15N-labeled RNA phosphoramidites building blocks we were able to examine the base pairing pattern of the hatchet ribozyme with state of the art NMR experiments. We could confirm some of the postulated secondary structure features, other substructures could not be observed but we found strong Watson-Crick like base pair interactions in postulated single stranded regions. Even if we are able to study atom- and site-specific labeled RNA constructs with sizes up to 86 nucleotides, one of the main focus points was the hairpin (P1) closest to the cleavage site which seem to be quite flexible in the full-length construct. Currently, a rigorous NMR study is carried out to establish a full secondary structure proposal for the hatchet ribozyme, a crucial pre-requisite for a full high resolution 3D structure elucidation.

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P163

Structure of d(GA) repetitions with i-motif anchor

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Alternating d(GA)n•(TC)n (n>15) sequences are unusually abundant in mammalian genome.1 It was shown that some proteins are able to selectively bind d(GA) repeats, e.g. PGB protein found in human fibroblasts.2 Microsatellites d(GA)22•(TC)22 enhance recombination in minichromosomes of polyomavirus SV40 found in monkeys and humans.3 Depending on experimental conditions, the d(GA)•(TC) sequence can adopt different conformational arrangements including triplex, loop-out d(TC)n duplex or parallel stranded d(GA)n homoduplex. In-depth description of the d(GA) duplex structure using NMR is complicated by its dynamics. To overcome this problem a C3 tract was inserted in the sequence. In acidic conditions, the cytosine tract forms an i-motif that acts like an anchor restraining the flexibility of the system. In this study we focus on sequences d(C3GAGA) and d(AGAGC3). NMR, CD spectroscopy and MD calculations were used for structure and stability determination and construction of global topology. Particular attention was devoted to identification of the i-motif topology and to the pairing of purine bases. The most common epigenetic cytosine modification 5-methylcytosine (mC) was used to break the i-motif symmetry and to facilitate unambiguous assignment of NMR spectra. Guanine to inosine mutations were employed to determine the pairing pattern of guanines. Both d(C3GAGA) and d(AGAGC3) sequences form i-motifs in 3'E topology. In the case of d(C3GAGA) methylation of cytosine do not change the i-motif topology. However, in d(AGAGC3) partial and complete conversion to 5'E is observed for mC5 and mC7 substitutions, respectively. Inosine mutations decrease melting temperature to a different degree although the change is significant in each sequence. Local position-dependent effects on purine pairing are discussed as well as driving forces that may determine the preferred i-motif topology.

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P164

G-quadruplex with unique combination of structural elements

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G-quadruplexes are noncanonical secondary structures formed by G-rich DNA sequences. Main building blocks of G-quadruplexes are stacked G-quartets, which are formed by four guanine residues in planar arrangement connected with Hoogsteen type of hydrogen bonds. Crucially, monovalent cations which are localized between stacked G-quartets reduce the repulsion of carbonyl oxygen atoms and promote stacking of G-quartets. In the present study, we structurally characterized two G-rich DNA oligonucleotides S3 d(GCG2AG4AG2) and S4 d(GCG2AG4AG2CG) which both have GC residues at 5'-end. Both sequences also contain three G-tracts of different lengths separated by adenine residues, with oligonucleotide S4 having additional CG residues at 3'-end. Such short G-rich oligonucleotides with GC ends were expected to form long G-wires on the basis of inter-quadruplex GCGC linkages1. Oligonucleotides S3 and S4 fold into single, well-defined G-quadruplex structures in the presence of sodium cations at 25°C and pH 6.8. The NMR data based simulated annealing derived high resolution G-quadruplex structures of S3 and S4 showed that both of them fold into symmetric dimers comprised of two G-quartets, an A(GGGG)A hexad, a GCGC-quartet and a N1-carbonyl G-G base pair. The only difference is that the G-quadruplex of S4 has two additional GC overhangs at the 3'-end compared to the structure of S3. Interestingly, in both the S3 and S4 structures the G1 residue (5'-end) is located in the middle of the G-quadruplex structures and is stacked on G-quartets from both sides. Additionally, GCGC-quartets and A(GGGG)A hexads are rare structural features with their presence in the same G-quadruplex structure described, to the best of our knowledge, for the first time in S3 and S4 structures.

P165

Resolving structural and functional properties of the pistol ribozyme by solution NMR spectroscopy

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Recently, Roland R. Breaker and his co-workers reported on a new class of self-cleaving ribozymes found by a bioinformatics search strategy. In detail, they characterized three new RNA sequences called twister sister, hatchet and pistol ribozyme [1]. In the last years, the pistol ribozyme was in the focus of intensive research in our group with a special interest in its structure and the catalytic mechanism. The env25 pistol ribozyme sequence that was used for the X-ray crystal structure determination by Patel and co-workers [2], was optimized to improve the NMR spectra quality. After several rounds of re-design, cleavage assays were performed to ensure that the mutant is still a catalytically active self-cleaving ribozyme. Then, our aim was to get a more detailed insight into the folding landscape of the mutant. Therefore, relaxation dispersion (RD) NMR spectroscopic methods have been applied to the pistol ribozyme complex. In detail, R1ρ RD, CPMG RD and chemical exchange saturation transfer (CEST) experiments were measured. With these powerful methods, a deeper understanding of functional dynamics could be gained. We have been able to localize three "dynamic hotspots" within the ribozyme complex inherently linked to its catalytic activity. Further NMR experiments to localize magnesium binding sites in this particular ribozyme were performed. Thereby, an on-going discussion on the functional role of divalent metal ions in the cleavage process could be addressed. Finally, we started to elucidate the solution structure of the pistol ribozyme complex. Combining all the NMR data will give us novel insights into the folding landscape and the functional dynamics of the pistol ribozyme.

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P166

Studying sparsely populated conformational states in RNA combining chemical synthesis and solution NMR spectroscopy

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Using chemical synthesis and solution NMR spectroscopy, RNA structural ensembles including a major ground state and minor populated excited states can be studied at atomic resolution. On this poster, the chemical synthesis of a 2,8-13C2-adenosine building block is presented to introduce isolated 13C-1H-spin topologies into a target RNA to probe structural ensembles via NMR spectroscopy. The 2,8-13C2-adenosine 2'-O-TBDMS-phosphoramidite building block was incorporated via solid phase synthesis into two target RNAs- a 9 kDa and a 15 kDa construct derived from the epsilon RNA element of the duck Hepatitis B Virus. The resonances of the isotope labelled adenosines of the 9 kDa 28 nt sequence could be mapped to the full-length 53 nt construct. The isolated NMR active nuclei pairs were used to probe for low populated excited/ground states via 13C-Carr-Purcell-Meiboom-Gill (CPMG)-relaxation dispersion NMR spectroscopy. The 13C-CPMG relaxation dispersion experiment recapitulated a unfolding event occurring on the millisecond time scale was found in the upper stem in-line with earlier observations. This unpaired conformational state is presumed to be important for the binding of the epsilon reverse transcriptase (RT) enzyme. Thus, a full description of an RNA's folding landscape helps to obtain a deeper understanding of its function, as these high energy conformational states often represent functionally important intermediates involved in (un)folding or ribozyme catalysis.

P167

Elucidating G-quadruplex - polyethylene glycol interactions

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G-quadruplexes are non-canonical structures adopted by G-rich DNA. Core of a G-quadruplex comprises stacked G-quartets, each formed by four Hoogsteen hydrogen-bonded guanine residues. These four-stranded structures are extremely polymorphic due to the versatility of how guanines in the core of the structure are interconnected, which furthermore depends on conditions, in particular the concentration and nature of cations in solution. The delicate susceptibility of G-quadruplex features to subtle changes in (micro)environment along with their distinguished characteristics compared to the canonical double-stranded helix points to unique biologically-relevant mechanism of responsiveness. Indeed, there are com-

pelling evidences on roles of G-quadruplex in a number of vital biological mechanisms, including gene expression, DNA replication and maintenance of genome integrity. Thus, unrevealing structural modulations of a G-rich DNA provides to better understanding of key biological processes. Additionally, high-resolution insights are required from aspect of drug design, which aim is to change gene expression profile(s) by targeting particular G-quadruplex structures formed within regulatory regions. Complexity of cellular environment together with limitations of experimental methodologies confines many biologically important phenomena to be explored under in vitro or at most in near cell-like environments. In this regard, cosolutes, which are added to aqueous solution to impose molecular crowding conditions and thus mimic biological environment,1, 2 have been used to investigate G-quadruplex structures as well as their interactions, e.g. with potential therapeutic ligands. Many researchers use polyethylene glycols (PEGs) of various molecular sizes and concentrations. Certainly, this approach enabled progress in understanding complexity of G-quadruplex structuring and the related biological impacts.3, 4 However, reports on non-inertness of PEG with respect to DNA as well as on preferential binding of the cosolute to DNA G-quadruplex5 call for caution and more critical consideration on the particular strategy to simulate the intracellular environment. By using NMR-based structural characterization we highlighted different modes of interactions between DNA G-quadruplex and (poly)ethylene glycols.6 In presence of ethylene glycol the increase in DNA G-quadruplex stability and small structural changes mainly correspond to dehydration effect. High molecular weight polyethylene glycol, on other hand, stabilizes DNA G-quadruplex by delicate direct interactions with terminal G-quartet. 1. Miyoshi D. and Sugimoto N., Biochimie, 90, 1040-1051, (2008) 2. Nakano S., Miyoshi D. and Sugimoto, N., N. Che. Rev., 114, 2733-2758 (2014) 3. Fujii T., Podbevsek P., Plavec J. and Sugimoto N., J. Inorg. Biochem, 166, 190-198, (2017) 4. Hänsel R., Löhr F., Foldynova-Trantirkova S., Bamberg E., Trantirek L. and Dötsch V., Nucleic Acids Res., 39, 5768-5775, (2011) 5. Buscaglia R., Miller M.C., Dean W.L., Gray R.D., Lane A., Trent J.O. and Chaires, J.B., Nucleic Acids Res., 41, 7934-7946, (2013) 6. Trajkovski M., Endoh T., Tateishi-Karimata H., Ohyama T., Tanaka S., Plavec J. and Sugimoto N., Nucleic Acids Res., 46, 4301-4315, (2018)

P168

Seeing the invisible: Transient low populated anionic Watson-Crick base pairs are stabilized by the 5-oxyacetic acid uridine (cmo5U) modification in RNAs

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Over the past years, natural modifications have been discovered to play a crucial role in many cellular processes such as protein translation or the development of severe genetic diseases(1). More than 100 alternations of nucleotides in both DNA as well as RNA have been uncovered so far and unravelling all their functions and properties in diverse biomolecular systems is a challenging task. Solution NMR spectroscopy, especially the relaxation dispersion methodology has been proven to be a valuable tool for dynamic studies in the field of nucleic acids and their natural modifications(2).

5-Oxyacetic acid uridine (cmo5U) is a well-known post-transcriptional modification, which is often found at the wobble position of tRNAs(3). In this work, we present a novel synthesis of a stable isotope labeled 15N2-cmo5U phosphoramidite building block which we incorporated into a 20 nt hairpin RNA via solid phase synthesis. Recently, it has been proposed that the U•G wobble base pair naturally occurs as a dynamic equilibrium of their respective enol tautomer mispairs, U•G(enol) and U(enol)•G(4).

With the help of NMR relaxation dispersion experiments, we are the first to report that the 5-oxyacetic acid modification stabilizes an anionic uracil form and furthermore increases the population of a short-lived Watson-Crick like cmo5U(anionic)•G mispair about three-fold. These results are supported by pKa measurements via NMR confirming the higher acidity of the imino proton of the cmo5U residue.

Various model RNAs were further characterized via UV spectroscopic melting curve analysis revealing a destabilization by the cmo5U modifier, which is in accordance with base pair stability data from CLEANEX-PM NMR data. Our results shall give a comprehensive picture on the influence of the cmo5U modification on the RNA folding landscape.

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P169

Investigating the structural basis for increased activity of TBA variants

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Preventing coagulation of blood cells leading to thrombosis is still an ongoing field of research. 1992 a first aptamer binding to thrombin (thrombin binding aptamer) was identified by Bock et al. [1]. This aptamer forms a G-quadruplex in solution. Here, we compare and investigate three aptamers binding to thrombin using NMR-spectroscopy: The original aptamer and two variants identified by Buff et al. [2], exhibiting increased activity compared to the original sequence. Based on our NMR data, we can show that while the original aptamer populates one conformation in solution [3], a higher degree of polymorphism is observed in the investigated variants. [1] Bock et al, Nature, 355, 564-566 (1992) [2] Buff et al. Nucl. Acid. res., 38, 2111-2118 (2009) [3] Macaya et al. PNAS, 90, 3745-3749 (1993)

P170

Investigation on the structural and dynamic influence of 2-Methyladenosine and Dihydrouridine in tRNA

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Post-transcriptional modified nucleotides play crucial roles to fine-tune structural and dynamic features of tRNAs. In many cases, however, the molecular details how these modifiers alter the folding landscape of RNA remain elusive. In this research project, we want to shed light on the physicochemical properties and functions of 2-methyladenosine (m2A) and dihydrouridine (DHU) within tRNAs. The synthesis of stable isotope labeled phosphoramidite building blocks of both modifications for RNA solid phase synthesis as well as their incorporation into oligonucleotides was achieved. These precursors were incorporated into target RNAs to investigate base pairing properties and internal dynamics via NMR spectroscopy. In case of m2A, a modification prevalent at position 37 in iso-acceptor tR-

NAs [1], NMR data revealed a Watson-Crick like base pair with in a 35 nucleotide (nt) long hairpin. The solution state WC like base pairing is additionally supported by crystallographic studies. In contrast, DHU, one of the most common RNA modification in all three domains of life, tends to disrupt helix formation due to its elevated conformational flexibility and the loss of planarity through saturation of the double bond between C5 and C6 [2]. DHU's role as a flexible hot-spot was confirmed by NMR spectroscopy on a 15 nt long hairpin mimicing the D-arm in a S. pombe initiator tRNA(Met). By the incorporation of a 5,5,6-D3-6-13C-DHU as well as 1'-13C-DHU residue we could demonstrate motional dynamics both in the nucleobase and the sugar moiety, with a clear preference of the C2'-endo sugar pucker.

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P171

Structural and theoretical insights into the RNA-recognition mechanism by Musashi-1

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Musashi-1 (Msi1) is a key player in the self-renewal of stem cells and in the development of cancer cells. Msi1 binds to the 3'-untranslated regions of the target mRNAs and either positively or negatively regulates the translation initiation. Msi1 has two RNA-binding domains (RBDs), RBD1 and RBD2. We previously determined the structure of the complex between RBD1 and an RNA, r(GUAGU) [NAR, 2012]. RBD1 uses two phenylalanines, which are on the β-sheet and the C-terminal region, to sandwich the adenine, while the first guanine is stacked onto tryptophan in the loop between β1 and α1. Recently, we have calculated the free-energy change upon the RBD1:r(GUAGU) binding, and the energetic and entropic components, using the currently most reliable hybrid method developed by Kinoshita et al. We showed that the binding is driven by a large gain of configurational entropy of water in the entire system. To achieve this, above mentioned sandwiching and stacking were revealed to be utilized effectively [PCCP, 2018]. On the other hand, we determined the structure of the RBD2:r(GUAGU) complex [Mol, 2017]. RBD2 recognizes the central r(UAG) using hydrogen-bonding and aromatic stacking interactions, but no sandwiching interaction is used. Finally, we constructed a structural model of consecutive RBDs with the minimal recognition sequence of Msi1, r(UAGGUAG). The model suggests that Msi1 can recognize r(UAGNnGUAG) (n = 0 - 50) that are found in the Msi1 target mRNAs.

P172

Investigation of Conformational Dynamics of a RNA-Chaperone-Complex by NMR Spectroscopy

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The term RNA chaperone describes a heterogeneous group of proteins that do not sample a common sequence, motif or fold, but are able to destabilize RNA structures and interactions. Due to stable folding intermediates that act as folding traps, the folding process of a functional RNA is rendered slow. By lowering energetic barriers between competing states, RNA chaperones accelerate RNA refolding processes. Characteristic of RNA chaperones is their independence of ATP or any other energy providing cofactor for their activity and non-sequence and non-structure specific activity regarding the RNA substrate. Consequently the chaperone is unfolding the RNA and enables a refolding of the RNA in an un-directional manner. However little is known about the mechanism details of this interaction and its driving force.(1),(2)

Here we show the investigation and characterization of fast and slow dynamics of the RNA and the RNA chaperone StpA during their interaction by 15N relaxation and 15N/13C CEST NMR spectroscopy to get a better insight into the open questions. During the interaction fast dynamics on the ps-ns timescale increase for the RNA, due to an increasing exchange with the environment and partial opening of the base pairs, and decrease for the backbone of StpA, which is adapting secondary structure elements for the interaction. By 15N and 13C CEST we were able to show that fluctuations between local different conformations on the ms-s timescale arise more frequently during interaction for both biomolecules.

During binding StpA has to maintain a certain conformation, resulting in a decreased conformational mobility and consequently reduced entropy. The RNA molecule gets more flexible during the interaction, because of base pair destabilizing and strand opening events. This potential entropy transfer could be the driving force of the activity that lowers the energetic barriers between folding states.

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P173

hnRNPA1 mediated specific recognition and unfolding of DNA quadruplexes

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hnRNPA1 is a modular ribonucleoprotein with two RNA Recognition Motifs (RRMs) (collectively called the UP1), an RGG-box, and a C-terminal region that contains the nuclear localization sequence. It is involved in RNA transport, trafficking, alternative splicing, and telomere DNA maintenance. hnRNPA1 interacts with ss and G-quadruplex DNA as well as RNA structures and also possesses DNA G-quadruplex unfolding activity. Using both X-ray and NMR spectroscopy methods, the UP1 domain has been studied previously for its structure and interaction with the DNA and RNA substrates in detail. The crystal structure of UP1 with single-stranded telomeric DNA has been solved, where two protein molecules were shown to form a complex with the single stranded DNA in an anti-parallel conformation. In solution, however, the two RRMs interact with each other and the relative orientations of them have been shown to resemble the nucleic acid bound form rather than the free form. The G-quadruplex unfolding mechanism of the hnRNPA1 is not fully understood. In this study, using CD, fluorescence, and NMR spectroscopy and ITC methods, the interaction and unfolding of telomeric DNA G-quadruplexes by hnRNPA1 domains was studied. Structure specific interaction of hnRNPA1 with DNA G-quadruplexes has been observed. Mutation studies have also been carried out to confirm these results. These results provide insight into the recognition, affinity, and destabilization of DNA G-quadruplex structures by hnRNPA1. Our results provide a comprehensive picture of hnRNPA1 interaction with telomeric G-quadruplex structures.

P174

New protein-DNA complexes in archaea: a small monomeric protein induces a V-turn structure

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In archaea the two major modes of DNA packaging are wrapping by histone proteins or bending by architectural nonhistone proteins. MC1 (Methanogen Chromosomal protein 1) is a small basic monomeric nucleoid-associated protein (NAP) which is structurally unrelated to other NAPs. Like most proteins that strongly bend DNA, MC1 is known to bind in the minor groove. The first model of a complex in which MC1 binds to the concave side of a strongly bent 15 basepairs DNA was obtained by two complementary docking approaches. This model allowed us to calculate the expected protein-DNA contacts and helped us to unambiguously identify 50 intermolecular distance restraints derived from NOEs. However, the use of NOEs restraints is generally not sufficient to determine global structural features such as bending in nucleic acid. RDCs constraints, known to improve the precision and accuracy of both the local and global structures of the double helix were measured on both the DNA and the protein. Their addition in the calculation was an absolute necessity to resolve the 3D structure of the complex. We report here the 3D solution structure of a new DNA-protein complex formed by MC1 and a strongly distorted 15 base pairs DNA. While the protein just needs to adapt slightly its conformation, the DNA undergoes a dramatic curvature and an impressive torsional stress due to several kinks caused by the binding of MC1 to its concave side, and thus adopts a sharp V-turn structure. On longer DNAs, MC1 stabilizes multiple V-turn conformations in a flexible and dynamic manner. It participates to the genome organization of some Euryarchaea species through an atypical compaction mechanism. It is also involved in DNA transcription and cellular division through unknown mechanisms. The V-turn conformations of the MC1-DNA complexes open new opportunities to studying and understanding the different roles of MC1 in Euryarchaea.

P175

NMR and Thermodynamic Studies on BAF250a ARID and DNA Interaction

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SWI/SNF chromatin remodeling complexes contain a subunit belonging to AT rich interacting domain (ARID) family of proteins through which they are recruited to nucleosomal target DNA with high affinity, allowing the transcriptional activation of normally silenced chromatin. In higher eukaryotes, SWI/SNF complexes contain two or more mutually exclusive ARID subunits e.g. ARID2 (BAF200) in the PBAF complex, while ARID1a (BAF250a) and its paralogous ARID1b (BAF250b) resulting in BAF-A and BAF-B complexes respectively. The central ARID in these proteins belongs to a family of highly conserved DNA binding domain. However, the exact contribution of ARIDs to SWI/SNF functions as well as their DNA binding specificities has not been established. Here we have probed the structure and interaction of BAF250A ARID with different ds-DNA sequences using NMR, ITC, and SPR methods. We used dsDNA sequences of varying sequences to understand the DNA binding properties of BAF250A ARID. A comprehensive biophysical study of the interaction between BAF250A ARID and various dsDNAs revealed complex nature of ARID–DNA interactions. Our results suggest that ARID binds to GC rich DNA sequences with better affinity (or lower Kd) than AT rich sequences. However, the interaction of AT rich DNA sequence with ARID proceeds with a large negative enthalpy (ΔH = -23 Kcal/mol) change upon complex formation compared to GC rich sequence, suggesting that this interaction is more specific in nature. Using NMR chemical shift perturbation experiments, we have identified DNA binding residues on ARID and mapped them on to the ARID structure. Further, we have generated the NMR data driven HADDOCK models of ARID-DNA complexes. Implications of BAF250A ARID binding to different DNAs will be discussed.

P176

Site-Specific Studies of Nucleosome Interactions by Solid-State NMR

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The nucleosome is a protein-DNA complex playing a central role in chromatin structure and function. Numerous proteins interact with the nucleosome, either regulating or driving crucial cellular processes such as DNA replication, regulation of gene expression and DNA repair. X-ray crystallography and more recently cryo-EM have successfully explored the nature of several interactions, yet the structural basis for such mechanisms remains largely elusive. NMR, through most notably Methyl-TROSY, brought important contributions; especially in the case of dynamic complexes and intrinsically disordered proteins. To further extend the toolbox of techniques available to study the nucleosome, we present here a new approach based on proton detected solid-state NMR of sedimented nucleosomes. We show the potential of sedimentation for preparation of nucleosome samples, yielding a concentrated gel of isolated nucleosomes. Additionally, the proton detection allowed us to obtain signals arising from the majority of the core residues, in parallel with J-coupling based experiments specifically observing the flexible histone tails. These results encouraged us to study nucleosome interactions though co-sedimentation. As exemplified on H2A, we obtained residue-specific structural information of the histone and its interface of interaction with LANA N-terminal peptide, in context of the whole nucleosome. We also show preliminary results on epigenetic reader proteins interacting with the histone tails. Compared to the established methyl-TROSY approach, our new method observes more structural reporters, evenly distributed along the sequence, and allows a lower deuteration level in sample preparation. We believe that this approach holds great promise for the observation of nucleosome-protein complexes and potentially even chromatin.

S. Xiang, U. B. le Paige, V. Horn, K. Houben, M. Baldus, H. van Ingen, Angew. Chem. Int. Ed. 2018, 57, 4571.

P177

Characteristics of the C-terminal domain of anti-viral factor APOBEC3G: mechanism behind a target search and inhibition by HIV-1 Vif protein

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APOBEC3G (A3G) deaminates a cytosine of a newly synthesized minus

DNA strand of human immunodeficiency virus 1 (HIV-1), and thereby restricts the infectivity of viral-infectivity-factor (Vif)-deficient HIV-1 strains. Deamination activity of A3G is highly specific; A3G favors the third position of a triplet cytosine (CCC); A3G prefers to edit a CCC located closer to the 5'-end than the one located less close to the 5'-end. A3G comprises two zinc-coordinating domains: a catalytically inactive N-terminal domain (NTD) and an active C-terminal domain (CTD). We have been characterizing the deamination mechanisms of A3G involving NMR-based enzymatic assay, so called "real-time NMR monitoring method". Using this method we previously found that the deamination preference is caused by sliding, and interestingly, both full length and A3G-CTD undergo sliding for target search. Here we investigated intersegmental transfer, which is known as a phenomenon by which a protein directly moves between two sites widely separated along the DNA contour but closely located in three dimensions. Deamination of two cytosines located separately on substrate ssDNA by the A3G-CTD were monitored using real-time NMR. The deamination preference between the two cytosines was lost when either the substrate or non-substrate competitive ssDNA concentration was increased. When the competitive ssDNA concentration increased, the deamination activity first increased, but then decreased. This indicates that even a single domain, A3G-CTD, undergoes intersegmental transfer for a target search. HIV-1 Virion infectivity factor (Vif) protein completely suppress the activity of A3G. Vif is an intrinsically disordered protein, forming a five-membered complex (Vif complex) by hijacking the components of human E3 ubiquitin ligase and a transcription factor. Vif reportedly binds to NTD of A3G, ubiquitinates CTD of A3G, then degrades A3G through ubiquitin-proteasome proteolysis; Vif is also known to directly inhibit deamination activity of A3G. Here we investigated the effect of Vif complex on deaminase activity by A3G. We inspected the interaction between A3G, Vif complex, and ssDNA. Vif complex exhibited DNA-binding affinity and also binding-affinity toward A3G, both of which were involved in the inhibition of A3G activity.

P178

The solution structure of FUS bound to RNA reveals both sequence and shape specificities.

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FUsed in Sarcoma (FUS) is an hnRNP protein which regulates pre-mRNA splicing, miRNA biogenesis, DNA damage and can drive subcellular phase transitions. Two neurodegenerative diseases Amyotrophic Lateral Sclerosis (ALS) and Fronto-Temporal Lobar Degeneration (FTLD) show neuropathological protein aggregates containing FUS and it is hypothesized that mis-regulation of RNA processing could play a major role in these diseases1. FUS consists of a C-terminal prion-like region and an N-terminal RNA/DNA binding region including an RNA Recognition Motif (RRM) and a zinc-finger (ZnF) domain interspersed between methylated RGG repeats. FUS associates with a large variety of RNAs2 including pre-mRNA, and lncRNA. Differing results from in vivo (CLIP) and in vitro (SELEX, binding assays) suggest that RNA binding by FUS is complex. Despite intense interest, to date there is no structural information on how FUS interacts with RNA.

We have used solution NMR spectroscopy to investigate the molecular mechanisms by which FUS recognizes RNA. We present the solution structures of FUS RRM and ZnF domains bound to RNA. The RRM domain bound to a stem-loop RNA3 shows an unusual binding mode in which the RRM binds in the 3' loop region using the β-sheet and C-terminal tail with limited sequence specificity and an $\alpha1$-$\beta2$ extension unique to the FUS family, contacts the stem-loop junction. The ZnF shows a sequence specific interaction with an NGGU motif and this interaction. Additional studies show that the RRM and ZnF can coordinate in binding a bi-partite RNA site with contributions from RGG repeat motifs and that both domains contribute to minor intron splicing events. These results give the first insights into the structural basis of RNA recognition by FUS.

P179

Structural studies of oxidative stress induced protection to telomeric DNA by telomere binding protein derived from Zebra fish

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Telomeres are protein-DNA elements that play an important role of maintenance chromosome stability by capping the ends of eukaryotic chromosomes. Telomere repeats binding factor 2 (TRF2), as a vital telomeric protein in shelterin complex against instability, binds specifically to double-stranded telomeric DNA [(TTAGGG)n]. TRF2 plays important roles in chromatin reorganization, telomere maintenance and DNA repair under Reactive oxygen stress (ROS) attacked. Even though the structural studies of TRF2 complexed with other biomolecular were well published, the detail of how TRF2 structural would change under oxidative environment has uncovered yet. Very recently, since zebrafish have human like short telomeres, using zebra fish in telomere research show several advantages for illustrating how telomere attrition contributes to cellular senescence organ dysfunction and disease. In the present studies, we use Zebra fish TRF2 (zTRF2) protein as research target protein to discuss the topic of the recognition and assembly mechanism of telomeric DNA and TRF2 protein under ROS caused oxidative environment which was the mainly reason of telomere shorting to a large extent. For mimicking the oxidative environment to observing the zTRF2 structural change after ROS attack, several ROS source were used in our study such as hydrogen peroxide, and different type of Plasma treatments. Plasma is an ionized gas can move the RNS and ROS from the gas to solution phase. We have determined that oxidative environment makes zTRF2 have conformational change indeed by the circular dichroism spectroscopy and fluorescence quenching after plasma treatment. Interestingly, the binding affinity between zTRF2 and human telomere was enhanced. By employing NMR, the residues affected by the ROS were mapped. Compared the crystal structure of non-treated zTRF2 with DBD plasma, JET plasma, and H2O2 treated zTRF2, the ROS-dependent structural change could be observed. Furthermore, the thermodynamic analysis indicates the overall structure of zTRF2 become unstable. For telomeric DNA, which is easily attacked by ROS, complexed with zTRF2 could reduce the potential to be oxidized. The decreased stability of zTRF2 caused stronger binding with telomeric DNA could prevent the telomere damage by ROS temporary until the environment back to normal ROS level. Our study reports the behavior of TRF2 when protecting telomere from ROS in structural studies, which could help molecular understanding for mechanism of telomere shorting and related diseases.

P180

NMR Kinetics Study of the Z-RNA binding of the Zα Domain of Human RNA Editing Enzyme ADAR1 More Slowly Binds to Z-RNA than Z-DNA

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Human RNA editing enzyme ADAR1 deaminates adenine in pre-mRNA to yield inosine. The Zα domains of human ADAR1 (hZαADAR1) binds specifically to left-handed Z-RNA as well as Z-DNA and stabilizes the Z-conformation. To answer the question of how hZα ADAR1 can induce both B–Z transition of DNA and A–Z transition of RNA, we investigated the structure and dynamics of hZα ADAR1 in complexes with 6-bp Z-DNA or Z-RNA. We performed chemical shift perturbation and relaxation dispersion experiments of hZαADAR1 upon binding to Z-DNA as well as Z-RNA. Our study demonstrates the unique dynamic feature of hZαADAR1 during A–Z transition of RNA, in which the hZαADAR1 protein forms thermodynamically stable complex with Z-RNA like Z-DNA but kinetically more slowly converts to Z-RNA than Z-DNA. We also found the distinct structural features of the hZα ADAR1 in the Z-RNA binding conformation. Our results suggest that the A–Z transition of RNA by hZα ADAR1 displays the unique structural and dynamic feature that may be involved in targeting ADAR1 for a role in recognition of RNA substrates.

P181

Tyrosine in Hydrophobic Packing: Key Feature of Psychrophilic Cold Shock Protein for Its Low Thermostability and Activity at Subfreezing Temperature

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Cold shock proteins (Csps) are expressed at lower-than-optimum temperatures, and they function as RNA chaperones; however, no structural studies on psychrophilic Csps have been reported. Here, we aimed to investigate the structure and dynamics of the Csp of the psychrophile Colwellia psychrerythraea 34H, (Cp-Csp). Although Cp-Csp shares sequence homology, common folding patterns, and motifs—including a five β-stranded barrel—with its thermophilic counterparts, its thermostability (37 °C) was markedly lower than those of other Csps. Cp-Csp binds heptathymidine with an affinity of 100 nM, thereby increasing its thermostability to 50 °C. Nuclear magnetic resonance spectroscopic analysis of Cp-Csp structure and backbone dynamics revealed a flexible structure with only one saltbridge and 10 residues in the hydrophobic cavity. Notably, Cp-Csp contains Tyr51 instead of the conserved Phe in the hydrophobic core, and its phenolic hydroxyl group projects toward the surface. The Y51F mutation increased the stability of hydrophobic packing and may have allowed the formation of a K3–E21 saltbridge, thereby increasing its thermostability to 43 °C. Cp-Csp exhibited conformational exchanges in its ribonucleoprotein motifs 1 and 2 (754 s-1 and 642 s-1), and heptathymidine binding markedly decreased these motions. Cp-Csp lacks saltbridges and has longer flexible loops and a less compact hydrophobic cavity resulting from Tyr51 compared to mesophilic and thermophilic Csps. These might explain the low thermostability of Cp-Csp. The conformational flexibility of Cp-Csp facilitates its accommodation of nucleic acids at low temperatures in polar oceans and its function as an RNA chaperone for cold adaptation.(Y. Lee, et al.(2018) Biochemistry57(26), 3625-3640. doi: 10.1021/acs.biochem.8b00144.)

P182

RecQ C-terminal domain of Human Bloom Syndrome protein binds and destabilizes the G-quadruplex DNA

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Bloom syndrome protein (BLM) is one of five human RecQ helicases that plays an essential role in DNA metabolism. It recognizes G-quadruplex (G4) DNA via its RecQ C-terminal (RQC) domain with high specificity. G4s are non-canonical DNA structures which are found in G-rich sequences such as telomeres and promotor sites. Although several studies have revealed the detailed mechanisms of duplex DNA binding and G4 unwinding process of BLM, how BLM RQC initially recognizes G4 structure is not elucidated. Here, we investigated the interaction between BLM RQC and the G4 DNA from the c-Myc promoter sequence by NMR spectroscopy. While signals got broadened upon mutual titrations, the β-wing region of BLM RQC has significant chemical shift perturbations and experiences intermediate time scale exchanges upon G4 binding. A point mutation in the β-wing region reduces G4 binding affinity. Our HDX data indicate that imino protons of G4 were exchanged with deuterium much faster in the presence of BLM RQC. We suggest BLM RQC binds to G4 by using the β-wing as a separating pin and it destabilizes the G4 DNA.

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Structural characterization of the cytoplasmic DNA binding domain of Vibrio cholerae transcription regulator ToxR

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ToxR is a periplasmic transcription regulator controlling the virulence gene expression in Vibrio cholerae, the causative organism of the diarrheal disease cholera. The protein contains a regulatory periplasmic domain, a transmembrane region and a cytoplasmic part (cToxR) homologous to the winged helix-turn-helix (wHTH) DNA binding domain. ToxR regulates the expression of various genes by using different mechanisms which are not completely understood. The production of two outer membrane proteins namely OmpU and OmpT is directly controlled by cToxR, in the case of OmpT cToxR acts as a repressor whereas OmpU expression is activated by cToxR. To activate the transcription of toxT, which is the main virulence regulator in V. cholerae, ToxR co-operates with another membrane located transcription factor named TcpP. Furthermore, there are indications that ToxR may also directly activate the transcription of the cholera toxin ctx. [1] In our work, we were able to solve the structure of cToxR by using a combination of NMR assignments (including NOEs) as well as Talos secondary structure prediction and Rosetta, a computational protein modeling

software. The wHTH family member YycF was used as a template, showing 31% sequence identity with cToxR. The calculated structure could be confirmed by assigned NOEs in the NMR spectra, revealing the typical wHTH topology and some special structural features. Additionally, we performed several NMR titration experiments by using ds DNA Oligos ctx, toxt, ompU, ompT containing one binding site for cToxR. By using 15N-HSQCS we analysed the resulting shifts and could observe the binding pocket in cToxR. Cholera is still an epidemic disease in several regions of the world and outbreaks still occur on a regular basis. [2] Research in this field is therefore highly important in order to understand how the virulence production in V. cholerae is controlled and the how the bacterium is able to survive even in harsh environments.

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P184

Dead End destabilizes mRNA targets through an unprecedented mode of tandem RRM - AU-rich RNA recognition

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Post-transcriptional gene regulation is orchestrated by an interplay between mRNA sequence and structure, microRNAs and RNA binding proteins (RBPs), which immediately cover mRNAs as they are transcribed and are essential for mRNA maturation, transport, cellular localization and turnover. Understanding how these RBPs specifically recognize their mRNA targets is therefore crucial to understand the complex gene regulatory networks involved in health and disease. The RNA binding protein Dead End (Dnd1) is a vertebrate-specific germ cell viability mediator. Mutations in the Dnd1 gene cause loss of germ line stem cells, male sterility and testicular tumors. It is thought that Dnd1 is crucial for germ line development by clearing certain mRNAs containing AU-rich sequences in their 3'UTR from the cell through recruitment of the CCR4-NOT deadenylase complex [1], but the molecular mechanisms for target selection are unknown. Like most RBPs, Dnd1 combines several copies of RNA binding domains to increase target specificity and affinity. Its domain structure is unique, carrying two RNA recognition motifs (RRMs), the second lacking conserved residues on its beta-sheet that are normally involved in canonical RRM-RNA binding, combined with a dsRBD (double-stranded RNA binding domain). To understand how Dnd1 recognizes its targets at the molecular level we have solved the solution structure of its tandem RRMs in complex with an AU-rich RNA recognition element (ARE) by NMR. Use of selectively ribose 13C–labeled RNA prepared by solid state synthesis resolved spectral overlap of critical residues, greatly aiding resonance assignments and collection of intermolecular restraints. Our RRM12-ARE structure shows that Dnd1 harbours a novel fold in RRM1 containing helical and beta-hairpin extensions to the canonical RRM fold, RNA recognition involves these non-canonical elements and the RNA interaction is stabilized by a novel RNA-interaction surface in RRM2. The structure explains a surprising increase in affinity to RNA upon addition of RRM2 to RRM1 even though it lacks a canonical RNA-binding surface and fails to bind RNA on its own. Furthermore, it explains Dnd1's preference to AU-rich over U-rich sequences. Finally, using biophysical and cell-culture based assays, we show that RNA binding and target destabilization is weakened by mutating essential residues in the RRM12-RNA interface. Although several structures of tandem RRM-RNA complexes have been solved previously, most of them concern combinations of canonical RRM-RNA binding surfaces. Therefore, this unprecedented mode of cooperative RNA binding by Dnd1's tandem RRMs not only improves our understanding of Dnd1 function but more generally extends our understanding of the versatility of tandem RRM-RNA interactions.

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P185

Dissecting the mechanism for oskar mRNA localization by structural biology

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During the development of cellular organisms, mRNA localization allows confined protein expression, thereby sustaining cell polarization. This process is essential to all developmental programs, such as cell movement, cell polarization and asymmetric cell division1-3. In Drosophila melanogaster, the oskar mRNA is synthesized during oogenesis and is mainly responsible for the formation of germplasm and posterior polarity. Oskar mRNA localization to the posterior pole of the oocyte determines where the abdomen and primordial germ cells form. The transport occurs along the polarized cytoskeleton. Trans acting factors recognize specific sequence in the oskar mRNA transcript and form ribonucleoprotein particles that interact with the kinesin motor for transport along the microtubules4. Recent studies have shown that splicing of the first of the three introns is an imperative event for the localisation5. The Spliced Oskar Localized Element (SOLE) RNA motif7, which is formed upon splicing of the first intron,6 is very much essential for localization, as well as deposition of the exon-exon junction complex (EJC) at the first splice site6. However the functional link between the SOLE RNA and the EJC is unclear. We aim to shed light on the role of the SOLE RNA for localization by studying the interaction of this RNA motif with its protein binding partners. To achieve this we apply solution state NMR, Small Angle Scattering (SAS), molecular modeling and biochemical assays.

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P186

Conformational landscape and active conformation of the mRNA decapping complex revealed by methyl TROSY NMR

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Enzymes are dynamic molecular machines. Many insights into the molecular details of their function have been gained from crystal structures. But in the case of highly dynamic enzymes crystal structures are prone to packing artifacts. They also hide dynamic information that is often crucial for the understanding of the enzymatic function.

A striking example is the bilobed decapping enzyme Dcp2. It catalyzes the removal of the protecting 5' cap from eukaryotic mRNAs and thereby regulates gene expression. The activity of the C-terminal catalytic domain (CD) of Dcp2 is increased in a stepwise manner by the N-terminal regulatory domain (RD) and the activator proteins Dcp1 and Edc1. The two domains of Dcp2 are connected by a flexible linker and Dcp2 has been shown to be highly dynamic. Several crystal structures of Dcp2 in the free state and in complex with these activator proteins have been solved. These structures can be divided into six groups with vastly different domain orientations. Due to the dynamic nature of Dcp2 it is challenging to determine whether the obtained crystal structures are adopted in solution. This explains why the mechanisms of activation and the structure of the catalytically active state of the enzyme still remain controversial despite this wealth of structural information.

To address these questions we explore the conformations that Dcp2 samples in solution using a suite of methyl TROSY based NMR experiments. By combining CSP, 13C-CPMG relaxation dispersion, NOESY and PRE experiments we show that Dcp2 samples three different structural states in solution: an open and a closed conformation and a catalytically active form. The apo and the activator bound enzyme complexes exchanges between catalytically impaired open and closed conformations. Substrate binding to the Dcp1:Dcp2 complex competes with the closed conformation and results in a highly dynamic assembly. The stable catalytically active state of the decapping complex is only formed in the presence of substrate and both activators, which is explained by a novel crystal structure of the quaternary complex. In summary, we provide a detailed model of how the conformational landscape of Dcp2 is modulated by decapping activators and how this increases the catalytic activity.

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Uniformly 13C-labeled carbohydrates for probing carbohydrate-protein interactions by NMR spectroscopy

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Carbohydrates used in structural studies by NMR are rarely 13C-labeled, despite severe resonance overlap in their 1H NMR spectra. This is caused by the lack of easily accessible isotope-labeled material, preventing exploitation of the 13C spectral dispersion in 3D or higher order NMR experiments.

In this presentation, we demonstrate the power of using a uniformly 13C-labeled trimannoside to delineate the carbohydrate-protein interface by tailored isotope-filtered experiments [1]. Our approach is applicable to systems that exhibit slow exchange on the NMR time scale, which therefore are not amenable to trNOE or STD experiments, commonly used in the fast exchange regime. As model system we selected the complex of a 13C-labeled Manα(1–2)Manα(1–2)ManαOMe trisaccharide, bound to cyanovirin-N (CV-N). NOE-based mapping of carbohydrate-protein contacts established that Manα(1–2)Manα(1–2)Manα OMe is bound more intimately with its two reducing-end mannoses into the domain A binding site of CV-N than with the non-reducing end unit. Using different isotope labels on the glycan and the protein our approach provides a versatile means for simultaneously mapping binding interfaces on both a carbohydrate and its protein binding partner.

We also identified four carbohydrate hydroxyl protons that form hydrogen bonds with CV-N backbone carbonyl oxygens [2]. Their resonances, surprisingly, were observable in the room-temperature spectra of the complex and were assigned via scalar couplings from the adjacent sugar ring protons. Intra- and intermolecular NOEs involving these hydroxyl protons permitted the determination of their orientation and hydrogen-bonding patterns. Further studies on similar mannoside/CV-N complexes also revealed such hydroxyl proton resonances, highlighting the general applicability of this novel approach for characterizing hydrogen bonding in carbohydrate-protein interactions.

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P188

Elucidating the molecular mechanisms by which the HNH endonuclease gp74 activates the terminases in bacteriophage HK97

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The last gene in the genome of the bacteriophage HK97 codes for gp74, an HNH endonuclease. HNH endonucleases digest DNA in the presence of metals and are characterized by two highly conserved His residues and an Asn residue. Gp74 is essential for phage head morphogenesis, likely because gp74 enhances the activity of the HK97 terminase enzymes toward the cos site. Enhancement of terminase-mediated cleavage of the phage cos site requires the presence of an intact HNH motif in gp74. Mutation of the canonical metal binding His in the HNH motif abrogates gp74 mediated-enhancement of terminase activity. Although phages are widely studied, there is no definitive structural or mechanistic evidence as to how the HNH endonuclease within gp74 functionally interacts with the terminase enzymes to facilitate phage morphogenesis. Further, gp74 possesses very low sequence similarity to HNH proteins for which the structure has been determined, making structural studies of gp74 necessary.

To gain structural information of gp74 we use nuclear magnetic resonance (NMR) spectroscopy. Current work reports NMR resonance assignments of 80% of the backbone and side chain C resonances of gp74. Analysis of the chemical shifts indicates that, as expected, gp74 contains the characteristic $\beta\beta\alpha$ motif found in HNH endonucleases. However, there are additional structural elements in gp74 outside the $\beta\beta\alpha$ motif that are not present in other HNH endonucleases, including the HNH endonuclease Gmet_0936 that is predicted to be structurally similar to gp74. In addition, NMR studies have elucidated residues within gp74 required for metal binding and terminase activity. These data are being used to assess the role of specific gp74 residues in phage morphogenesis. Together, this work will identify how metal binding to the HNH endonuclease gp74 is crucial in the

replication and morphogenesis of phages.

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The long forgotten Intermolecular transferred-NOE.

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Transferred NOE (trNOE) can be used to study intermolecular interactions in large protein complexes exceeding even 100 kDa, when the ligand is considerably smaller and exhibits a fast off-rate. In the past we used a T1ρ-filter to abolish intramolecular NOEs within the large protein and to observe intermolecular interactions between the aromatic protons of a 50 kDa antibody fragment and a 1.8 kDa peptide antigen as well as intramolecular interactions within the bound peptide. However, when the peptide ligand contains several aromatic residues, the spectrum becomes dominated by intramolecular trNOE interactions within the peptide, limiting the applicability of this approach. The isotope-edited/isotope-filtered (edited/filtered) technique is the most powerful method to study intermolecular interactions in macromolecular complexes. However, the lengthy delays in the experiment limit the applicability of this method to proteins up to 30 kDa. To optimize the detection of intermolecular trNOE we use a combination of the two approaches to study intermolecular interactions in protein complexes ranging from 9 kDa to over 100 kDa. In a typical experiment the protein is uniformly labeled with 13C and 15N and the peptide is unlabeled. A 4-6 fold excess of a peptide ligand is used. The 12 ms filtering step of the regular edited/filtered experiment is applied on the peptide magnetization before the acquisition period. The peptide exhibits a T2 in the 100 ms range averaged with the T2 of the peptide in the bound state, long enough to retain considerable magnetization after the filtering step. The order of the editing and filtering steps enables one to use a shorter acquisition time in the indirect dimension and a long acquisition time for the peptide magnetization, optimizing resolution, sensitivity and signal-to-noise ratio. The 13CH3-labeling in otherwise uniform deuteration of the protein extends the applicability of our approach to much larger systems. We demonstrate the power of trNOE approach combined with the edited/filtered experiment for the 11 kDa RANTES complex with an N-terminal CCR5 peptide (Nt-CCR5) and a 40 kDa complex of the protein kinase p38 with a KIM peptide. For studying intermolecular interactions in the 110 kDa ternary complex of HIV-1 gp120 with a CD4-mimic peptide and Nt-CCR5 (1:1:7 molar ratio) we used commercially available 13CH3-labeled methyl containing amino acids which are otherwise deuterated. gp120 cannot be uniformly deuterated because of its expression in mammalian cells and therefore the filtering step is not applied. Instead, we use the asymmetric deuteration approach. The aromatic amino acids of gp120 and the methyl containing residues of the peptide are deuterated. This approach eliminates intramolecular interactions between aromatic protons and methyl containing residues in both the protein and the peptide ligand, and the trNOE intermolecular interactions enhanced by the presence of the peptide excess are clearly observed.

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Interaction of angiogenin with heparin, DNA and the p53 TAD2 domain and its implication for inhibitor discovery

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Angiogenin (Ang), a ribonucleolytic enzyme, has been implicated in various biological processes, such as angiogenesis, cell proliferation, migration, and invasion in both normal and cancer cells. The heparin complex of rat angiogenin revealed that a heparin strand is fitted into a positively charged groove formed by the dual binding site of rat angiogenin. However, the positively charged surface with a 50KRSIK54 motif is the main interaction site of human angiogenin for both heparin and DNA binding. The 50KRSIK54 motifs were also identified as p53-binding sites that could interact with p53 TAD2 domain suggesting play a critical role in the regulation of p53-mediated apoptosis and angiogenesis in cancer cells. The study identifies potential target sites for screening angiogenin specific inhibitors for p53 binding, cell binding, internalization, DNA binding, and nuclear translocation of human angiogenin.

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Remodelling of the conformational landscape of the EGFR extracellular domain after nanobody binding prevents receptor activation

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Ligand binding to transmembrane receptors is an essential process for cellular activation, molecular transport or cell-cell communication and are therefore common targets for therapy. For example, the Epidermal Growth Factor Receptor (EGFR) is vital for the regulation of cell proliferation, migration and differentiation and has consequently major implications in tumorigenesis. Significant evidence (REF) including our previous solid-state NMR studies1 suggest that the extracellular domain (ECD) of EGFR exhibits significant dynamics prior to ligand binding and becomes rigid upon binding to its natural ligand the Epidermal Growth Factor (EGF). These dynamics have significant implications on receptor functioning and subsequent signalling events and therefore the role of dynamics in receptor activation needs to be well understood. Here, we describe a solid-state Nuclear Magnetic Resonance (ssNMR) approach that allows us to study the dynamics of EGFR in native plasma membrane vesicles using non-activating nanobodies. In particular, we show that binding of nanobody EgA1 does not affect ECD dynamics but prevents receptor activation without blocking the EGF binding site. We propose that binding of EgA1 modulates the conformational entropy of the ECD and prevents the occurrence of conformations necessary for EGF-induced EGFR activation.

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NMR characterization of allosteric pathways and a post translational modification regulating HBGA recognition in GII.4 human Norovirus

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Human noroviruses (hNoV) recognize histo-blood group antigens (HBGAs) as cellular attachment factors. Recently, it has been reported that norovirus infection can be significantly enhanced by HBGA and bile salts.[1] Yet the attachment process, and how it promotes host-cell entry is only poorly understood. We have recently reported that HBGA recognition in GII.4 hNoV P dimers follows a cooperative process.[2] Here, we extend the methodology to the study of the allosteric networks controlling the recognition of hNoV towards their attachment factors.

We have successfully assigned 86% of the backbone NH signals and 100% of the methyl groups in a U-[2H,15N] stereo-selectively AILProS-MVProS methyl group labeled hNoV GII.4 P dimers (72 KDa). Chemical shift perturbations (CSPs) provided the binding epitope for bile acids, which is located c.a. 30 Å apart from the canonical HBGA binding pockets. Titrations of HBGAs and bile acids yielded long-range effects up to 45 Å, indicating the presence of a densely connected allosteric network. Paramagnetic relaxation enhancements (PREs) induced by titrations with Gadolinum-contanining H-disaccharide excluded the presence of additional low-affinity binding pockets on the P dimers. Finally, a CHESCA-like[3] analysis combined with Link community[4] revealed a functional connectivity between HBGAs and bile acid epitopes, suggesting a joint role in viral infection.

To understand the architecture of the allosteric network we generated a set of 17 naturally occurring soft mutants, which yielded distinct chemical shift perturbation patterns. Mutations caused long-range effects and led to altered binding profiles towards blood group B trisaccharide. Affected regions include HBGA and bile acids epitopes, as well as previously reported antibody blockade epitopes.[5] A 2nd order Markov[6] analysis revealed the structure of the network, reflecting the flow of allosteric signals. We aim at using this information for the identification of protein "hot-spots", which could be targeted as allosteric modulators of hNoV infection.

Unexpectedly, our NMR analysis revealed a highly specific post translational modification (PTM) forming over time. This finding is supported by mass spectrometry and crystallography. In particular, this PTM involves the irreversible deamidation of N373 and subsequent formation of an iso-aspartate residue (isoD373), significantly affecting HBGA binding and reshaping the structure of the allosteric network. At 310 K, this transformation proceeds with a half-life of two to three days. The analysis of all crystal structures and sequences available for hNoV P dimers shows that N372/N373 is a highly conserved motif among GII.4 and the currently predominant GII.17 hNoV strains, suggesting an important role for this post translational modification in the processes of infection and immune evasion.

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Structural basis of AtGrp7 from Arabidopsis thaliana in RNA binding and inactivation

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The glycine-rich RNA-binding protein AtGrp7 was first determined as a component of a negative feedback loop in the circadian clock regulation of Arabidopsis thaliana [1]. This protein is composed of an N-terminal RNA recognition motif (RRM) and a C-terminal unstructured glycine-rich domain. The RRM domain functions as an RNA chaperone in RNA metabolism, while the glycine-rich domain acts as a shuttle for RNA transport between the nucleus and cytoplasm. AtGrp7 can bind to a set of transcripts such as 5' UTR, 3' UTR, and intron RNA in Arabidopsis plants, including its own transcript. Through interaction with the RNA targets, AtGrp7 can promote or affect alternating splicing, pri-miRNA processing, flowering, and stomata opening and closing. AtGrp7 can be modified and inactive in RNA binding by the type III effector HopU1, a mono-ADP-ribosyltransferase from Pseudomonas syringae pv. Tomato DC3000. But how AtGrp7 recognizes its numerous RNA targets and how its binding to RNA blocked by the effector are still unanswered. In this study, we first solved the high-resolution NMR structures of AtGrp7 RRM domain (aa. 1-90) and its complex with a 6-nt RNA from its 3' UTR. The AtGrp7 1-90 had a high structural similarity to an RRM domain from Nicotiana tabacum with PDB code 4C7Q. Binding with the 6-nt RNA resulted in large conformational change of the C-terminal tail of AtGrp7 1-90, and mutagenesis and binding analysis suggested that AtGrp7 1-90 belongs to a canonical RRM domain. To understand the glycine-rich domain's contribution on RNA binding, we investigated the RRM domain extended with additional (H/Y)RGG/RS motifs (H95RGG and Y103RS) in AtGrp7, by the combination of mutagenesis, ITC, NMR and different DNA counterparts of the RNA from the 3' UTR of AtGrp7. An explanation was then given for the roles of the two RGG/RS motifs on AtGrp7-RNA binding. Finally, we investigated the interaction of the AtGrp7 RRM domain and the effector HopU1 by NMR and docking calculation. As revealed by NMR titration experiment of labeled AtGrp7 1-90 and unlabeled HopU1, the residues at the L1($\beta1\alpha1$) and L2($\beta2\beta3$) loops mainly contribute to HopU1 binding. Unfortunately, reverse NMR experiment was inhibited by one third of signals missing in the finger-print 1H-15N HSQC spectrum of HopU1. After referring to a exhaustive mutation work on HopU1 in the study of its interaction with AtGrp7 [2], we generated the complex model of AtGrp7 RRM domain and HopU1 using HADDOCK, which provides some clues of AtGrp7's recognition by the effector.

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Deciphering a novel mechanism regulating galectin-glycoprotein lattice assembly/disassembly during cell-cell interactions

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Multivalent protein-carbohydrate interactions lead to the formation of lattices on cell surfaces which regulate essential cellular events, including cell adhesion, cell proliferation, modulation of cell signaling, cell death and pathogen recognition of host cells. Consequently, cells "fine-tune" the regulation of these functions by modulating the assembly/disassembly of protein-carbohydrate lattices. One family of multivalent lectins that can organize cell surface lattices is the galectins. Altering protein glycosylation or lectin expression are two well-described ways for a cell to dynamically modulate the strength of the lectin-glycoprotein lattice interactions. Recently, we suggested a third new mechanism of modulation of such crucial interactions (1). During B-cell development, bone marrow stromal cells secreting galectin-1 (Gal1) constitute a specific niche for pre-BII cells. Besides binding glycans, Gal1 is also a pre-B cell receptor (pre-BCR) ligand which induces pre-BCR clustering, the first checkpoint of B cell differentiation. The Gal1/pre-BCR interaction is the first example of a Gal1/unglycosylated protein interaction in the extracellular compartment. We performed in-depth NMR structural characterization of this interaction (2) and showed that Gal1/pre-BCR interaction modifies Gal1/glycan affinity towards specific glycan epitopes. NMR structural studies using 13C-labeled glycan bound to Gal1 in the presence and in the absence of the pre-BCR binding sequence revealed the structural basis of this new mechanism of regulation of Gal1 interactions. Moreover, our investigations led us to demonstrate that, while bound to stromal cell membranes, the carbohydrate binding site of Gal1 undergoes structural modifications in the presence of the pre-BCR. These results provide evidence that Gal1/pre-BCR interaction acts as a physiological regulator of the Gal1/glycoprotein lattice. Therefore, this fine tuning of GAL1/glycan interactions may be a strategic mechanism for allowing pre-BCR activation and pre-BII cells departure from their niche. But, beyond the pre-BCR case, it was also tempting to wonder if Gal1 lattice modifications induced by binding to an unglycosylated protein sequence could be used in other Gal1 dependent processes to regulate specific cellular functions and responses. Therefore, we screened for other potential unglycosylated protein candidates for Gal1 binding and investigated there binding to Gal1 using NMR spectroscopy. The results obtained suggest that the protein interacting site on Gal1 serves as tuning platform of lattice assembly/disassembly for several signaling pathways and cellular processes.

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P195

NMR Based Structural and Dynamic Studies of a Self-assembling Polypeptide Nanotube

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Protein self-assembly is the best representation of molecular self-assemblies and a potent research tool to employ in biomedical applications due to their high biocompatibility and biodegradability. The self-organization is achieved due to the structural compatibility and chemical complementarity of the atoms and molecules involved in covalent bonding (disulfides) or weak interactions (hydrogen bonding) (Gonen, S et al., Science, 2015; Suzuki, Y et al., Nature, 2016). Self-assembled structures possess advantages over disordered or random coiled protein monomers, as they provide higher stability and mechanical strengths. In addition, the periodic occurrence of the functional motif of monomer in its self-assembled form enhances its biological functions (Welsh, D. J. et al., Org Biomol Chem, 2013). We have previously reported a self-assembling nanotube system derived from Insulin like growth factor binding protein 2 (Swain et al., Chem. Commun., 2010). Self-assembly is driven by intermolecular disulfide bonds that renders redox responsiveness to the nanotubes. They can be loaded with suitable drugs to be utilized as drug carrier and can be covalently tagged with various probing molecules to be integrated as a part respective nanodevices. The presentation will emphasize on the NMR-based study conducted to characterize the formation of the self-assembling nanotubes and their bio-applications. The structural insights and capturing of intermediates of self-assembling nanotubes obtained using protein-NMR techniques will be described (Asampille et al., manuscript under preparation 2018).

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Conserved asymmetry underpins homodimerization of Dicer-associated double-stranded RNA-binding proteins

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Not all double-stranded RNA-binding domains (dsRBDs) function by interacting with dsRNA. Two varieties of dsRBD exist: canonical Type-A dsRBDs interact with dsRNA, while non-canonical Type-B dsRBDs lack essential RNA-binding residues and have instead evolved to interact with proteins. In higher eukaryotes, the microRNA biogenesis enzyme Dicer forms a 1:1 association with a dsRNA-binding protein (dsRBP) that contains a conserved Type-B dsRBD. Human Dicer associates with HIV TAR RNA-binding protein (TRBP) or protein activator of PKR (PACT), while Drosophila Dicer-1 associates with Loquacious (Loqs). The Type-B dsRBD in each of these proteins interacts with the RNA helicase domain of Dicer. While recent studies have revealed how Dicer interacts with Type-B dsRBDs, we know considerably less about how and why these domains homo- and heterodimerise.

We will present data that show that the Type B dsRBDs of Loqs, PACT and TRBP self-associate to form homodimers that have significant and unusual structural asymmetry. We have elucidated the 3D structures of the Type B dsRBDs of TRBP and PACT, which reveal an asymmetric self-association mechanism that involves a parallel β-strand at the homodimer interface. This interaction motif means that each dsRBD has two non-equivalent self-association modes. NMR analysis of the Type B dsRBDs of Loqs, PACT and TRBP reveal that asymmetric self-association is conserved from flies to humans. The three dsRBDs have different self-association affinities, which can be attributed to evolutionary divergence of their homodimerization interfaces. Mutation of a single conserved leucine residue on this interface abolishes self-association in all three dsRBPs. Moreover, mutating the TRBP homodimer interface to render it more PACT-like enhances self-association, whereas the reciprocal mutations in PACT reduce self-association. Finally, we have determined that the Type-B dsRBDs of TRBP and PACT preferentially heterodimerise. Unlike the two modes of

interaction that are observed in self-association, NMR analyses reveals that heterodimerisation of these Type B dsRBDs involves only a single mode of interaction.

Our structural and NMR studies show that dsRBD-dsRBD interactions involving Loqs, PACT and TRBP utilize the same surface that is required for binding Dicer. These data suggests that dissociation of homo- or heteromeric interactions involving Type B dsRBDs may be a key step in the assembly of a functional Dicer complex.

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P197

Structural and functional analyses of oxidative stress response by Keap1-Nrf2 system

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Keap1-Nrf2 system plays critical roles in the response against oxidative and electrophilic stresses in human. Keap1 is a cysteine-rich adaptor protein and acts as a sensor of redox insults. Under quiescent conditions, Keap1 suppresses Nrf2, a transcription factor that regulates cytoprotective gene expression. When oxidative stress stimulates this system, Nrf2 suppression by Keap1 is attenuated and the expression of cytoprotective genes are activated. In this study, we have analyzed molecular mechanisms how oxidative stress response activates the Keap1-Nrf2 system by means of nuclear magnetic resonance spectroscopy. We expressed the sensor region (BTB-3box domains) of Keap1 and investigated interactions of this region with thee types of electrophilic inducers. We found that these electrophilic inducers interact with similar residues of Keap1, indicating that molecular mechanism of the Nrf2 induction by these electrophilic inducers is basically similar with each other. We are currently investigating details of the interaction of Keap1 with Nrf2 and will discuss mechanisms of the interaction.

P198

Escaping the Groove: Structural Plasticity of MHC I

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The control of the immune system plays a key role in host defense. The presentation of peptides at the surface of most nucleated cells by major histocompatibility complex class I molecules (MHC I) is crucial for eliciting or evading immune responses. The long established view that MHC I, unlike MHC II, imposes strict length restrictions on the repertoire of MHC bond peptides is being challenged by recent advances in determining the complement of cell surface presented peptide antigens that consistently show a significant proportion of peptides that are longer than the canonical 8–10mers. (1) Our recent investigations in the trimming of N-terminally extended single chain MHC I peptide complexes indicated that MHC I may allow the partial dissociation of peptides in vivo (2). Here we are using a combination of extensive (100 μs) molecular dynamics simulations together with NMR observations of the protein and the ligand to investigate the plasticity of the peptide-binding domain of the mouse allele H2-Db. The analysis of chemical shift, RDC and relaxation data together deuterium exchange data give insight into the conformational dynamics of the peptide bound state of MHC I on a wide rage of time scales. The use of extensive MD simulations provide an atomistic interpretation of the data and together with the NMR data challenge the notion of a deeply buried ligand in the MHC I groove.

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P199

Structural aspects of interaction between heparin sulfate and programmed cell death protein 5 (PDCD5) from pathogenic protozoan Toxoplasma gondii

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Toxoplasma gondii (T. gondii), an obligate pathogenic protozoan parasite, can infect most of all warm-blooded animals and has been a great threat to public health. The development of drugs against T. gondii infection is urgent, since severe encephalitis and retinochoroiditis occur when T. gondii infecting immunocompromised patients. T. gondii programmed cell death protein 5 (TgPDCD5), a secreted protein leading to increased apoptosis of host macrophage, was considered as a promising drug target, while the translocation of TgPDCD5 into cell originated from its binding with heparin/heparan sulfate proteoglycans. The molten globular structural identity of TgPDCD5 was first characterized, which supported by CD, ANS fluorescence, and SAXS profiles. NMR structure of TgPDCD5 reveals a flexibly folded structures, adopting an extended triple-helix bundle fold that is connected to a mobile but structured alpha-helix in the N-terminus by means of a highly flexible linker. In addition to C-terminal HSPG-binding motif, NMR perturbations of TgPDCD5 by heparin sulfate implied more critical residues for heparin sulfate binding. Combined analysis of the results may not only provide a novel HSPG binding mode but also imply the inhibitory strategy of TgPDCD5/HSPG binding for therapeutic drug development.

P200

Molecular basis of beta chaperone activity of Lipocalin type prostaglandin D synthase

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Aggregation of amyloid β peptides is considered as one of the most important events associated with Alzheimer's disease (AD). This aggregation can be controlled by molecular chaperones which are protein machineries that aid in protein folding. Here, we investigate one such protein called Lipocalin type Prostaglandin D synthase (LPGDS), the second most abundant protein in the cerebrospinal fluid, which is found to have chaperone activity for the Alzheimer's amyloid β peptide. We establish that LPGDS binds to monomeric Aβ40 and Aβ(25-35) peptides, and inhibits its aggregation as seen in Thioflavin T assay and Transmission electron microscopy (TEM). The binding interface of protein-peptide complex was studied by 15N HSQC titrations of LPGDS with monomeric Aβ40. Small angle X-ray scattering experiments show increase in radius of gyration for the LPGDS-Aβ40 complex compared to the apo form and the overall shape shows an extra domain probably occupied by the monomeric peptide. Besides its protective role, LPGDS also shows disaggregase activity by dismantling the preformed amyloid fibrils in ThT assay and TEM images. TEM also helped in giving insight into the possible mechanism of fibril disaggregation by LPGDS. As a validation of disaggregase activity, LPGDS was able to extract out a number proteins (commonly found in protein aggregates of AD) from protein aggregates collected from human AD brain tissue. Since LPGDS exhibits both inhibitory and disaggregase activity for monomers and fibrils respectively, we propose LPGDS as a potential candidate for chaperonotherapy for AD.

P201

Role of tryptophan-chromophore interaction for colour tuning in the red/green photoreceptor AnPixJ

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Light perception is crucial for all organisms. In order to adapt to the environmental illumination conditions and to respond to its changes, chromophore-carrying photoreceptor proteins have been developed. The chromophore includes a π-system and therefore is able to absorb light. Cyanobacteriochromes (CBCRs) compose a group of these photoreceptor proteins. They are found in cyanobacteria and are part of a superfamily of photoreceptors commonly summarized as phytochromes. All photoreceptors of this family covalently bind a tetrapyrrole chromophore, in most cases in a GAF-domain of the protein. While phytochromes switch between a resting state absorbing red light and an activated state absorbing far-red light, the absorption range of CBCRs is much broader.[1] Our work focusses on a CBCR GAF-domain called AnPixJ_GAF2 (from Anabaena sp. PCC7120) that absorbs red light (648 nm) in its resting state and green light (543 nm) in its activated state. [2] In order to understand what determines the absorbed wavelengths on a molecular level, tryptophan sidechains are labelled using labelled indole. In addition, the chromophore will be labelled using labelled aminolevulinic acid. 2D-NMR-spectroscopy will then be applied to examine correlations between the tryptophan and the tetrapyrrole. Focus will be set to the presence or absence of π-stacking between the aromatic rings of the amino acid and the chromophore. This π-stacking might be responsible for a twisted conformation of the chromophore which could lead to a n altered π-system and therefore to a changed absorbance behaviour.

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P202

Nonenzymatic acetylation of ubiquitin Lys side chains is modulated by their neighboring residues

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Nonenzymatic acetylation of Lys side chains (Lys-SCs) by various in vivo reactive molecules has been suggested to play novel regulatory roles. Ubiquitin (UB) has seven Lys residues that are utilized for synthesis of specific poly-UB chains. To understand the nature of these Lys-SC modifications, the chemical acetylation rate and pKa and Hill coefficient of each UB-Lys-SC were measured. Mutagenesis studies combined with the determination of activation energy indicated that specific neighboring residues of the Lys-SCs have a potential catalytic activity during nonenzymatic acetylation. Based on the shared chemistry between nonenzymatic Lys acetylation and ubiquitylation, the characterized chemical properties of the UB-Lys-SCs could be a reference for deciphering both mechanisms. Our NMR approaches could be useful for studying general nonenzymatic Lys acylations of various proteins.

P203

Structural studies of the calcium-dependent transport of vitamin A via STRA6

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Vitamin A is an essential nutrient for all mammals and it is vital for vision. Retinol delivery to cells is via the retinol binding protein receptor, which is encoded by a gene named stimulated by retinoic acid 6 (STRA6). STRA6 is expressed widely with abundance in the eye, and its mutation in humans is linked to Matthew Wood syndrome (MWS). The STRA6 vitamin A transporter is a 75-kDa multi-pass transmembrane (TM) protein, and a recently determined 3.9-Å resolution cryoEM structure shows it to be a dimer with each of its subunits bound to the calcium-binding protein calmodulin (CaM). CaM binds to the cytoplasmic side of STRA6 in an unconventional arrangement with a helix, termed CaMBP1, of STRA6 found to bind exclusively to the main hydrophobic cleft in the N lobe of CaM. Another helix of STRA6, termed CaMBP0 (BP0) binds to the N lobe, on a surface between CaM helices 1 and 4, and interacts additionally with CaMBP1 (BP1) in a helix-helix crossing mode. The major interaction surface at the CaM-Stra6 interface involves another helix of STRA6 termed CaMBP2 (BP2), which interacts with the hydrophobic groove in the C lobe of CaM much like the canonical 1-5 interaction of the myosin light-chain kinase (MLCK) [2]. With this structure in hand, the effect that peptides derived from Stra6 (CaMBP0, CaMBP1, and CaMBP2) had on the structure, dynamics, and calcium binding to CaM were measured using a combination of NMR spectroscopy and ITC. These data show that CaMP2 binding to CaM increases the Ca2+-binding affinity of CaM by more than 25-fold and are consistent with a "Binding and Functional Folding" model, which will be discussed. Support for these studies in funded by the NIH (PI: Mancia; 1R01EY027405).

P204

Preparation of START Domain of the Ceramide Transfer Protein (CERT) for Nuclear Magnetic Resonance (NMR)

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Ceramide, an important component in the metabolism of sphingolipids, plays a significant role in proliferation and apoptosis of cells.1 De novo synthesis of ceramide takes place at the cytosolic surface of the endoplasmic reticulum (ER), and then, ceramide is transferred to the Golgi apparatus for conversion into both sphingomyelin and glucosylceramide by either vesicular trafficking or non-vesicular trafficking. Non-vesicular transport of ceramide is carried out by the ceramide transfer protein (CERT) that consists of peptidic motifs and multiple domains.1 The C-terminal (StAR)-related lipid transfer (START) domain is the most important domain given the fact that it is capable of extracting and accommodating ceramide in its deep hydrophobic cavity. CERT could be an attractive pharmacological target because of its involvement in common pathological processes such as Alzheimer's disease, infectious diseases and cancer2. A well-known antagonist of CERT is N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl) dodecanamide (HPA-12)3, however, there is only limited structure-activity relationship (SAR) data available. In this study, we aim to explore the interaction between CERT and HPA-12 to establish SAR of this compound class by nuclear magnetic resonance spectroscopy (NMR) in order to improve the inhibition activity of the ligand for a potential drug design. Herein, we optimized the established expression and purification protocols to get a moderate yield of the monomer of the N-terminally His tagged START domain. In order to get an idea about the suitability of the protein of interest for ligand and receptor-based NMR experiments, the target protein was labeled with 15N and purified using an optimized two-step purification method. The 15N labeled START domain's monomer was used to record initial 1H-15N HSQC and TROSY NMR spectra in phosphate buffer. Results of both HSQC and TROSY experiments were promising for further receptor-based NMR experiments.

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P205

NMR study of iron binding of magnetite interacting sequences from magnetotactic bacteria magnetite biomineralization proteins

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Biomineralization of iron is an essential process of mineral deposition for magnetotactic bacteria. The specialized biomineralization process takes place in a subcellular organelle, magnetosome. The magnetosome contains a unique set of magnetite associated proteins (MAPs) that control magnetite formation through regulating nucleation, and particle size and shape. These MAPs are predicted to be small membrane proteins containing sequences specifically targeting irons in solution. Here, we focused on three different MAPs and study how the individual magnetite interacting sequences (MISs) interact with irons. We prepared isotope-labeling peptides and employed comprehensive NMR methods to evaluate the interactions. We noticed the significant effect from Fe2+ and Ni2+, where intensity attenuation occurred due to the paramagnetic property of irons but with no pseudo-contact chemical shift. In studying conventional protein-protein interaction, [1H, 15N]-HSQC happens to be the most powerful spectra. However, in iron titration, the detection based on amine proton becomes less reliable since the proton continually exchanges with solvent. The derived resonance intensity change actually reports not only interaction but also solvent exchange that is influenced by the paramagnetic irons. Therefore, a series of 2D spectra based on 13C detection, including [13C, 15N]-CON, [13C, 15N]-CAN and [13C]-CACO was employed to dissect the direct effect only from binding. The binding-leading intensity attenuation and the derived binding affinities (KDs) could be used to more accurately report the interactions. Here, we would establish a better model to define the interaction between the irons and the MISs.

P206

Insights into Molecular Recognition Processes of Norovirus Capsid Proteins Using NMR Spectroscopy

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Human noroviruses (NoV) recognize glycans as cellular attachment factors. Still, their role in attachment and host-cell entry processes remains poorly understood. Existing structural models have revealed details of glycan-capsid interactions, but there is growing evidence that a static picture of glycan recognition is insufficient. Recently, we have demonstrated that binding of NoV to human histo-blood group antigens (HBGAs) is a complex and cooperative multi-step process using nuclear magnetic resonance (NMR) spectroscopy. Here, we present an almost complete assignment of NH backbone resonances of a GII.4 NoV capsid protein, which at a molecular weight of 73 kDa still is a major task. To shine more light on HBGA-binding to human noroviruses we have engaged chemical shift perturbation (CSP) experiments. Our findings have been complemented by 15N relaxation measurements. Unexpectedly, our NMR analysis also revealed a highly specific post translational modification of the capsid pro-

tein that has a significant impact on glycan recognition.

P207

Protein-Cucurbit[6]uril Interactions

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Cucurbit[n]urils (CBn) are "molecular doughnuts" capable of highly selective complexation with biomolecules, including proteins, in aqueous solution. For example, N-terminal phenylalanine residues are high affinity targets - with CB8 capable of accommodating two such groups [1]. Lysine residues are also promising targets for CBn-complexation, with affinity increasing with methylation of the ammonium group [2]. A recent NMR and crystallographic study revealed CB7 binding to a dimethylated lysine and showed exciting potential for complexation of non-(N-terminal) residues [3]. CB6 may be better suited to complexation with native lysine residues, based on its affinity for aliphatic amines [4,5]. Here, we report initial NMR investigations into protein-CB6 complexation. The influence of mono- and di-valent cations on the protein-CB6 established interaction was investigated. Our data hint at complexation with the most sterically-accessible lysine, as well as the N-terminus. Interestingly, interaction of CB6 with the dimethylated variant was significantly reduced - the opposite effect to that observed with CB7.

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P208

Dimethylarginine binding by SMN Tudor domains: a case of conformational selection

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Tudor domains are the only known proteins that can bind symmetrically N-dimethylated arginine (sDMA), a post-translationally modified amino acid that is central for regulating RNA processing in eukaryotic cells. The ligand-binding sites of Tudor domains proteins are highly specific, and comprise a characteristic aromatic cage containing several aromatic side chains. We will discuss the energetics and dynamics of methylated arginines in solution and bound to the basic Tudor domain of human survival motor neurons (SMN) protein. Dynamic NMR data are used to characterize the conformational equilibrium of free sDMA in solution. From quantum chemical calculations, we can compare this to the different bound states observed in NMR and x-ray structures of different Tudor domains. We propose that the aromatic cage in the SMN domain leads to a stronger cation–π interaction within the active site, and can result in significant selectivity for anti-anti sDMA vs. the anti-syn conformation. Furthermore, our findings are reconciling the structural differences observed between the different classes of Tudor domains, i.e., the anti-anti sDMA-binding basic SMN Tudor domain and the anti-syn sDMA-binding extended Tudor domains. The difference in the bound states of sDMA can be explained by the composition of the aromatic cage, as demonstrated by NMR data of a Trp-to-Phe mutant of the SMN Tudor domain.

P209

Towards Protein Encapsulation via Charge-Charge Interactions

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Abstract

Currently, there is a great interest in developing protein encapsulation for biocatalysis and drug delivery applications [1,2]. We are developing an approach towards encapsulation based on charge-charge interactions with synthetic macrocycles [3,4]. The model lectin RSL [5], was modified to contain additional arginine residues, thus raising significantly its pI. This increased pI enhances charge-based interactions with anionic p-sulfonatocalix[4]arene (sclx4) and similar ligands. Additionally, the versatility of the arginine sidechain in intermolecular interactions further improves the host-guest interactions. A combination of NMR spectroscopy and X-ray crystallography has been carried out to characterize RSL-Rful interactions with sclx4. A plethora of protein-ligand interactions, involving non-covalent bonds contribute to formation of calixarene clusters at protein-protein junctions. This data provides a stepping stone towards achieving the goal of protein encapsulation.

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P210

Proline restricts loop 1 conformation of the high affinity WW domain from human Nedd4-1 to a ligand binding-competent type 1 β-turn

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The E3 ubiquitin ligase human Nedd4-1 (hNedd4-1) (neuronal precursor cell expressed developmentally downregulated gene 4-1) mediates the transfer of ubiquitin from the cognate E2 ubiquitin-conjugating enzyme to the substrate. The four WW domains from hNedd4-1 interact with PPxY

(PY) motifs of the human epithelial Na+ channel (hENaC) subunits, and have high sequence similarity amongst themselves. However, affinity to the PY motif varies significantly, with the third domain (WW3*) showing the highest affinity. We have previously reported that WW3* domain recognizes the α-hENaC peptide through coupled folding and binding equilibria (1), which also exist in presence of the neighboring WW domains (2). The highest sequence diversity amongst the four hNedd4-1 WW domains lies within the loop connecting the first two β-strands (loop I). Loop I of WW3* adopts a type I β-turn with a highly statistically preferred proline at i+1 position (P433), absent in the other hNedd4-1 WW domains. Using a combination of MD simulations and NMR spectroscopy, we show that exchange of proline in loop I from WW3* into the corresponding residue from WW4 (threonine) resulted in a dynamic seven residue loop rather than the wild-type type I β-turn of the apo-protein (3). In presence of the ligand, the structure of the mutated loop I is locked into a type I β-turn. Besides altering the local stability, the P433T mutation also affects the overall domain stability, evident from a 6 °C decrease in melting temperature. However, the affinity between the mutant domain and α-hENaC peptide, quantified using chemical shift perturbations of resonances from WW3* P433T mutant in presence of increasing concentrations of the α-hENaC peptide, remains near identical. Interestingly, affinity measured using ITC was four fold higher for the WW3* P433T mutant in comparison to the wild-type. 15N CPMG relaxation dispersion experiments revealed that the P433T mutation causes the WW3* domain to fold significantly slower, with nearly two-fold increase in the minor-state population, in comparison with the wild-type domain. The results indicate that proline contributes to high affinity WW3*-PY motif interaction by ensuring a stable type I β-turn, rather than by providing a direct peptide-interaction interface. This suggests that amino acid sequence modulates local flexibility to tune binding preferences and stability of dynamic interaction motifs.

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P211

Thermodynamic characterization of cooperative substrate binding by the homodimeric enzyme human thymidylate synthase

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Allostery, or changes in distal locations of a biomolecule in response to a perturbation, is utilized by macromolecules throughout biology to achieve functional regulation and can even be essential for biomolecular function itself. Allostery is currently understood in terms of changes in the free energy landscape of a biomolecule resulting from a perturbation such as ligand binding, including both enthalpic (structural) and entropic (dynamic) contributions. Appreciation of both structural and dynamic contributions to allostery makes NMR spectroscopy an ideal method to probe this phenomenon. Allosteric communication between subunits in the homodimeric enzyme thymidylate synthase (TS) is evidenced by its property of "half-the-sites reactivity", meaning that it performs catalysis in only a single subunit at a time. TS catalyzes the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate, making it an essential enzyme for the cell as it lies along the only metabolic pathway producing the nucleotide thymine. In this work, we quantify the thermodynamics of the two substrate binding events in the 72 kDa human TS enzyme using NMR spectroscopy and isothermal titration calorimetry. Resonance "quartets" from titration spectra, which report on all binding states of the protein, are analyzed using the software TITAN (Waudby et al. Sci Rep 2016). TITAN performs 2D lineshape analysis by simulating and fitting two-dimensional spectra. Interestingly, our data show positive cooperativity in substrate binding by human TS, in contrast to the independent binding observed in the structurally similar E. coli enzyme.

P212

Interaction between IL-18 and the propeptide that suppresses its proinflammatory activity

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Interleukin (IL)-18 is a proinflammatory cytokine that belongs to the IL-1 family, which potently stimulates interferon (IFN)-γ production to protect hosts against infections. In cells, IL-18 is synthesized in its precursor forms (proIL-18) with N-terminal propeptide sequences (PPs), whose lengths is 36 amino acid residues. ProIL-18 remains inactive in the cytosol until a large protein complex termed the inflammasome is formed. The inflammasome is a multi-protein assembly composed of three proteins, NLR, ASC and caspase-1, that is formed upon the detection of pathogens or other harmful substances such as reactive oxygen species (ROS) and urate crystals in the cytosol. The inflammasome formation activates caspase-1, which is known to catalyze removal of PPs from ProIL-18 to yield active IL-18. In this study, we characterized the structure of proIL-18 by using NMR spectroscopy and revealed that proIL-18 adopts a structure distinct from that of IL-18. Notably, the PP region extensively interacted with the mature region of proIL-18 in an intramolecular manner, which may affect the observed structural difference. We also discovered that a peptide harboring the PP sequence can bind IL-18 essentially in the same manner as in proIL-18. Intriguingly, this peptide binding toward IL-18 suppressed its function. Namely, NF-κB activation and IFN-γ production induced by IL-18 in cells were significantly moderated in the presence of the PP peptide. These results suggest that the PP peptide can become a scaffold to develop a new class of IL-18 inhibitors for the treatment of various IL-18-associated inflammatory diseases.

P213

Interactions of GHK and GHTD peptides as promiscuous binding partners with human serum albumin by means NMR spectroscopy.

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Human serum albumin (HSA 585 a.a. long, molecular mass 60 kDa) is a universal carrier of fatty acids, metabolites, drugs and also metal ions, including Ca(II), Zn(II) and Cu(II) [1]. It is generally assumed that HSA transports Cu(II) via its N-terminal binding site characterized with Kd nearly 1 pM at pH 7.4. The GHK and GHTD peptides, presented in human blood serum at sub-micromolar concentrations, demonstrated Kd of

Cu(II) ions around 0.2 pM. Quantitative calculations reveals that such Kd values and concentrations should not be enough to peptides to bind Cu(II) ions which contradicted with experimental observations. We speculate that GHK or GHTD peptides facilitate transfer of Cu(II) ions between carrier proteins (HSA) and cellular receptors combining high thermodynamic stability with rapid kinetics of transfer.

NMR spectroscopy presented couple effective techniques for screening and characterization of binding small ligands to high molecular weight biomolecules in solution. In presented study we explore the interactions between two - GHK and GHTD - peptides with HSA protein without presence of Cu(II). The binding peptides to HSA has been confirmed with Diffusion Ordered SpectroscopY (DOSY) recorded as Double Polar Field Gradient Double Stimulated Echo (DPFGDSTE) as 128 accumulations with 25 gradient steps using 100 ms diffusion time (Δ) and 2 ms gradient time (δ). All experiments were conducted on Varian Inova 500 NMR spectrometer equipped with Performa IV z-gradient unit. Processing of experimental data were done with CONTIN algorithm [2] embedded in VnmrJ 4.2 (Agilent Inc., USA) and MestreNova (version 12.0.2) software. Four samples characterized with 20 : 1, 10 : 1, 5 : 1 and 3 : 1 ratio peptides to HSA were prepared in 50 mM phosphate buffer pH 7.4 to acquire experimental data and compared with the pure GHK and GHTD solved in the same buffer. Evaluated results exhibited decrease of translational diffusion for both GHK and GHTD peptides from 3.2 down to 2.9 x 10-10 (m2/s) under increase concentration of HSA. Additional data about interactions GHK and GHTD with HSA were extracted from T1 and T2 relaxation times which exhibit increase overall tumbling for both peptides. Our results provides evidences interaction of GHK and GHTD with HSA, which were also confirmed by other spectroscopic techniques.

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P214

Structural and functional analysis of phosphoinositide binding specificity of divergent FYVE domains using NMR spectroscopic techniques.

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FYVE domains are highly conserved membrane recognition modules which typically bind phosphatidylinositol 3-phosphate (PI3P) lipids, thereby directing proteins to the surface of early endosomes. The FYVE domains in the Faciogenital dysplasia (Fgd) protein family are particularly divergent, with amino acid substitutions in the motifs that facilitate phosphoinositide phosphate (PIP) lipid binding. We hypothesize that alterations in these key motifs confer a unique lipid binding specificity. Saturation Transfer Difference (STD) and water-Ligand Observed via Gradient Spectroscopy (waterLOGSY) experiments are used to identify protein-ligand interactions between purified Fgd FYVE- GST constructs and lipid molecules. Chemical shift mapping is being used to identify the groups that contribute to protein:lipid interactions. Aberrant cell signaling caused by altered Fgd protein-mediated pathways, either by mutations or overexpression, have been associated with Faciogenital dysplasia or Aarskog-Scott syndrome, and various forms of cancer. Future analysis of disease-associated mutations will identify their effects on lipid binding and organelle targeting.

P215

Investigation of RPA interaction of human Werner Syndrome protein and Fanconi Anemia Group J

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Human DNA helicases, WRN (Werner Syndrome Protein) and FANCJ (Fanconi Anemia Group J) contribute to maintain genomic stability and have common interaction partner, Replication Protein A (RPA). Interaction of both helicases with RPA stimulates helicase activities which are crucial in DNA repair processes. Although these interactions are of importance in cancer cells, the precise binding site of each interaction are not well understood. In this study, we observed interactions of RPA-WRN and RPA-FANCJ by NMR spectroscopy and Fluorescence Polarization anisotropy assays. The chemical shift perturbations of RPA70N by WRN were more prominent than those by FANCJ. The perturbed regions by WRN and FANCJ were partially overlapped and located in the basic cleft of RPA70N. We defined minimal acidic peptide region of both helicases for RPA70 binding. Our analysis shows that the common electrostatic interactions with precisely tuned hydrophobic interactions mediate each helicase-RPA binding.

P216

Non-canonical Docking Mediates the Phosphorylation of a Transcription Factor by the MAP Kinase ERK2

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The transcription factor Ets-1 possesses neither canonical sequence, D- or F-site, considered necessary to associate with, and be phosphorylated by the MAP kinase ERK2. Yet Ets-1 engages regions of ERK2 (D-recruitment site, DRS and F-recruitment site, FRS) that form docking sites for the canonical sequence motifs and is efficiently phosphorylated. Using NMR methodology, we provide the structural basis for the unique, bipartite recognition of ERK2 by Ets-1 and establish its role in driving Ets-1 phosphorylation. A set of hydrophobic residues on the disordered N-terminus of Ets-1 engages a part of the ERK2 DRS that normally accommodates the hydrophobic sidechains of a canonical D-site sequence. While the Ets-1 sidechains that directly contact the ERK2 DRS become locally ordered, significant dynamics persist at the Ets-1 N-terminus indicating a "fuzzy" association. In contrast, the C-terminal pointed (PNT) domain of Ets-1 takes part in a largely rigid body interaction with the C-lobe of ERK2 utilizing a surface that encompasses a part of the ERK2 FRS, though the mode of interaction deviates significantly from that involving a canonical F-site sequence. Thus, Ets-1 utilizes the cumulative effects of two sub-optimal interactions to localize its phospho-acceptor near the ERK2 active-site, rather than the common mechanism involving individual canonical D-site/DRS or F-site/FRS binding. In spite of significant disorder around the Ets-1 phospho-acceptor, and indeed in the phosphorylatable sidechain itself, in the pre-chemistry ternary complex (Ets-1•active-ERK2•Mg2+-ATP), approximately 40% of the ERK2 binding events lead to Ets-1 phosphorylation, suggesting a highly optimized proximity-mediated mechanism. This efficiency appears to be the result of a high ratio of productive to non-productive collisions with the ERK2 active site of the dynamic phospho-acceptor facilitated by its optimal location relative to the two docking sites and further by the destabilization of a

flexible helix that bridges it to the rigid PNT domain.

P217

Influence of membrane environment on the energy of transmembrane protein-protein interaction using the solution NMR

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Being involved in a large variety of processes in living organisms, membrane proteins are the most challenging object for the modern structural biology and they are still poorly explored. The most of membrane proteins are constituted by a number of transmembrane (TM) α-helices, interacting with each other and thus forming the tertiary structure inside the lipid bilayer. Folding of helical segments into a TM domain together with the functional mobility of certain helices often determine functional characteristics of the full-size membrane protein. In this aspect, it is necessary to study principles, underlying the TM helix-helix interactions. Bitopic proteins (e.g. receptors of type I such as receptor tyrosine kinases) having only single-span α-helical TM domain separating ecto- and cytoplasmic domains are a class of biologically significant membrane proteins that are the most convenient to study helix-helix interactions in the membrane. Activity regulation of these proteins is mostly associated with their lateral dimerization in cell membranes. The properties of membrane environment are an important factor, influencing the free energy and mode of TM protein-protein and helix-helix contacts. However, to the date, the studies of the lipid-related effects on the free energy and structural mode of TM helix-helix interactions are represented mainly by the computer simulations, performed mostly in the coarse-grained regime, which definitely need to be verified experimentally. In the present work, using the recombinant ErbB4 receptor TM fragment embedded into different phospholipid bicelles we provide the approach to study the TM helix-helix interactions of membrane proteins in various lipid environment with the aid of high-resolution NMR spectroscopy. ErbB4 is a ubiquitously expressed member of the HER/ErbB family of the growth factor receptor tyrosine kinases that is essential for the normal development of different human tissues. The technique is based on the ability of bicelles to form particles with the size, depending on the lipid/detergent ratio. To implement the approach, we provide the experimental parameters of "ideal bicelle" models for four kinds of zwitterionic phospholipids, which can be also used in other structural studies. We show that size of bicelles and type of the rim-forming detergent does not affect substantially the spatial structure and stability of the model TM dimer. On the other hand, the effect of the bilayer thickness on the free energy of the dimer is dramatic, while the structure of the protein is unchanged in various lipids with fatty chains having length from 12 to 18 carbon atoms. The obtained data is analyzed from the viewpoint of hydrophobic mismatch and lipophobic effects, and sheds light on the folding determinants of α-helical membrane proteins. The work is supported by the Russian Science Foundation, grant #14-14-00573.

P218

Probing Substrate Selectivity and Binding Kinetics in HIV-1 Protease-Gag Interactions by Chemical-Exchange NMR

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The conversion of immature non-infectious HIV-1 particles to infectious virions is dependent upon the sequential cleavage of the precursor group-specific antigen (Gag) polyprotein by HIV-1 protease. The mechanism whereby protease recognizes distinct Gag cleavage sites, located in the intrinsically disordered linkers connecting the globular domains of Gag, remains unclear. The dynamics of the interaction of large fragments of Gag and various variants of protease is quantitatively probed by Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion and chemical exchange saturation transfer (CEST) NMR experiments. The conformational dynamics within the flaps of HIV-1 protease that form the lid over the catalytic site, play an important role in substrate specificity and ordered Gag cleavage. Rapid inter-conversion between closed and open protease flap conformations facilitates the formation of a transient, sparsely-populated productive complex between protease and Gag substrates. Flap closure traps the Gag cleavage sites within the catalytic cleft of protease. Modulation of flap opening through protease-Gag interactions fine-tunes the lifetime of the productive complex and hence the likelihood of Gag proteolysis. A productive complex can also be formed in the presence of non-cognate substrates but is short-lived owing to lack of complementarity between the active site cleft of protease and the substrate, resulting in rapid flap opening and substrate release, thereby enabling the differentiation between cognate and non-cognate substrates.

P219

Elucidating protein-protein interactions in an antibiotic polyketide synthase

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Many natural products, such as antibiotics and anti-tumor agents, are synthesized inside bacterial cells by gigantic modular multienzyme complexes such as polyketide synthases (PKSs) and nonribosomal polyketide synthases (NRPSs). Enacyloxin is an antibiotic produced by a type I modular polyketide synthase (PKS), which is active against multidrug-resistant bacteria, such as Acinetobacter baumanii, which is on the top of the World Health Organization 'priority pathogens' list [1]. However, enacyloxin is not suitable to be directly used as a drug - its analogues preserving the activity but with improved pharmacological properties need to be synthesised. In this context, it is attractive to achieve that through biosynthetic rather then chemical methods. The modular nature of PKSs and NRPSs makes them amenable to rational engineering to produce novel drugs [2]. One of the prerequisites for effective bioengineering of such systems is to elucidate protein-protein interactions involved in the control mechanism for biosynthesis. A key step in enacyloxin biosynthesis is the transfer of the polyketide chain from an acyl carrier protein (ACP) domain in the last PKS module to a standalone peptidyl carrier protein (PCP) by a non-elongating ketosynthase (KS0) domain. It has previously been shown in our laboratory that this transfer is necessary for the condensation (C) domain to catalyse polyketide chain release via intermolecular esterification

with 3, 4-dihydroxycyclo-hexane carboxylic acid. No esterification occurs when the substrate is attached to the ACP domain. NMR experiments provided evidence that esterification does not occur due to the lack of specific protein-protein interactions between the ACP domain and the C domain. In contrast, specific protein-protein interactions are observed by NMR spectroscopy between the PCP and the C domain. Thus, the KS0 transfers the substrate from a carrier protein that is unable to interact with the C domain to one that it is able to interact with. Here, for the first time, we report structural and dynamical studies of specific interactions between the ACP:KS0 and PCP:KS0 complexes combining solution and solid-state NMR spectroscopies, together with their biochemical and biophysical characterization.

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P220

Trajectory of changes in conformation and dynamics of acid-stress bacterial chaperone HdeA as it transitions at low pH to its unfolded and activated state

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HdeA is one of the smallest known chaperones. It is located in the periplasm of several common pathogenic bacteria and is a major contributor to the infectivity of these organisms in causing dysentery. The chaperone activity of HdeA is stimulated when the bacteria pass through the low pH environment of the stomach: HdeA associates with other proteins in the periplasm to prevent their acid-induced aggregation and to aid in bacterial survival as the organism travels through the stomach and on to infect the intestinal region of the host.

HdeA is an inactive dimer at physiological pH and transitions to an active, unfolded monomer below pH 2.5. It has already been determined that neutralization of Asp and Glu residues does not trigger unfolding. The goal of this study, therefore, has been to use NMR spectroscopy to investigate the changes in conformation and dynamics of HdeA to determine what initiates the transition from fully folded at pH 3 to fully unfolded at pH 2. We observe an increase in flexibility between pH 3.0 and 2.5; in some cases, the motion shifts between intermediate and fast timescale, particularly in the B-C loop region, which is part of the dimer interface and is likely to be be a site of unfolding initiation. Further decreases in pH reveal a trajectory of increased dynamics which may be associated with the mechanism of unfolding. Unexpectedly, hydrogen exchange experiments have revealed a significant increase in solvent protection in the N-terminal region with decreased pH, suggesting the formation of transient structure in a region that is unstructured at higher pH. CS Rosetta calculations and simulation experiments have provided support for the formation of a small helix at the N-terminus. Our ongoing studies continue to provide insight into the mechanism of unfolding and activation of this unusual bacterial chaperone.

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P221

Contributions of the EB1 disordered region into the binding affinity and specificity: advantages of dynamic interactions.

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Microtubule-bindig protein EB1 is one of the key adapter molecules of signaling networks associated with the plus-ends of microtubules. It is involved in the regulation of microtubule dynamics and recruitment of other proteins to the mictrotubule growing ends. EB1 is up-regulated in a range of cancers and is considered to be a potential target for anticancer drugs. The C-terminal target recognition EC1c domain is a homo-dimer that consists of the N-terminal leucine-zipper coiled-coil region terminated on a small 4-helix bundle of the end-binding homology (EBH) domain. A large group of EB1 targets are recognised through a conserved SxIP motif that interacts with the SxIP binding pocket located at the interface between the EBH helices. Part of the binding pocket is formed by the C-terminal region of EB1c that is disordered in the absence of the ligand. The main contribution to the EB1 interaction with the SxIP targets was thought to be associated with the SxIP motif. However, we found that the SKIP peptide binds to EB1c very weakly (Kd 14 mM), suggesting critical contributions from other, less conserved, target regions. Using a combination of different ligand peptides and EB1 fragments we defined how different interactions and folding of the disordered EB1 region affect the strength and dynamic of the interaction. In the solution structure of EB1c with 11-residue MACF peptide KPSKIPTPQRK containing SxIP motif, the unstructured in the free form C-terminus of EB1 becomes immobilised and forms a range of hydrophobic contacts with the TPQ region of the peptide. Deletion of the EB1 C-terminus reduces the affinity of the interaction 10-fold, while mutation of PTP to a more hydrophobic sequence VLL increases affinity 20-fold, resulting i a Kd=0.15 uM. Truncation of the peptide to the 6-residue SKIPTP fragment leads to a very weak interaction with Kd 2 mM, with the C-terminus of EB1 remaining unstructured. The effects on the 15N-EB1 HSQC spectra on the peptide additions demonstrate a corresponding change from a very fast to a slow exchange regimes on the affinity increase, with particularly large differences associated with the C-terminus. Using line-shape analysis implemented in TITAN software (Waudby ea, Sci. Reports, 2016, 6, 24826) and CEST experiments (Vallurupalli, JACS, 2012, 134, 8148), we conducted systematic residue-based exchange rate analysis of different complexes and related them to the thermodynamic parameters determined by ITC. We summarise the results in the multi-step model of the interaction, where SxIP initially interacts with a partially formed binding pocket, followed by the C-terminal part of the peptide ligand making contact with the proteins surface and establishing hydrophobic interface for docking of the EB1 disordered region and formation of the full binding pocket.

P222

Effect of Binding and Association on Internal Protein Dynamics: The intriguing case of EphA1/2 and SHIP2 SAM Domains

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It is commonly recognized that the internal fluctuations of proteins are in-

tegral to their function, either as enzymes or as binding partners. In the latter case, protein dynamics can contribute critically to binding affinity as well as specificity via entropy changes that occur upon protein association. Our laboratory has been developing the EphA2 – SHIP2 SAM : SAM complex as a model system of a dynamic protein complex [1-3], demonstrating that the degeneracy of binding surfaces (one almost entirely positively, the other entirely negatively charged) leads to dynamics within the protein complex between multiconfigurational states. These were seen in long timescale all-atom molecular dynamics simulations [2] and are a feature of a step-wise dissociation process [3]. Recently we have been successful in predicting native-like complexes, starting from separate randomly oriented SAM domain from all-atom simulations [4], further emphasizing the role of electrostatic steering. This presentation concerns recent unpublished NMR relaxation data for the free proteins and complexes on the pico-nanosecond as well as micro-millisecond timescale. In collaboration with the Lee laboratory, high power relaxation dispersion experiments [5,6] were used in order to seek the fast transitions seen by molecular dynamics. While the multiconfigurational states of the EphA1/2 : SHIP2 complexes may still be too fast for detection, we show that EphA1 homodimerizes utilizing an interface that is novel for SAM domains. While adjacent to the SHIP2 binding surface, it may still be utilized for the formation of larger dynamic protein networks, even in the absence of SAM domain phosphorylation [7].

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P223

Differential 19F labelling as a Tool to Monitor the Behaviour of Multiple Proteins Simultaneously in Complex Mixtures

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The ability to monitor the behavior and interactions of multiple proteins of interest in a complex environment has many potential uses. Biological samples, biopharmaceutical formulations and the intracellular environment are all examples of situations where interesting behavior of the individual protein components can be challenging to analyze by NMR. Differential labelling NMR approaches, traditionally using 15N or 13C isotope incorporation during recombinant expression, are not always practical in cases when endogenous proteins are obtained from an organism, or where the expression system does not allow for efficient labelling. Here we show a method of differential labelling by post-translational covalent modification with 19F groups having distinct chemical shifts. This gives each protein of interest a unique spectral signature which can be monitored by 19F NMR without signal overlap, even in the presence of many other proteins, and without any interfering signals from the buffer or other unlabelled components. By monitoring NMR measurable parameters such as relaxation rates, translational diffusion coefficients and signal integrals, it is possible to detect behavior such as complex formation, transient self- or hetero-association, and aggregation for each labelled protein independently and simultaneously. This technique is practical even for large proteins, such as 140 kDa monoclonal antibodies, and challenging environments such as extremely high protein concentrations similar to that found in the intracellular environment.

P224

Investigation on the flexibility of multi-domain protein An application on a protein complex of the lysosomal degradation pathway.

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In the lysosomal degradation pathway, protein ubiquitination constitutes a trafficking signal that is deciphered and executed by the ESCRT machinery. The ESCRT-0 sorting complex is constituted of the STAM (signal transducing adaptor molecule) and Hrs (hepatocyte growth factor-regulated substrate) proteins and each of them harbours several UBDs (Ubiquitin Binding Domains). STAM protein provides three UBDs, the VHS, UIM and SH3 domains with long flexible linkers between them. The domain structures of STAM have been explored by means of different biological assays. However, an in depth structural and dynamical information describing the flexibility determinants of STAM and its interaction with ubiquitin is still missing. Here we investigated a structural basis of the complex with the emphasis on the flexible and partially (un)structured inter-domain linker regions of the STAM protein and the influence of which upon the ubiquitin-binding ability of the protein. We carried out such efforts firstly by introducing specific mutants altering the linker region between the 2 domains UIM and SH3. The truncated versions have their linker shortened for 7 and 14 amino acids, that we call UST1 and UST2 respectively. NMR experiments such as R1 and R2 Relaxation, hetero-NOEs and SAXS experiments (Small Angle X-ray Scattering) are carried out to investigate the change of flexibility and conformation of these mutants comparing to the wild-type UIM-SH3. NMR titration of the 15N proteins UST1, UST2 and UIM-SH3 are also performed with 14N K62 Di-ubiquitin to look for affinity change. Our preliminary data have shown that the flexibility of the protein is indeed altered and therefore their binding capacity. Effectively, we have observed a decrease in the affinity of the SH3 domain toward Di-ubiquitin when the linker region is shortened. In short, with SAXS, NMR and Ion-mobility spectrometry (upcoming works) combined, we believe that the flexibility of multi-domain protein with partially disordered linkers could play a very important role in the recognition within a protein complex and a well understanding of this dynamic will provide new insights into the field of protein interaction.

P225

Detection of pair-wise intermolecular interactions in large protein complexes using transferred nuclear Overhauser exchange spectroscopy and specific methyl labeling: HIV-1 gp120 in complex with a CCR5 peptide

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The interaction between HIV-1 envelope glycoprotein gp120 and the CCR5 chemokine receptor is crucial for viral entry and thus serves as an ideal target for inhibitors. The molecular details of gp120 interactions with the N-terminal segment of CCR5 (Nt-CCR5) are undescribed due to

the lack of experimental data on intermolecular pair-wise interactions and lack of crystal or cryo-EM structure. We have reported on the use of the transferred NOE (TRNOE) experiment for the detection of pair-wise interactions between a mostly dimeric gp120 complexed with a CD4 mimic peptide (CD4M33) in fast exchange with a 27-residue peptide, Nt-CCR5(1-27), representing the N-terminal segment of CCR5. Intermolecular interactions in this 110kDa complex were identified using asymmetric deuteration. The high molecular weight and the fact that gp120 is glycosylated and can only be expressed in mammalian or insect cells, creates significant challenges. Here we report a strategy that uses TRNOE and 13C edited NOESY to circumvent some of these challenges. Specific methyl labeling has revolutionized NMR of large proteins by incorporating 1H,13C, labeled methyl groups in a fully deuterated background. 1H,13C methyl labeled methionine, valine, threonine and alanine were successfully incorporated in gp120 where other methyl containing residues as well as aromatic residues were deuterated, and 1H,13C correlation spectra of the ternary complex of these proteins with CD4M33 and Nt-CCR5(1-27) were recorded. Good signal to noise ratios and fairly narrow linewidths were obtained. Using HMQC-NOESY experiments on methyl-labeled gp120 in complex with a 7-fold excess of Nt-CCR5 peptide, intermolecular TRNOE cross peaks were detected for Met, Val and Thr but not for Ala. Specific assignment of these interactions is in progress using available structural information, site-directed mutagenesis and13C edited NOESY measurements. The information from this study will be combined with the findings from the asymmetric deuteration strategy and used to calculate a gp120/NtCCR5(1-27) structure.

P226

The interaction of CCR5 sulfated tyrosine residues with the RANTES/CCL5 protein

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RANTES (CCL5) is an 8 KD C-C chemokine that plays an important role in the immune response. It exerts its biological activity by binding to the chemokine receptor, CCR5 (C-C chemokine receptor type-5), which is a major co-receptor for HIV. CCL5 also suppresses HIV -1 replication, hence, elucidating its binding mechanism may contribute significantly to the development of novel HIV-1 entry inhibitors .Four tyrosine residues are present in the N-terminus of CCR5 (Nt-CCR5). Sulfation of at least two of these tyrosines is essential for optimal CCL5 binding, and the co-receptor functions of CCR5. The tyrosines of Nt-CCR5 are heterogeneously sulfated by tyrosylprotein sulfotransferases in-vivo. The purpose of this project was to investigate the effect of different Nt-CCR5 sulfation patterns on the mode and affinity of CCL5 binding to Nt-CCR5. Peptide surrogates (Nt-CCR5 residues 8-20; G-PIYDINYYTSEPA-G) with different patterns of sulfation were synthetized and used in 1H-15N HSQC titration experiments with 15N labeled CCL5. Chemical shift perturbations were used to map the binding epitope of CCL5 interacting with the differentially sulfated Nt-CCR5 peptides. It was found that the chemical shift perturbation pattern of monomeric CCL5 titrated with Nt-CCR5, depends on the sulfation pattern of the peptide. Binding curves for chemical shift changes upon titration were used to determine dissociation constants (KDs) of the peptide/CCL5 complex. We found that interactions between CCL5 and Nt-CCR5 peptides, sulfated at two Tyr residues, are all characterized by relatively strong binding (KDs 20-35 $\neq$ M), compared to singly-sulfated CCR5s (KDs =200-500 $\neq$ M). This shows that the sulfation pattern on the N-terminus of CCR5 is important and may provide a powerful mechanism to regulate receptor interactions with the same and different chemokines.

P227

Detection of Pairwise Interactions of p38α complexed with a STEPKIM peptide by Transferred Nuclear Overhauser Effect (TRNOE) Spectroscopy

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The mitogen-activation-protein-kinase p38α plays an important role within signaling cascades that are activated by a wide variety of extracellular stimuli. Ultimately the cascade results in the dual-phosphorylation of the regulatory Thr and Tyr residues of the Thr-Gly-Tyr domain in the MAPK activation loop. Three protein tyrosine phosphatases (PTPSL, STEP, and, HePTP) that have kinase-interaction motifs (KIMs) regulate the activation of p38α. The KIMs of PTP are 13-17 amino acids long linear segments recognized by p38α. Structural information on complexes of p38α with KIM peptides is highly valuable for understanding the molecular basis of the specificity and affinity of binding at the atomic level. NMR spectroscopy has been extensively used for structural studies of protein-protein and protein-peptide complexes exhibiting weak to moderate binding affinities. Measurements of nuclear Overhauser enhancements (NOEs) provide information about through space interactions between hydrogen nuclei and are essential for identifying contacts between components and for the structure determination of bimolecular complexes. In particular, the Transferred-NOE (TRNOE) experiment is very well suited to study protein complexes exhibiting weak to moderate binding and fast off-rates usually characterized by KD > 1 μM. Intramolecular TRNOE is used extensively to study the conformation of small ligands and peptides bound to large proteins. Our group has developed the 2D-TRNOE difference spectroscopy and the T1ρ-filtered NOESY experiments to exploit the benefits of TRNOE to study intermolecular interactions in biomolecular complexes. Using 13C-edited/13C-filtered NOESY combined with the TRNOE effect, we studied the intermolecular interactions between p38α (38 kDa) and a STEPKIM peptide (RLQERRGSNVSLTLDM, 16 AAs). p38α was 13C-methyl labeled on Ile (δ1) or Leu/Val (ILV) residues in a fully deuterated background. The STEPKIM peptide was unlabeled. The results demonstrate that V10, L12, T13, L14, and M16 of the STEPKIM peptide interact with L74, V83, I116, L122, L130, and/or V158 of p38α.

P228

NMR Studies on Oligomeric Transmembrane Domain of Syndecan Proteoglycan

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Syndecans are cell surface adhesion receptors that initiate intracellular signaling events through receptor clustering mediated by their highly conserved transmembrane domain (TMD). Syndecan functions are controlled by their oligomerization, which is regulated by its TMD. Three constructs of syndecan-2, Syn-2TMD, Syn-2eTC, Syn-2eTC-F167I, and three constructs of syndecan-4, Syn-4TMD, Syn-4eTC, Syn-4eTC-I169F, are designed to study their homo- and hetero-dimerization by NMR spectroscopy and biochemical experiments. NMR experiments are performed for six syndecan constructs to investigate the structural difference between syndecan-2 and -4's homo- and hetero-dimer. We completely as-

signed Syn-2TMD and Syn-4TMD using data form 15N HSQC, HNCACB, CBCACONH. From our data, we found that Syn-2 and Syn-4 make heterodimer formation and Syn-2 have higher heterodimeric tendency than Syn-4. We have performed MR titration experiments using heterodimer mimic mutant, Syn-2eTC-F167I. NMR resonances of Syn-2TMD are dramatically changed upon Syn-2eTC-F167I interaction, whereas Syn-2eTC is not, vice versa, on Syn-4TMD. These results indicate that heterodimer mimic complexes, Syn-2TMD/Syn-2eTC-F167I and Syn-4TMD/Syn-4eTC-I169F, experience conformational changes than both homo-dimer complexes, Syn-2TMD/Syn-2eTC and Syn-4TMD/Syn-4eTC. In conclusion, we showed that the hetero-dimerization between Syn-2 and -4 in atomic level and the unique F167 residue of Syn-2 play an important role in the interaction between TMD of the Syn-2 homo-dimer. Our data provides offers new insights into the signaling mediated by the TMD of syndecan family members.

P229

Interaction between NOXA1 and NOXO1 in NOX1 complex using NMR and X-ray crystallography

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NOX1 is activated by two regulatory cytosolic proteins that form a heterodimer, NOXO1 (NOX organizer 1) and NOXA1 (NOX activator 1). The interaction between NOXA1 and NOXO1 is critical for activating NOX1. NOX1 (NADPH oxidase), homolog of NOX2 (also known as gp91phox), is expressed mainly in colon epithelium which is responsible for host defense against microbial infections by generating ROS (Reactive Oxygen Species). ROS have been involved in important pathological processes which are associated with aging, cancer and several inflammatory as well as cardiovascular diseases. Both NOXA1 and NOXO1 are cytosolic proteins having one and two SH3 domain respectively. Here, we determined structure of NOXA1 SH3 domain and how the intramolecular interaction may occur between NOXA1 SH3 domain and NOXO1. 15N/13C labeled SH3 domain of NOXA1 was purified for NMR experiments (HNCACB, CBCACONH, HNCA, HNCO and HSQC). Solution structure of SH3 domain showed a typical SH3 topology dominated by four β-sheets connected by flexible loops. Pulldown assay confirmed that there was intermolecular-interaction between NOXO1 PRR and NOXA1 SH3 domain. Further NMR titration between SH3 domain and PRR peptide showed perturbed residues (namely Gln407 and Ala412) on the loop region connecting second and third β-sheet, together with residues (Cys340 until Leu437) from first and fourth β-sheets. These regions take part in during the interaction between NOXA1 SH3 and NOXO1 PRR. Our result will help to understand ROS generating system in NOX1 complex and molecular interaction providing an insight on their cytoplasmic activity mediated functioning.

P230

Parkin activation through synergistic binding of its phosphorylated ubiquitin-like domain and an E2 Ub conjugate

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Implicated in 50% of all autosomal recessive juvenile parkinsonism cases, understanding the mechanism of activation and ubiquitin conjugation by the RBR E3 ligase parkin has become pivotal for determining the molecular pathology of Parkinson's disease. Parkin is a multidomain enzyme comprising an ubiquitin-like domain and C-terminal RING0, RING1, IBR and RING2(Rcat) domains. The RING2(Rcat) domain houses a catalytic cysteine needed to accept ubiquitin from a bound E2 Ub conjugate and transfer it to a substrate. Parkin requires two-fold activation by the kinase PINK1 through phosphorylation of a S65 residue in its N-terminal, ubiquitin-like domain (pUBL), as well as binding of an equivalently S65-phosphorylated ubiquitin (pUb). These events together cause disengagement of pUBL from the rest of parkin and this reveals the canonical RING1 binding site for an incoming E2 Ub conjugate. We used chemical shift perturbation and HADDOCK modelling to determine the structure of the C-terminus of phosphorylated parkin (35 kDa) in complex with an E2-Ub conjugate (25 kDa) and pUb (8 kDa). Further, NMR spectroscopy was used to investigate the role for the pUbl domain after its release and how this might contribute to parkin activation. NMR-based titration and T2-filter experiments showed that pUbl does not bind (or binds very weakly) to the C-terminus of parkin bound to pUb. With addition of UbcH7 Ub to this mixture, chemical shift changes were observed in pUbl corresponding to pSer65 and the surrounding residues suggestive of low affinity binding. We hypothesize that the binding site corresponds to a basic patch in parkin's RING0 domain, previously thought to bind pUb. Ubiquitin accessibility experiments showed that both parkin phosphorylation and E2 Ub binding led to rapid availability of the catalytic cysteine in Rcat. We concluded that binding of the E2 Ub conjugate and pUbl synergistically induce a conformational change that results in release of Rcat and subsequent parkin ubiquitination activity.

P231

Structural basis of the interaction between Cyclophilin A and RIG-I

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Cyclophilin A (CypA), a peptidyl-prolyl cis/trans isomerase, binds to CypA and the complex suppresses immune response. CypA also known as an interactor of various protein involved in virus replication. Retinoic acid inducible gene-I (RIG-I) protein, is a pattern recognition receptor involved in the recognition of viral double-stranded RNA. RIG-I is a key protein in the innate immune response to RNA virus, including the induction of type I interferons and pro-inflammatory cytokines. CypA acts as a positive regulator of RIG-I-mediated antiviral immune responses.[1] In this study, we characterized the interaction between CypA and RIG-I by nuclear magnetic resonance experiment. In order to figure out the binding interface for RIG-I on CypA, 15N HSQC titration experiments were performed. Backbone dynamics for the free and bound form of CypA with RIG-I were investigated by the spin relaxation experiments. Our study provides the structural basis of the interaction between CypA and RIG-I, that gives the structural insights into the regulation of antiviral immune responses.

[1] Liu et al. eLife (2017) 6:e24425.

P232

Preliminary structural studies of the tumor reversion TCTP protein in interaction with Bcl-2 family members

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The role of TCTP in cancer is mainly described in the context of the tumoral reversion program. Cells that undergo such program spontaneously loose their malignant phenotype, leading to the recovery of characteristics associated to benign cells, such as susceptibility to apoptosis. Apoptosis is mediated by the interplay between pro/anti-apoptotic Bcl2 family proteins and BH3-only proteins. TCTP contains a BH3-like domain and binds to the Bcl2 family proteins Bcl-xL and Mcl-1, reinforcing their anti-apoptotic properties by two distinct suggested mechanisms. Indeed, TCTP/Bcl-xL interaction increases the Bax-mediated anti-apoptotic function of Bcl-xL, suggesting that the binding of TCTP to Bcl-xL could increase Bcl-xL affinity for Bax through a yet unexplained mechanism. The structure of Bcl-xL in complex with the BH3 peptide of TCTP was obtained by X-ray crystallography but the structure of Bcl-cL in complex with full-length TCTP is not well characterized yet. For Mcl-1, TCTP is known to decrease its turnover, by reducing its ubiquitinylation and thus proteasome-mediated degradation. However, no structural information of TCTP/Mcl-1 complex is available at the moment. Here we used solution-state NMR and other biophysical methods (CD, DLS, SEC,...) to get deeper structural characterization of full-length TCTP in complex with Bcl-xL and Mcl-1. Overall, this work will help to enrich the comprehension of apoptosis in the context of the tumor reversion program, TCTP biology and on-going therapeutical efforts.

P233

Trimethylsilyl (TMS) tag for probing protein – protein interactions in bacterial conjugation mechanism

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Antibiotic resistance is becoming a serious and dangerous problem in all parts of the world. The misuse and overuse of antibiotics in animal husbandry the food industry and hospitals can leads to rapid spread of resistance genes not only through a single species but also through unrelated species. This makes the treatment of infections and diseases with conventional antibiotics less efficient or even impossible. The mechanism of bacterial gene transfer is called bacterial conjugation. This rapid dissemination of genetic material requires two main proteins, TraM (relaxosome) and TraD (hexameric ring ATPase). TraD interacts with TraM through its intrinsically disordered region (IDR) containing 165 residues. However, the binding site resides only within a stretch of 10 residues in the C-terminal end of these 165 residues. TraM is a tetramer providing four binding sites for TraD[2][3]. In the presented study, we used a cysteine reactive TMS tag to estimate, for the first time, the affinities between two proteins, TraD and TraM. TMS tag have been successfully used in protein – ligand interactions [1]. To estimate the affinity we used a commercial synthesized peptide containing the last fourteen residues of TraD IDR and added N-terminal glycine and cysteine. The cysteine of the peptide was successfully tagged with TMS and a 1D proton chemical shift mapping experiment was carried out by adding increasing amounts of TraM. The TMS's intensity, narrow line shape and distinctive chemical shift at around 0 ppm allowed us to monitor the interactions between TraD and TraM. Thus, we were able to estimate the KD value of around 29 ± 18 μM. Acquired 2D experiments confirmed the KD value of 36 ± 7 μM. This approach shows that the TMS tag might be used also as an additional tool to study protein-protein interactions in the field of structural biology. Therefore, a more detailed mechanistic picture of bacterial gene transfer will allow us to find new ways to fight against untreatable infection diseases caused by antibiotic resistance.

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P234

Exploiting conformational flexibility in the Interleukin-2 cytokine to potentiates regulatory T cells

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Interleukin-2 (IL-2) is a pleiotropic cytokine that regulates immune cell homeostasis and has been used to treat a range of disorders including cancer and autoimmune disease. IL-2 signals via interleukin-2 receptor-α(IL-2Rα):IL-2R heterodimers on cells expressing high (regulatory T cells, Treg) or low (effector cells) amounts of IL-2Rα (CD25). Previous studies have shown that interaction of mouse IL-2 with an anti-mouse IL-2 monoclonal antibody (JES6-1) preferentially enhance Treg populations through a unique mechanism whereby IL-2 is exchanged from the antibody to IL-2Rα. Using single-quantum methyl CPMG relaxation dispersion experiments we characterized a dynamic conformational equilibrium of IL-2 with implications for the JES6-1 and CD25 binding. Additionally, using methyl-HMQC NMR we structurally characterized the IL-2/JES6-1 and IL-2/CD25 complexes to elucidate the distinct allosteric mechanisms through which these molecules modulate IL-2 function. Our results suggest that conformational exchange observed in the free IL-2 state involves a transient twisting or ''breathing'' of the α-helices, which may induce negative allosteric coupling between the JES6-1 antibody and IL-2Rα receptor binding sites, through the selection of distinct conformations from an ensemble of states sampled by the free form. Furthermore, the conformational plasticity of IL-2 is explored by NMR-based fragment screening in an attempt to identify small molecules to improve its therapeutic potential by modulating its ability to selectively target Tregs. These results provide a molecular basis for an allosteric mechanism in IL-2 signaling with potential implications for modulation of IL-2 function by drug molecules.

P235

Structural basis of hierarchical protein secretion in the type III secretion system

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Type III secretion system (T3SS) is a multi-protein nanomachine protruding from the bacteria membrane, implementing the assembly and function of bacteria flagellum and injectisome. Proteins for flagellum assembly or bacteria virulence are specifically chaperoned and sequentially transported from the cytosol to the export apparatus and then to bacterial outer membrane or host cells through Type III secretion pathway. The export apparatus provides the targeting site of chaperone-substrate complex and determines the order of substrate secretion. FlhA, the major export platform protein recognizes the substrate-chaperone using similar mechanism, while structural insight of sequential regulation of substrate secretion remains elusive. Here we dissected specific recognition of chaperone-substrates by export gate component both in flagellar and pathogenic T3SS, FlhB and EscU rescpectively. FlhB adopts a highly conserved, hydrophobic and dynamic site to specific recognize the N-terminal disordered part of hook-associated protein FlgK, FlgL and filament-capping protein FliD, but donot directly interact with the cognate chaperone. However, FlhB specific binds to FliS, chaperone of flagellin, but not flagellin itself. Auto-cleavage of FlhB at NPTH motif do not change the structure of FlhB, but allosterically changed the dynamics of the binding site to favorably recognize the substrate or chaperone. Similar mechanism was proved in pathogenic T3SS. The structural and biochemical data reveal the general basis of the switch controlling substrate secretion hierarchy in type III secretion system.

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Interactions of Bacterial Type III Secretion ATPase FliI with FliJ

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The Salmonella flagellum is a large, specialized organelle that is composed of many protein subunits that are assembled via a dedicated flagellar Type III export apparatus. High resolution information on the interaction between many of these proteins has remains elusive as well as the sorting mechanism. FliI is a key component of the export system and a flagellar-specific ATPase that shares significant structural homology to the α and β subunits of F0F1-ATP synthase, forming a 318 kDa homohexameric ring. FliJ is a 17 kDa cytoplasmic protein that forms a two-stranded coiled-coil, very similar to the γ subunit of the F0F1-ATP synthase. As such, FliJ can interact with residues in the central channel of the FliI ring. In addition, FliJ performs protein export chaperone functions for late flagella export substrates. Using NMR spectroscopy, we obtain atomic insights into the interaction between FliJ and other flagellar proteins and map the binding epitopes. Interestingly, FliJ has also been implicated in trafficking and recycling of chaperones FliT and FlgN between the FliI ATPase and the sorting platform FlhA. Current progress in elucidating these key interactions are outlined.

P237

Disordered Segments in the Dynein Machinery as Key Players in Neurodegenerative Diseases

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The essential molecular motor cytoplasmic dynein 1 (dynein) powers the transport of proteins, mRNA, and organelles towards microtubule minus ends. Malfunctions and mutations in dynein machinery is reported in many neurodegenerative disease such Spinal Muscular Atrophy (SMA). Cargo-specific adaptors engage with dynein to recruit and activate the motor, but the molecular mechanisms are poorly understood. Here, we use structural and dynamic NMR analysis to demonstrate that the C-terminal region of human dynein light intermediate chain 1 (LIC-C) is disordered and to identify two transiently formed short α helices in LIC, which bind to the adaptor proteins Spindly, BICD2 and HOOK3. In addition, a short segment preceding the first α helix, which is conserved in LIC among different species, appears to convey binding specificity. We demonstrate that the first helix in LIC-C is essential for adaptor binding in vitro and extend this finding to Ninein, FIP3, and RILP. The LIC-C helix is sufficient for binding to RILP, but other adaptors require additional LIC-C segments that make multiple contacts with the flexible LIC-C scaffold. Point mutations in the short LIC-C helix of C. elegans, introduced by genome editing, severely affect development, movement, and life span of the animal, and disrupt the distribution and transport kinetics of cargo in axons. Identical defects are observed when the entire LIC-C is deleted. We conclude that a highly conserved interaction between cargo adaptors and a C-terminal LIC helix is essential for dynein function in vivo.

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Structure and dynamics of an NMR-invisible disordered protein that partially folds upon binding

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Intrinsically disordered proteins (IDPs) and regions have emerged as a major class of proteins that are involved in a wide range of cellular processes. The inherent flexibility of IDPs allows them to interact with a variety of protein partners. These interactions often lead to folding of a local motif while large parts of a disordered protein retain some flexibility. Characterizing the structure and dynamics of long IDPs in biomolecular complexes is of fundamental importance to understand their function at the atomic level. NMR is the method of choice to investigate the conformational space of IDPs free in solution. Yet, when bound to a large protein, the unfavourable relaxation properties of the ordered parts of an IDP lead to weak and broad NMR signals that are difficult to identify among the many intense signals from the regions that retain disorder. In addition, many IDPs engage in fuzzy complexes in which motions on the microsecond-millisecond time scale lead to dramatic line broadening. There is thus a need for a general method to characterize both fully and partially ordered regions in the bound states of IDPs, in particular when the NMR signals of these bound states cannot be directly observed. Here, we introduce an approach based on chemical-exchange NMR to investigate the conformation and dynamics of a disordered region that partially folds upon binding through the observation of the free state of the protein. Backbone chemical shifts and site-specific dynamics of the bound state are obtained from the analysis of chemical-exchange saturation transfer (CEST) and Carr-Purcell-Meiboom-Gill (CPMG) experiments recorded in the presence of a

small amount of the protein partner. The conformation of the interacting region was determined and docked onto the structure of the protein partner using chemical shift perturbation (CSP) data. We apply the method to investigate the interaction between a 96-residue disordered fragment of the protein Artemis and the DNA-binding domain (27 kDa) of Ligase IV. Artemis is a nuclease, which plays a key role in adaptive immunity and DNA repair through its participation in the Non-Homologous End Joining pathway (NHEJ). We show that our approach can accurately reproduce the structure of the core of the complex determined by X-ray crystallography and identify a broader interface that includes additional transient contacts. This study demonstrates the power of chemical-exchange NMR to determine the conformation and dynamics of protein states that are not directly observable. Its application to the Artemis-Ligase-IV complex provides new molecular insights into the recruitment of Ligase IV by Artemis onto DNA double-strand breaks.

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P239

Order within disorder: exploring a locally super-compact state in the intrinsically disordered TEAD-binding domain of YAP

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Yes-associated protein (YAP) contains intrinsically disordered protein (IDP) regions that play a major role in the Hippo pathway that regulates organ size, cell proliferation, apoptosis, and is associated with a wide range of cancers. Therefore, the binding between YAP and transcriptional enhanced associate domain (TEAD) proteins is an interesting target for cancer therapy. For further characterization of the apo state of the YAP 50-171 fragment containing the TEAD-binding domain, we applied paramagnetic relaxation enhancement, selective labeling with late metabolic precursors, utilization of 15N relaxation, and site-directed mutagenesis of key residues for the interaction. Our findings reveal a compact state in YAP that is even more compact than the bound form of YAP. The preformation of this super-compact state seems to facilitate the interaction with TEAD. The site-specific 13C labeling of Phenylalanine residues enables us to detect long-range NOEs within this compact state and identify the preformation of a non-canonical secondary structure element in YAP. Furthermore, observed preformed secondary structure elements, which are known to be crucial for the YAP:TEAD interaction, exhibit an interdependence in the locally super-compact apo state. In summary, these findings provide novel major insights into the YAP:TEAD interaction, reveal a surprisingly high degree of order for an intrinsically disordered system, and suggest that YAP needs to de-compact to bind to TEAD.

P240

NMR study of the structure and dynamics of the intrinsically disordered tail of ErbB2, phosphorylation and interaction

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ErbB2/HER2/neu is a member of the ErbB family of receptor tyrosine kinases. These receptors are located upstream of major signaling pathways, controlling cell proliferation, cell migration and apoptosis. ErbB2 is the only member of the family for which no ligand is needed for efficient signaling. Its overexpression is correlated with the occurrence of several types of cancer, and especially poor prognosis in breast cancer. Signal transduction is triggered by homo- or hetero-dimerization with ErbB proteins, leading to activation of the kinase domain and phosphorylation of the C-terminal end of the protein, which we showed to be an intrinsically disordered region (IDR). This tail, which we call CtErbB2, is the hub for the interactions that will determine cell fate. CtErbB2 is a 268-residue, proline-rich IDR. We determined the structural and dynamic features of unphosphorylated CtErbB2 using high-field NMR. It was shown to retain very little residual structure and to be locally particularly extended. However, a N-to-C terminal contact was observed, potentially modulating the accessibility of certain sites to partners. In addition to phosphorylatable tyrosines prone to interact with SH2 or PTB domains, CtErbB2 contains PxxP motifs that could favor interaction with SH3-containing proteins. Grb2, a central protein in Ras-dependent pathways and cell proliferation, contains both SH2 and SH3 domains and could therefore be subject to particular interaction mechanisms. We determined the solution organization of Grb2 domains, and then studied the interaction between unphosphorylated CtErbB2 and Grb2, as well as between Grb2 and phosphorylated peptides of ErbB2, showing that the SH3 domains indeed modulate the interaction. Strategies to obtain the phosphorylated form of CtErbB2 are also tested, to have a more global idea of the interdependence of phosphorylation events, behavior of the modified protein, and role of phosphorylation in regulating interactions.

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Structure-function relationship in disordered proteins: Microtubule Associated Protein 2c

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We used NMR to reveal structural features important for function of 49.2 kDa intrinsically disordered Microtubule Associated Protein 2c (MAP2c), regulating microtubule dynamics in neurons of developing brain. The major questions addressed in this study were (i) how much is the structure-function relationship visible in the ensemble-averaged data of the studied intrinsically disordered protein; and (ii) what are the key differ-

ences between MAP2c and Tau, which has highly homologous C-terminal microtubule-binding domain, but differs in the N-terminal domain, cellular localization, biological function, and is (unlike MAP2c) associated with the development of the Alzheimer's disease. Non-uniformly sampled experiments allowed us to obtain chemical shifts of all 466 amino acids of MAP2c. We probed long-range contacts in MAP2c using paramagnetic relaxation enhancement (PRE) and used small angle X-ray scattering (SAXS) to describe the average global shape of MAP2c. We applied the ASTEROIDS analysis to obtain a quantitative description of conformations adopted by MAP2c. In addition, we recorded relaxation data describing dynamics of MAP2c, monitored phosphorylation of MAP2c by cAMP-dependent protein kinase (PKA), and observed interactions of MAP2c with its binding partners. The results clearly showed that regions with known or proposed biological function have distinct conformational behavior. In the C-terminal region, we observed similar conformations as described for Tau. Also, interactions of the C-terminal domain with microtubules and its competitor, 14-3-3 protein, are similar for MAP2c and Tau. Analysis of the N-terminal domain was more interesting, providing a new insight into the structural basis of interactions of MAP2c with other proteins. We identified several regions with high populations of helical and poly-proline II conformations. The most distinct structural features were observed in the binding sites for SH3 domains (different from the SH3-binding site of Tau) and for the regulatory subunit of PKA. Moreover, the latter site is much more ordered (based on relaxation data) than the rest of the protein, and interacts with a specific sequence close to the N-terminus (based on PRE). These findings show that the interaction sites of MAP2c have specific conformations, present in the free form of the protein. The PKA phosphorylation study also revealed unexpected differences between MAP2c and Tau. High sensitivity allowed us to follow kinetics of phosphorylation in real time. Surprisingly, patterns and rates of phosphorylation of individual residues differ dramatically between MAP2c and Tau, despite of high sequence similarity. We confirmed that the phosphorylated residues of MAP2c strongly bind to 14-3-3. Differences between MAP2c and Tau in phosphorylation and consequent 14-3-3 binding suggest that both MAP2c and Tau respond to the same signal (phosphorylation by PKA) but have different downstream effects, indicating a possible signaling branch point for regulation of microtubule stability.

P242

Energetic Frustration: Allosteric regulation by the modulation of correlated segmental fluctuations in IDPs

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Intrinsically disordered proteins (IDPs) are considered to be ever more important for their central role in protein interaction networks. Still, molecular recognition of IDPs is an elusive phenomenon often described by folding upon binding or the formation of "fuzzy complexes". Using correlation analysis for the interpretation of paramagnetic relaxation data we are able to show changes of concerted fluctuations and therefore, of the conformational ensemble of IDPs upon ligand binding. In detail, we investigated the interaction between α-Synuclein and calmodulin as well as the interaction between the extracellular matrix IDP Osteopontin and the Glucosaminoglycan Heparin. On both systems, binding leads to a reduction of correlated long range motions. This indicates an overall loss of compaction upon complex formation. In addition, we find anti-correlated fluctuations of preformed segments indicating the existence of states in which these segments cannot be simultaneously formed and which were relieved in the bound state. This points towards the existence of a novel form of allosteric regulation in IDPs by energetic frustration between different segments of the protein and has important implications for their biological functionality.

P243

The heterogeneous structural behaviour of the oncogenic protein E7 from HPV 16

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The importance of local flexibility in determining the function of proteins has been recognized long ago and also widely scrutinized. If the extent of local flexibility is taken to its extreme conditions it leads to completely random coil behaviour of a polypeptide chain, indicated as intrinsic disorder, through a wide variety of intermediate cases both in terms of extent of mobility or in terms of protein stretches involved. Many examples of intrinsically disordered proteins (IDPs) appeared in the literature showing how their structural plasticity and intrinsic flexibility can be key features to enable them to interact with a variety of different partners and to adapt to different conditions. IDPs are extensively used by viruses to infect healthy cells since, in virtue of their small genomes able to code only a limited number of proteins, they need economic ways to interfere with the host hijacking cell regulation.

When present, structural disorder makes it very challenging to characterise the conformational properties of proteins. This is particularly the case of proteins, such as the oncogene protein E7 of human papillomavirus type 16 (HPV 16), which contain both ordered and disordered domains, and that can populate monomeric and oligomeric states under physiological conditions.

NMR spectroscopy is emerging as a powerful method to study these complex systems, most notably in combination with molecular dynamics simulations. Here we use NMR chemical shifts and residual dipolar couplings as structural restraints in replica-averaged molecular dynamics simulations to determine the free energy landscape of E7. This landscape reveals the presence of equilibrium between a high populated dimeric state and a low populated monomeric state of the protein. In both states, the picture that emerges is that of a folded but highly dynamical C-terminal domain and a disordered N-terminal domain that forms transient secondary structure. The results are validated by other biophysical methods and provide compelling evidence of the complex conformational heterogeneity associated with the behavior and interactions of this disordered protein associated with cancer.

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P244

An atypical mechanism of split intein recognition and assembly.

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Protein trans-splicing (PTS) is a post-translational autoprocessing event mediated by split inteins that has widespread application in protein engineering and biotechnology [1,2]. In canonical inteins, the chemical steps of PTS are preceded by an association event whereby two intrinsically disordered polypeptides, a larger N-intein and a smaller C-intein, interact and fold into the functional intein through a process mediated by electrostatic interactions [3]. Atypically split inteins have been reported in which the N-intein is the smaller peptide, resulting in a change in the topological requirements for assembly [4,5]. Here we introduce a more robust consensus engineered intein with atypical architecture (Cat) as a model to examine the association of atypically split inteins. We discovered that individual fragments of Cat are partially disordered prior to interaction and undergo an order to disorder transition upon binding. Furthermore, we have solved the NMR structure of the Cat intein complex and find a fold which is similar to that of other split inteins. However, the Cat intein fragments associate strongly with minimal salt-dependence and have very few inter-fragment electrostatic interactions. Protection from proteolysis is also distinct for the individual Cat fragments compared to canonical split inteins, suggesting an altered distribution of local order in the unbound state. Taken together our results demonstrate the utility of consensus engineering to circumvent the problem of aggregation while studying intrinsically disordered proteins and that Cat adopts a unique mechanism of assembly while still sharing a common structural identity with canonical inteins.

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P245

Structure of Radical-Induced Cell Death1 Hub Domain Reveals a Common Alpha-Alpha-Scaffold for Disorder in Transcriptional Networks

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Communication within cells relies on a few protein nodes called hubs, which organize vast interactomes with many partners. Frequently, hub proteins are intrinsically disordered conferring multi-specificity and dynamic communication. Conversely, folded hub proteins may organize networks using disordered partners. We solved the structure of the RST domain, a unique folded hub, by NMR spectroscopy and small- angle X-ray scattering, and its complex with a region of the transcription factor DREB2A through data-driven HADDOCK modeling and mutagenesis analysis. The RST fold is unique, but similar structures are identified in the PAH (paired amphi- pathic helix), TAFH (TATA-box-associated factor homology), and NCBD (nuclear coactivator binding domain) domains. We designate them as a group the alpha-alpha-hubs, as they share an alpha-alpha-hairpin super-secondary motif, which serves as an organizing platform for malleable helices of varying topology. This allows for partner adaptation, exclusion, and selection. Our findings provide insights into structural features enabling signaling fidelity.

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Protein-protein interactions of intrinsically disordered microtubule associated protein 2c in the context of neurite initiation

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Microtubules are flexible polymers constituting the eukaryotic cytoskeleton and taking part in fundamental cellular processes such as morphogenesis, cytokinesis, and mitosis. Microtubule-associated proteins (MAPs) regulate the microtubule assembly and disassembly, the processes generally termed dynamic instability, in a phosphorylation dependent manner. The microtubule dynamics in the neuronal cells is mainly modulated by two members of the MAP family, intrinsically disordered MAP2c and Tau, which play key roles in the neuronal outgrowth. It has been reported that MAP2c bind and stabilize microtubules, but phosphorylation by cAMP-dependent protein kinase A (PKA) induces dissociation of MAP2c from microtubules, which leads to microtubule destabilization. Another important protein family expressed in the brain, 14-3-3, has been proposed to be involved in the phosphorylation dependent control of microtubule dynamics by competing for phosphorylated Tau with tubulin.

In this study, we used high-resolution non-uniformly sampled HNCO experiments to monitor interaction of MAP2c with microtubules. We found that microtubules and the regulatory protein 14-3-3ζ bind to the same region of unphosphorylated MAP2c, as described for Tau. However, detailed analysis of phosphorylation kinetics by SOFAST experiments revealed striking differences between Tau and MAP2c, suggesting that phosphorylation of MAP2c by PKA controls influences interaction of MAP2c with 14-3-3ζ, but not in the microtubule-binding domain. Consequently, we hypothesized that the regulation of microtubule dynamics by 14-3-3ζ binding is not modulated by PKA phosphorylation (as reported for Tau) in the case of MAP2c. This hypothesis was confirmed by microtubule polymerization assay which showed that 14-3-3ζ competes with MAP2c for microtubules regardless of MAP2c phosphorylation, in contrast to the behavior of Tau. Moreover, we employed cryo-electron microscopy with single particle reconstruction analysis to complement the NMR analysis of the MAP2c-microtubule complexes.

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Combining NMR relaxation and MD simulations to characterize proteins containing ordered and intrinsically disordered regions

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Many proteins that play key roles in processes such as molecular recognition, regulation of transcription, or are related to neurodegenerative diseases, contain intrinsically disordered regions (IDRs) that are tethered to ordered domains (ODs). These hybrid systems represent a serious challenge for experimental and computational studies. In our study, we investigated δ-subunit of RNA polymerase with a flexible and highly repetitive C-terminal part and a well-structured N-terminal domain. We used non-uniformly sampled 3D HNCO-based relaxation experiments to study dynamics of the partially disordered protein with sufficient resolution. To interpret the data, we used a framework termed Average of Blocks Selected by Using Relaxation Data (ABSURD) that filters out those segments of the trajectories that are not compatible with the experimental NMR relaxation data. A series of several short (100 ns) molecular dynamics (MD) simulations were used in combination with experimental 15N relaxation measurements to characterize the ensemble of dynamic processes contributing to the observed rates. By accounting for the distinct dynamic averaging present in the different conformational states sampled by the equilibrium ensemble, we were able to accurately describe both dynamic time scales and local and global conformational sampling. The method is robust, systematically improving agreement with independent experimental relaxation data, irrespective of the actively targeted rates. Our results demonstrate applicability of the ABSURD analysis to hybrid proteins consisting of well-ordered and disordered regions. This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 692068.

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Characterizing the WIP-Cortactin Interaction through NMR

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WASp-Interacting Protein (WIP) is a 503-residue Intrinsically Disordered Protein (IDP) involved in regulation of cytoskeleton assembly, with binding sites for globular actin, WASp, and various adaptor proteins. Its central region binds to cortical actin-binding protein (cortactin), a 550-residue actin nucleation promoting factor, via its C-terminal SH3 domain. This interaction is essential for podosome formation and extracellular matrix degradation. Cells with a deletion mutant of WIP that lacked the cortactin-binding domain were defective in both. [1] Because these processes are necessary for the normal motility of certain immune cells and for the invasiveness of cancer cells, the question of how WIP binds to cortactin may have clinical significance. Here we use NMR, well-suited to study IDP biophysics, to investigate the WIP-cortactin interaction on the molecular level. Backbone resonance assignment of the WIP fragment followed by analysis of SH3-induced Chemical Shift Perturbations (CSPs) revealed the binding motif, showing that it deviates from the consensus binding sequence. [2] NMR spectra are consistent with a system in fast-to-intermediate exchange on the NMR timescale, a finding confirmed also by Isothermal Titration Calorimetry (ITC). In addition, the cortactin Proline-Rich Region (PRR) preceding the SH3 domain may affect WIP binding. CSPs of PRR-SH3 (compared to SH3 alone) indicate an intramolecular interaction between these two domains. This suggests a potential auto-inhibitory effect of PRR or modulation of SH3 affinity to its interaction partners. Future experiments such as transferred NOE spectroscopy and CPMG relaxation dispersion measurements will provide information on the elusive SH3-bound conformation of WIP, providing a biologically important structural view of how WIP engages its cellular binding partner cortactin.

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P249

Disorder and Order: Looking into Protein Misfolding with (DNP-enhanced) Solid-State NMR

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We exploit high-resolution solid-state MAS NMR spectroscopy as well as DNP enhanced MAS NMR-spectroscopy for the study of structure and distribution of conformational ensembles of intrinsically unfolded as well as aggregated proteins. Low-temperature Nuclear Magnetic Resonance (NMR) spectra usually suffer from severe line broadening due to freezing out different conformations. While this is usually accounted for as an unwanted side-effect of DNP-NMR, inhomogeneously broadened lines also contain valuable information about conformational ensembles of (disordered) proteins. We have studied the conformational ensembles of α-syn in frozen solution under different conditions: the intrinsically disordered monomer, fibrillar α-synuclein with flexible ends, and α synuclein in contact with lipid bilayers (1,2). We could probe the conformational ensembles of all valine residues in a selectively labeled sample of α-syn by evaluating the inhomogeneously broadened line-shapes of the Cα/Cβ cross peak. We could estimate the amount of disordered regions in fibrillar α-syn and delineate the membrane binding regions of α-syn in contact with membrane surfaces in in different protein to lipid ratios. Furthermore, secondary chemical shifts of neighboring amino acids tend to be correlated, indicative of formation of transient secondary structure elements. Our approach thus provides accurate quantitative information on the propensity to sample transient secondary structures in different functional states. We also present MAS NMR results obtained for one type of Aβ(1-42) fibrils (3). In contrast to previously described fibril polymorphs, resonances for all 42 amino acids could be observed in NMR spectra based on dipolar transfer, and site-specific resonance assignments were obtained for all residues. Fibrils are characterized by a high degree of homogeneity: doubled resonance peaks, representing local disorder, were observed only for three residues in the sequence. Strikingly and in contrast to previously described fibrillar Aβ(1-42) structures, the N-terminus consists of two extended β-strands and thus also belongs to the core region. A 3D structure with a resolution of 4 Å was obtained on this identical fibril sample by cryo-EM; all secondary structure elements as well as partial disorder of Phe20 as well as the turn region around Ser26 perfectly agree with the NMR data. Chemical shifts of charged amino acid residues, indicative of the corresponding protonation state, confirmed locations of salt bridges. Additionally, signal intensities obtained with different homo- and heteronuclear recoupling techniques, as well as spin lattice relaxation times in the rotating frame (T1ρ) were exploited to elucidate local dynamic variations in the fibrillar structure. Finally, these results are compared to dynamic nuclear polarization (DNP) measurements to probe structural heterogeneity at low temperatures. Uluca, B. et al. Biophys. J. 2018, 114, 1614-1623.

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P250

Structural dissection of TDP-16, TDP-35 and TDP-43 amyloid fibrillar aggregates

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Self-assembly and deposition of proteinaceous fibrillar aggregates are associated with many neurodegenerative diseases. Mislocalization and aggregation of the essential nuclear protein TDP-43 (TAR DNA binding protein of 43 kDa) are observed as a pathological hallmark of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar dementia (FTLD) (1). TDP-43 is composed of an N-terminal domain, two RNA recognition motif domains (RRM1 and RRM2) and a glycine-rich C-terminal domain. The prion-like C-terminal domain possesses most of the pathologically relevant mutations, and it also plays a critical role in the spontaneous aggregation of TDP-43 and associated proteinopathy (2, 3). The Q/N-rich segment of the C–terminus is proposed to adopt a beta-hairpin structure that aggregates to form an amyloid-like structure in a parallel beta-turn configuration (4). For detailed structural analysis of the amyloid-forming C-terminal region, we have created three different TDP constructs namely TDP-43 (1-414), TDP-35 (TAR DNA binding protein of 35 kDa) and TDP-16 (TAR DNA binding protein of 16 kDa). TDP-35 (90-414) contains the two RRM domains and the intrinsically disordered C-terminus, whereas TDP-16 (267-414) includes only the latter, consisting of the hydrophobic (318–340) and Q/N rich (341–367) segments. The molecular organization of the different fibrillar assemblies is analyzed using a combination of electron microscopy, X-ray diffraction, and solid-state NMR spectroscopy. The three TDP constructs exhibit similar fibril morphology and the typical amyloid cross-beta diffraction pattern. However solid-state NMR revealed that TDP-43 and TDP-35 share the same polymorphic molecular structure, while TDP-16 comprises of a well-organized amyloid core. Our results provide some preliminary structural insights about fibrillar assemblies of different TDP constructs.

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P251

Molecular mechanism of β2-microglobulin amyloid formation and its inhibition using affimers

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Aggregation of β2-microglobulin into amyloid fibrils is associated with the dialysis related amyloidosis disorder. Studies on these mechanisms involves the understanding of structure and dynamics of the intermediates in amyloid assembly leading to fibril formation. Such intermediates or interfaces involved in β2m amyloid formation can be mapped, enabling the development of protein affimers that are able to inhibit amyloid formation. Kinetic assembly reactions in conjunction with NMR, negative stain EM and other biophysical methods was employed in studying such molecular events, and the effect of affimers was tested in vitro. The inhibition of β2m fibril formation by the different affimers is discussed in detail.

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A novel approach to enable the structural and dynamics characterisation of huntingtin protein

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Huntington's disease (HD) is a hereditary neurodegenerative disorder that is caused by an expansion of the CAG triplet beyond a threshold of 35 repeats. This results in an abnormally long poly glutamine (poly Q) tract within the N-terminus of the huntingtin protein (htt exon1) that renders the protein aggregation prone and leads to neuronal degeneration. While the presence of neuronal inclusions containing htt exon1 aggregates is a hallmark of HD, there is growing evidence that the toxic properties of mutant htt exon1 arise from interactions involving soluble forms prior to their aggregation into visible aggregates. Unfortunately, the mechanisms by which soluble forms of mutant htt exon1 give rise to toxicity and cell death are still poorly understood. This is mostly due to the technical challenges encountered in high-resolution structure/function studies of poly Q regions that are often intrinsically disordered, precluding crystallisation. Furthermore, the assignment of NMR spectra is complicated by the chemical similarity and thus poor peak dispersion of the residues within the repeats. In order to understand the structural and conformational properties of monomeric htt exon1, to assess the impact of poly Q length and the adjacent poly P tract, we used a combination of cell-free protein expression and nonsense suppression to site-specifically incorporate a single 15N/13C-labelled amino acid into the poly Q and poly P regions of htt exon1, and thereby simplified the NMR spectra. This enabled us to unambiguously monitor structural features of the labelled residue in the context of the poly Q and poly P tracts. By integrating all data we are now able to decipher local structural propensities, structural cooperativity and possible intra- and inter-molecular interactions, that may be crucial for disease development.

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Combining DNP NMR with Segmental and Specific Labeling to Study the Quaternary Structures of Yeast Prion Protein Strains

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Yeast prions are self-templating protein-based mechanisms of inheritance whose conformational changes lead to the acquisition of diverse new phenotypes. The best studied of these is the prion domain (NM) of Sup35, which forms an amyloid that can adopt several distinct conformations (strains) that produce distinct phenotypes. Despite intense study, there is no consensus on the organization of monomers within Sup35NM fibrils. Some studies point to an α-helical arrangement, whereas others suggest a parallel in-register organization. Intermolecular contacts are often determined by experiments that probe long-range heteronuclear contacts for fibrils templated from a 1:1 mixture of 13C- and 15N-labeled monomers. However, for Sup35NM, like many large proteins, chemical shift degeneracy limits the usefulness of this approach. Segmental and specific isotopic labeling reduce degeneracy, but experiments to measure long-range interactions are often too insensitive. To limit degeneracy and increase experimental sensitivity, we combined specific and segmental isotopic labeling schemes with dynamic nuclear polarization (DNP) NMR. Using this combination, we examined two of the amyloid forms of Sup35NM that do not have a parallel in-register structure as general pathological disordered proteins do. The combination of a small number of specific labels with DNP NMR enables determination of architectural information about polymeric protein systems.

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Zinc-induced oligomerization of the amyloid-β peptide fragments

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Zinc-induced oligomerization of amyloid-β peptide (Aβ) is a hallmark molecular feature of Alzheimer's disease (AD). Interactions of zinc ions with Aβ are mediated by the N-terminal Aβ1-16 domain. Mutations and modifications in the metal binding domain Aβ1-16 crucially affect its zinc-induced oligomerization by changing intermolecular mediated interface. We studied interaction of zinc ions with the metal binding domains derived from several Aβ variants. NMR techniques were used to identify the centers of chelation of metal ions and stoichiometry of zinc-peptide interaction, to determine structure of the formed complexes, and to elucidate dynamics of their formation. It was found that the native Aβ1-16 isoform and the fragments carrying familial English (H6R) and Taiwanese (D7H) mutation undergo zinc-induced dimerization [1-3]. Zinc-induced peptide interaction is governed by conformational changes in the minimal zinc binding site 6HDSGYEVHH14 [2]. Novel binuclear zinc interaction fold in the dimer structure of the D7H-Aβ fragments has been discovered [3]. We determined solution structure of the formed peptide dimers [2, 3]. Zinc coordination site in the peptide that contains the phosphorylated Ser8 (pS8) residue has been determined [4]. In an attempt to reveal the mechanism of rats' resistance to Alzheimer's disease, the structure of the metal binding domain of rat beta-amyloid in the presence of zinc ions has been determined [5]. Zinc-induced oligomerization of the all the studied Aβ fragments has been shown to follow the same molecular mechanism: (i) peptide dimer is formed through the primary zinc-mediated interface 11EVHH14; (ii) residues H6, H13, H7, pS8 and E3 in various Aβ isoforms are realigned creating the second zinc-dependent interface in each subunit; (iii) the dimer becomes a seed of subsequent zinc-dependent oligomerization [2]. Targeting of Aβ zinc-mediated interfaces provides a therapeutic route for AD treatment [6]. Work was supported by the Russian Science Foundation (grants 14-24-00100 & 14-14-00598). References 1. Kozin, S.A., Kulikova, A.A., Istrate, A.N., Tsvetkov, P.O., Zhokhov, S.S., Mezentsev, Y.V., Kechko, O.I., Ivanov, A.S., Polshakov, V.I., and Makarov, A.A., Metallomics, 2015. 7(3): 422-5. 2. Istrate, A.N., Kozin, S.A., Zhokhov, S.S., Mantsyzov, A.B., Kechko, O.I., Pastore, A., Makarov, A.A., and Polshakov, V.I., Sci Rep, 2016. 6: 21734. 3. Polshakov, V.I., Mantsyzov, A.B., Kozin, S.A., Adzhubei, A.A., Zhokhov, S.S., van Beek, W., Kulikova, A.A., Indeykina, M.I., Mitkevich, V.A., and Makarov, A.A., Angew Chem Int Ed Engl, 2017. 56(39): 11734-11739. 4. Kulikova, A.A., Tsvetkov, P.O., Indeykina, M.I., Popov, I.A., Zhokhov, S.S., Golovin, A.V., Polshakov, V.I., Kozin, S.A., Nudler, E., and Makarov, A.A., Mol Biosyst, 2014. 10(10): 2590-6. 5. Istrate, A.N., Tsvetkov, P.O., Mantsyzov, A.B., Kulikova, A.A., Kozin, S.A., Makarov, A.A., and Polshakov, V.I., Biophys J, 2012. 102(1): 136-143. 6. Tsvetkov, P.O., Cheglakov, I.B., Ovsepyan, A.A., Mediannikov, O.Y., Morozov, A.O., Telegin, G.B., and Kozin, S.A., J Alzheimers Dis, 2015. 46(4): 849-853.

P255

Conformation and dynamics of soluble prefibrillar repetitive domain of spider dragline silk proteins: insights into β-sheet formation

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Spider dragline silks are fibrous proteins, which are recognized for their superior mechanical properties. These proteins are composed of long repetitive domain, flanked by conserved N-terminal (NTD) and C-terminal (CTD) domains. Repetitive domain of spider dragline silk are mainly composed of poly-ala region (4-12 alanine residues) and glycine-rich region (-GGX-). In silk fiber, polyalanine region forms β-sheet structure in a crystalline region, which is a key structure underlying mechanical properties of spider silk (Keten, S, et al. Nature Material, 2010).

Prior to spinning, spider silk proteins (spidroins) are stored at high concentration in the spinning dope and they are transformed into insoluble fiber after passing pH- and ions-gradient across the spider gland. Spinning dope contains high concentration of chaotropic ions (Mg2+, Na+, Cl-) and pH in this region is close to neutral (pH 7), while closer to spinning duct, chaotropic ion concentrations and pH decrease (pH 5) while kosmotropic ion (PO43-) concentration increases (Andersson, M, et al, PLOS Biology, 2014; Knight, D.P and Vollrath, naturewissenschaften, 2001)

Conformation of NTD and CTD of spidroin display strong pH-dependence and these domains are essential for controlling the pH-dependent of fiber assembly (Askarieh G, et al, Nature, 2010; Hagn F et al, Nature, 2010). Despite the essential role of NTD and CTD in spider silk self-assembly, conformation and dynamics of the soluble repetitive domain, which dominate the length of protein chain, at different pH and ions are still not completely understood.

Here, we report conformation and dynamics of soluble repetitive domain of Nephila clavipes spider dragline silk in different repeat units, pH, temperature, and ions using solution-state NMR, far-UV CD and vibrational CD (VCD). At pH 7 and temperature 10°C, soluble repetitive domain of spider dragline silk consist of two major populations, which are 65% random coil and 24% PPII helix. PPII helix conformation is distributed over glycine-rich region, which exhibits more limited flexibility compared to

polyalanine region. The PPII helix in the glycine-rich region is proposed as a soluble prefibrillar, which can subsequently undergo intramolecular interaction (Oktaviani, N.A et al, Nature communication, in press). Contrary to CTD and NTD, conformation of soluble repetitive domain is pH independent, indicating that the prefibrillar form of the repetitive domain is not affected by pH (Oktaviani, N.A et al, Nature communication, in press). Our NMR data also showed that chaotropic ions prevent intra- and inter-molecular interactions, which explains the role of chaotropic ions in improving the solubility of spidroin. In contrast, in the presence of kosmotropic ion (PO43-), β-sheet propensity in the polyalanine region increases, which is supported by the increase of rigidity of the repetitive domain. Together, these findings provide the insight into β-sheet formation of spider silk protein.

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Investigating the binding interactions of Amyloid Beta 1-42 with molecular tweezers and peptidomimetics by solution NMR

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Alzheimer's disease is one of the most prevalent debilitating and fatal neurodegenerative disorders in the developed world. Patients affected by this disorder exhibit cognitive impairment and acute loss of memory function due to loss of neuron cells. Pathological indicators which confirm AD include brain shrinkage, plaques and neurofibrillary tangles. These plaques are mainly comprised of the amyloid-β isoforms Aβ40 and Aβ42, whereby the latter is the more pathogenic isoform. Currently, it is believed that the Aβ oligomers, and not the subsequent plaques, are responsible for neuronal cell death. The aggregation rate of the more amyloidogenic Aβ42 is significantly faster than that of the less-toxic Aβ40. [1] This, coupled with Aβ42's lower aqueous solubility often leads to the Aβ40 peptide being chosen as a model system for studying primary and secondary nucleation, aggregation kinetics and inhibitor studies.

The lysine-specific molecular tweezer, CLR01, has previously been studied with respect to Aβ binding and its capability of inhibiting and reversing aggregation. [2] These NMR studies were performed using Aβ40. Here, we report the effects of CLR01 with respect to Aβ42 binding and aggregation determined by solution state NMR. Furthermore, a comparative analysis of the binding modes of the known Aβ KLVFF sequence derived peptide inhibitors, OR1 and OR2 [3] is presented.

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P257

NMR reveals anchor role of Threonines in the self-association of alpha-synuclein: Triphala, a herbal preparation inhibits fibrillation

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The process of assembly and accumulation of the intrinsically disordered protein (IDP), alpha-synuclein (aSyn) into amyloid fibrils is a pathogenic process leading to several neurodegenerative disorders such as parkinson's disease, multiple system atropy, etc. While several molecules are known to inhibit aSyn fibrillation, the mechanism of inhibition of the fibrillation process, for IDPs in general, is beginning to emerge. In this work, using NMR along with other biophysical tools such as ThioflavinT-binding assay, transmission electron microscopy and circular dichroism spectroscopy we report interesting observations and mechanistic insights into the fibrillation process of aSyn. The threonine residues at several locations, prominently in the NAC region seem to play anchor roles in the self-association process of aSyn. We demonstrate that Triphala, a herbal preparation in the traditional Indian medical system of Ayurveda, inhibits fibrillation of aSyn by interfering with the threonine anchors. The polyol constituents of Triphala, which are the major components as determined from mass spectrometry based metabolite screening studies, interfere with these anchors to bring about the inhibition. Based on literature and our results, we hypothesize that the polyols may be working together in a synergistic fashion to interfere with the threonine based association. On the other hand, it was observed that Triphala does not disaggregate preformed aSyn fibrils.

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Human serum albumin and α-synuclein partnership: dissecting the interaction modes by NMR

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The overexpression, misfolding and uncontrolled aggregation of α-synuclein (α-syn) is linked to the onset and progression of a branch of neurological disorders named synucleinopathies. Monomeric and oligomeric α-syn have been found to be present in biological fluids such as saliva, cerebrospinal fluid (CSF)[1] serum and plasma. Increasing evidences are emerging about the misfolded α-syn cell to cell transmission trough biological fluids and its implication in the development of neurological disorders.[2] Detecting pathological aggregates or altered levels of α-syn in biological fluids would be of great interest in developing new assays for the early-stage diagnosis of synucleinopathies. Of notable mention are the recent developments of RT-QuIC and α-syn PMCA for the diagnosis of synucleinopathies,[3] these techniques rely on the modulation of the in vitro α-syn aggregation due to the addition of aliquots of biological fluids. To this purpose, it is necessary to obtain information about α-syn proteostasis in biofluids and its interaction with compounds that can influence its aggregation. Since human serum albumin (HSA) is the most common

protein found in serum, plasma and CSF, we characterized its interaction with α-syn, also considering that high levels of HSA are found in advanced PD patients[4] and a recent published study showed the inhibitory effect of HSA on α-syn aggregation.[5] For this work, we tested the inhibitory effect of HSA on α-syn aggregation with ThT fluorescence and by high field solution NMR. In our experimental conditions HSA inhibited α-syn aggregation and solution NMR experiments showed that HSA interacts both at the C and N termini of α-syn. The addition of NaCl in our KPi buffer hindered the interaction at the C-terminus. The N-terminus interaction is still visible by solution NMR both in KPi + 150 mM NaCl and in PBS.

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P259

Structural Insight into hIAPP Self-Assembly by NMR Methods

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Islet amyloid deposits, associated with type 2 diabetes, are primarily composed of an aggregated form of the human islet amyloid polypeptide (hIAPP). In spite of great efforts, there are only a few studies published on the structures of the fibrillar aggregates at atomic resolution because of their extensive polymorphism and polymeric properties.1,2 Taking advantage of developments in proton-detected MAS solid-state NMR methods, our work aims at determining high-resolution 3D structures of different hIAPP fibrils. To investigate the effects of an N-terminal disulfide bond on the fibrillar structure, we generated hIAPP aggregates composed of oxidized and reduced forms of the protein. Preliminary solid-state NMR results will be presented. In the second part of the poster, we explore the conformation behavior of hIAPP in blood plasma. Our previous study indicates that sugars and lipids of plasma interact with hIAPP resulting in β-sheet rich toxic oligomers.3,4 Interestingly, sugars induce the formation of hIAPP-rich droplet-like colloidal structures. We use solution-state NMR complemented with imaging methods to characterize these structures and to detect possible exchange between sugar-induced oligomeric state and a monomeric state. These studies provide better insight into the amyloid formation in vivo-like environment and should facilitate optimization of therapeutic strategies to interfere with this pathological process.

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P260

Structure and molecular association of Aux/IAA17 for the auxin-response transcriptional regulation in plants

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Auxin is a central hormone in plant development and adaptive growth. Auxin signaling is regulated by auxin response transcription factors (ARFs) and Aux/IAA transcriptional repressors. Aux/IAA consists of four domains. Domain I recruits TOPLESS(TPL)/TPL-RELATED (TPR) corepressors, domain II binds to E3 ubiquitin ligase for degradation, and domain III-IV is a PB1 domain that interacts with ARF and Aux/IAA family proteins. Domains III-IV of Aux/IAA and ARF interacts with each other in a front-to-end manner to form a high-order oligomer. It has been unclear, however, how the presence of Domains I and II affect the oligomeric elongation of Domain III-IV. We observed that full-length Aux/IAA17 with mutations at a positively or negatively charged interface expressed as inclusion bodies. We could refold the full-length protein to obtain a soluble form, but the protein was in general prone to aggregation. We suppose that Domains I-II may interfere with the folding of Domains III-IV or participate in protein oligomerization via a yet unknown mechanism.

P261

Structural and kinetic studies of oligomeric aggregates of α-synuclein

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Amyloid aggregates of the protein α-synuclein are a hallmark of a family of neurodegenerative diseases known as Lewy body pathologies, which include Parkinson's. Recent evidence suggests that early oligomeric forms may have more of a pathogenic effect than mature fibrils. [1-3] In this study, high performance liquid chromatography (HPLC), together with an array of spectroscopic techniques, was used to investigate the formation of α-synuclein oligomers in real time. The main advantage of HPLC over other methods being its ability to quantify the size distribution of a heterogeneous mix of molecules as individual measurements rather than as a population averaged value. The hydrodynamic radii of α-synuclein at different stages of aggregation were also calculated under native and denaturing conditions. The results obtained were cross-verified using atomic force microscopy (AFM) and transmission electron microscopy (TEM). The effects on long term potentiation of the α-synuclein oligomers produced were studied using in vivo electrophysiology experiments on rats, and the oligomers but not the monomers of α-synuclein were found to have a significant baseline effect on long term potentiation.

P262

Mycobacterium tuberculosis β-lactamase BlaC is highly ordered in solution

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The pathogen Mycobacterium tuberculosis is resistant to β-lactam antibiotics due to its native β-lactamase, BlaC. We studied the dynamics of BlaC in solution by measuring the NOE, T1 and T2 relaxation of its backbone amides, as well as their relaxation dispersion. We found that the protein is extraordinarily rigid on the pico- to microsecond timescale, where-as dynamic behaviour on the micro- to millisecond timescale was found to cluster around the active site and the omega loop. Mechanistic interpretation of these results is the subject of further research. Eventually, we hope that increased understanding of this enzyme will contribute to the development of more efficient inhibitors to be used in tuberculosis treatment.

P264

Dynamical Characterization of the Disordered N-Terminal Domain of Amyloid-Beta Fibrils Using Deuterium Static Solid-State NMR

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The disordered N-terminal domain of amyloid-beta protein (Abeta) is believed to be important in the control of aggregation propensities of the protein. The domain has been difficult to characterize at site-specific level due to its lack of structure. Using 2H static solid-state NMR techniques in combination with site-specific labeling, we investigated the side-chain dynamics of the N-terminal subdomain in mature Abeta fibrils. The results suggest concerted motions of the N-terminal subdomain on microsecond-millisecond time scales, and point to a specific residue along the sequence (G9) at which the motions begin to diminish drastically. We determined fractions of the side-chain populations that participate in the global motion of the domain as well as rate constants at selected sites, using deuterium line shapes and longitudinal relaxation measurements over a broad temperature range. Interestingly, the results also suggest a possibility of interactions between the flexible N-terminal domain and the more rigid amyloid core. The data enable us to estimate the rate constant for these motions. The isolated N-terminal domain sequence is considerably less flexible than the same region in the context of the fibrils, indicating that interactions with the core play a role in inducing the flexibility of the N-terminal domain. The determination of the rate constant of the N-terminal domain motion is also approached from another angle using novel 2H NMR R1rho relaxation measurements. Overall, these results provide a thorough dynamical characterization of this disordered and functionally important domain.

P265

Narrowing the Gap Between NMR Relaxation and Molecular Dynamics Simulations of Methyl Dynamics in Proteins

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Molecular dynamics (MD) simulations and nuclear magnetic resonance (NMR) spin relaxation experiments have become increasingly powerful to study protein dynamics at atomic resolution due to steady improvements in physical models and computation power. Good agreement between generalized Lipari-Szabo (S2NH) order parameters derived from experiment and simulation is now observed for the backbone dynamics of a number of proteins. Unfortunately, the agreement for side chains, as e.g. probed by S2CH3 for methyl-containing side chains, is much poorer. In this work we discuss several issues with methyl side chains that need to be addressed to close the gap between NMR and MD. Accounting for protein tumbling is one very important factor to obtain a good agreement. In our hands, the application of improved water force fields with an appropriate way of including anisotropic overall protein tumbling improves the prediction of experimentally measured dynamic observables by MD simulations. We demonstrate these aspects for T4 lysozyme as a representative example. Our results guide the way for extracting from the NMR relaxation data the most accurate parameters that describe protein side chain dynamics and report on conformational entropy. Additionally, we reparametrized side chain dihedral angle energy barriers for methyl rotation in the Amber99SB*-ILDN force field. We demonstrate that this leads to a much more realistic dynamics of methyl groups and is another important feature to close the gap between spectral densities from NMR relaxation and MD simulation[1]. Finally, we show how much entropy is actually contained in backbone and sidechain order parameters measured in NMR relaxation experiments and its implications for the entropy meter.

[1] Accurate Methyl Group Dynamics in Protein Simulations with AMBER Force Fields Falk Hoffmann, Frans A. A. Mulder, and Lars V. Schäfer The Journal of Physical Chemistry B 2018 122 (19), 5038-5048 DOI: 10.1021/acs.jpcb.8b02769

P266

Dynamics, Hidden States and Stress Induced Unfolding of Small Disulphide Proteins

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Antimicrobial, disulphide proteins like PAF[1,2] are generally believed to exist as rock hard entities, though they have many charged residues. We have recently shown[3,4] by thermal unfolding, 15N-relaxation, 15N chemical exchange saturation transfer (CEST) and ensemble molecular dynamics that this is not the case. Even under the conditions of maximum protein stability, considerable amount of NMR invisible protein states may per-

sist due to conformational and dynamical heterogenity. The hidden nature of these states for everyday NMR methods may be explained by the low populations and/or fading effects due to exchange between two or more states in the intermediate time scale (ms-us) regime. Partially unfolded states can be biologically relevant, e.g. connected to disulphide shuffling or chirality switching. In general, less structured intermediates can be useful for more efficient conformational-selection upon molecular recognition. In fact, there is no sharp boarder between the folded and „disordered" protein world (IDPs). Practical consequences may have impact on the validation of MD simulations or protein concentration measurements. The present overview is an extension of our earlier work to new PAF variants and related antifungal proteins (AFP, PAFB[5], NFAP, NFAP2[6]). NMR monitoring of chemical unfolding caused by DMSO is equipped now with molecular dynamics calculations and DSC microcalorimetry.

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P267

Novel insights into the binding properties of monomeric relaxin peptide with LDLa-LRR linker of RXFP1

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The Relaxin Family Peptide Receptor 1 (RXFP1) is a unique class A GPCR with a ligand-induced self-activation mechanism that involves binding of H2 relaxin hormone to the large ectodomain (ECD) that leads to conformational changes and activation by the N-terminal LDLa module. While relaxin is reported to bind with high affinity to the Leucine Rich Repeat (LRR) domain we recently showed that the 32-residue linker, that tethers the N-terminal LDLa module to the LRR domain, comprises a second low affinity-binding site. This linker was found to adopt a stable helical structure after binding to H2 relaxin. The N-terminal region of the linker was also found to be critical for interaction with exoloop-2 of the transmembrane domain (TMD) hence vital for receptor activation. Nevertheless, our study was complicated by the tendency of relaxin to dimerize at micromolar concentrations. Here we have analyzed this secondary binding site using the monomeric amidated form of H2 relaxin and found the key residues on the linker that are involved in binding, also provided insight into the formation of the activation complex that potentially involves the binding of LDLa and linker to exoloop-2 of the TMD. Based on the measurements of 1H solvent exchange rates, 1H temperature coefficients, and 15N relaxation parameters and CPMG dispersion rates of backbone amides in the apo- and amidated H2 bound-state of LDLa-LRR linker, the mechanism of the formation of a helical structure in the linker is reported. We hypothesize that LDLa-LRR linker and relaxin binding is a two-step mechanism in which partially ordered conformations of the linker form a complex with relaxin and then rapidly rearrange to form a stable helical structure, that potentially may involve the LDLa module.

P268

Localized Allele-Specific MHC-I Dynamics Drive Chaperone Association and Peptide Exchange

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Conformational dynamics and protein plasticity are known to play pivotal roles in driving biomolecular interactions. However, the precise underlying mechanisms that govern these phenomena are less understood. Here, we uncover how conformational exchange dictates the protein-protein interaction between class-I major histocompatibility complexes (MHC-I) and the peptide-editing chaperone TAPBPR, which is important for the proper functioning of the adaptive immune system. Previous computational studies have suggested that preferential association of peptide-editing chaperones with certain MHC-I alleles can be attributed to differential dynamics within the groove, as the result of either peptide occupancy and specific amino acid polymorphisms within the MHC-I groove. Here, we apply single-quantum methyl relaxation dispersion experiments at two NMR fields to characterize allele-specific conformational exchange in a representative set of common MHC-I molecules with varying affinities for the chaperone. Our NMR relaxation experiments establish that regardless of affinity for TAPBPR, each MHC-I molecule samples a minor, sparsely populated, microstate conformation of 5% population with exchange rate constants ranging from 800-1000 s-1. These findings suggest the sampling of a minor state conformation alone is not sufficient for TAPBPR binding. Instead, we find that allele-specific dynamics in localized "hotspots" of MHC-I molecules, including the heavy chain α2 helix, the pleated β-sheet adjacent to the F-pocket and α3 domain, correlate with binding affinity and kinetic off-rate of TAPBPR for the MHC-I in vitro. We corroborate our NMR results with μs time-scale all-atom molecular dynamics (MD) simulations, where we additionally use a combination of MD trajectories, NMR chemical shifts and Rosetta modeling to examine the structure of the minor MHC-I state. Our results highlight a peptide-editing mechanism where TAPBPR exploits localized structural adaptations, both near and distant from the MHC-I groove, to recognize different molecules in a dynamically-driven conformational selection-like mechanism. Mutational studies of pMHC-I molecules with restricted groove dynamics support a role for conformational exchange in driving chaperone association.

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Dynamics in a divalent cation channel by NMR at >100 kHz MAS

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In recent years, faster and faster magic-angle spinning (MAS) rates have paved the way for proton-detection in the solid state, enabling the acquisition of resolved proton resonances in fully protonated samples available in sub-milligram amounts. This technical progress revolutionizes the atomic-level investigation of proteins, expanding the range of information exploitable for the determination of protein structures and opening new horizons for the investigation of protein dynamics. Here we demonstrate that MAS rates above 100 kHz permit the measurement of a variety of observables connected to local and global dynamics in protonated proteins

of diverse molecular sizes and aggregation states, from microcrystalline to membrane proteins. Notably, we evaluate the feasibility of measurements of backbone and side-chain order parameters in a fully protonated environment and investigate the coherent effect on 13C longitudinal relaxation rates at fast MAS rates. In addition, we explore the potential of 1H R1ρ relaxation dispersion experiments at amide sites, in samples of different deuteration levels, to obtain information on μs-ms dynamics. These methods are first benchmarked on the model microcrystalline protein GB1. Subsequently, we extend the investigation to a bacterial divalent cation channel CorA reconstituted in lipid bilayers, a 5x42 kDa pentamer comprised of two transmembrane helices and a large cytoplasmic domain hosting a metal binding site (usually Mg2+ or Co2+). The combination of high magnetic fields and fast MAS rates allows the acquisition of well-resolved spectra for backbone and side-chains resonance assignment and the measurement of residue-specific dynamic parameters, providing insights on the transport mechanism of cations through the CorA channel. This progress represents a concrete step forward in the study of challenging biological systems by solid-state NMR.

P270

NMR Dynamics Reveals Allostery in Ubiquitination <=> HARD and Geometric Approximation access Faster Rates

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Many biological processes derive from the interplay of two or more proteins. This interplay is often driven by allosteric and dynamic changes. NMR (structure and dynamics), combined with other structural (X-ray, SAXS) and biophysical techniques, is unique in the ability to dissect these interactions. However, generally, these tools provide ground-state structures, while it is often the case that low-population excited states are the keys to the binding interactions. The field of NMR dynamics is expanding, such that both a wider range of rate processes can be measured, and more complex exchange models can be analyzed (Chao & Byrd, Emerging Topics in Life Sciences (in press, 2018). We have focused on ubiquitin-conjugating enzymes (E2s) interacting with ubiquitin ligases (E3s) to create post-translational modification by ubiquitination (Mol. Cell 2009, 2013; Structure 2012; EMBO J. 2014), which is critical to protein regulation in ERAD. RING finger proteins constitute the majority of E3s and function by interacting with E2s charged with ubiquitin. How low-affinity RING:E2 interactions result in highly processive substrate ubiquitination remains largely unknown. The RING E3, gp78, represents an excellent model to study this process. gp78 includes a high-affinity secondary binding region, G2BR, for its cognate E2, Ube2g2. Structural analyses reveal two allosteric events critical to the recognition, ubiquitin-transfer, and release of these proteins. These processes suggest a role for conformational dynamics in this biological machine; however, recent attempts to apply CPMG techniques to similar E2 systems have proven refractory. We have been able to overcome these difficulties and are beginning to understand the role of conformational dynamics in the recognition of the E2 by the E3. CPMG experiments combined with molecular dynamics are revealing new insight into the interconversion between the various states of the E2 along the reaction pathway (Structure 2017). Overall, these studies reveal that gp78:Ube2g2 is a ubiquitination machine where multiple E2-binding sites coordinately facilitate processive ubiquitination. Continuing studies involve examination of more complete functional complexes, which places stringent demands on experimental methods. In addition to the gp78 G2BR, other secondary binding domains have been identified for Ube2g2, and we are exploring the comparative allosteric effects in these systems.

In order to explore a broader range of exchange rate processes, we have continued development of the heteronuclear adiabatic relaxation dispersion (HARD) experiments combined with the novel analysis tools of geometric approximation (Chao & Byrd, JACS 2016, JMR 2017, Emerging Topics in Life Sciences (2018). These tools indicate the processes approaching 20-60 x 10^3 sec^{-1} can be detected and quantified. We will describe these tools and discuss the challenges associated with analyses of more complex exchange models/processes using geometric approximation methodology. This approach is enabling the examination of fast dynamic processes in biomolecules at relevant temperatures and molecular sizes.

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The Dynamics of Lactaptin in solution by NMR and PRE

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Lactaptin is a recombinant analogue of the proteolytic fragment of the human milk protein κ-casein, has antitumor activity and is also a membrane-active peptide capable of direct penetration into eukaryotic cells. Casein proteins belong to the class of proteins that do not have an ordered structure (IDP). We studied Lactaptin dynamics in solution by NMR and PRE and identified protein polypeptide chain fragments which differ in their local mobility. The work is supported by a grant from the Russian Science Foundation [14-14-00922].

P272

Assortment and optimization of NMR relaxation techniques for 13CH2, 13CH3 and NH2 groups of the protein side chains.

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Internal dynamics of the protein side chains is an optimal interaction sensor because they have a wider conformational space than the backbone and are directly affected by the interaction effects. This increases sensitivity and information value of the NMR relaxation data. Thus, experimental monitoring of the side chain mobility by means of NMR relaxation allows better understanding how the protein surface adapts to the interaction with the biological partner, which is especially important for understanding the functioning of the protein hot-spots at the atomic level. Up to date, for uniformly 15N/13C-labeled proteins, NMR relaxation measurement methods for aromatic, carboxil, carbonyl, NH and CH groups of the side chains are widely used. Herewith, for 13CH2, 13CH3 and NH2 groups, the classical approach of measuring R2, R1 and NOE encounters a number of experimental difficulties associated with 1JCC' and R2 immeasurability due to the multiexponential decay of the heteronuclear transverse magnetization caused by impossible effective suppression of cross-correlation contributions. For these groups, measurement of the R2 relaxation parameter can be replaced with direct observation of the cross-correlation contributions

itself: dipole-dipole cross-correlation contributions to R2 (Γ2CH,CH) and R1 (Γ1CH,CH), as well as CSA-dipole cross-correlation contributions to R2 (Γ2C,CH) and R1 (Γ1C,CH). The optimization of the pulse sequences for measuring the parameters Γ2CH,CH, Γ1CH,CH, Γ2C,CH and Γ1C,CH and analysis of the results allow us to reveal the systematic and accidental errors depending on the measurement method. The suggested graphical methods of interpretation made it possible to assess the informational value of the parameters as a sensors of the side chain internal dynamics. Examination of the effects of systematic and accidental errors and informational value allowed to arrange the parameters in order of usefulness depending on the task assigned. Appraisal of the designed techniques was carried out both on water-soluble proteins (with τc 3ns) as well as on membrane proteins (with τc 15ns), obtaining additional structural and dynamic information, overcoming the limitations and supplementing the conventional solution NMR approaches.

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P273

Ligand-induced variations in structural and dynamical properties within an enzyme superfamily

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Enzymes enhance rates of chemical reactions over 20 fold relative to uncatalyzed reactions. Evidence continues to emerge linking the functionally and evolutionary role of conformational exchange processes in optimal catalytic activity. Ligand binding changes the conformational landscape of enzymes, inducing long-range structural and dynamical changes. Using functionally distinct members of the pancreatic ribonuclease superfamily as a model system, we characterized the structural and dynamical changes associated with the binding of two mononucleotide ligands. By combining NMR chemical shift titration experiments with the chemical shift projection analysis (CHESPA) and relaxation dispersion experiments, we show that biologically distinct members of the RNase superfamily display discrete chemical shift perturbations upon ligand binding that are not conserved even in functionally-related members. Amino acid networks exhibiting coordinated chemical shift displacements upon binding of the two ligands are unique to each of the RNases analyzed. Our results highlight the contribution of conformational rearrangements to the observed chemical shift perturbations upon ligand binding. These observations provide important insights into the contribution of the different ligand binding specificities and dynamical effects on the observed perturbations associated with ligand binding for functionally diverse members of the pancreatic RNase superfamily.

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15N Transverse Relaxation Measurements are Deteriorated by the Deuterium Isotope Effect on 15N resulting from Solvent Exchange

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15N R2 relaxation measurements are key for the elucidation of the dynamics of both folded and intrinsically disordered proteins (IDPs). In this study, we show on the example of the intrinsically disordered protein a-synuclein and the folded domain PDZ2, that at near physiological pH and temperatures amide - water exchange can significantly change 15N R2 relaxation measurements, unless deuterium oxide is omitted as internal reference substance and the evolution of the antiphase Nx/y Hz coherence is sufficiently suppressed during the relaxation period of the NMR pulse sequence. The solvent exchange with deuterium in the sample buffer modulates the 15N chemical shift tensor via the deuterium isotope effect, adding to the apparent relaxation decay which leads to systematic errors in the relaxation data. This results in an artificial increase of the measured apparent 15N R2 rate constants which should not be mistaken with protein inherent chemical exchange contributions, Rex, to 15N R2. For measurements of 15N R2 rate constants of IDPs and folded proteins at physiological temperatures and pH, we recommend therefore the use of an external D2O reference in combination with the presented modified 15N R2 Hahn-echo based experiment which is also applicable to high-molecular-weight complexes. The combination allows for the measurement of Rex contributions to 15N R2 originating from conformational exchange in a time window from us to ms.

P275

Disrupting the ”primed orientation” of cardiac troponin C can lead to dilated cardiomyopathy

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Introduction The heritable cardiomyopathies are genetic conditions associated with abnormal cardiac muscle growth, fatal arrhythmias, and heart failure. Dilated cardiomyopathy (DCM) is a type of cardiomyopathy associated with thinned dilated ventricles that contract weakly. A number of DCM-associated mutations localize to cardiac troponin (cTn), a trimeric complex consisting of cardiac troponin I (cTnI), troponin C (cTnC) and troponin T (cTnT). The regulatory N-terminal domain of troponin C (cNTnC) is the central player in turning cardiac muscle contraction on and off in a calcium-dependent manner. The calcium sensitivity of cardiac contraction is regulated by phosphorylation of the N-terminal tail of cTnI [1-37]. The region is invisible in the X-ray crystal structure of the cardiac troponin complex. However, we previously used solution NMR to demonstrate that it interacts electrostatically with the cNTnC domain to position it to a “primed” orientation and indirectly increases calcium affinity by optimally positioning cNTnC to bind the cTnI switch peptide, cTnI [146-158].

Methods Cardiac troponin C (cTnC), cTnC D75Y, cTnC G159D, cTnC Q50R, cTnI [1-77], cTnI [1-77] K35Q, and cTnI [1-77] S41/43D protein constructs were produced in E. coli with 15N, 13C-isotopic enrichment, facilitating biophysical studies of their complexes by solution NMR.

Results 15N relaxation rates provide a window into nanosecond to picosecond (10−9 to 10−12 s) timescale conformational fluctuations in a protein. Using 15N T2 relaxation analysis, we demonstrate that in the absence of cTnI [1–77], the two domains of cTnC tumbled independently (T2 100 ms at 30 degrees Celsius). Upon addition of cTnI [1–77], the two domains became rigidly fixed, and the complex tumbled as a single unit (T2 35 ms). Pseudophosphorylation of cTnI Ser41/Ser43 by mutation to aspartate resulted in intermediate T2 times (T2 50 ms). Intermediate T2 times were also observed in complexes containing DCM-associated mutations: cTnI K35Q, cTnC D75Y, and cTnC G159D (T2 40 - 45 ms).

Conclusion The positioning of cNTnC is critically important because it binds to the switch region, cTnI [148–158], to initiate cardiac contraction. 15N relaxation studies revealed that pseudophosphorylation at cTnI Ser41/Ser43 or DCM mutations release the cNTnC domain from the primed orientation. Thus, we postulate that the primed orientation is needed for optimal activation of the cardiac troponin complex, and its dis-

ruption by mutations is what leads to DCM.

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The highly dynamic, helical structure of human growth hormone formed under acidic conditions

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Human growth hormone (HGH) adopts a four helical bundle fold with an up-up-down-down connectivity and long cross-over loops. Under acidic conditions, the backbone flexibility of the protein increases significantly with the experimental NMR 1H-15N order parameter values in some regions of the loops being below 0.2, comparable to those seen for an unfolded protein [1]. Molecular dynamics simulations of HGH at pH 3, with restraining to the 1H-15N order parameter data [2], provide a model for this highly dynamic state. The simulations show that low order parameter values seen for a few residues in the centre of the main helices in the structure arise from fluctuations between alpha-helical and 3-10 helical hydrogen bonding patterns while the loop regions contain some fluctuating secondary structure consistent with available experimental data [3]. The work provides a clear example of the advantages of combining MD simulations with experimental NMR data to obtain a structural interpretation of the latter.

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P277

Molecular Basis of Ice-Binding and Cryo-Preservation Activities of Type III Antifreeze Proteins

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Antifreeze proteins (AFPs) refer to a class of polypeptides produced by certain vertebrates, plants, fungi and bacteria that permit their survival in subzero environments. The type III AFPs have been categorized into two subgroups, quaternary-amino-ethyl (QAE) and sulfopropyl (SP) sephadex-binding isoforms, based on differences in their isoelectric points, and the QAE proteins can be further divided into QAE1 and QAE2 subgroups. The QAE1 isoforms exhibit full thermal hysteresis (TH) activity, whereas the SP and QAE2 isoforms are incapable of preventing the growth of ice crystal. We determined the antifreeze activity of three isoforms, AFP6 (SP), AFP8 (QAE1), AFP11 (QAE2) of the Japanese notched-fin eelpout (Zoarces elongates Kner) AFPs and characterized the structural and dynamics properties of their ice-binding surface using NMR. We found that the AFP6, AFP11 were unable to stop the growth of ice crystals and exhibited structural changes, as well as increased conformational flexibility in the first 310 helix of the sequence. Based on their backbone Dynamics properties, we designed three triple mutants nfeAFP6_tri (P19L, A20V, G42S), nfeAFP8_tri (Q9V/L19V/V20G), nfeAFP11_tri (V9Q/V19L/G20V) and compared their TH activities with Wild type protein. Our results suggest that the inactive nfeAFPs are incapable of anchoring water molecules due to the unusual and flexible backbone conformation of their primary prism plane-binding surface.

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Facilitated Protein Association via Engineered Target Search Pathways visualized by Paramagnetic NMR Spectroscopy

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Protein-Protein association can be described as a two-step process, with the formation of transient encounter complexes followed by a functional complex. We previously reported that the histidine-containing phosphocarrier protein (HPr) and the N-terminal domain of enzyme I (EIN) could form productive and non-productive encounter complexes. Here we demonstrate that protein association can be significantly enhanced by introducing alternative on-pathways using paramagnetic NMR spectroscopy and calorimetry. Modulations of surface charges away from the binding interface reshaped the target search process, leading to a 10-fold increase in binding affinity. The target search process revealed previously unexplored on and off-pathways, and blocking the on-pathway by countering mutations reverted the binding affinity. Our results illustrate that protein interactions can be engineered by rewiring the target search pathways.

P279

First kinetics description of the EcHU chain exchange system

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Found in all bacteria, HU is the major protein of the bacterial nucleoid (30,000copies/cell). In Escherichia coli, this protein exists in three dimeric forms, HUα2, HUβ2 and HU$\alpha\beta$, that exhibit different biological functions and different thermodynamic properties (specifically they don't exhibit the same temperature stability). They present a chain exchange mechanism conducting to the formation of the heterodimer from homodimers (HUα2+HUβ2$\leftrightharpoons$2HU$\alpha\beta$). The heterodimer formation is strongly temperature dependent and can be described as a three steps mechanism. First, The homodimers change from a "closed" native conformation (N2) to an "open" intermediate conformation (I2). Second, a transient tetramer is formed by associating the I2 homodimers. Finally, the tetramer splits into two heterodimers. N2 and I2 conformations of each dimer are in slow-chemical exchange at the NMR timescale. The hetero-tetramer can only be observed by native mass spectrometry with a population of less than 1%. It has also been shown that the homodimers themselves exchange their chains through a homo-tetrameric form leading the kinetics mechanism to be extremely difficult to decipher. In order to fully characterize this complex mechanism, extensive relaxation studies of the chain exchange were performed in function of the temperature. - 15N relaxation (R1, R2, NOE) was used to characterize the internal dynamics of the three dimers in their

N2/I2 conformations. - EXSY (zz exchange) was used to study the kinetics parameters of the first step of this complex mechanism. The exchange rates of the N2/I2 HUα2 transition at 310 K are kN2$\rightarrow$I2=2.7 s-1 and kI2$\rightarrow$N2=6.8 s-1. - 15N relaxation dispersion experiments were used on the three dimers to potentially bring out the tetramer signals or "hidden" I2 signals. -The global chain exchange mechanism was studied by NMR and native Mass Spectrometry (MS). It is a quasi-total 2nd order reaction exhibiting an observing rate of 8.5 10-5 s-1 at 293 K.

We have determined that: 1/ The intermediate conformation is more dynamic than the native dimer. Indeed, this dynamical property is related to the partial unfold of the intermediate conformation compared to the native one. 2/ The equilibrium between the two conformations at each temperature is rapidly reached. The HUβ2 I2 conformation is in excess compared to HUα2 I2 whatever the temperature is and serves as a reservoir to pump HUα2 I2 population to slowly form the tetramer. 3/ The limiting step is the association of the I2 dimers. The chain-exchange mechanism is driven by the affinity of the α-chain for the β-chain (and inversely). Knowing that the chain exchange also exists in the homodimers leads us to hypothesize that this mechanism is a necessity to form the heterodimer without a high energetic cost, and is also a type of self-chaperoning mechanism for the HU proteins.

P280

High-pressure NMR reveals water-protein interactions coupled with protein conformational transition

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Although motions of polypeptide chains on nanosecond-to-second timescales can be revealed by solution NMR spectroscopy, the location and dynamics of hydrated water in many proteins have not been fully understood. Here, we used phase-modulated clean chemical exchange (CLEANEX-PM) NMR approach to investigate pressure-induced changes in water-to-amide proton exchange occurring at sub-second time scale. With the transition of ubiquitin from its native conformation (N1) to an alternative conformation (N2) at 250 MPa, proton exchange rates of residues located at the C-terminal side of the protein were significantly increased. These observations can be explained by the destabilization of the hydrogen bonds in the backbone and partial exposure of those amide groups to solvent in N2.

P281

Dynamic salt bridge behaviour stabilizes alpha helices

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Single alpha-helical (SAH) domains are found in many different proteins. They are rich in Glu, Arg and Lys residues and their stability is suspected to arise from a network of stabilizing ionic interactions (salt bridges) between the charged side chains of these residues. Here we characterised the SAH domain from mouse myosin 7a using a combination of approaches. The salt bridge behaviour was interrogated by NMR using relaxation, through hydrogen bond couplings and intra-residual sidechain j-couplings and in silico modelling. Together the data show that the salt bridges formed within SAH domains are not fixed or long lasting, but exhibit dynamic behaviour.

P282

Conformational transitions in Polypyrimidine tract binding protein: coupling dynamics with function

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Polypyrimidine Tract Binding (PTB) protein is a key player in many processes in post transcriptional gene regulation such as alternative splicing and internal ribosomal entry site (IRES) mediated translation. Upon binding to a stemloop RNA with a UCUUU pentaloop occurring in the encephalomyocarditis virus IRES, the N-terminal RNA recognition motif domain (RRM) of PTB forms an additional helix α3 extending the canonical $\beta\alpha\beta\beta\alpha\beta$ RRM topology. Helix α3 docks to the β2-strand at one edge of the β-sheet. NMR relaxation studies show the free protein is highly dynamic exhibiting motions in the C-terminal half of the domain characteristic of partly folded proteins including a partially formed α3. Furthermore chemical shift perturbation of resonances from PTB RRM1 upon binding to a series of variant stemloop RNAs indicates that the degree of α3 helix formation is determined by the loop sequence. MD simulations provide insight into the coupling of stemloop RNA binding and organization of the α3 helix. Cellular assays using full length PTB and IRES RNA variants suggest that the degree of IRES activation is correlated with the formation of helix α3 and may indicate that this represents an allosteric mechanism to control assembly of activated IRES.

P283

Unusual temperature dependence of protein dynamics - towards understanding the enzymatic cold-adaptation by NMR

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The principles of protein cold-adaptation allowing enzymes to fulfil their functions under low (below 15C) and extremely low temperatures (around 0°C) constitute scientifically interesting and still largely unanswered question. Here we use the NMR spin relaxation methods to study dynamics of the cold-adapted Archeal analogues of the human Pin1 cis-trans isomerase[1] in the wide range of time scales, ranging from ps-ns down to us-ms, under different temperatures. To facilitate the analysis of the protein dynamics we use our newly developed methods of fast evaluation of the 15N spin relaxation data sets [2, 3]. We find that the cold adapted analogues are showing the optimal and functional dynamics pattern at their native temperatures. We discuss the origins of the sophisticated temperature dependences of the cold-adapted protein dynamics and associated

structural changes. These findings put new light on the protein motions underlying the pivotal importance of the protein dynamics, thus the functioning of enzymes under low temperatures.

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P284

Profits from nuclear magnetic relaxation - studies of motions in macrobiomolecular from deficient and robust experimental data sets

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The unsurpassed power of NMR spectroscopy among other high-resolution biophysical methods relies on the detection of molecular motions by means of nuclear spin relaxation in the broad time scale spanning 10 orders of magnitude. The most common 15N relaxation applied to proteins allows to track the backbone motions from ps-ns down to us-ms. Despite the numerous successful applications up to now, the biomedical problems require studies of more challenging proteins, with MW > 25 kDa, at low concentration < 150 uM, and high ionic strength >=150 mM NaCl. Moreover, their spectra are characterized by a great spectral dynamic range from signals arising from rigid and flexible parts of the protein. Here we will demonstrate novel fast method of NMR spin relaxation data analysis yielding the description of protein motions in the fast and slow time scales (1) and validate them with the multiple magnetic field data sets (2) determined for proteins ranging from 6.3 to 42 kDa. Presented method yields results closely similar to the traditional multiple field analyses (R2 > 0.95) while demands less computational power than other existing fast 15N spin relaxation data analysis methods. The limitations and applications to the high-molecular weight systems > 26 kDa of the presented approach will also be discussed (2).

P285

Automatic methyl assignment in large proteins by the MAGIC algorithm

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Selective methyl labeling is an extremely powerful approach to study the structure, dynamics and function of biomolecule systems by NMR, however, methyl resonance assignment remains one of the barriers to widespread use of the technique. Brute force mutagenesis approaches are time consuming and expensive and, for that reason, computational approaches have emerged. Assignment is achieved by correlating NOESY crosspeak patterns to crystal structure or an in silico template model of the protein using stochastic- or graph-theory-based algorithms. We have developed a computational approach MAGIG (Methyl Assignment by Graphing Inference Construct) that utilizes raw NOESY peak lists and exhaustive search. Methyls are not equally distributed in the protein hydrophobic core but instead are clustered into densely-connected local networks interspaced by aromatic and polar residues. MAGIC ranks the NOE networks based on their connectivity density and performs local exhaustive search hierarchically from high to low density based on the value of the element in the 'NOE density' matrix. In so doing, MAGIC alleviates the combinatorial problem. Following local network identification, the final global search step combines all the local assignment into the full output. MAGIC 2.0 now handles a wider array of methyl NOESY types including 3D HCH- and CCH- and 4D HCCH- HMQC-NOESY-HMQC for enhanced flexibility. When amide assignment is available, MAGIC 2.0 can utilize methyl-amide NOESY such as 3D NCH- or CNH- HMQC-NOESY-HMQC and 3D N,C- NOESY-HMQC data as an independent matching parameter. We evaluate and optimize the impact of backbone NOESY data on the assignment accuracy.

P286

If I only had an R!

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An important distinction between NMR spectroscopy and crystallography is the availability of an R-factor quantifying how well structural models explain the input data. This opens up a range of methodologies based on testing specific conformational hypotheses, where the R-factor represents an independent target function. Despite several attempts over many years, no analogous measure has been established in NMR spectroscopy, restricting the available palette of analysis and structure calculation methods. Here we demonstrate that an effective R-factor can be calculated for proteins is solution using the 3D CNH-NOESY1, which displays contacts to 15N-bound protons in a well-resolved 13C dimension. This spectrum exploits the higher dispersion and more homogeneous effective linewidths of the 13C dimension, while suppressing water-exchange cross-peaks and diagonal peaks. These represent decisive advantages in the accuracy of back calculation. The R-factor is calculated as the RMS difference between experimental and back-calculated data on a residue-by-residue basis for each amide proton. We show that it is highly sensitive to local conformational parameters. Further we show how an effective R-factor opens a range of new techniques in model building, refinement and validation, including novel spectral decomposition techniques designed to produce accurate representations of conformational ensembles. Combined, these techniques form a complete ab initio structure determination protocol, based on direct comparison of experimental and back-calculated data. Thus, interpretation of the data in terms of peak picking or conversion to distance or dihedral restraints is avoided and conformational diversity can be assessed in unrestrained molecular dynamics simulations. We anticipate the method will be of use in a wide range of cases where subtle conformation changes are important determinants.

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P287

Simultaneous assignment and characterization of proteins by integrated analysis of time-domain NMR data

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In traditional analyses of proteins by NMR, it is general to assign signals first and then analyze protein characteristics, such as structure, dynamics, and interaction. Chemical shifts of interested atoms, which are determined at the assignment step, are used to identify signals at the characterization step. We propose a new method by integrating these two steps so that we no longer require identification of signals according to the chemical shifts. In this study, we demonstrate an application to 15N relaxation measurements. For the assignment, we employed amino-acid selective isotope labeling (AASIL). In dual selective AASIL, observing amide signals with a 13C-15N correlation spectrum such as 2D HN(CO), one can assign a signal from an amino acid pair which appears only once in the sequence. Various combinatorial selective labeling (CSL) methods have been proposed to overcome a disadvantage that a simple dual selective AASIL requires many kinds of labeled samples. We also proposed a CSL method, named Stable isotope encoding (SiCode), using fractional labeling and quantitative analysis of signal intensities to enable discrimination of 19 non-proline amino acids with as few as three kinds of labeled samples. In our proposed method, spectra for the characteristic are acquired not with a uniformly labeled sample but with SiCode samples. A whole dataset, regarded as a single tensor, is decomposed. Each component, which corresponds to a single amide, contains information of both for the assignment and the characteristic. The "information for assignment" here means signal intensities among SiCode samples which can be converted to the amino-acid information. Therefore we can obtain the desired characteristic of amides which are assignable with the dual selective AASIL. We adopted canonical polyadic (CP) decomposition (also known as CANDECOMP or PARAFAC) model, which assumes that the tensor is a sum of components and that each component is a direct (outer) product of vectors. These assumptions are appropriate when intensities of elements of the tensor are modulated with the desired information. SiCode is suitable for this model since amino acid information is encoded on signal intensities. As well as 15N relaxation, we can measure other characteristics by intensity modulation, for example, paramagnetic relaxation enhancement (PRE), hydrogen-deuterium (H-D) exchange, and cross saturation There are two advantages of this method. First, overlapped signals can be separated according to differences in either or both of the amino acid and the characteristic, which is, in this case, the time constant of relaxation. Second, time-domain raw data, including non-uniformly sampled (NUS) data, can be directly analyzed, i.e. without spectral reconstructions, unless chemical shift values themselves are demanded information. This contributes to reduction of machine time especially in case that the measurement of the characterization is time-consuming.

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Product Operator Diagrams: Nice and Precise Pulse Sequences on the Back of an Envelope

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Understanding how pulse sequences work for the wider biological audience presents a complicated problem. Typically two approaches present themselves. In the most complete approach you start with the maths of product operators which gives a rigorous and unambiguous analysis at the cost of a high degree of abstraction. Alternatively vector models, possibly in combination with energy level diagrams, give a less abstract but somewhat incoherent explanation of what is occurring during a pulse sequence.

Here I present a new non classical vector diagram or product operator diagram (POD) that provides a nice and precise method for calculating the effects of pulse sequences using a purely graphical approach. This formalism provides a complete description of any pulse sequence working with weakly coupled spin 1/2 nuclei. It doesn't require recourse to formal mathematical approaches when thinking about a pulse sequence, but at the same time can always be converted to mathematical notation unambiguously. As an example I will use a simplified on resonance INEPT sequence that contains only three pulses to demonstrate how an HSQC works and how 1H-15N coherence transfer increases signal intensity. Other examples will be given and simple python scripts will be presented which provide interactive animated diagrams and translation between the mathematical and graphical formalisms.

At heart PODs are a complete description for product operators because they model two of the most important properties of product operators which were previously neglected. Specifically: they account for and follow the sign of operators during calculations and have the ability to label component operators (eg Iz and Sx in the term 2IzSx) of the product operators so they are distinguishable. With these additional tools in hand and working on the foundation provided by Donne and Gorenstein (1) and other previous approaches it is possible to think rigorously about product operators graphically. Therefore clear and complete diagrams of pulse sequences and pulse sequence fragments can be built without recourse to the formal maths previously required, while at the same time not losing sight of the formal maths required to make numerical predictions.

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P289

Inference of backbone dihedral angle distributions in intrinsically disordered proteins using cross-correlated relaxation

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Unlike folded proteins, intrinsically disordered proteins (IDPs) are characterized by high conformational flexibility. Consequently, experimental observables represent ensemble averaged quantities not sufficient to capture the underlying ensemble in full detail. The present study considers this problem in terms of the classical Ramachandran map: While backbone dihedral angles of folded proteins are readily accessible by NMR, their distributions in IDPs are inherently underdetermined.

In light of this ambiguity, the questions arises how experimental observables are to be interpreted in an epistemologically sound and coherent fashion. For this purpose, a general and robust Maximum Entropy framework is derived from established principles of statistical inference in order to assess the experimental accessibility of backbone dihedral angles in folded

and unfolded proteins alike.

The general feasibility of the approach is illustrated using cross-correlated relaxation (CCR) experiments which prove highly proficient and versatile in probing dihedral angles. The diverse range of backbone interactions accessible by CCR cannot be matched by more conventionally used scalar couplings. This key feature allows for the assessment of previously unexplored interactions and the development of novel pulse sequences specifically fit for IDP studies. Considering both the structural and dynamic insights CCR experiments provide, we argue that their potential in IDP characterization has long been overlooked. Applications to selected model systems will serve as illustrations.

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Comparison of N-site approximations for CPMG and R1ρ experiments

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N-site chemical exchange in proteins and nucleic acids can be analyzed by R1ρ and CPMG experiments. Most N-site examples explore the 3-state linear case, most likely due to the availability of appropriate models and methods. A triangular kinetic scheme has been discovered previously for E-Cadherin, which can proceed to the dimer form either directly or via an intermediate that has been termed the X-dimer (Li, Y, Altorelli, NL, Bahna, F, Honig, B, Shapiro, L and Palmer, AG (2013), Proc Natl Acad Sci USA 110). Another potentially large group of N-site, particularly 4-site, processes arise through coupled equilibria for allosteric processes (Li, P., Martins, IR, and Rosen, MK (2011), J Biomol NMR 51). Complex N-site exchange processes were qualitatively identified in the mechanism of dynamic allosteric communication in PLCγ1 (Koss, H, Bunney, TD, Esposito, D, Martins, M, Katan, M, Driscoll, PC (2018), Biophys J, in press). Lowering the practical and theoretical hurdles to perform and analyze R1rho and CPMG experiments will help to shed light on N-site processes and further the application of these powerful techniques to biological systems. Previously, we have obtained general analytical expressions for arbitrary N-site exchange models in R1rho relaxation, as long as the population at one site dominates (Koss, H, Rance, M, and Palmer, AG, 3rd. (2017), J Magn Reson 274, 36-45). Closed-form expressions for CPMG relaxation rates are more difficult to obtain because evolution of magnetization is more complex in the presence of a refocusing pulse train than during spin locking. Nonetheless, we are now presenting analytical expressions for arbitrary kinetic schemes in context of CPMG relaxation. The new equations also improve the accuracy of expressions for R1ρ experiments. Numerical fitting of data to more complex schemes can be difficult (Li, P, Martins, IR, and Rosen, MK (2011), J Biomol NMR 51). Our approximations can be used in efficient fitting algorithms, but they also assist to qualitatively understand the relationship between various kinetic parameters and characteristic features in the experimental data. With a compact expression for CPMG rates in a triangular kinetics scheme we are able to rationalize certain features in the CPMG curve at the fast and slow pulsing limits. One question arising from the ability to model arbitrary N-site kinetic schemes is whether and how best to distinguish between different N-site schemes in order to facilitate the choice of the appropriate kinetic models. We show that analytical expressions are useful to address this question, and we supplement them with numerical calculations. Our studies help to not only facilitate data analysis, but also support choice appropriate experimental conditions. We also provide interactive tools which can be used when being confronted with the possibility of N-site exchange in the context of CPMG and R1ρ experiments.

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Application of Deep Neural Networks to Highly Accurate NMR Structure Analysis by Deep-MagRO

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Deep neural networks are a rapidly developing technology that has enabled a computer to gain a visual inspection capability like the human eye. We have developed a new function for noise/signal discrimination using Convolutional Neural Networks (CNN) in an integrated tool for NMR analysis named "Deep-MagRO". The new function enables fully automated peak identification even in the presence of a huge number of artifacts caused, for instance, by sinc-function type noise, base-line noise, satellite noise from incomplete decoupling of hetero-nuclear spins, and bulk water noise. In combination with the external program FLYA, one can perform fully automated NMR chemical shift assignments and structure calculation using the filtered peak lists from a limited number of 2D and 3D NMR spectra. In the presentation we will demonstrate the feasibility of convolutional neural networks for obtaining highly accurate assigned chemical shifts and three-dimensional structures for several small proteins.

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The Chemical Shift Z-score for assessing Order and Disorder in Proteins

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The chemical shift is the single most easy obtainable parameter from NMR experiments, can be measured with very high precision, and carries important information on molecular structure and dynamics. Here we introduce the Chemical Shift Z-score for assessing Order and Disorder in Proteins (CheZOD Z-scores), which quantifies the deviation from random coil chemical shift (RCCS). We demonstrate that the CheZOD Z-score is closely related to local order/disorder of a protein. Here we reveal four new important findings: (i) By analyzing sequence specific CheZOD Z-scores for 117 proteins, we reveal the underlying patterns in disorder profiles: that there is order in disorder: a full spectrum of disorder, but still, largely a segregation into two classes1. (ii) We present a new method, POTENCI, for the prediction of RCCSs that outperforms the currently most authoritative methods2. (iii) Principle component analysis of the signed deviation from RCCS is used to discriminate between the different secondary structures and identify hydrogen bonding. (iv) We apply the database of the 117 proteins with derived CheZOD Z-scores to benchmark and rank the performance of a large set of widely used software for predicting disorder from sequence in proteins.

P293

A fast computational method for predictions of allostery and conformational coupling using rigidity theory

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Advancements in the field of rigidity theory have led to developments of fast computational predictions of protein flexibility and their dynamics. Allostery (distant conformational coupling) can be viewed as an effect of binding at one site of the protein to a second, often significantly distant functional site, enabling regulation of the protein function. In spite of its importance, the molecular mechanisms that give rise to allostery are still poorly understood. We have recently developed rigidity-transmission allostery (RTA) algorithm, an extremely fast computational method based on mathematical algorithms in rigidity theory. RTA algorithm provides a mechanical interpretation of allosteric signaling and is designed to predict if mechanical perturbation of rigidity (mimicking ligand binding) at one site of the protein can transmit and propagate across a protein structure and in turn cause a transmission and change in conformational degrees of freedom at a second distant site, resulting in allosteric transmission. We will illustrate our method, identification of novel allosteric sites and a detailed mapping of allosteric pathways, which are in agreement with NMR data studies on 3 different class of proteins: GPCRs [Nature Communication 2018], enzyme fluorocatate dehalogenase [Science 2017], eukaryotic translation initiation factor eIF4E and others. RTA method is computational very efficient (takes minutes of computational time on standard PC) and can scan many unknown sites for allosteric communication, identifying potential new allosteric sites.

P294

A novel chitin-binding platform of the chitin binding domain of chitinase A1 from Bacillus circulans WL-12 revealed by solid-state NMR

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Chitin is a huge insoluble complex of beta-1,4-linked homopolymer of N-acetyl-D-glucosamine found in many kinds of biological architectures such as shells of crabs and shrimps, exoskeletons of insects, eggshells of nematodes, and cell-walls of fungi. Bacillus circulans WL-12 chitinase A1 is efficient in hydrolyzing insoluble chitin. It comprises three kinds of domains, namely, an N-terminal catalytic domain, a C-terminal chitin-binding domain, and two fibronectin type III domains in the middle. The high efficiency of this chitinase comes from its unique chitin-binding domain (ChBDChiA1). ChBDChiA1 composed of 45 amino acid residues binds to only insoluble chitin. It cannot bind to any other polysaccharides or soluble derivatives of chitin. Furthermore, its binding did not fit to the mechanism accepted for major carbohydrate-binding modules (CBMs), suggesting that its chitin binding mechanism is unique. However, it was difficult for solution NMR and X-ray crystallography to analyze the chitin-binding of ChBDChiA1 because the complex was huge and difficult to be crystallized. We could directly analyze the complex of ChBDChiA1 and insoluble chitin by the use of solid-state MAS-NMR (ssNMR). First of all, the assignment of 13C and 15N signals of microcrystalline ChBDChiA1 was carried out. On the basis of assignment, the dihedral angles phi and psi of the main chain were estimated from the chemical shifts of 13CA, 13CB, 13C', and 15NH by TALOS+. The result was consistent with the reported solution structure composed of five beta-strands [1], suggesting that the ChBDChiA1 structure in crystal was similar to that in solution. Then, we examined the chemical shift perturbation on chitin-binding, using powder chitin and ssNMR. Chemical shift changes induced by the chitin binding could be identified for thirteen residues. Among them, A685, E688, N691, and V692 are located around the W687 ring. The perturbation was the greatest at W687. This is consistent with the lethal effects of mutations at W687 on binding, but inconsistent with the reported binding modes, involving multiple aromatic rings on the CBM surface. Furthermore, a mutation work suggested the involvement of Q679 in the binding to insoluble chitin [2]. Q679, E688, N691, and V692 line up in parallel to the W687 ring. Therefore, these residues are expected to work together in binding to insoluble chitin, providing a novel platform for the interaction with insoluble chitin. References 1. Ikegami, T., Okada, T., Hashimoto, M., Seino, S., Watanabe, T., and Shirakawa, M. (2000) J. Biol. Chem. 275, 13654-13661. 2. Hara, M., Sugimoto, H., Uemura, M., Akagi, K., Suzuki, K., Ikegami, T., and Watanabe, T. (2013) J. Biochem. 154, 185-193.

P295

Structural characterization of human microtubules by solid-state NMR

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Microtubules (MTs) are associated with many biological processes including cell migration, mitosis, and polarization. They are assembled by α- and β-tubulin heterodimers and represent highly dynamic polymers with a fast-growing plus end and the slow-growing minus end. The MT dynamic instability depends on the GTP/GDP state of tubulin on the plus ends. Many microtubule-associated proteins (MAPs) interact with MTs and regulate MT assembly and disassembly. Atomic insight into these processes has so far been limited.

Recently, we have shown how solid-state NMR (ssNMR) can be used to study binding of an isotope-labeled protein ligand to MTs at atomic level1. Extending such studies to the direct ssNMR-based analysis of MT's has so far been precluded by the difficulty of protein purification and obtaining adequate isotope labeling. We have developed a novel protocol which yields approximately 2 mg of 13C, 15N labeled MTs from 2 L mammalian cell culture, allowing us to perform structural studies on MTs by using fast magic angle spinning (MAS) and Dynamic Nuclear Polarization (DNP) supported ssNMR. In our contribution we present first results highlighting the potential of our approach to study structure and chemical modifications of human microtubules at atomic level.

1. Atherton, J. et al. A structural model for microtubule minus-end recognition and protection by CAMSAP proteins. Nature Struct. Mol. Biol. (2017).

P296

Probing water accessibility in membrane proteins

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Water is a crucial part of every protein's native environment and water accessibility contributes to the themodynamic stability and function of the protein. Using solid state NMR spectroscopy, the method of choice for studying membrane proteins in near-native conditions, we present new 3 and 4 dimensional experimental schemes for probing the environment of a protein in a residue specific manner at ultrafast magic angle spinning (>55000 kHz). We explored different magnetization transfer schemes to yield information about water exposed residues of a membrane embedded protein. The application of the method is demonstrated on several membrane proteins.

P297

Rapid L1 signal reconstruction for multidimensional MAS solid-state NMR

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Magic angle spinning (MAS) solid-state NMR can exhibit tremendous power for large and/or insoluble proteins e.g. membrane proteins, amyloid fibrils and protein-antibody complexes. Recently, many informative spectra have been obtained from the challenging targets. However, signal assignment prevented by signal overlapping in 3D spectra is one major bottleneck of the analysis. A promising way to solve this problem is measuring 4D spectra. To record 4D spectra in sufficient digital resolution in practical time, fast data sampling methods is required. We developed a rapid signal reconstruction technique by L1-norm minimization that can utilize a lower dimensional spectrum as prior information, named as SIRUP.

To demonstrate NMR data reconstitution ability of SIRUP, 4D-CANCOCA and CONCACO measurements of GB1 were performed by uniform sampling. To reduce the measurement time, indirect 15N, 13CA and 15CO dimensions were recorded at 10 complex points, then the maximal evolution times were 2.8, 1.6 and 2.8 ms, respectively. The experiment time of CANCOCA and CONCACO with 8 transient scans and 3.0 s repetition delay was 57 h. These spectra were reconstructed using CANCO and CONCA spectra as the prior information. The computational time of the SIRUP reconstruction was 10 times shorter than conventional iterative soft-thresholding (IST) method. This clearly shows the prior information is effective to save computational cost of compressed sensing. The obtained 4D peaks in the list were assigned in combination with a 3D NCACX peak list by an automated signal assignment program, FLYA. These results were compared with manually assignments performed for CANCO, NCACO and NCOCA spectra. The reconstructed CANCOCA and CONCACO spectra provided all peaks excepting for a K10CO-T11N-CA-CO peak on the CONCACO spectrum. Thus, the reconstructed spectra gave almost complete sequential connectivities.

Recent sensitivity improvement in MAS solid-state NMR by 1H direct detection or dynamic nuclear polarization allows us to measure 4D or higher dimensional spectra. For such measurements, fast data acquisition and signal reconstruction techniques should be used mandatorily. Our SIRUP is a helpful method for upcoming multidimensional measurements.

P298

Elucidating Structure and Dynamics of the Nucleosome by Solid-state NMR

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DNA in eukaryotic cells is organized in nucleosomes formed by 145-147 bp DNA wrapping around the histone octamer (HO) composed of the four histone proteins, H2A, H2B, H3 and H4. Both the flexible histone tails and the globular domains play key roles in genomic regulation. We have implemented solid-state NMR (SSNMR) to investigate the detailed structure and dynamics information for human histone H4 (hH4) in the nucleosome core particle (NCP) and in a nucleosome array. Remarkable spectral resolution was achieved and near complete backbone resonances were assigned for residues N25-G101, enabling the successful secondary structure prediction for the hH4 in the NCP precipitated with Mg2+. Furthermore, site-resolved dynamics on nanosecond to microsecond and microsecond to millisecond timescales were elucidated, revealing the existence of diverse internal motions in the hH4 protein. Relatively higher flexibility was observed for residues participating in the regulation of chromatin mobility and DNA accessibility. In addition, our SSNMR study revealed that hH4 in the nucleosome array adopts the same structure and show similar internal dynamics as that in the NCP assembly while exhibiting relatively restricted motions in several regions consisting of residues in the N-terminus, Loop 1 and the α3 helix region. The current research results shed new light on the contribution of dynamics to nucleosome stability, sliding and unwrapping as well as DNA accessibility.

P299

Multi-dimensional solid-state NMR on the light-driven Sodium Pump KR2

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Krokinobacter eikastus rhodopsin 2 (KR2) is the first light-driven sodium pump which translocates Na+ to the extracellular side [1] whereas protons are only pumped in the absence of Na+ [2]. As compared to only proton pumping rhodopsins such as bacteriorhodopsin (BR) and proteorhodopsin (PR), KR2 contains two neutral residues at positions occupied by proton donors and acceptors, i.e. N112 and Q123 (D85/D96 in BR; D97/E108 in PR) [3,4,5]. Due to successful application of other microbial rhodopsins as optogenetic tools [6], KR2 can be considered as a promising neural silencer. Solid-state nuclear magnetic resonance (ssNMR) is a powerful tool for unveiling the ion transfer and selectivity mechanisms. So far, only three other species of seven transmembrane rhodopsins have been assigned via ssNMR so that the here presented, first chemical shift assignment on KR2 is a first step towards linking its 3D structure with functional data. High magnetic fields applied to uniformly, reverse and forward labelled KR2 proteoliposomes in combination with advanced methodological concepts such as non-uniform sampling and optimal control pulses allowed the acquisition and analysis of up to 4D highly resolved homo- and heteronuclear MAS-NMR spectra. The current assignment covers more than 60% of the overall amino acid sequence and includes numerous functional important residues which are supposed to be mainly involved in the ion selectivity and pumping mechanism, such as the unique D116 and Q123.

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P300

Characterization of microbial poly(γ-glutamic acid) and its polymer complex by solid NMR

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Molecular structure analysis of microbial poly(γ-glutamic acid) (γ-PGA) with different D/L ratio and its polymer complex was done by IR and solid NMR. γ-PGA is a naturally occurring biopolymer consisting of a glutamic acid, in a certain combination of D-form and L-form (γ-(D,L)-PGA), all D-form (γ-D-PGA), or all L-form (γ-L-PGA). Sodium salt of γ-(D,L)-PGA (D/L = 8/2, 7/3, and 5/5) and γ-L-PGA (D/L = 0/10) were used as received. γ-(D,L)-PGA (free acid form) does not dissolve in water, but its sodium salt γ-(D,L)-PGANa is water soluble. However, γ-L-PGA (free acid form) and its sodium salt γ-L-PGANa are both water soluble. Free form of γ-(D,L)-PGA and γ-L-PGA were obtained as a precipitate or film cast from acidic aqueous solution, respectively, in which pH is lower than pKa. Carbonyl carbon peak in 13C solid NMR spectrum of γ-(D,L)-PGA differs from that of γ-L-PGA. γ-(D,L)PGA has an sharp peak at 171 ppm in addition to that of γ-L-PGA. Conformations of γ-(D,L)-PGA and γ-L-PGA in water is reported as follows: γ-(D,L)-PGA takes parallel β-sheet form in an acidic solution, and random coil form in an alkaline solution. γ-L-PGA (free acid form) takes α-helix, and γ-L-PGANa random coil form. In a free acid form of both γ-(D,L)-PGA and γ-L-PGA have strong IR peek at 1735cm-1. There may be intra- and intermolecular carboxyl group dimer. In acidic solution, γ-(D,L)-PGA forms parallel β-sheet conformation, and γ-L-PGA forms α-helix conformation. Thus, γ-(D,L)-PGA has strong intermolecular hydrogen bonding and shows sharp peak at 171ppm. On the other hand, γ-L-PGA does not have intermolecular hydrogen bonding and does not show such peak. Sodium salt of both γ-(D,L)-PGA and γ-L-PGA take random coil form. Thus, γ-(D,L)-PGA and γ-L-PGA show similar spectrum without peak at 171ppm in solid NMR and at 1735cm-1 in IR spectra. Structural analyses of poly-ionic complex of γ-PGA will also be discussed.

P301

Study of bacterial LPS and outer membranes using solid state and DNP NMR

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Cell membranes form the defining interface of life. They are essential for cell integrity, mediate cell signalling, regulate cellular conditions and support the function of membrane proteins. Modern biofermentative production of industrial chemicals places high demands and stress on the membranes of production organisms. We seek to understand the mechanism of membrane toxicity in the presence of industrial chemicals to inform bacterial chassis design and membrane engineering for high levels of tolerance to toxic chemicals. The cell envelope in Gram-negative organisms, often used as cell factories, consists of plasma membrane (IM) and outer membranes (OM), encompassing an important periplasmic compartment. In this study we use solid state and DNP MAS NMR to investigate the structure and stability of model membranes, as well as outer bacterial membranes in the presence of high levels of industrial chemicals. The outer bacterial membranes are asymmetric bilayers with a lipopolysaccharide (LPS) outer leaflet and a phospholipid inner leaflet. We use solid state wideline and MAS NMR to characterise the stability of model membranes in the presence of industrial chemicals. We also use dynamic nuclear polarisation (DNP) enhanced spectroscopy to investigate the structure of E. coli LPS-containing model membranes. We use 31P filtered 13C-13C correlation spectroscopy to impose spatial selectivity and highlight the LPS core region, where we seek to identify the location of phosphorylation and pyrophosphorylation, responsible for stability and integrity of bacterial OMs. This selection allowed identification of a few phosphorylated saccharides on the background of hundreds of units forming the core and O-antigen regions of LPS. Solid state NMR is an ideal tool for investigating cell membrane stability in the presence of industrial chemicals. Modern developments in "green" manufacturing of monomeric precursors of plastics, using non-food biomaterials aiming to replace petrochemical sources, demand a detailed understanding of membrane stability and toxicity. We investigate the effect of methacrylate esters (MAEs) and model membranes with varied chain composition, exploring chain length and unsaturation. Wideline 31P NMR was used to monitor lipid bilayer integrity in the presence of MAEs, while 13C MAS NMR was used to follow changes in lipid phase transition. Our results show MAE incorporation and high tolerance of membranes to MAE. Membrane saturation with MAE has allowed us, for the first time, to uncouple the chain melting contributions from trans-gauche isomerization and cooperative chain dynamics.

P302

Making sense of antisense. High-resolution structural information of oligonucleotides: in-vitro to in-cell NMR

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Synthetic antisense oligonucleotides (AONs) can be designed to target specific mRNAs and thereby effectively silence the expression of those genes, making them prime candidates for therapeutics against cancer and other diseases. However progress has been slow due to unspecific off-target-effects, and the difficulty in understanding the cellular uptake and the mRNA targeting mechanism of AONs.[1,2]

Firstly, we present how in-vitro NMR can shine a light on this targeting process. RNAs and/or proteins, which are relevant for the AON's function, were identified and complexes of the oligonucleotide with those RNAs/proteins were studied.

While these experiments can give detailed in-sight on structure and dynamics of an oligonucleotide in this reconstituted minimal complex, a clear limitation is that simplified aqueous solutions do not allow full comprehension of the cellular environment and how it influences the behaviour of oligonucleotides in the living cell. Currently there is a distinct lack of methods for understanding structures and function of target-engaged antisense drugs with atomic precision directly in living cells.[3]

We hypothesize that in-cell NMR can deliver exactly this missing tool. Therefore, we have secondly investigated the oligonucleotide in intact human cells. Sample stability over time, as well as the AON being present in a large complex with other (big) molecules in its relevant state, represent the main challenges. We will present methods allowing us to tackle those challenges and obtain in-cell NMR data on previously invisible AONs in cells, in their biologically relevant complex with RNAs/proteins, e.g. their target engaged state.

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P303

Conformational Variation in Cellular Proteins Observed by In-Cell NMR Spectroscopy

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In-cell NMR spectroscopy is a technology that can investigate atomic-scale protein structure in living cells. In the presentation, we firstly demonstrate the utility of in-cell NMR for monitoring cyclical biochemical reaction states and conformational changes in a membrane-associated protein of biological and clinical relevance. We site-specifically labelled the 21 kD tumorigenic oncogene product H-Ras with p-trifluoromethoxyphenylalanine (OCF3Phe) at Tyr32 of the switch I region, one of key residues in intracellular signaling pathways. High-yield soluble expression of full-length H-Ras protein, which has been difficult with living E. coli expression system due to highly reactive cysteine residues at its C-terminal hyper variable region (HVR), therefore almost all of in vitro structural studies were carried out with H-Ras without HVR, was successfully achieved by using E. coli cell-free protein synthesis. In mammalian cells in vivo, we used 19F NMR to measure the integration of exogenous H-Ras into endogenous metabolic lipid and trafficking pathways. The sensitivity of 19F in-cell NMR enabled the identification of distinct GTP-bound active and GDP-bound inactive forms of wild-type H-Ras in endoplasmic compartments and plasma membranes. In addition, a Ras mutant (C181S/C184S), which is unable to be transferred to plasma membranes so that stays only in endoplasmic compartments, exhibited conformational multiplicity. It strongly suggested that guanin-nucleotide exchange of Ras occurs in endoplasmic compartments as well as in plasma membranes. In contrast, dominant-negative Ras mutant (S17N), which was reportedly predominant in GDP-bound form in vitro, showed conformational stereotypy. In-cell NMR can thus be used to observe fine-scale conformational changes in large membrane-associated proteins, presaging the use of this technique in integrated structural and functional studies. The utility of this method in eukaryotic cells has remained limited to the determination of small cytosolic proteins and their molecular crowding properties. Next, we demonstrate the utility of in-cell NMR for investigating the correlation between the cellular health condition and the protein folding state in living cells. We used the bioreactor system to maintain appropriate cell culture conditions during the experiments by continuously supplying fresh medium. We employed the centrifuge-based alginate gel encapsulation method for cell immobilization to establish highly reproducible system. We have then investigated human Adenylate Kinase 1 (hAK1), which is a cytosolic enzyme catalyzing the reversible phosphoryl transfer reaction of adenine nucleotides in cells. Interestingly, the folding state of hAK1 in cells was significantly influenced by the cellular health condition. hAK1 was induced to be denatured in cells by exposed to stressful culture conditions without fresh medium supply, while it remained functional and properly folded for at least one day in healthy cells by continuously supplying with fresh medium. Our results indicated that the proper regulation of the physiological condition is crucial and will provide a critical guide to in-cell NMR analyses.

P304

Atomic resolution insights of the molecular interactions of proteins in living cells

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In the last decade, an enormous effort has been made in order to obtain structural and functional information on biological macromolecules in their native cellular environment. It led to the development of several methods for the study of molecules inside living cells. Due to the advantage of providing structural insights at atomic resolution, NMR spectroscopy has been recently applied to study proteins within prokaryotic as well as eukaryotic cells.

In order to improve current methods for in-cell NMR studies of human proteins, we recently developed a novel system to monitor the delivery of isotopically-labeled recombinant proteins into mammalian cells. This tool allowed us to experimentally test the best conditions for the delivery and integrity of proteins in a fast and reliable way. Using an optimized protocol that resulted from these analyses, we successfully delivered several proteins with markedly different structural properties into cultured mammalian cells. Our data indicates that the function of proteins introduced into mammalian cells is preserved. However, it strongly depends on the experimental conditions used. In agreement with this, heteronuclear (1H-15N HMQC) multidimensional in-cell NMR experiments revealed that certain proteins retain their structure when introduced in human-derived cells. But more importantly, structural changes resulting from the physical interaction of the delivered proteins with intracellular partners are also observed

and quantified.

As an example of this, we found that alpha-Synuclein (αSyn), an IDP related to Parkinson's disease with roles in vesicle trafficking and dynamics, physically interacts with certain molecular chaperons in the cytoplasm. This interaction is transient but sustained in time, and is critical for the disordered nature of αSyn in the cytoplasm. Indeed, when chaperons are inhibited or silenced, αSyn strongly associates to intracellular vesicles adopting a compact structure mainly in its N-terminus. As binding of αSyn to membranes leads to its aggregation into insoluble high molecular weight species, a process believed to be a causative role for Parkinson's disease, elucidating this interaction by in–cell NMR might uncover the mechanisms that underlie this neurological disorder.

P305

CMOS transceivers for compact architectures and nL to sub-nL high performance NMR micro-sensors.

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CMOS technologies allow for the implementation of miniaturized electronics and they can be used to realize pulsed NMR probes that are compact and versatile. In one implementation, single-chip CMOS transceivers have been interfaced with external (i.e., off-chip) excitation-detection resonators, proving to be valuable tools for the manufacturing of compact instrumentation. In probes with sample volumes ranging from a few microL down to 100 nanoL, such configuration delivered state-of-art performance and was used to demonstrate high-resolution multi-nuclear measurements [1] and broadband magnetometry, also including quadrature detection capabilities [2]. As a result, CMOS chips interfaced with external resonators simplify the construction of compact NMR probes, reduce their costs, and enable novel architectures.

CMOS technologies were also used to implement ultra-compact probes, where multilayer micro-coils are co-integrated on the same chip with the transceiver electronics. This type of probe allows for an exceptional degree of versatility and state-of-art performance for the analysis of microscopic samples, i.e. having volumes ranging from 10 nanoL down to 100 picoL. By using an ultra-compact probe having a sensing region of about 200 picoL and a spin sensitivity of about 10^{13} spins/sqrt(Hz) at 7 T we demonstrated direct reading of endogenous compounds in sub-nL eggs of microorganisms [3]. More recently, the combination with cutting-edge microfabrication techniques also allowed for spectroscopy of sub-sections of intact C. elegans worms [4]. In this poster we focus on the advantages of CMOS ultra-compact architectures with respect to alternatives by comparing performance and geometrical features, indicating the path towards high-sensitivity CMOS-based NMR micro-sensors that will enable NMR on samples that are currently out of reach, such as large unicellular microorganisms, micro-tissues derived from stem-cells, and even mammalian embryos (humans included).

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P306

Measuring Real-Time Metabolism in Mammalian Cells by NMR

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We have recently proposed real-time NMR as an approach to monitor biochemical events by following a continuous time course of metabolism in living human cancer cells. In contrast to other metabolic methodologies that sample at discrete time points the continuum of data from real-time NMR avoids loss of information between data points. This has previously been demonstrated by the Günther group at the University of Birmingham by embedding primary chronic lymphophatic leukaemia cells into agarose to measure the kinetics of metabolic turn-over during a 24 hour period [1]. This experiment has now been considerably improved by integrating the InsightMR reaction monitoring system, supplied by Bruker, enabling real-time NMR experiments for mammalian cells over extended periods of time. The approach we propose consists of a closed-loop flow system integrated with a bioreactor and cell culture incubator, suitable to examine human cells in an NMR laboratory without exposure to the surrounding environment. Using NMR to monitor cellular metabolism offers unprecedented opportunities to study human cells and is directly applicable to cells derived from human blood including cells from blood cancers. Future applications may facilitate the testing of drugs on primary patient cells in a personalised disease setting.

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P307

Planar waveguide NMR for in-vitro metabolomics in 3D-cell culture models

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Along with the other fields of omics research metabolomics has accelerated the elucidation of fundamental cellular functions and their corresponding biochemical pathways. The concentrations and dynamics of the metabolites not only give direct evidence of the cells' physiological condition like normal or pathogenic. The metabolome shows also the fastest and most sensitive response to the changes in the microenvironment which can be used to study the cellular response to pharmaceuticals or other stress factors. Nevertheless, the role of metabolites is complex and dynamic and they are involved in many essential life processes. Resulting from this, new measurement techniques must be invented or improved to enable in vitro studies on appropriate cell culture model systems within suitable microenvironment conditions. The planar microslot NMR probes we are presenting enable us to measure the metabolites of a multicellular 3D spheroid in.[1,2]Spheroids consisting of approximately 9000 living cells were placed inside a microfluidic glass device with dynamical media and oxygen supply. An on-board temperature sensor and heater accurately controls the temperature in order to maintain the viability of the cells at near physiological conditions for the long-term in vitro studies. In order to study transport, a B1 gradient NMR detector technique delivers spatially resolved NMR information, which can be used to study cellular spheroids patho-physiolgical gradients. This method preserves the anatomical and functional integrity of the cells and enables a time resolved analysis of the living spheroids' metabolic profile and can give a dynamic picture of the

biochemical reaction inside a single spheroid. With our instrumental and methodological development the complex metabolic profiles as well as the metabolic adaptation of the cells to their (changing) microenvironment can be studied in vitro over extended temporal periods. The technique has no special sample requirements, i.e. tissues, tissue like samples, cells grown in monolayers, and even cell organelles like mitochondria can be measured adequately. Labor-intensive sample preparations and pretreatments like metabolite extraction are not necessary. [1] Krojanski HG, Lambert J, Gerikalan Y, Suter D, Hergenroeder R., Anal. Chemi., 2008, 80, 8672. [1] Kalfe A, Telfah A, Lambert J, Hergenröder R., Analytical Chemistry, 2015, 87,7402.

P308

Pure shift heteronuclear 2D J-resolved spectroscopy for improved isotopic profiling in fluxomics

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Measurement of the carbon isotopic content of different metabolites is a powerful tool to quantify fluxes in living systems. In principle, this information is inherently present in 1H NMR spectra through both one-bond and long-range 1H-13C J-coupling constants. However, in practice the analysis of these couplings is strongly hindered by two issues. First, especially in complex metabolite mixtures, coupling information from each 1H site is difficult to resolve because of overlap between their multiplets, which are composed of both 1H−1H and 1H−13C couplings. A conceptually simple way to avoid this issue is 2D J-resolved (2DJ) spectroscopy, which disperses the multiplets along a –45° axis in the 2D spectrum, effectively separating chemical shift and coupling information. However, the classical experiment suffers from the phasetwist lineshape, which is highly unfavorable for resolution and coupling accuracy. Current heteronuclear 2DJ methods for 13C fluxomics therefore apply adiabatic z-filtration to obtain pure absorption mode lineshapes, but at the severe cost of no longer separating coupling and chemical shift information. [1,2] Second, even in the absence of multiplet overlap, long-range 1H-13C couplings are very challenging to resolve, since (as opposed to one-bond couplings) they are similar in magnitude to 1H-1H couplings.

Here, we present a new heteronuclear 2DJ experiment building on developments in pure shift NMR spectroscopy [3] that solves all these issues. First, by combining the Pell-Keeler method [4] and the PSYCHE element, [5] a heteronuclear 2DJ spectrum is obtained with pure absorption mode lineshapes that retains complete separation of coupling and chemical shift information. This allows resolving one-bond 1H-13C couplings at pure shift resolution and thus quantification of the 13C content. Furthermore, by introducing a pure shift acquisition scheme — similar to the recent PSYCHEDELIC experiment [6] — we can suppress all 1H-1H couplings in the 2DJ spectrum. This resolves long-range 1H-13C couplings, providing access to isotopomer distributions. We demonstrate the power of this method on cell lysates from different bacterial cultures and investigate in detail the branched chain amino acid biosynthesis. [7]

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P309

Workflow optimization of metabonomic NMR spectra processing at the Taiwan Biobank

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Metabonomic profiling has become a powerful tool to discern normal vs. abnormal patterns of biofluids with many potential applications. One-dimensional proton NMR-based methods are particularly suitable for this purpose owing to their robustness and reproducibility. However, spectral processing such as phasing, baseline correction and peak alignment remain tedious tasks, which has resulted in the development of multiple competing methods for each processing step. Choosing the best combination of processing methods and incorporating them into an automated workflow is important for large-scale metabonomic initiatives. Here we present an optimized workflow for the processing of 1D proton NMR metabonomic data from blood plasma stored at the Taiwan Biobank. The resulting workflow incorporates manual phase correction, automated baseline correction with airPLS (adaptive iterative reweighted Penalize Least Squares) and peak alignment using the CluPA (Cluster-based Peak Alignment) algorithm. We compare the current workflow with alternative combinations of other processing methods and discuss their strengths and weaknesses.

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Derivative-Based STOCSY Recovers Structural Information Masked by Broad Signals and Distorted Baseline

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Statistical spectroscopy has been established as a useful tool in analytical chemistry and systems biology. One typical statistical spectroscopy in NMR is Statistical Total Correlation Spectroscopy (STOCSY). STOCSY is extendable beyond 1H NMR both to other metabolic profiling techniques and to multiple 'omics' technologies. Thus a diverse set of tools have been developed based on STOCSY thereafter, in order to improve the performance of STOCSY by enhancing the information recovery. However, the distorted baseline and broad signals from macromolecules or tail of intense peaks in normal 1D spectra is prevalent and may weaken correlation, which remains a problem unsettled to the best of our knowledge. Here, we propose a STOCSY based on derivative spectra to solve the problem. Savitzky-Golay method is applied and optimized without sacrificing the spectral quality. Our simulation tests indicate that the quantification characteristic of the derivative spectra is well preserved. It is demonstrated that compared with conventional STOCSY, the STOCSY based on derivative spectra successfully recovers structural information masked by broad signals and distorted baseline.

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An Automated Quantification Algorithm (AQuA) for high-throughput NMR-based metabolomics

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The lack of rapid tools for absolute metabolite quantification severely limits the throughput of NMR-based metabolomics. We therefore designed an automated quantification algorithm (AQuA) for targeted quantification of metabolites in complex 1H NMR spectra. AQuA is library-based processing approach where one preselected signal is used for the quantification of a specific metabolite while accounting for interferences caused by other metabolite signals. Experimental spectra from human plasma were used to evaluate AQuA. The accuracy of AQuA was tested against an approach for manual spectra fitting using the ChenomX software. Out of 67 human plasma metabolites quantified in 30 experimental spectra, 61 metabolites displayed a goodness-of-fit (r2) value close to or above 0.9. The high efficiency of AQuA was demonstrated by quantifying 67 human plasma metabolites in 1342 experimental spectra, where the AQuA computations were done in less than 1 second on a standard personal computer. In addition, we demonstrate that quality indicators generated by AQuA (e.g., occurrence, interference and positional deviation) can be used to evaluate the results each time the algorithm is operated.

P312

NMR Metabolomics to Detect Huntington's Disease at Early Stage

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Huntington's disease is an inherited progressive neurodegenerative disorder associated with cognitive deficits, involuntary abnormal movements and psychiatric disturbances. This disease is due to an abnormal expansion of a CAG repeat (coding for Gln) in the gene encoding the Huntingtin protein (Htt). However, the mechanisms underlying neurodegeneration in Huntington's disease are not totally elucidated. Identification of metabolic pathways disrupted by the disease will help to consider future therapeutic targets. This study has been designed to identify metabolites of Huntington's disease modeled in Drosophila melanogaster. The expression in transgenic flies of the pathogenic polyglutaminated huntingtin fragment (exon1 with 93 polyQ repeats) induces neuropathology that recapitulates most of the characteristics of the human disease. NMR analyses were performed on several sets of adult drosophila carrying the disease at the presymptomatic stage (10 days-old flies) and advanced stage (16 days-old flies). In order to optimise sensitivity and acquisition times, each sample was prepared from the metabolic extraction of 5 flies, in 3mm tubes. All 1H-NMR spectra were recorded on a BRUKER advance III HD 700 MHz NMR spectrometer equipped with a 5mm cryoprobe, processed with Topspin, and bucketed with nmrprocflow before the multivariate statistical analyzes (done with workflow4metabolomics.org). Discriminant metabolites were unambiguously identified according to their chemical shifts, and by additional COSY, TOCSY, 13C-HSQC and HMBC spectra or spikes addition, if necessary. The metabolites identified in this study report perturbations of i) synaptic signaling, ii) of energy metabolism or energy storage; iii) of the TCA cycle, and iv) of muscle fibres and/or brain tissues.

We demonstrate in this study that: A small set of identified metabolites allow the prediction of Huntington's disease before the appearance of the symptoms, which is encouraging to develop early diagnostics (data with the 10 days-old flies modelling this pre-symptomatic stage). The discriminant metabolites evolve at a more advanced stage of the disease (data with the 16 days-old flies), which will help to better understand the evolution of the disease at the molecular level.

P313

NMR-based Metabolomics Study on Infectious disease in White leg shrimp

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The shrimp industry in Korea is thriving as to meet the demands. However, there is a growing concern over the industry that infectious disease causes a massive economic loss. It is necessary to set up new strategy to prevent the disease. Among the infectious disease in white leg shrimp (Litopenaeus vannamei), White spot syndrome virus (WSSV) and Vibrio harveyi was studied in this research. In this study, white leg shrimp was infected with WSSV and Vibrio harveyi for a week. The blood and the hepatopancreas of white leg shrimp were analyzed by High Resolution Magic Angle Spinning Nuclear Magnetic Resonance (HR-MAS NMR) based Metabolomics. Spectral binning was performed at the Chenomx NMR suite 7.1 (Chenomx Inc., Edmonton, AB, Canada) and the binning data was analyzed by the Principal component analysis (PCA), Partial least squares discriminant analysis (PLS-DA) and Orthogonal Partial Least Squares discriminant analysis (OPLS-DA) using the SIMCA-P+ 12.0 software (Umetrics, Umeå, Sweden). The metabolites were assigned using the Chenomx 600 MHz library database. With the multivariate analysis, choline and uracil was the most significant metabolite in the study. This was supported by the Variable Importance Projection VIP) Score that the score was over 2.0. In the result of the correlation analysis, glycine-serine-threonine metabolism was affected.

P314

The study of metabolic changes caused by RBIV infection using NMR

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Rise in temperature of water affects the immune system and susceptibility of fish. It leads to an infectious disease and causes an enormous financial loss in the aquaculture industry. When the disease starts to spread, it is quick. Thus, rapid diagnosis for infection in fish is necessary. In this study, Rock Bream (Oplegnathus fasciatus) was naturally infected with Rock Bream Iridovirus (RBIV) for 3 weeks. Control groups which are not infected and infected groups were divided into two groups and compared each other using NMR-based metabolomics. Liver, spleen, and kidney of Rock Bream were measured with High Resolution-Magic Angle Spinning Nuclear Magnetic Resonance (HR-MAS NMR). The metabolites were assigned using the Chenomx 600 MHz library database. The data was analyzed by the Orthogonal Partial Least Squares discriminant analysis (OPLS-DA) using the SIMCA-P+ 12.0 software (Umetrics, Umeå,

Sweden). OPLS-DA score plots showed the clear separation between control groups and infected groups in all organs. Spleen, the target organ of the RBIV, had the largest number of metabolites changed in infected group. Also, the pattern in the stages of infection was discriminated by the heatmap.

P315

Studies on the detection and quantification of post-translationaly modified peptides.

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Pathological levels of oxidative stress (OS) have been implicated in many diseases including diabetes mellitus, neurodegenerative diseases, inflammatory diseases, atherosclerosis, and cancer. Studies of oxidative stress are however complicated by the low concentration of oxidation products. Therefore, studies on the detection and quantification of post-translationaly modified peptides (e.g., carbonylated or glycated) require defined, analytically pure standards of modified peptides. Here we have indicated that peptides, obtained solid phase synthesis, comprising the aminoadipic semialdehyde derivative are reactive and under acidic conditions tend to undergo side reactions, whose products were characterized in detail with the use of the 2D NMR spectroscopy as well as MS techniques. Obtained results put some new light on the possible posttrantional modifications of proteins.

P316

Magnetic resonance microscopy of Apis mellifera

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Domestic honey bees (members of genus Apis) are an important part of our ecosystem mostly because of their role in crop pollination as well as their honey production. The anatomy of the internal organs of the honey bee is well known, mainly through dissection but also through in-vivo non-invasive magnetic resonance microscopy (MRM). MRM provides soft-tissue contrast in small biological samples, for instance 3D structure of the brain, and thus fat distribution and fat consumption of living insects can be investigated with time. NMR spectroscopic methods can be used beyond imaging for example for identification, production and characterization of silk proteins. Domestic honey bees and drones were examined by magnetic resonance microscopy. The method's feasibility to study the pupation was studied and preliminary tests show promising results in terms of image quality and spatial resolution in a time frame that is sufficiently short to enable dynamical following of the process. Images were made with a standard 3D spin echo method with imaging parameters: echo-time of 2 ms, repetition time of 200 ms, with 40 μm spatial resolution, field of view 10 mm × 10 mm × 20 mm, 256 × 256 × 512 matrix for single insects imaged in a 10 mm inner diameter microimaging coil. Honeycombs were imaged with 117 μm resolution, 30 mm × 30 mm × 30 mm field of view and 256 × 256 × 256 matrix in a 30 mm inner diameter coil. The method also enables the detection and study of effect of parasites such as Varroa destructor on the evolution and degradation of honey bees.

P317

Tracking Drug Delivery Systems in vivo by Magnetic Resonance Spectrscopy

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Poor solubility of new chemical entities (NCE) is emerging as a major problem in the pharmaceutical industry. It increases chances of attrition and adds to costs in drug development. Poor solubility is an issue, especially for oncology NCEs as they tend to have high doses and often require intravenous (IV) formulations. Nano drug delivery vehicles are a promising solution and polymeric systems such as PLA-PEG nanoparticles (NP) allow to encapsulate hydrophobic drugs, protect the drug from rapid clearance, facilitate passive targeting and active targeting. A major roadblock in successfully developing such systems is the lack of real-time monitoring of the bio-distribution and clearance of the vehicle. We propose a novel method to track these NP in vivo and in real-time using MRI. This will provide new insights into the bio-distribution and clearance of these systems: information which has been lacking so far. With this feedback it is possible to iteratively modify the NP topologies to enhance targeting and circulatory half-lives. We aim to tailor these drug delivery vehicles for critically important cancer drugs which are under development but suffer from poor solubility, very short half-lives and/or off-target effects.

P318

Image registration of electron and nuclear resonance imaging of the murine model of glioblastoma.

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Purpose The authors used magnetic resonance imaging (MRI) and electron paramagnetic resonance imaging (EPRI) to register anatomical and functional images of the murine orthotopic model of glioblastoma. Methods MRI and Continuous wave (CW) EPRI methodologies were used to obtain three – dimensional images of the murine model of glioblastoma multiforme. The authors also examined different approaches to tumour implantation to establish a stable mouse model of glioblastoma. For image registration, 3D printing technology was used to ensure the same positioning of the animal head in both imagers. Results Software for reconstruction, registration and analysis of images from both methodologies was generated and tested using phantoms. We established a successful tumour implantation method lasting less than 30 minutes in a single animal. We also developed CW EPR imaging protocols with acquisition time as low as 11 min for 3D and 38 min for 4D. For registration procedure, we used 3D-printed holder from PLA polymer. Preliminary results demonstrated sufficient precision of tumour implementation and high repeatability of EPRI imaging. The efficacy of registration process is currently being tested on glioblastoma tumours growing in the brain of C57bl mice. Conclusions MRI and EPRI have different areas of applicability. Image registration of both methods provides a new tool for obtaining anatomical and functional information which are impossible to achieve using these methods independently. The described procedure may also be easily extended to other imaging modalities.

P319

Design, synthesis and application of double armed Cobalt (II) paramagnetic NMR Probes

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Paramagnetic NMR spectroscopy is a powerful technique for biomolecular studies, during to a variety of paramagnetic effects, like pseudocontact shifts (PCS), residual dipolar couplings (RDCs), and paramagnetic relaxation enthancements (PREs). However, most proteins do not have a paramagnetic center or metal binding site. Thus, it is required to introduce a paramagnetic center to protein artificially, without affecting protein properties. In this work, we designed and synthesized two cobalt(II) paramagnetic complexes. These two probes were tagged to T4 lysozyme by two cysteine residues to yield PCSs. According to the PSCs, magnetic susceptibility anisotropy ($\Delta\chi$) tensors of the probes were calculated. EPR and XPS measurements were applied to investigate the spin states and charge of the Co complexes.

P320

Lanthanide-binding tags as a valuable tool for in-cell investigation of protein structure and function

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In a biological environment most proteins function not as monomers but rather in complexes with other proteins or distinct binding partners. Based on those interaction pathways a lot of research has been conducted to gain new insights in disease nascency and for the identification of new drug targets. NMR spectroscopy is a powerful method for structure determination and investigation of structural changes at atomic resolution caused by binding events. Yet, the characterization of larger protein complexes still remains challenging.

For this purpose lanthanide-binding tags (LBTs) are a versatile tool, allowing site-specific labeling and detection. They consist of an encodable peptide-sequence of 17 amino acids and are genetically engineered into a loop region of a given protein.(1) Their ability to bind Ln3+-ions in a low nM range combined with multiple physical properties of the different lanthanides makes them applicable to several methods including NMR and EPR spectroscopy, X-ray crystallography or luminescence-based studies.

To address biological relevant questions we aim to measure proteins under in-vivo conditions. The model protein for our in-cell approach is the double-loop construct of human Interleukine-1-beta (IL1b-S2R2), as described before.(2) Our aim is to improve existing in-cell experiments by the usage of LBTs in NMR and EPR spectroscopy.

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P321

A site-specific TEMPO-labeling protocol via derivatization of functionally tethered oligonucleotides (FTOs)

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In this work, we present a reliable new strategy applicable for the site-specific attachment of paramagnetic centers within nucleic acids to meet the demand of high sample amounts for NMR spectroscopic investigations. To this end, a 5-trifluoroacetamido-propargyl uridine phosphoramidite building block was synthesized and site-specifically incorporated during the RNA chain elongation on an automated synthesizer using standard solid phase synthesis. The subsequent work-up procedure yields a functionally tethered oligonucleotide (FTO) with a propargyl amine moiety. This amino functionality was used to attach a paramagnetic center by treating the RNA with an electrophilic 4-thioisocyanato-TEMPO reagent. Almost quantitative yields were obtained in the derivatization reaction making the approach suitable for NMR spectroscopic applications relying on high sample amounts. The introduction of the paramagnetic TEMPO radical sets the stage for PRE-NMR experiments giving valuable information on structure and dynamics in biomolecules. Such measurements have proven to be a powerful tool in the solution structure determination and for the investigation of dynamics within transitions between a sparsely populated high energy- and a global minimum state. The novel methodology was tested on an 18 nt RNA hairpin – a substrate for the ribosomal N6-adenosine methyltransferase RlmJ. Exploratory NMR and EPR experiments suggest that the approach will be useful to map the RNA binding site on the RlmJ protein.

P322

Deciphering the cellular copper trafficking mechanism in order to develop a new generation of antibiotics

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Copper's ability to accept and donate single electrons makes it an ideal redox cofactor, and thus one of the most essential metal ions to the survival of the cell. However, copper ions are also involved in the Fenton reaction and hence capable of driving the generation of deleterious hydroxyl radicals, which are deleterious to the cell. Hence, both prokaryotic systems as well as eukaryotic system have developed a considerable regulation mechanism to maintain negligible copper concentration, in the femtomolar concentration. E.coli cells, in common with the vast majority of bacterial cells, require copper for several important enzymes such as ubiquinole oxidases, Cu,Zn-superoxide dismutases, or cytochrome c oxidase. However, as was mentioned above, copper can be deleterious, making protective mechanisms necessary. Deciphering this regulation mechanism in bacteria, is tremendously important from two specific reasons: one over 70% of the putative cuproproteins identified in prokaryotes have homologs in eukaryotes, and thus resolving the copper cycle in prokaryotic systems will also shed light on the copper cycle in eukaryotic systems. Second, copper has been used throughout much of the human civilization as an antimicrobial agent. In this work, we will shed some light on two important copper regulation systems in E.coli: the copper periplasmic efflux system, CusCFBA, and the Cu(I) metal sensor, gene expression regulation system, CueR. Using Electron Paramagnetic Resonance (EPR) spectroscopy, together with biochemical experiments, cell experiments, and computational methods we will show the essentiality of methionine and lysine residues to the interaction between two proteins in the CusCFBA system and for Cu(I) coordi-

nation. We will also present a structural model for the CueR-Cu(I)-DNA complex, shedding light on the transcription mechanism of the CueR protein. Last, we will demonstrate how molecular level understanding on the function of these systems can assist in designing new class of antibiotics.

P323

Conformational Coupling and trans-Inhibition in the heterodimeric ABC exporter TmrAB observed with PELDOR

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ATP-binding cassette (ABC) transport complexes use ATP binding and subsequent hydrolysis to actively translocate chemically diverse substrates across biological membranes. Their malfunction leads to several human diseases. Many ABC exporters contain asymmetric nucleotide-binding sites (NBSs) and some of them are inhibited by the transported substrate. The functional relevance of the catalytic asymmetry or the mechanism for trans-inhibition remains unknown. Here, we investigated the heterodimeric ABC exporter TmrAB[1,2] from Thermus thermophilus, a functional homolog of the human antigen translocation complex TAP using pulsed electron-electron double resonance (PELDOR) spectroscopy. In the presence of ATP, TmrAB exists in a tunable equilibrium between inward- and outward-facing conformations. The two NBSs show pronounced asymmetry in the open-to-close equilibrium. The closed conformation is more preferred at the degenerate NBS and closure of one of the NBSs is sufficient to open the extracellular gate. We elucidated the mechanistic basis for trans-inhibition, which operates by a reverse transition from the outward-facing state via an occluded conformation. This work uncovers the central role of reversible conformational equilibrium in the function and regulation of an ABC exporter and establish a mechanistic model for new investigations on other medically important transporters with asymmetric NBSs. In addition, this study shows for the first-time the possibility to resolve equilibrium populations at multiple domains and their coupling for global conformational changes in a large membrane protein complex.[2] [1] A.Nöll, et al., Proc. Natl. Acad. Sci. USA 114, (2017) [2] K.Barth, et al., J. Am. Chem. Soc. 140 (2018)

P324

EPR study of human ribosome complexes modeling ribonucleoproteins formed during ribosomal biogenesis and translation

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The human ribosome is a special cellular molecular machine that performs the final stage of the realization of genetic information, protein biosynthesis or translation. Structural organization of complexes modeling different states of ribosomes of higher organisms during translation is actively studied by X-ray crystallography and cryo-electron microscopy, which allows the structures of complexes to be deciphered at a sub-atom level. However, the above approaches have a number of significant limitations. For example, it is difficult or even impossible to obtain a number of complexes as crystals suitable for their analysis by X-ray crystallography. Also, both approaches do not allow the visualization of flexible structural elements of the complexes and deciphering the structures of labile easily dissociating complexes. In this work we used EPR methods and spin- labeled nucleic acid derivatives to study a wide variety of complexes that simulate different states of ribosomes in the course of translation, including those that have not yet been studied with X-ray crystallography and cryoelectrom microscopy. mRNA analog - oligiribonucleotide UGUGUUCGACA, with nitroxide labels attached at C5 5'atom of U- residue and C8 3' atom of A residue was used to study complexes of human ribosomes and codon-anticodon interaction with tRNA using DEER, ESEEM and CW EPR [1]. Model 80S ribosomal complex with mRNAs were assembled with participation of tRNAPhe that targets triplet UUC (coding for Phe) into P site of ribosome. The distances between spin labels were measured in 5 model complexes of mRNAs analog with 80S ribosomes, where tRNA was located at A site and/or at E-site. The data confirmed the cooperative character of the coupling between molecules of tRNA at A-, P- and E-sites of ribosome and allowed to make conclusion concerning the absence of codon-anticodon interaction in the E-site of ribosome. We also detected labile binary complexes of the small ribosome subunit with unstructured RNAs, which are formed due to their interaction with the fragment of ribosomal protein uS3 exposed on the subunit surface and which have not been visualized by any other methods. We also prepared derivatives of short single-stranded RNAs and DNAs of various length (6 to 15 nucleotides) that contain nitroxide and trityl spin labels at the 3'- or 5'-termini and complexes of these derivatives with 40S ribosomal subunits bound with tRNA and 3'-spin-labeled mRNA were obtained. These complexes were investigated by CW EPR spectroscopy that allows us to detect labile complexes of DNA and RNA derivatives with the 40S ribosomal subunit.

[1] Malygin, A. et al., Structural Rearrangements in mRNA upon Its Binding to Human 80S Ribosomes Revealed by EPR Spectroscopy. Nucleic Acids Res.,2018, 46, 897–904

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P325

Determining conformational ensembles of highly flexible protein assemblies using exhaustive conformational ensembles based on EPR distance distributions

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The investigation of large molecular complexes and how they assemble and rearrange in the cell is important for understanding of cellular regulation. For many complexes, structures of the complex building proteins are known in an isolated state and can be used in computational approaches to predict the complex structure. In addition, the fast-growing field of high-resolution cryo-electron microscopy (cryo-EM) allows the determination of the structure of large molecular complexes with continuously increasing resolution. However, for many cellular processes the dynamic assembly, disassembly, and rearrangement of protein complexes are of great importance. Due to their dynamic nature, these systems cannot be fully investigated by classical methods, e.g. because these complexes cannot be crystalized or show a too high variability when investigated by cryo-EM. Although in many cases structural information can be acquired, the dynamic nature of the system, important for its function, cannot be captured. Such systems must rather be described by conformational ensembles than by a single structure. This could be observed by methods such as EPR and FRET, which do not reveal atomistic information about the whole complex but allow to measure specific distances between the components of a large complex. As these methods use covalently attached labels, it is possible by combinatorial labeling to gain information about rearrangements of each part of the large complex. If the structures of the isolated components of the complex are known and a sufficient number of long-distance measurements are available, computational methods allow the determination of the conformational ensemble of the complex. We present an approach using the software CYANA to generate large conformational ensembles of dynamic protein complexes based on multiple EPR distance distributions. The approach was applied to a highly flexible di-ubiquitin system to understand the influence of different modulators in the protein degradation pathway. We established a protocol for determining conformational ensembles weighted by a normalized combined pseudo-probability derived from all available experimental distance distributions. The pseudo-probability weighted ensemble provides a more general picture of the ensemble. Comparison to X-ray structures showed that they occupy high-probability regions, as expected for the low energy states observed in X-ray structure. However, in addition other high-probability regions and rearrangements of the poly-ubiquitin chain in the presence of different modulators could be observed. Thereby, the conformational space of di-ubiquitin revealed conformational selection or remodeling as mechanisms for chain-recognition during elongation or hydrolysis, respectively.

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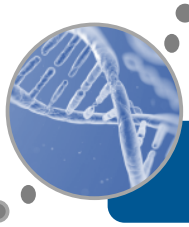

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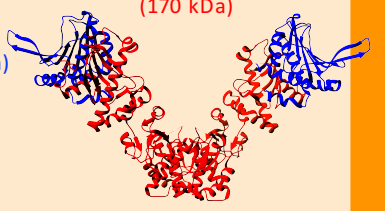
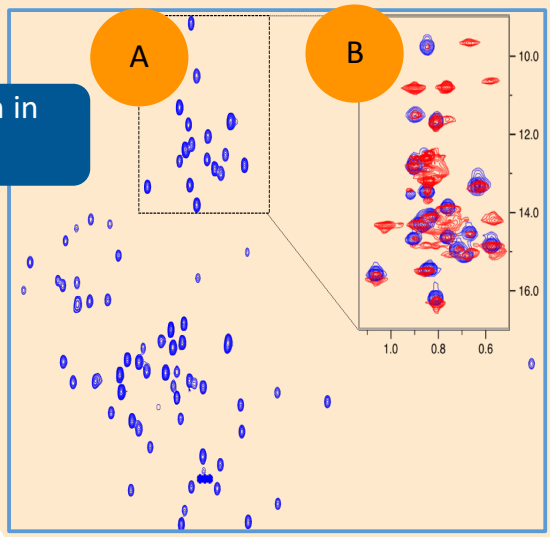
For expression in bacteria

Human HSP90 (170 kDa)

NTD (30 kDa)





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(4) Methyl groups assignment.

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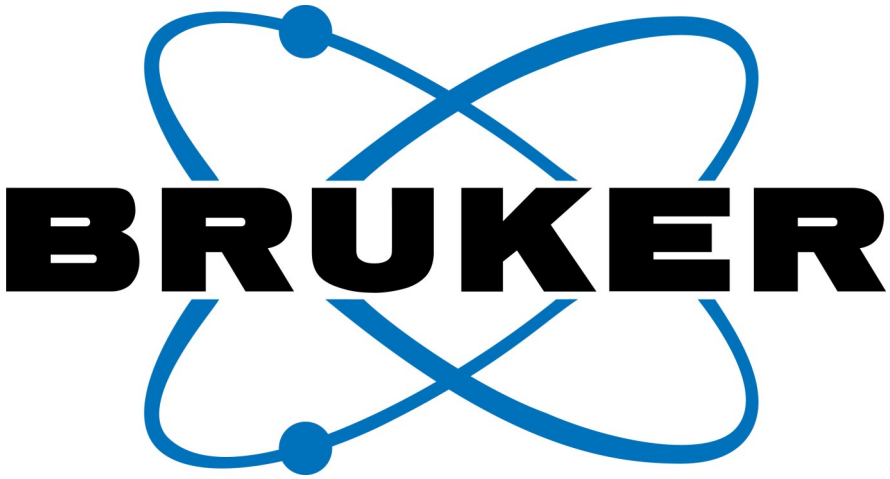

Aug 19 Sun - O'Reilly Hall	
ICMRBS Opening - Chandralal Hewage	16.00-16.10
Chair: Peter Wright - Keynote Lecture: Lewis Kay	16.10-17.00
Chair: Mei Hong - Founders' Medal Lecture: Sebastian Hiller & Lynette Cegelski	17.00-18.00
Welcome Reception - O'Brien Science Centre	18.15-20.15

Aug 20 Mon				Aug 22 Wed		
O'Reilly Hall				O'Reilly Hall		
Chair: K Gardner - Plenary: A McDermott			08.30-09.15	Chair: M Delepierre - Plenary: H Schwalbe		
Chair: R Griffin - Plenary: G Pintacuda			09.15-10.00	Chair: J Dyson - Plenary: Song-I Han		
O'Brien Science Centre				O'Brien Science Centre		
O'CONNOR A	ELAN B	ACCENTURE C		O'CONNOR A	ELAN B	ACCENTURE C
F Ferrage	V Sklenar	H Mott		A Böckmann	A Gronenborn	D Nietlispach
New Methods I	Protein DNA/RNA	Biomolecular Interactions I		Biological Solids I	Dis. Proteins - Aggregation	Macromol. Complexes
G Wagner	F Allain	P Gooley	10.50-11.20	H Oschkinat	P Wright	C Kalodimos
B Vögeli	M Katahira	H Möller	11.20-11.40	T Cross	A De Simone	B Bersch
G Jeschke	J Iwahara	B Kragelund	11.40-12.10	M Duer	R Riek	J Boisbouvier
T K Dayie	D Neuhaus	B Burmann	12.10-12.30	Tuo Wang	K Frederick	N Sgourakis
C Tang	J Plavec	R Griffin	12.30-13.00	T Polenova	D Eliezer	R De Guzman
O'Brien Science Centre				O'Brien Science Centre		
O'CONNOR A	ELAN B	ACCENTURE C		O'CONNOR A	ELAN B	ACCENTURE C
L Brennan	M Ikura	Gerhard Wagner		A Palmer	M Overduin	T Carlomagno
Dis. Proteins & Imaging	EPR/ESR	Paramagnetic Systems		Computational NMR	Biomol. Struc. & Function	Interactions & Folding
K Brindle	D Goldfarb	L Banci	15.50-16.20	P Guntert	C Partch	A Mittermaier
K Han	T Smirnova	Yin Yang	16.20-16.40	T Madl	E Lescop	F Hagn
L Frydman	G Lorigan	D Brougham	16.40-17.10	W Kozminski	R Bruschweiler	M Clore
D Cowburn	M Mahawaththa	D Lacabanne	17.10-17.30	F Mulder	M Latham	G Veglia
S Aime	D Norman	M Ubbink	17.30-18.00	A Bonvin	I Landrieu	J Christodoulou
O'Reilly Hall				O'Reilly Hall		
Chair: D Goldfarb - Plenary: M Bennati			18.15-19.00	Chair: R Norton - Plenary: A Watts		
O'Brien Centre				Downtown		
BRUKER Hospitality				Student Nightout in Dublin		

Aug 21 Tue				Aug 23 Thu		
O'Reilly Hall				O'Reilly Hall		
Chair: K Mok - Plenary: M Caffrey			08.30-09.15	Chair: N Izadi-Pruneyre - Plenary: I Felli		
Chair: M Kainosho - Plenary: I Shimada			09.15-10.00	Chair: J Markley - Plenary: K Gardner		
O'Brien Science Centre				O'Brien Science Centre		
O'CONNOR A	ELAN B	ACCENTURE C		O'CONNOR A	ELAN B	ACCENTURE C
M Williamson	J Plavec	M Buck		G Pintacuda	C Kalodimos	J Boisbouvier
New Methods II	Nucleic Acids	Biomolecular Interactions II		Biological Solids II	Dis. Proteins - Interactions	Membrane Proteins
Ad Bax	Bin Xia	W Lee	10.50-11.20	Mei Hong	J Forman-Kay	S Prosser
H Arthanari	J Marchant	D Fushman	11.20-11.40	Y Ishii	F-X Theillet	D Nietlispach
F Ferrage	T Carlomagno	C Griesinger	11.40-12.10	A Barnes	T Mittag	M Mobli
M Kowalska	C Dominguez	R Rosenzweig	12.10-12.30	A Gallo	J Chill	A Lange
R Kitahara	H Al-Hashimi	R Norton	12.30-13.00	A Böckmann	M Blackledge	F Marassi
	JEOL SEMINAR		13.00-13.50			
O'Brien Science Centre				O'Brien Science Centre		
O'CONNOR A	ELAN B	ACCENTURE C		O'CONNOR A	ELAN B	ACCENTURE C
S Matthews	M Baldus	P Malthouse		P Guntert	C. van Heijenoort	P Crowley
Structural Biology	Metabolomics	Pharm & Drug Development		Relaxation & Dynamics	Enhanced Polarisation	Metabolism & In-Cell NMR
M Sattler	H Keun	M Scanlon	15.50-16.20	J Lewandowski	B Corzilius	P Giraudeau
J Prestegard	J Markley	H R Kalbitzer	16.20-16.40	K Takeuchi	S Bibow	H Kadavath
A Gronenborn	L Brennan	Bong-Jin Lee	16.40-17.10	D Yang	A Lesage	Y Ito
C Waudby	M Kennedy	M McCoy	17.10-17.30	K Petzold	D Shishmarev	A Shekhtman
P Crowley	D Wishart	D Kojetin	17.30-18.00	D F Hansen	Xin Zhou	M Baldus
O'Reilly Hall						
Chair: I Shimada - Plenary: A Webb			18.15-19.00			
O'Brien Centre				O'Brien Centre		
MERCK Hospitality				Farewell Reception		