AILM2019

Advanced Isotopic Labelling Methods for Integrated Structural Biology

March 26th to 29th
Grenoble – France.
Over the past few years, isotopic labelling technologies have undergone tremendous developments enabling their applications in a large set of biological systems. The appropriate use of recent labelling methods allowed to address some fundamental biological questions by revealing the dynamics of molecular systems regardless of their size or their location inside a cell. It is expected that the application of labelling technologies will continue to expand as the possibilities offered by these methods have just started to be explored. The AILM 2019 workshop aims at providing a survey of current developments of isotopic labelling techniques and their applications to the study of biomolecular structure and dynamics. This interdisciplinary meeting brings together researchers from NMR spectroscopy, mass spectrometry, neutron scattering and diffraction to promote scientific exchanges about the latest labelling technologies. The number of attendees as well as the very high quality of the communications is a rewarding sign of the high interest raised by such an event in the biophysical research community. We hope that this meeting, at the heart of the French Alps, will meet your expectations and contribute to disseminate a productive use of labelling technologies for integrated structural biology.

The organizers want to warmly thank Drs. M. Sattler, G. Wagner, and S. Wiesner for their contribution to the preparation of the AILM2019 program, the ESRF for giving us access to the auditorium, and our generous sponsors and exhibitors for their support to AILM2019. We also acknowledge the IBS-NMR group members and the IBS staff for their help with the organization.

We wish you an enjoyable stay in Grenoble and fruitful scientific exchanges.

The AILM2019 organizing committee,
Jérôme Boisbouvier (IBS – Grenoble )
Bruno Kieffer (IGBMC – Strasbourg)
Michael Plevin (University of York)
Carine Tisné (IBPC - Paris)
Registration and Venue

The AILM2019 Conference takes place on the EPN Campus in Grenoble. To enter the site, you need to pass at the Site Entrance (access map) with a valid identity document (passport or ID card, driving licence refused). Badges that will allow access to the conference site as well as to lunch buffets and the conference dinners will be given at the Registration Desk.

Registration:

Registration takes place at the ESRF Central Building on Tuesday, March 26th from 8 to 11 am. Late registration will be possible at the Help Desk situated in the ESRF Entrance Hall.
Venue:

- from Lyon Airport: Lyon Saint Exupery Airport is a one-hour drive from Grenoble and regular shuttle buses (‘Navette Aéroport’) operate to and from Grenoble bus station (‘Gare Routière’), which is next to Grenoble railway station. Buses run hourly, seven days a week. People under 25 pay a reduced rate. The LYS Express shuttle bus is now run by the OUIBUS company. You should book your seat via the link below in advance. The bus will stop near the ESRF/ILL/EMBL site on request. You must tell the bus driver when boarding at the airport that you want to be dropped off at "Presqu'île - Résistance". The bus stop is within walking distance to the EPN Campus (see access map on previous page). https://www.lyonaeroports.com/en/access-transport/shuttle-buses/grenoble

- from Geneva Airport: Geneva Airport is a two-hour drive from the EPN campus. Shuttle bus is run by the OUIBUS company (https://www.gva.ch/fr/Downloads/Passagers/Horaires-autocars-GVA-Grenoble-Ouibus.pdf). In addition, train connections from Geneva Central Station (Genève Cornavin) to Grenoble exist but are not very frequent. https://www.sncf.com/en

- from Grenoble train/coach station: take the Tram Line B to its terminus ("Presqu'île") which is within walking distance from the EPN Campus. (See access map on previous page).

- from Lyon or Valence by car: stay on the A48 when approaching Grenoble, following signs for "Grenoble Bastille-Gares-Europole". Leave the A48 at exit N°16 ("Polygone Scientifique") and cross the suspension bridge over the Isère river. At the end of the bridge filter right and turn right, heading north along the Rue des Martyrs. The entrance to the site is on the left after a few hundred meters.

- from Geneva by car: take the "Rocade Sud/N87" (the ringroad) direction "Lyon", which takes you onto the A480. Take the A480 direction "Lyon". Leave the A480 at exit N°1 for the "Polygone Scientifique". At the first roundabout turn right. The entrance to the site will be on your right after a few hundred meters.

Accommodation on the EPN Campus

Arrival

The EPN Guesthouses are located close to the IBS building on the EPN campus. Keys can be recovered at the reception in the central guesthouse during the opening hours on Sunday, 2-8 pm; Monday to Friday, 7 am-3 pm; and Saturday, 7 am-12 pm. For those who arrive outside the opening hours, the keys are kept at the EPN entrance site. The rooms will be available for luggage drop-off during the day preceding the first booked night.

Departure

Keys have to be returned to the reception before 12 pm (weekdays) or 11 am (Saturday)
Public Transportation

Tram:

The B line connects Grenoble City Center with a frequency of 3 to 10 minutes from 6 am to 8 pm. In the evening, trams run every 20 minutes until 1 am. The tram stop is only 400 m from the new EPN site entrance, and corresponds to the terminus of tram B called 'Presqu’île'. Tickets are available from a machine (cash or credit card). Tickets have to be stamped at each access to the tram (on the platform) or bus (in the bus).

Bike rental:

Yellow Métrovélo bikes can be rented close to the Central Station for 3 € a day. You need an ID card and credit card for the security deposit.
Conference Organization
Lectures:
Lectures will be held in the ESRF conference hall in the basement of the Central ESRF Building.

Poster Sessions and Exhibition:
Company booths will be in the IBS Entrance Hall. Posters will be on display in the IBS Conference room and should be set up on Tuesday during lunch time. Odd and even numbered posters will be presented on Tuesday (7-9 pm) and Wednesday (12-2 pm), respectively. A third poster session is scheduled Thursday (12-2pm) during lunch time.

Visit of the cryoEM / NMR / ILL / ESRF facilities
Participants are given the opportunity to visit the local facilities Thursday March 28th between 1 and 2 pm. Online pre-registration is required: https://framadate.org/Tm1aDzoe3hQIFPAa

Coffee Breaks:
Coffee will be served in the ESRF Entrance Hall.

Lunch:
A lunch buffet is proposed in the IBS Entrance Hall from Tuesday to Thursday. On Friday, lunch will be served at the common restaurant on the EPN campus. You will need to present your AILM2019 badge at the check out

WiFi Access:
Free access is provided by eduroam in EPN guest houses, ESRF and IBS buidings using your personal login and password (https://www.eduroam.org/). For those who do not have an eduroam access, a visitor login and password can be requested at the IBS Help Desk (IBS buiding only). In EPN guest houses and ESRF buidings you can also use EPN_visitor Wifi with login: AILM_2019 and password: aVP19_0304

The Conference Dinners:
A Wine-and-Cheese reception will take place on Tuesday evening in the IBS entrance hall during poster session 1 (7-9 pm). On wednesday evening, dinner will be served at the common restaurant on the EPN campus from 7:30 pm. Thursday evening between 8 pm and 10:30 pm, you will have the opportunity to visit the Grenoble Museum of Fine Arts around a buffet. Guides will be available to introduce you to its collections of ancient, modern and contemporary art. The museum is located in Grenoble downtown and can easily be reached directly with the Tram B (stop at station Notre Dame – Musée). Two tram tickets will be given with your conference bag. For AILM attendees who have booked a night in the EPN guesthouse on Monday 25th or Friday 29th, a dinner will be proposed in the EPN campus restaurant at 7:30 pm.
Satellite Symposium “40 years of NMR Development for Biology”

Symposium in honor of Dr. Dominique MARION's retirement - Monday March 25th 2019

Organised as a satellite event to AILM2019, the symposium takes place at the ILL conference hall and opens at 10 am on Monday, 25th. The participation to this satellite symposium is free of charge but preregistration is required. Participants to this symposium can have access to rooms at the ESRF/ILL guesthouse for Sunday and Monday nights. Lunch will be offered to all participants on Monday in the IBS entrance hall. For registered attendees who have booked a night in the EPN guesthouse on Monday 25th a dinner will be proposed in the EPN campus restaurant at 7:30 pm.
Monday March 25th

Satellite Symposium “40 years of NMR Developments for Biology”
Symposium in honor of Dr. Dominique MARION's retirement

9:50 Opening / Welcome by IBS Director

10:00 Muriel Delepierre (Institut Pasteur - Paris - FR): Biological NMR in France in the 80’s

10:35 Marc-André Delsuc (IGBMC - Illkirch - FR): Dominique, in Phase with NMR, in Resonance with Biology, to Transform modern Science

11:10 - Coffee break

11:40 Françoise Guerlesquin (CNRS Marseille - FR): “NMR of metalloproteins : a wonderful life”

12:15 Jo Zaccai (IBS/ILL - Grenoble - FR): “The early days of Structural Biology in Grenoble”

13:00 - Lunch in IBS Hall

15:00 Ad Bax (NIH - Bethesda- USA): “1, 2, 3, ... How many dimensions do we need?”

15:35 Mitsu Ikura (University of Toronto - CA): "The Birth of Triple-Resonance Experiments for Protein NMR at NIH"

16:10 - Coffee break

16:40 Gerhard Wagner (Harvard Medical School - Boston - USA): "Observing the development of protein NMR from the beginning"

17:15 Dominique Marion (IBS Grenoble - FR): “Spin and science evolution”
Tuesday March 26th

8:00-9:00 Arrival / Registration / Coffee

9:00 Opening – Welcome - Winfried Weissenhorn (Head of IBS) and Michael Plevin

Integrated Structural Biology of Protein Complexes I – Chair: Carine Tisné

9:20 Sheena Radford (University of Leeds – GB) – The structural molecular mechanism of amyloid formation

10:05 Mitsu Ikura (University of Toronto – CA) – Targeting K-RAS4B on Biological Membranes

10:40 Beate Bersch (IBS – FR) – Structural Basis of Membrane Protein Translocation Through the Mitochondrial Intermembrane Space

11:00 Silke Wiesner (University of Regensburg – DE) – The importance of being inactive: Controlling the activity of ubiquitin ligases through restriction of conformational dynamics

11:45-13:30 Lunch in the IBS Hall – Poster set up

Advanced Isotopic Labelling Methodologies I – Chair: Gerhard Wagner

13:40 Pau Bernado (CBS – FR) – Access to Atomic Resolution Structural Information of Homobeta (s) Repeats by NMR: The Huntington Case

14:15 Takanori Kigawa (RIKEN – JP) – Cell-free protein synthesis for advanced isotopic labeling

14:35 François-Xavier Theillet (I2BC – FR) – α-Deuteration makes kinases more amenable to NMR studies

14:55 Galia Debelouchina (UCSD – USA) – A Molecular Engineering Toolbox for Integrated Chromatin Structural Biology

15:15 E. Luchinat (CERM – IT) – Recent developments and applications of in-cell NMR in mammalian cells

15:50-16:20 Coffee

Chaperones, Folding & Dynamics – Chair: Bernhard Brutscher

16:20 Malene Ringkjöbing Jensen (IBS – FR) – Elucidating the Dynamics of Intrinsically Disordered Protein Complexes by NMR Exchange Spectroscopy

16:55 Sigrid Milles (IBS – FR) – The Measles virus phosphoprotein – an intrinsically disordered chaperone that regulates nucleocapsid assembly

17:15 Ad Bax (NIH – USA) – Studies of protein folding, unfolding, and misfolding by rapid pressure jump NMR

17:50 Loïc Salmon (CRMN – FR) – Looking into chaperone assisted protein folding using NMR spectroscopy, X-ray crystallography and Molecular Dynamic Simulations

18:10 Jerome Boisbouvier (IBS – FR) – Structural and Functional investigation of a 1MDa chaperonin in action by solution NMR

19:00-21:30 : Wine & Cheese in the IBS Hall – Poster Session 1
Wednesday March 27th

Advanced Isotopic Labelling Methodologies II – Chair : Ad Bax

9:00 Gerhard Wagner (Harvard Medical School – USA) – Use of specific $^{19}$F, $^{13}$C and $^2$H labeling for studies of large biopolymers

9:45 Jacob Anglister (Weizmann Institute – IL) – Observation of Intermolecular Transferred-NOE interactions in a 40 kDa Complex: MAP Kinase p38α interactions with its recognition motif

10:05 Ricarda Törner (IBS – FR) – Selective Edition of Intra- vs. Inter- Subunit NOEs : A New Tool to Study Large Protein Complexes

10:25 Haribabu Arthanari (Harvard Medical School – USA) – Resolution beyond the Nyquist grid: Combining the amino acid specific information from pyruvate labeling with band selective pulses to extend the resolution for large systems

11:00 Charalampos Kalodimos (St Jude Children’s Research Hospital – USA) – Approaches for the structure determination of large, dynamic protein complexes by NMR

11:45-13:45 Lunch in the IBS Hall – Poster Session 2

Perdeuteration for Neutron Studies – Chair : Trevor Forsyth

14:00 Frank Gabel (IBS – FR) – Small angle neutron scattering for structural biology and dynamics of large biomacromolecular complexes

14:35 Sarah Waldie (ILL – FR) – Cholesterol Deuteration and Exploitation for the Study of HDL/LDL Exchange Phenomena in Atherosclerosis

14:55 Vinardas Kelpšas (Lund University – SE) – Development of Escherichia coli Strains for Protein Perdeuteration

15:15 Janosch Hennig (EMBL – DE) – Segmental and sub-unit selective isotope labeling of protein-RNA complexes for structure determination, combining small-angle neutron scattering and other techniques

Structural Biology of Nucleic Acid/Protein complexes – Chair : Michael Sattler

15:50 Julie Feigon (UCLA – USA) – Integrative structural biology of regulatory RNPs: telomerase and 7SK

16:25-16:55 Coffee

16:55 Olga Vitsyna (Leibniz University Hannover – DE) – Structural studies of the 5'-UTR of HCV RNA by solid-state NMR based structural biology

17:15 Emil Spreitzer (Medical University of Graz – AT) – Deciphering the Molecular Regulatory Mechanisms of FUS Phase Separation

17:35 Junji Iwahara (UTMB – USA) – Ion labeling for NMR investigations of protein-DNA interactions

18:10 Andres Ramos (University College London – GB) – Understanding combinatorial protein-RNA recognition in RNA regulation

19:30 Dinner at the EPN campus restaurant
Thursday March 28th

Integrated Structural Biology of large complexes – Chair: Silke Wiesner

9:00 Michael Sattler (BNMRZ – DE) – Decoding regulatory protein-RNA recognition and dynamics using integrative structural biology

9:45 Sophie Zinn-Justin (CEA – FR) – How does the nucleoskeleton interact with the inner nuclear membrane and chromatin?

10:20 Nadia Izadi-Prunery (Institut Pasteur – FR) – Integrative Structural Biology of bacterial nano-machines

10:40 Adrien Favier (IBS – FR) – Integrated NMR and cryo-EM atomic-resolution structure determination of a half-megadalton enzyme complex

11:00 Tobias Madl (Medical University of Graz – AT) – Protein structure determination using surface-accessibility data

11:45-13:45 Lunch in the IBS Hall – Poster Session 3

13:00-14:00 Visit of cryoEM / ILL / ESRF / NMR Facilities

Mass-Spectrometry & Metabolomics – Chair: Bruno Kieffer

14:00 Justin Benesch (University of Oxford – GB) – Quantitative biophysics – weighing the evidence for mechanism

14:35 Hermann Heumann (Silantes GmbH – DE) – In vivo-stable isotopic labeling of animal models such as mice, zebrafish for quantitative proteomics and metabolomics

14:55 Roman Zubarev (Karolinska Institutet – SE) – Isotopic Resonance - the First 10 Years

15:30 Guy Lippens (LISBP – FR) – Pure Shift methodology for fluxomics

16:05-16:35 Coffee

Isotopic Labelling of RNA & modified RNAs – Chair: Michael Plevin

16:35 Pierre Barraud (IBPC – FR) – Time-resolved NMR monitoring of tRNA maturation

17:10 Hannes Feyrer (Karolinska Institutet – SE) – Making RNA at maximum purity and yield with minimal effort and cost

17:30 Christoph Kreutz (University of Innsbruck – AT) – Chemical tools to study nucleic acids via NMR spectroscopy

18:05 Katja Petzold (Karolinska Institute – SE) – MicroRNA’s Dynamics Influence Targeting of mRNA

20:00-22:30 Dinner at Grenoble Museum
Advanced Isotopic Labelling Methodologies III – Chair: Alvar Gossert

9:00  Masatsune Kainosho (Tokyo Metropolitan University – JP) – Recent Advances in Isotope-Aided Methods for Studying Protein Structures and Dynamics – Historical Background and Future Perspectives

9:45  Roman Lichtenecker (University of Vienna – AT) – Late metabolic precursors – advantages in selectivity and efficiency concerning cell-based protein isotope labeling

10:05 Denis Lacabanne (Université de Lyon – FR) – Selective unlabeling strategies of a 120 kDa membrane protein for solid-state NMR spectroscopy

10:25 Daniel Häussinger (University of Basel – CH) – Intrinsic paramagnetic $\chi$-tensors of lanthanide tags by isotopic labelling

10:45-11:15 Coffee Break

NMR of Large Proteins Using Advanced Isotopic Labelling – Chair: J. Boisbouvier

11:15 Alvar Gossert (ETH – CH) – NMR studies of proteins produced in eukaryotic cells – challenges in expression and NMR spectroscopy

11:50 Ichio Shimada (The University of Tokyo – JP) Function-related Dynamics of High Molecular Proteins

12:25 Closing of AILM2019

12:45-13:30 Lunch at the EPN Campus Restaurant
S 01 Sheena E. RADFORD

The structural molecular mechanism of amyloid formation

Sheena E Radford

ASTbury Centre for Structural Molecular Biology, University of Leeds

ABSTRACT

Understanding how misfolded proteins aggregate, and how aggregated species cause cellular dysfunction and cell death, remain significant challenges. Whilst it is generally accepted that protein misfolding is required for the initiation of formation of amyloid, the structures of oligomers that are on- and off-pathway to amyloid fibrils, why and how oligomers cause cytotoxicity and cell death, and how these species convert into the cross-beta structure of amyloid, remain obscure. The structure of amyloid fibrils themselves, generated in vitro or formed in vivo, also remained elusive, until the advent of cryo-electron microscopy (cryo-EM) and advanced methods in solid state NMR methods, which now offer the opportunity to see amyloid structures in all-atom detail.

In this talk I will describe how we are using different structural and biophysical methods to delineate the mechanism by which the normally soluble protein β2-microglobulin converts into amyloid fibrils that deposit in the joints, causing dialysis-related amyloidosis. By combining detailed kinetic analysis of the progress of amyloid formation with experiments using solution NMR and other methods, I will show how we have been able to determine the structure of β2m oligomers that are on-pathway to amyloid fibrils in all-atom detail. In addition, I will show the structure of the amyloid fibrils themselves, obtained by combining cryo-EM and solid state NMR data. Together, we are beginning to piece together the entire pathway of amyloid formation in all-atom detail. Such knowledge is not only transforming our understanding of these amazing protein structures and how they form at a fundamental level, but it may also open the door to new strategies to combat dialysis-related amyloidosis and other amyloid diseases.

REFERENCES:


S 02 Mitsu IKURA

Targeting K-RAS4B on Biological Membranes

Mitsu Ikura

Princess Margaret Cancer Centre, University Health Network and Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada, M5G 1L7

ABSTRACT

RAS proteins are frequently mutated in cancer (~30% of all human tumours) and an estimated world-wide death pole of RAS-associated cancers exceeds 2 million/yr. Despite of enormous efforts in the RAS research over three decades, there is no clinically approved RAS inhibitor and the RAS protein remains to be a challenging target for cancer therapy development. In order to overcome this challenge, we ought to better understand how RAS functions under physiological conditions and alters related signaling pathways in the mutant RAS-driven tumours. Fully-matured protein K-RAS4B, the major target for cancer therapeutics, is prenylated and methylated at the carboxy-terminus, which enables K-RAS4B to anchor to the plasma membrane where it receives an upstream signal and transmits the signal to a number of downstream pathways. There is, however, a large gap in our understanding of how the matured K-RAS4B protein functions at the surface of the plasma membrane. In order to tackle this challenge in RAS-driven cancer research, we have been extensively employing isotope-aided NMR spectroscopy and have developed new conformational and functional assays for the RAS protein on lipid bilayers using the nanodisc platform developed by Sligar et al. We elucidated how the membrane environment dictates the conformation of K-RAS4B and how oncogenic mutations influences the membrane-dependent conformational states of the protein (Mazhab-Jafari et al. PNAS 2015). More recently, we have been investigating multiple aspects of K-RAS4B functions and I will discuss (i) how the biological membrane influences K-RAS4B interaction with a binding domain of RAF kinases and (ii) how we could inhibit K-RAS4B at the membrane surface by small molecules (Fang et al. Cell Chem Biol 2018) and an engineered protein. Supported by CCS, CIHR, CFI & PMCF.
S 03 Beate BERSCH

Structural Basis of Membrane Protein Translocation Through the Mitochondrial Intermembrane Space

B. Bersch1, K. Weinhäupl1, C. Lindau2, Y. Wang3, C. Schütze2, T. Jores4, H. Kalbacher4, A. Hessel1, D. Rapaport4, M. Brennich5, K. Lindorff-Larsen3, N. Wiedemann2, Paul Schanda1

1. Institut de Biologie Structurale, Univ. Grenoble Alpes, CEA, CNRS, France; 2. Institut für Biochemie und Molekularbiologie, Universität Freiburg, Germany; 3. The Linderstrøm-Lang Centre for Protein Science, University of Copenhagen, Denmark; 4. Interfaculty Institute of Biochemistry, University of Tübingen, Germany; 5. European Molecular Biology Laboratory, Grenoble, France

ABSTRACT

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. 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. After translocation through the TOM complex, the precursors are taken over by ATP-independent complexes of small Tim chaperones 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 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 or TIM8/13 chaperones. We employed an integrated structural biology approach, combining NMR, SAXS, in-vivo experiments and various biophysical methods with data-guided molecular dynamics simulations to determine the structural ensemble and dynamics of the chaperone-preprotein complexes. Our findings decipher how the small TIM hexameric complexes transport both α-helical and β-barrel proteins across the mitochondrial intermembrane space. The precursor protein binds to a conserved cleft on the chaperone in a highly dynamic manner, ensuring high overall affinity through avidity of multiple binding sites. The stoichiometry of TIM9/10 or TIM8/13 in complex with different preproteins depends on the substrate-protein length, revealing thus a modular architecture of this versatile chaperone unique in the mitochondrial intermembrane space.

REFERENCES:

S04 Silke WEISNER

The importance of being inactive: Controlling the activity of ubiquitin ligases through restriction of conformational dynamics

Silke Wiesner¹,², Magnus Jäckl², Carsten Stollmaier¹,², Mira Schütz-Stoffregen¹,², Samira Anders²

1 Institute for Biophysics and Physical Biochemistry, University of Regensburg, Germany
2 Max Planck Institute for Developmental Biology, Tübingen, Germany

ABSTRACT

Enzyme activity is inherently linked to protein motions. Yet, how conformational dynamics correlates with enzyme activity is poorly understood. HECT-type ubiquitin ligases (E3s) covalently attach ubiquitin to substrates and thereby regulate the localization and abundance of proteins in the cell. Inhibition of HECT E3s prevents untimely ubiquitination and is essential for cellular homeostasis. In line with this, deregulation of HECT activity is closely associated with diseases such as cancer. HECT domains consist of two lobes that are tethered by a flexible linker allowing the lobes to sample a wide range of conformations with respect to each other. I will discuss mechanisms that we have uncovered using methyl-NMR spectroscopy, x-ray crystallography and biochemical assays that underlie the down-regulation and activation of HECT-type ligases. I will delineate mechanistic commonalities and differences among different classes of HECT ligases and focus on the conformational dynamics of the lobes that is essential for HECT activity.
Access to Atomic Resolution Structural Information of Homo-Repeats by NMR: The Huntingtin Case

Annika Urbanek¹, Anna Morató¹, Matija Popovic¹, Alejandro Estaña¹, Frédéric Allemand¹, Carlos Elena-Real¹, Aurélie Fournet¹, Anabel Jiménez³, Carlos Cativiela³, Juan Cortés², Stéphane Delbecq⁴, Nathalie Sibille¹, Pau Bernadó¹

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ABSTRACT

Homorepeats (HRs), protein fragments composed by the same amino repeated multiple times, are very common in eukaryotes and are involved in key biological processes and multiple pathologies. HRs are enriched in particular biophysical properties that enables them to perform very specialized functions but that can also trigger disease. Despite their interesting properties, the high-resolution structural characterization of HRs has been impaired due to their inherent flexibility and polymeric nature, which give poorly dispersed NMR spectra. Huntingtin (Htt), the causative agent of Huntington’s disease (HD), is the prototypical example of a HR hosting protein. Htt has a poly-Glutamine tract of variable length that becomes toxic when the number of consecutive glutamines exceeds 35. Moreover, Htt contains two Poly-Proline tracts with 11 and 10 consecutive prolines. The long-term aim of our study is to decipher the structural perturbations exerted by the extension of Poly-Glutamine tract beyond the pathological threshold, and the role that flanking regions, including the Poly-Proline tracts, have in the pathology.

To overcome challenges posed by HRs, we have developed a chemical biology strategy to isotopically label individual glutamines and prolines within HRs by combining nonsense tRNA suppression and cell-free expression. Our method disentangles the spectroscopic complexity of the HR and has enabled the NMR investigation of huntingtin exon1 with a 16 residue-long poly-Glutamine tract. The resulting chemical shift analysis indicates the presence of a partially α-helix at its N-terminal region that encompasses part of the HR. The application to poly-Proline has allowed us to precisely explore the proline cis/trans isomerization in these HR regions. Implications of these observations to understand the structural bases of HDs will be discussed.
S06 Takanori KIGAWA

Cell-free protein synthesis for advanced isotopic labeling

Takanori Kigawa¹

1. RIKEN Center for Biosystems Dynamics Research (BDR)

ABSTRACT

We have been developing E.coli cell-extract based cell-free protein synthesis system (CF) for protein production, which has been used not only for a small-scale expression in microgram quantity on 96-well plates but for a large-scale expression in milligram quantity even with the fully automated expression system. It is especially useful for preparing sophisticatedly stable isotope (SI)-labeled proteins for the NMR analysis of complex biomacromolecules. Recently, we have proposed a combinatorial selective labeling (CSL) method named Stable isotope encoding (SiCode), which is based on the idea that amino-acid residue information encoded in the SI-labeling pattern of the residue can be decoded by observing the NMR spectrum [1]. SiCode requires accurate and precise SI labelling, which can only be achieved through CF with metabolic inhibitors to fully suppress isotopic scrambling and dilution [2]. SiCode enables us to combine NMR analysis of protein with information techniques such as Bayesian inference to achieve high tolerance to noisy NMR spectra [3,4]. We have also introduced a new approach called “Stable-isotope-assisted Parameter extraction (SiPex)”, integrating the NMR signal “assignment” step with the “characterization” step, such as 15N relaxation analysis, by combining SiCode and tensor factorization [5]. This enables the decomposition of overlapped signals, and the direct analysis of sparsely sampled time-domain NMR data without Fourier transform. These SI-labeling strategies achieved with CF would be particularly useful for analyzing difficult targets under challenging conditions, such as higher molecular size, lower solubility, lower signal intensity, and molecules in living cells (in-cell NMR).

REFERENCES:

α-Deuteration makes kinases more amenable to NMR studies

Theillet Francois-Xavier1, Roche Maxime2, Ghouil Rania1, Bouguechtouli Chafiaa1

1. Institute for Integrative Biology of the Cell (I2BC), CNRS/CEA/Univ. Paris-Saclay, Av. de la Terrasse, Gif-sur-Yvette 91198, France; 2. CortecNet, CEA-Saclay, Gif-sur-Yvette 91191, France.

ABSTRACT

Kinases are effectors of cell signaling and drive cell fate to a large extent, from cell cycle progression, proliferation, differentiation etc.. They are important drug targets with no doubts. However, they are not always easily amenable to structural investigations because of their dynamic, multiconformational behavior. Regarding NMR studies, they are relatively large proteins of ~35-40kDa, which calls for advanced labeling strategies.

In this context, we have explored the potential benefits of α-deuterated amino acid labeling. α-protons are responsible for about half of dipolar relaxation. Homogeneous deuteration is beneficial but generates longer T1-relaxation, hence long interscan delays and low signal-to-noise per experimental time. Selective α-deuteration would thus be attractive, but is subject to enzymatic back protonation during recombinant expression in living organisms.

Using 15N-amino acids α-deuterated1 and an E.coli cell-free expression system, we have produced 2Dα/15N-Ala/Ser labeled 40kDa MAP-kinases. These samples generates exploitable 1H-15N SOFAST-HMQC spectra (with ~30% slower T2(1H)) that require 10 times less recording time than standard TROSY-HSQC approaches. This permits to distinguish kinases conformations and to detect drug interactions, which should be helpful for pharmacological investigations.

ABSTRACT

Histone post-translational modifications and the proteins that install, erase and interact with them are key players in chromatin biology and DNA transactions such as replication, transcription and repair. Elucidating their role in biological processes and their mechanism of action, however, require access to biochemical substrates that reproduce the chromatin environment and the multitude of DNA and protein interactions that characterize this complex polymer. Here, I will describe strategies to produce synthetic chromatin polymers that contain multiple nucleosomes and post-translationally modified histones that are ideally suited for integrated structural biology investigations. To produce and study these polymers, we utilize state-of-the-art protein engineering tools such as unnatural amino acid incorporation, segmental isotopic labeling, native chemical ligation, cysteine alkylation and cross-linking. These techniques have allowed us to produce 2.4 MDa modified chromatin polymers suitable for solution and solid-state NMR, hydrogen-deuterium exchange and mass spectrometry. I will illustrate the use of these synthetic polymers in the study of histone ubiquitylation by NMR spectroscopy. Our work has illuminated key ubiquitin-chromatin interactions that play a role in chromatin higher order structural organization and dynamics.

REFERENCES:

**S09 Enrico LUCHINAT**

**Recent developments and applications of in-cell NMR in mammalian cells**

*Enrico Luchinat*

1. Magnetic Resonance Center – CERM and Department of Experimental and Clinical Biomedical Sciences “Mario Serio”, University of Florence, Italy.

**ABSTRACT**

In-cell NMR provides insights on biological macromolecules in their native cellular environment at atomic resolution. At CERM, we have developed an approach for expressing and labelling proteins directly in mammalian cells, which is ideally applied to monitor functional processes occurring right after protein synthesis, such as protein folding and maturation events, ligand binding, and changes in redox state. In addition to protein maturation, the approach can also be applied to observe chemical and conformational changes in response to external stimuli. With this approach, we show that exposing human cells to a small reactive molecule, ebselen, affects superoxide dismutase 1 maturation and rescues the correct folding of pathogenic mutants. The effect of toxic substances can also be investigated: cadmium exposure alters the redox state of superoxide dismutase 1 via a mechanism modulated by zinc and metallothioneins. Finally, a modular bioreactor design is shown that allows cells, either in suspension or embedded in gel, to remain viable for longer acquisition times in the NMR spectrometer to allow in principle real-time measurements of biological events.

**REFERENCES:**

**S10 Malene RINGKJØBING JENSEN**

Elucidating the Dynamics of Intrinsically Disordered Protein Complexes by NMR Exchange Spectroscopy

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**ABSTRACT**

Intrinsically disordered proteins (IDPs) display a number of interaction modes including folding-upon-binding, binding without structural transitions or binding through dynamic complexes. The majority of information about IDP binding modes has been inferred from crystal structures of proteins in complex with peptides corresponding to linear motifs of IDPs, however, crystal structures provide a mainly static view of the complexes. Knowledge of the dynamics of IDP complexes is of fundamental importance to understand how IDPs engage in specific interactions without concomitantly high binding affinity.

NMR spectroscopy can be used to access both the structure and dynamics of IDP complexes at atomic resolution (1-2). However, titrations of IDPs with their binding partners often lead to line broadening of the NMR resonances due to exchange occurring on the micro- to millisecond time scale between free and bound forms of the IDP. In these cases, NMR exchange techniques can be used to visualize the interaction trajectory. We combine CPMG relaxation dispersion as well as chemical exchange saturation transfer (CEST) to decipher the dynamic interaction profile of an IDP in complex with its partner. We apply the approach to the dynamic signaling complex formed between the MAP kinase p38α and the disordered regulatory domain of the MAPK kinase MKK4.

Our study demonstrates that MKK4 employs a subtle combination of interaction modes in order to bind to p38α, leading to a complex displaying significantly different dynamics across the bound regions (3-4).

**REFERENCES:**

**S11 Sigrid MILLES**

The Measles virus phosphoprotein – an intrinsically disordered chaperone that regulates nucleocapsid assembly


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**ABSTRACT**

Measles virus genome encapsidation is essential for viral replication and is controlled by the phosphoprotein (P) maintaining the nucleoprotein (N) in a monomeric form before nucleocapsid assembly. All paramyxoviruses harbor highly disordered amino-terminal domains of P (P\textsubscript{NTD}) that are hundreds of amino acids in length and whose function remains unknown. We previously demonstrated how the presence of a short 50 residue peptide of P\textsubscript{NTD} can prevent premature assembly of N during expression by forming a monomeric N\textsubscript{0}P construct that self-assembles into NC-like particles upon addition of RNA in vitro [1]. We now describe the structure and dynamics of the 90-kDa N\textsubscript{0}P complex comprising the full N and P\textsubscript{NTD} with a total of 450 disordered amino acids, at atomic resolution [2]. NMR relaxation dispersion reveals the existence of an ultraweak N-interaction motif, hidden within the highly disordered P\textsubscript{NTD}, that allows P\textsubscript{NTD} to rapidly associate and dissociate from a specific site on N while tightly bound at the amino terminus, thereby hindering access to the surface of N. Mutation of this linear motif quenches the long-range dynamic coupling between the two interaction sites and completely abolishes viral transcription/replication in cell-based minigenome assays comprising integral viral replication machinery. This description transforms our understanding of intrinsic conformational disorder in paramyxoviral replication. The essential mechanism appears to be conserved across Paramyxoviridae, opening unique new perspectives for drug development against this family of pathogens.

**REFERENCES:**

Studies of protein folding, unfolding, and misfolding by rapid pressure jump NMR

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ABSTRACT

The equilibrium between a protein’s folded and unfolded state is strongly impacted by hydrostatic pressure. Many proteins can be unfolded by applying a modest amount (≤2.5 kbar) of hydrostatic pressure or can be mutated to generate small internal cavities, such that the volume difference between the folded and denatured states becomes sufficiently large to permit pressure-induced unfolding inside an NMR spectrometer. Rapidly and repeatedly dropping the pressure from denaturing conditions (i.e. 2.5 kbar) to 1 bar makes possible a range of experiments to monitor the actual folding process under native conditions. By building a device that allows such rapid (ms) and repeated (>100,000 times) switching, it is now possible to monitor directly the folding process by two- and three-dimensional NMR. Measurements on the model system ubiquitin show that the spectrum of the unfolded state disappears at a rate that is faster than the appearance of the folded spectrum, providing evidence of a meta-stable, NMR-invisible intermediate state. Its structure is investigated by pressure-jump NOE and pressure-jump RDC measurements. High pressure is also able to resolubilize peptides and proteins embedded in amyloid fibrils and, as demonstrated for the Alzheimer’s related Aβ peptide, pressure-jump NMR experiments therefore can provide atomic information on the residues involved in the very initial steps of amyloid formation and on the growth of oligomers from less than 100 kD to larger than 1000 kD.
Looking into chaperone assisted protein folding using NMR spectroscopy, X-ray crystallography and Molecular Dynamic Simulations

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ABSTRACT

Chaperones are essential elements in the regulation of protein folding, aggregation prevention and cellular stress response. Despite their critical biological function, their mechanisms of action remain elusive, in part due to the important role played by conformational disorder\(^1\).

Here, we describe the interaction between the small ATP-independent periplasmic chaperone Spy and Im7, an in vivo substrate\(^2\). A possible role of Spy conformational dynamics in its activity was suggested as Spy mutants with higher flexibility present improved chaperone activity\(^3\).

To understand how Spy and Im7 interacts, we developed two parallel approaches, combining coarse grain modelling with either NMR spectroscopy or X-ray crystallography. In the first approach, NMR data were used to create NMR-informed molecular models that were applied to characterize at the residue-specific level the two partners either individually or in interaction\(^4\). In the second approach, classical crystallography revealed only Spy structure due to the high degree of Im7 dynamics in the complex. To overcome this problem, we implemented a strategy based on multiple site-specific iodine labelling\(^5,6\), that allowed us to obtain key information about Im7 behaviour while bound to Spy. The two approaches provide a coherent and convergent picture of a chaperone-substrate interaction allowing for an unprecedented description of the mechanism by which chaperone proteins act.

REFERENCES:

S14 Jerome BOISBOUVIER

Structural and Functional investigation of a 1 Mda chaperonin in action by solution NMR

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ABSTRACT

Chaperonins are ubiquitous protein assemblies present in bacteria, eukaryota and archaea, facilitating the folding of proteins, preventing protein aggregation, and thus participating in maintaining protein homeostasis in the cell. During their functional cycle, they bind unfolded client proteins inside their double ring structure and promote protein folding by closing the ring chamber in an ATP dependent manner. Although the static structures of fully open and closed forms of chaperonins were solved by X-ray crystallography or electron microscopy, elucidating the mechanisms of such ATP-driven molecular events requires studying the proteins at the structural level under working conditions. We introduce an approach that combines site-specific NMR observation of very large proteins, enabled by advanced isotope labelling methods (1), with an in situ ATP-regeneration system. Using this method, we provide functional insight into the 1 MDa large hsp60 chaperonin while processing client proteins and reveal how nucleotide binding, hydrolysis and release control switching between closed and open states (2). 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.

REFERENCES:


S15 Gerhard WAGNER

Use of specific $^{19}$F, $^{13}$C and $^2$H labeling for studies of large biopolymers

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ABSTRACT

Introducing stable isotope in proteins and nucleic acids had a dramatic impact on NMR spectroscopy of biological macromolecules. This has become increasingly important for studies of larger systems. Recently, we became quite interested in studying structure, dynamics and function of membrane proteins. While we initially focused on detergent micelles as membrane surrogates we now focus on nanodiscs, which can provide a near-native bilayer environment. In this context we became particularly interested in structure and dynamics of side chains in addition to protein backbones. Thus, we focused on specific labeling of aromatic side chains to enable the use of the aromatic TROSY and NOEs between aromatics and methyl groups. To utilize aromatic TROSY we need specific isotope labeling, which can be achieved for example by the choice of the growth media. Furthermore, we discovered that relaxation properties of aromatic side chain signals can be dramatically improved by introducing $^{19}$F and betting on the large CSA of $^{19}$F for TROSY experiments. Theoretical calculations and initial experimental results revealed the field-dependence of fluorine/carbon TROSYs for aromatic amino acid side chains and also nucleotide bases. To utilize these effects, specific labeling methods of aromatic moieties need to become available. In addition, executing the experiments requires specific probes that can pulse all nuclei involved.

REFERENCES:

S16 Jacob ANGLISTER

Observation of Intermolecular Transferred-NOE interactions in a 40 kDa Complex: MAP Kinase p38α interactions with its recognition motif

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ABSTRACT

NMR is a very powerful tool to study weak protein-ligand interactions exhibiting dissociation constants in the 1 µM – 1 mM range. This weak binding is usually associated with fast ligand off-rates. One of the most widely used NMR technique to study such complexes is the measurement of chemical shift perturbation upon binding. This is being used to map the residues in the binding protein affected by ligand binding. However, this method is limited as it only provides information on which residues are possibly in the binding site but not on specific interactions with the ligand. It is our claim that transferred NOE measurements can be implemented to study intermolecular interactions for such complexes to obtain more informative structural data that can be used for the structure determination of these complexes. The isotope-edited/isotope-filtered technique is a powerful NMR method used to study intermolecular interactions. However, due to the duration of the editing and filtering steps, which are of the order of 10 ms each, the signal-to-noise ratio drops rapidly for complexes with a molecular mass exceeding 35 kDa and exhibiting tight binding. Uniform deuteration of the proteins labeled with protonated $^{13}$C-methyl groups, considerably enhances the signal-to-noise ratio in such spectra. A further improvement in signal-to-noise ratio can be obtained for protein complexes with small ligands like peptides by using transferred-NOE, TRNOE, when the ligand is in large excess in comparison to the protein. When the ligand is in fast exchange and in large excess the average T2s of the peptide protons are dominated by that of the free peptide thus mitigating the relaxation loses in the filtering step. Here we demonstrate the applicability of the intermolecular transferred NOE effect for studying intermolecular interactions between the MAP-Kinase p38α and a kinase interaction motif (KIM) from the STEP phosphatase. The isotope-edited/isotope-filtered experiment was optimized to eliminate artifacts. At a 1:1 protein:peptide molar ratio the observed NOEs were weak and the signal-to-noise ratio was low. By increasing the molar ratio to 1:3.5 the signal-to-noise ratio improved 3.5-fold and many intermolecular NOEs could be clearly observed. Only a small improvement was achieved by further raising the molar ratio to 1:5. Numerous interactions were observed between the methyl groups of Ile, Leu and Val of p38α and protons of the STEP peptide. Additional intermolecular NOEs were detected using a uniformly $^{13}$C/$^{15}$N labeled p38α sample and an unlabeled STEP KIM peptide, that together provide restraints to generate structures of the complex.
Selective Edition of Intra- vs. Inter- Subunit NOEs : A New Tool to Study Large Protein Complexes

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ABSTRACT

Specific labeling of methyl probes, combined with optimized NMR spectroscopy is a powerful method to study large protein assemblies¹. However, the sequence specific assignment of methyl groups in high molecular weight protein complexes remains a major bottleneck. Recently, computational methods, based on analysis of inter methyl NOEs and known structures, were proposed²-⁵. Yet, their application to protein assemblies remains limited, due to the presence of inter-chain NOEs detected simultaneously with intra-chain NOEs. Misanalysis of these intermolecular connectivities can affect significantly the robustness of the assignment approaches, precluding accurate assignment.

To speed up assignment of homo-oligomeric complexes, we have developed a new combinatorial labeling approach, allowing to edit only intra-chain methyl NOEs. It relies on the labeling of half of the subunits with ¹³CH₃ isotopomers in an otherwise (²H,¹²C)-uniformly protein background, while the other subunits have both methyl group and first covalently bound carbon ¹³C-labeled. Combined with 3D-NOESY edited in constant time in both ¹³C dimensions, this strategy allows to filter out inter-chain NOEs, while preserving intra-chain NOEs edited in a high-resolution 3D spectrum.

The distinction between intra- and inter-chain NOES, obtained from comparison with conventional 3D NOESY spectra, serve as an important tool to cross-validate the assignment. Additionally, this approach can be used in hetero-oligomeric complexes to edit simultaneously both intra-chain and inter-chain NOEs with an opposite sign, allowing immediate and unambiguous identification of precious intermolecular distance restraints.

 Principle and application of this new optimized labeling strategy will be demonstrated on prefoldin, an 86 kDa heterohexameric complex, composed of two α and four β subunits. This strategy improved the assignment process and enabled the study of interaction between prefoldin and amyloidogenic proteins.

REFERENCES:

Resolution beyond the Nyquist grid: Combining the amino acid specific information from pyruvate labeling with band selective pulses to extend the resolution for large systems

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Backbone resonance assignment of proteins is a crucial first step in determining structures, probing intermolecular interactions or investigating dynamics. The resonance assignment is often accomplished by using a standard set of triple resonance experiments. In large molecular weight systems (> 30 kDa), HNCA is the most sensitive experiment that provides sequential connectivity. However, HNCA suffers from poor dispersion in $^{13}$Cα chemical shifts and limited resolution due to a $^{1}J$CaCb coupling. To extend the resolution we biochemically suppressed the $^{1}J$CaCb coupling by employing a new labeling scheme using a mix of $^{13}$C labeled pyruvate isotopomers (mixed pyruvate labeling) during protein expression. This labeling scheme results in a predominant central, uncoupled, narrow peak for all amino acids. This method not only provides highly resolved uncoupled peaks but also provides amino acid specific peak shapes from the residual scalar coupling which adds an extra dimensionality, crucial to breaking degeneracies. Using this we assigned ~85% of the 42 kDa protein MBP using a combination of high resolution and peak shape matching with a single TROSY-HNCA experiment. We have now combined “mixed pyruvate labeling” with band selective sculpting of the chemical shift space to extend this resolution. Results from our recent efforts to use a combination of labeling and optimal control pulse design with relaxation optimized pulse sequence to assign proteins will be presented.
S19 Charalampos Babis KALODIMOS

Approaches for the structure determination of large, dynamic protein complexes by NMR

Charalampos Babis Kalodimos

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ABSTRACT

I will discuss how isotope labeling techniques can be used, combined with 3D and 4D pulse sequences, to determine the atomic resolution structure of dynamic protein complexes. Examples of complexes between molecular chaperones and non-native client proteins will be presented.
S20 Frank GABEL

Small angle neutron scattering for structural biology and dynamics of large biomacromolecular complexes

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ABSTRACT

Small angle neutron scattering (SANS) provides structural information on solubilized biomacromolecules at the nanometer length-scale. In combination with solvent contrast variation (H2O:D2O exchange) and macromolecular deuteration, individual subunits in protein-protein, protein-RNA/DNA or membrane protein complexes can be studied [1]. Here, I present two recent examples in order to illustrate the capacity of SANS to provide structural and dynamic insight into large biomacromolecular complexes. In a first example, long distance and shape restraints from SANS are used to complement and guide the NMR structural refinement of the 390 kDa Box-C/D complex which methylates ribosomal RNA [2]. In addition, the stoichiometry and conformational changes upon substrate titration were determined by this combined NMR-SANS approach. In a second example, the ATP-mediated unfolding of GFP by the archaeal unfoldase PAN is observed by time-resolved SANS, in combination with online fluorescence [3]. By alternating substrate and unfoldase deuteration and using temperature activation (55-60ºC), the evolution of both partners could be followed at a time resolution of 30 seconds. The results show the reversible contraction of PAN during the unfolding process and the aggregation of GFP in the absence of a proteolytic 20S proteasome partner.

REFERENCES:

S21 Sarah WALDIE

Cholesterol Deuteration and Exploitation for the Study of HDL/LDL Exchange Phenomena in Atherosclerosis

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ABSTRACT

Atherosclerosis, the main killer of the west (1,2), is directly associated with the plasma levels of low and high-density lipoproteins (LDL and HDL) (3,4). These particles have been traditionally considered as bad and good cholesterol, respectively, as they either deposit or remove lipids from the vessel wall. These water-soluble aggregates stabilise and transport cholesterol and other fats in the blood. The ratio of LDL to HDL is of greater importance in the development of atherosclerosis than the absolute blood lipoprotein concentrations of the LDL and HDL separately (5). Therefore, lipid exchange between lipoproteins and the cell membrane is of crucial significance for an understanding of cardiovascular disease. It has been shown previously that neutron reflection excels at following lipid exchange processes between human fractions of LDL and HDL. The exchange is dependent on lipid charge and lipoprotein type (6,7). As cholesterol is of such importance in cell membranes for modulating the properties of lipids and further interactions with proteins, understanding the position of cholesterol in bilayers (8,9) and its role in these systems is also crucial to understand.

We have carried out studies on the surface interaction of human HDL and LDL particles with supported lipid bilayers via neutron reflection. In order to follow exchange of lipids, deuterated materials of varying composition with differing levels of deuteration can be used. Results show kinetics of lipid exchange to be dependent on the bilayer composition and the lipid type present; slower exchange was seen in the presence of cholesterol and when using an unsaturated lipid compared to a faster exchange seen when using a fully saturated lipid. These results highlight the effect the lipid environment has on the interaction with lipoprotein particles - notably the level of saturation of the lipids and incorporation of cholesterol on the degree of exchange.

Initial results from small angle neutron scattering and neutron reflectometry structural studies will be discussed alongside technical developments, for
example of the production of deuterated cholesterol, that have been undertaken to provide a clear biomarker within the lipoprotein systems.

REFERENCES
S22 Vinardas KELPŠAS

Development of *Escherichia coli* Strains for Protein Perdeuteration

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**ABSTRACT**

Neutron macromolecular crystallography (NMX) offers a unique approach for locating individual atoms by leveraging the neutron scattering properties of the hydrogen isotope deuterium (D). However, production of deuterated proteins and growing large crystals of proteins is a bottleneck in neutron crystallography. While recombinant production of perdeuterated proteins is relatively straightforward, improvements are needed. Bacteria in heavy water based growth medium experience severely reduced growth rate and biomass yield. We provide examples of improving protein perdeuteration by evolving *Escherichia coli* in deuterated conditions, pinpointing genetical adaptations and developing such strains for production of recombinant perdeuterated proteins. Developed strains have higher growth rate in deuterated conditions, with recombinant protein yields comparable to the parental strain. This is the first step in developing better strains for perdeuterated protein production as these strains are suitable for further genetical improvements.

The improved strains were used to perdeuterate *Leishmania mexicana* Triosephosphate Isomerase (TIM) for NMX. TIM is a key enzyme in glycolysis and it catalyzes the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate via proton transfer. Thus, NMX could provide additional information on protonation state of active site. We have crystalized perdeuterated TIM and collected high-resolution neutron diffraction data. In conclusion, we have developed faster growing *E. coli* strains for production of perdeuterated recombinant proteins and provided proof of usability by perdeutering TIM and collecting neutron diffraction data.
S23 Janosch HENNIG

Segmental and sub-unit selective isotope labeling of protein-RNA complexes for structure determination, combining small-angle neutron scattering and other techniques

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ABSTRACT

Structure determination of protein-RNA complexes is an important undertaking to understand the complex life of RNAs, which is often making the difference between health and disease. However, multi-domain and multi-subunit RNA-binding proteins (RBPs) and their complexes with RNA often include longer flexible sequence regions and transient binding. Also, RNA is inherently more flexible than their protein partners. This makes structure determination by conventional methods, e.g. X-ray crystallography and cryo-EM difficult. Nuclear magnetic resonance spectroscopy (NMR) by itself has size limitations and is therefore suitable for smaller complexes. However, combining NMR especially with small-angle X-ray and neutron scattering can overcome this size limitation and provide important structural insights into these complexes. I will discuss three examples, i) the published translation repression complex, consisting of Sex-lethal, Upstream-of-N-Ras, and msl2 mRNA, ii) the published multi-domain RBP TIA-1 bound to fas intron mRNA, and iii) the unpublished large translation repression complex, consisting of Brat, Pumilio and Nanos, bound to hunchback mRNA. In each example I will focus on the sub-unit selective and sub-domain selective labeling schemes we used and discuss strengths and pitfalls of the methods. Lastly, I will explain how we integrated this hybrid data into structure calculations to derive structural models.
S24 Juli FEIGON

Integrative structural biology of regulatory RNPs: telomerase and 7SK

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ABSTRACT

Telomerase maintains the DNA at the ends of linear chromosomes, thereby preventing genomic instability. It is an RNA-protein complex that contains a catalytic core comprised of a non-coding telomerase RNA (TER) and a unique telomerase reverse transcriptase (TERT), and other proteins involved in biogenesis, assembly, and recruitment of other proteins of the DNA synthesis machinery and to telomeres. TERT uses a template complementary to ~1.5 telomere repeats in TER to repetitively synthesize the telomere repeat sequence at the 3’ end of the DNA (TTGGGG in ciliates, TTAGGG in vertebrates), but this template alone is insufficient for activity with TERT. Multiple steps of ssDNA template binding/realignment, nucleotide addition, strand separation, and template translocation are required for synthesis of a single telomere repeat and telomere repeat addition processivity (RAP). We have been using an integrative structural biology approach combining NMR, crystallography, mass spectrometry, and electron microscopy to study the structure and function of telomerase from the ciliate Tetrahymena and from humans. I will discuss how our NMR studies of telomerase RNA structure and dynamics have been combined with cryoelectron microscopy to help elucidate the roles of TER in this remarkable enzyme. If time permits, I will also present recent NMR and X-ray crystallography results on another important RNP, the human IncRNA 7SK and two of its protein partners Larp7 and MePCE. 7SK is an important regulator of eukaryotic transcription.

REFERENCES:

S25 Olga VITSYNA

Structural studies of the 5'-UTR of HCV RNA by solid-state NMR based structural biology

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ABSTRACT

Hepatitis C virus (HCV) is a single-stranded plus strand RNA virus that belongs to the Flaviviridae family. Liver abundant human microRNA miR-122 acts in a non-typical fashion by stimulating HCV replication and translation upon interaction with the two binding sites within the highly organized 5' end (domain I) upstream of the IRES (Internal ribosome entry site) of HCV genome and leads to changes in architecture of the HCV 5' UTR. Extensive functional and structural studies have been performed on the IRES region of the HCV RNA, however precise structural information and exact mechanism of action of miR122 is missing. In this project we aim to decipher the interaction of miR-122 with the 5' UTR using a broad range of structural biology techniques.

Large RNAs are challenging objects for structural biology. While solution-state NMR has an intrinsic molecular weight limit, X-ray crystallography and cryo-EM are not applicable due to high flexibility of the RNA. Solid-state NMR is an emerging technique that can provide structural information for large biomolecules at atomic resolution. Recently we have developed a protocol for structural characterization of RNA by ssNMR and obtained the first de novo structure of RNA. In the current study we propose a combined approach that utilizes small-angle scattering methods and ssNMR together with segmental labelling of RNA that allows to study a more than 100 kDa large 5' UTR RNA. While ssNMR provides us with local structural information, SAXS allowed us to study overall structure of the 5' UTR in the apo state and in complex with miR-122 at low resolution, while SANS on segmentally deuterated samples can provide with a selective view of single domains. Taken together such integrative approach should provide in depth structural information about a large and flexible complex of the HCV 5'UTR and miR-122.

REFERENCES:

Deciphering the Molecular Regulatory Mechanisms of FUS Phase Separation

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ABSTRACT

Cytoplasmic aggregates of the RNA-binding protein FUS are characteristic for a subset of patients with frontotemporal dementia (FTD) or amyotrophic lateral sclerosis (ALS). A key step that is disrupted in these patients is nuclear import of FUS mediated by the import receptor Transportin 1 (TNPO1). In patients with ALS, this is caused by weakened binding of FUS to TNPO1 due to mutations in the nuclear localization signal (NLS). In FTD caused by FUS, post-translational arginine methylation of FUS is lost, which regulates the FUS – TNPO1 interaction, causing aggregation of TNPO1.

Recently we described the crucial function of TNPO1 and arginine methylation to suppress RGG/RG-driven phase separation and stress granule formation of FUS. The C-terminal RGG3-PY domain and arginines in RGG motifs of FUS are essential for phase separation of FUS, in consequence of RNA-binding. Using NMR-based studies on isotope labeled FUS RGG3-PY we observed the liquid-liquid phase separation upon addition of RNA and show that TNPO1 directly interacts with the PY NLS and arginines in the RGG3-PY domain, thereby displacing RNA from phase separated FUS droplets and dissolving them. TNPO1 acts as a FUS chaperone, suppressing the phase separation and stress granule association of FUS.

Arginine methylation of FUS reduces phase separation and stress granule association of FUS, as seen in FTD-FUS patients, and directly promotes FUS aggregation. Mutation of the FUS-NLS P525L, is associated with ALS, comprises the FUS-TNPO1 interaction, thereby preventing nuclear import and enhancing phase separation and stress granule accumulation of mutant FUS.

Our findings reveal two novel regulatory mechanisms of liquid phase homeostasis that are disrupted in FUS-associated ALS and FTD. This supports the view that phase separation and stress granule accumulation of FUS are crucially involved in ALS/FTD pathogenesis.

REFERENCES:

S27 Junji IWAHARA

Ion labeling for NMR investigations of protein-DNA interactions

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ABSTRACT

DNA recognition by proteins involves highly dynamic processes. For example, transcription factors dynamically scan DNA through nonspecific interactions to locate particular DNA sequences. In the target DNA search process, the proteins change their location on DNA from one site to another not only through dissociation and reassociation, but also through sliding and intersegmentation transfer. Protein-DNA interactions involve dynamics of ions as well. Counterions condensed around DNA are released upon protein’s binding to DNA. Intermolecular ion pairs between protein side-chain and DNA phosphate groups undergo dynamic transitions between the contact ion-pair state and the solvent-separated ion-pair state. Using various NMR methods, which involves labeling of ions, we study these dynamics of protein-DNA interactions. The presentation will cover applications of paramagnetic and isotopic labelings of ions.

REFERENCES:

S28 Andres RAMOS

Understanding combinatorial protein-RNA recognition in RNA regulation

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ABSTRACT

RNA regulation provides a finely-tuned and highly-coordinated control of gene expression. Regulation is mediated by multi-functional RNA-binding proteins which often interact with large sets of RNAs¹. In this talk I will focus on our work to understand how the proteins use multiple RNA-binding domains to interact selectively with the different RNA targets, and perform the different functions. I will discuss how the molecular understanding of the key determinants of recognition can be achieved using a range structural and molecular tools and different labelling strategies, and how the information obtained can be used to establish accurate models for protein-RNA recognition and protein target selectivity.

REFERENCES:

S29 Michael SATTLER

Decoding regulatory protein-RNA recognition and dynamics using integrative structural biology

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ABSTRACT

RNA plays essential roles in virtually all aspects of gene regulation, where single-stranded or folded regulatory RNA motifs are recognized by RNA binding proteins (RBPs). Most eukaryotic RBPs are multi-domain proteins that comprise multiple structural domains connected to mediate protein-RNA or protein-protein interactions. Thus, molecular mechanisms of regulatory protein-RNA complexes often involve dynamic structural ensembles and are controlled by population shifts between inactive and inactive conformations. The domains in these proteins are often connected by flexible linkers or flanked by intrinsically disordered regions, where posttranslational modifications can further modulate the protein-RNA interactions and to regulate the biological activity.

We employ integrative structural biology combining solution techniques such as NMR, small angle scattering (SAXS/SANS) and FRET with X-ray crystallography to unravel the molecular recognition and dynamics for the assembly and molecular function of regulatory RNP (ribonucleoprotein) complexes.

Examples will be presented that highlight the role of intrinsically disordered regions and dynamics in RNA recognition during spliceosome assembly and in miRNA pathways. These data provide unique insight into conformational dynamics underlying the regulation of essential biological processes.

REFERENCES:

How does the nucleoskeleton interact with the inner nuclear membrane and chromatin?

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ABSTRACT

The molecular mechanisms that regulate genome organization in the interphase nucleus are largely unclear. At the interface between the nuclear envelope and chromatin, the inner nuclear envelope contains both nucleoskeleton filaments (lamins) and transmembrane proteins (NETs). Lamins tether heterochromatin to the nuclear envelope and modulate chromosome territory positions. Tissue specific expression of NETs also influences genome organization. Phosphorylation regulates interactions at the nuclear envelope during cell cycle and after a mechanical stress. We focused on a complex formed by lamin A/C, emerin (one of the best characterized NETs) and the chromatin binding protein BAF. We showed that BAF dimer mediates the interaction between lamin A/C and emerin, we solved the 3D structure of the complex\textsuperscript{1}, and we analysed the impact of cell cycle-dependent BAF phosphorylation on BAF structure and complex assembly (Figure 1). We also described the impact of mechano-dependent emerin tyrosine phosphorylation on emerin structure and binding properties. Finally, we identified defective phosphorylation and binding events associated to muscular dystrophy\textsuperscript{2} and premature ageing syndromes\textsuperscript{1}.

Figure 1. Cell cycle-dependent phosphorylation of BAF Ser4 and mechano-dependent phosphorylation of emerin tyrosines regulate the formation of a ternary complex involving lamin A/C, emerin and BAF at the nuclear envelope.

REFERENCES:


S31 Nadia IZADI-PRUNERYE

Integrative Structural Biology of bacterial nano-machines

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ABSTRACT

Bacterial pili are thin flexible filaments that extend and retract on the cell surface to promote adherence, motility and transport of macromolecules. Pili are membrane anchored polymers of protein subunits assembled in helical fibers. These nano-machines are major virulence determinants of many plant, animal and human pathogens. Medical, environmental and fundamental relevance of these molecular machines can explain the growing interest in their study. Due to their dynamic, polymeric and membrane nature, their structural study is only possible by an integrative approach. Here, by combining NMR, cryo-EM and sophisticated modeling, we determined the structure of two bacterial pili at 5 and 8 Å resolution (1,2). The structural data together with mutagenesis and functional assays allowed us to identify residues crucial for assembly and function of these nano-machines.

REFERENCES:

**S32 Adrien FAVIER**

**Integrated NMR and cryo-EM atomic-resolution structure determination of a half-megadalton enzyme complex**

Adrien Favier\(^1\), Diego F. Gauto\(^1\), Leandro F. Estrozi\(^1\), Charles D. Schwieters\(^2\), Gregory Effantin\(^1\), Pavel Macek\(^1,\(\^6\)), Remy Sounier\(^1,\(\^7\)), Astrid C. Sivertsen\(^1\), Elena Schmidt\(^3\), Rime Kerfah\(^1,\(\^6\)), Guillaume Mas\(^1,\(\^8\)), Jacques-Philippe Colletier\(^1\), Peter Guntert\(^3,\(\^4,\(\^5\)), Guy Schoehn\(^1\), Jerome Boisbouvier\(^1\) & Paul Schanda\(^1\)

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**ABSTRACT**

Atomic-resolution structure determination is the key requirement for understanding protein function. Cryo-EM and NMR spectroscopy both provide structural information, but currently cryo-EM does not routinely give access to atomic-level structural data, and, generally, NMR structure determination is restricted to small (<30 kDa) proteins. We introduce an integrated structure determination approach\(^1\) that simultaneously uses NMR and EM data to overcome the limits of each of these methods. The approach enabled determination of the high-resolution structure of the 468 kDa large dodecameric aminopeptidase TET2 to a precision and accuracy below 1 Ångstrom by combining secondary-structure information obtained from near-complete magic-angle-spinning NMR assignments of the 39 kDa-large subunits, distance restraints from backbone amides and specifically labelled methyl groups, and a 4.1 Ångstrom resolution EM map. The resulting structure exceeds current standards of NMR and EM structure determination in terms of molecular weight and precision. Importantly, the approach is successful even in cases where only medium-resolution (up to 8 Ångstrom) cryo-EM data are available, thus paving avenues for the structure determination of challenging biological assemblies.

**REFERENCE:**

Protein structure determination using surface-accessibility data

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ABSTRACT

Characterization of structure and dynamics of large biomolecules by NMR spectroscopy is hampered by severe overlap of NMR signals and the limited number of structural restraints due to low proton density. As a consequence, the number of available NMR data is often sparse and alternative approaches providing complementary NMR data are needed. Paramagnetic relaxation enhancements obtained from inert and soluble paramagnetic probes (sPREs) provide detailed quantitative information about the solvent-accessibility of NMR-active nuclei. sPREs can be easily measured without modification of the biomolecule for any kind of isotopic labeling scheme, are sensitive for molecular structure and dynamics and are therefore becoming increasingly powerful for the study of (dynamic) biomolecules and their complexes in solution. Here we will present the applicability of sPREs for structural and dynamic studies of proteins. We will present the applicability of NMR-derived surface accessibility data for both protein structure determination and de novo structure prediction in the frameworks of XPLOR-NIH, and the structure prediction package Rosetta, respectively. We show that high-quality sPRE data can be obtained in a straightforward manner and substantially improves accuracy and precision of structure prediction and determination approaches. Moreover, we will present and discuss the applicability of sPREs for characterization of dynamic biomolecules. We show that sPREs provide a new class of restraints that are easily accessible and applicable to any kind of biomolecule. In particular for challenging systems and in cases when only sparse data is available, our approach promises significant time savings and significantly improved quality of de novo structure predictions and structure determination based on NMR data. 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 contribute to the integration of different structural biology techniques.
S34 Justice BENESCH

Quantitative biophysics – weighing the evidence for mechanism

Justin Benesch

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ABSTRACT

We have been developing and applying mass-spectrometry-based approaches to interrogate directly the structure and dynamics of proteins. Here I will focus on the insights this has enabled in studying the evolution of specificity in assembly of molecular chaperone proteins, and how they interact with target proteins under the application of force. I also present mass photometry, a new method we have developed that allows the quantitative, label-free interrogation of proteins in solution.

REFERENCES:

**S35 Hermann HEUMANN**

*In vivo*-stable isotopic labeling of animal models such as mice, zebrafish for quantitative proteomics and metabolomics.

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**ABSTRACT**

**Stable Isotopic Labeling of Amino Acids** (such as lysine and arginine) in Cell culture (**SILAC**) and **Stable Isotopic Labelling of Mammalians** (**SILAM**) uniformly with \(^{13}\)C and \(^{15}\)N are approaches facilitating data collection for quantitative proteomic and metabolomics. It will be shown the essentials of SILAC- and SILAM-approaches respectively, their strength and weaknesses. We will present the SILAC-food chain concept, a method for isotopic labeling of higher organisms as mass reference (EP 2 786 153).
S36 Roman A. ZUBAREV

Isotopic Resonance - the First 10 Years

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ABSTRACT

Isotopic resonance (IsoRes) is a postulation that the rates of chemical and biological reactions are affected nonlinearly and non-canonically by the specific ratios between different stable isotopes. The average terrestrial isotopic compositions of the elements C, H, N and O happen to be close to the resonance values. The IsoRes hypothesis formulated in 2008 predicted that the terrestrial IsoRes enhanced the chances of early life to either emerge or take root on our planet [1]. For the following decade, the existence of the IsoRes phenomenon in biology and biochemistry has been extensively tested experimentally and verified by at least two independent groups. Of the important IsoRes isotopic compositions, besides the terrestrial one, is the monoisotopic resonance as well as the resonances at around 350 ppm deuterium [2] and another one at 3.5% 15N, with all other isotopic ratios being normal. The fact that both Mars and Venus seem to have no resonance, underlies the potential importance of the IsoRes phenomenon in space exploration and astrobiology.

REFERENCES:
S37 Guy LIPPENS

Pure Shift methodology for fluxomics

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ABSTRACT

Quantitative information on the carbon isotope content of metabolites is essential for flux analysis. Whereas this information is in principle present in proton NMR spectra through both direct and long-range heteronuclear coupling constants, spectral overlap and homonuclear coupling constants both hinder its extraction. We demonstrate here how pure shift 2D J-resolved NMR spectroscopy can simultaneously remove the homonuclear couplings and separate the chemical shift information from the heteronuclear coupling patterns¹.

A synthetic biology approach whereby the essential natural enzymes for the branched chain amino acid biosynthesis are replaced by small artificial proteins² can be investigated by this methodology, and we will show results how this methodology on the Ile biosynthesis.

REFERENCES:

S38 Pierre BARRAUD

Time-resolved NMR monitoring of tRNA maturation

_Pierre Barraud^1, Alexandre Gato^1, Matthias Heiss^2, Marjorie Catala^1, Stefanie Kellner^2 & Carine Tisné^1_

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ABSTRACT

Decades of exploration in the field of RNA modification have revealed more than 90 different ribonucleoside structures incorporated as post-transcriptional modifications in tRNAs, which not only display the largest variety of post-transcriptional decoration among RNA molecules, but also the highest density of modification per RNA transcript. For instance in yeast, 13 modifications are typically found per tRNA molecule, which corresponds to ~12-20% of its nucleotides bearing a chemical modification. Many of these modifications are introduced at positions in the neighbourhood of others, enabling potential interplay in their incorporation. The study of these circuits of modifications remains difficult since monitoring the maturation of tRNA in real-time at a single nucleotide level is technically challenging. Here, using an original methodology, we show that nuclear magnetic resonance spectroscopy (NMR) is a powerful tool to monitor tRNA maturation events in a non-disruptive and continuous fashion. Time-resolved NMR measurements of different modification events in complex environments such as yeast cell extracts revealed a defined chronology in the incorporation of tRNA modifications. Furthermore, detailed analyses of snapshots measured along the tRNA maturation route under various conditions combined with mass spectrometry measurements (LC-MS/MS) revealed a complex circuitry of modifications where several modifications are seen to have positive and/or negative impact on the incorporation of other ones. We believe that the NMR-based methodology presented here can be adapted to investigate many aspects of tRNA maturation and RNA modifications in general.
S39 Hannes FEYRER

Making RNA at maximum purity and yield with minimal effort and cost

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Abstract

Structural biology, and especially NMR spectroscopy puts some harsh requirements at its RNA samples, that go further than the needs of classical molecular biology. Many techniques require large amounts, often isotope labels and do not tolerate even small heterogeneity in construct length. The standard method for in-house production of RNA is in vitro transcription with T7 RNA polymerase (T7RNAP) from a DNA template, which can lead to problems for many RNAs. T7RNAP tends to produce transcripts inhomogeneous 5’ and 3’ ends from certain sequences. The desired full-length construct requires laborious purification from those transcripts that are too long or too short, which further lowers the overall yield of the RNA obtained.

In the need of large amounts of pure RNA, we developed a protocol to improve T7 transcription reaction at reduced cost and time. It is based on transcription of a repetitive template of the target sequence and successive cleavage with E. Coli RNase H guided by a DNA-based chimeric splint. The robustness of RNase H allows the transcription and cleavage reactions to happen in the same tube, only separated by annealing of the cleavage guide. Simple purification with HPLC or an analogous method gives the full-length product without detectable side products as well as the cleavage guide for reuse. Alternative template routes can be pursued to reduce time and cost for template design for a specific application. For NMR samples with high demand, we used a plasmid with a repetitive insert, while other routes are based on linear synthetic templates with few repeats or rolling circle transcription. Even though the initial cost is slightly higher due to design of template and cleavage guide, this method makes RNA sample preparation for structural biology simpler, easier and cheaper than with traditional in vitro transcription.

References:

S40 Cristoph KREUTZ

Chemical tools to study nucleic acids via NMR spectroscopy

Christoph Kreutz

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ABSTRACT

Our recent additions to $^{13}$C/$^{15}$N-stable isotope labeled RNA and DNA phosphoramidites will be presented and their application to study the structure and dynamics of larger RNAs and DNAs (> 60 nucleotides) via site-specific labeling by chemical means. The high potential to study complex nucleic acid systems by the incorporation of atom-specific $^{13}$C and $^{15}$N labeled residues will be highlighted. In detail, the simplification of NMR spectra of a 61 nt RNA in slow exchange on the NMR chemical shift time scale will be showcased. The residue-specific isotope labeling also allowed to characterize the kinetic signature of the slow exchange process. Furthermore, a direct and robust approach to introduce radical PROXYL/TEMPO tags into larger RNAs will be presented allowing paramagnetic relaxation enhancement (PRE) NMR and electron paramagnetic (EPR) spectroscopy. A procedure for TEMPO labeling of a precursor micro RNA to study RNA protein interactions via PRE NMR will be presented.

REFERENCES:

S41 Katja PETZOLD

MicroRNA’s Dynamics Influence Targeting of mRNA

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Affiliation

ABSTRACT

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

MiRNAs are non-typical RNA samples, as one cannot modify the starting nucleotides, hence improved T7-production is required, which we present here based on repetitive sequence usage and cleavage with RNaseH and chimeras. We then employ R₁ρ relaxation dispersion NMR spectroscopy, and molecular dynamics to dissect the conformational flexibility of the miR-34a binding to Sirtuin 1 (SIRT1) mRNA. R₁ρ NMR data of the central bulge reveals that the weak 7nt long seed (ground state – GS) is in equilibrium with a transient and low populated excited state (ES) 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, without impairing K_d or T_m. 3D structures of GS and seed +1 constructs are calculated from NMR-informed molecular dynamics and docked into the crystal structure of human Argonaute 2 (hAgo2), revealing 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. The functional impact of the miRNA seed +1 ES was studied human cells by luciferase reporter assay showing that the trapped ES exhibits stronger repression, pointing towards the seed +1 state is guiding hAgo2 protein to its active state from the initial encounter complex.

REFERENCES:

S42 Masatsune KAINOSHO

Recent Advances in Isotope-Aided Methods for Studying Protein Structures and Dynamics – Historical Background and Future Perspectives
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ABSTRACT

Isotope-labeling techniques have continually contributed to the development of protein NMR spectroscopy during the past half-century. In the late 60s, selective deuterium labeling approaches were successfully applied for small proteins to obtain structural information from the 1D $^1$H-NMR spectra that were severely overlapped, even at the highest magnetic field-strengths at that time, e.g. 220MHz. The situation was dramatically improved after the mid-80s by the concomitant advent of efficient preparation of uniformly isotope-labeled proteins using recombinant DNA methods and multi-nuclear, multi-dimensional NMR spectroscopy. These technological breakthroughs made NMR methods the main structure determination tool, along with X-ray crystallography, in the structural genomics projects that flourished around the beginning of this century. The stereo-array isotope labeling (SAIL) method was born during this period, as the optimal isotope-aided NMR method for efficient and accurate structure determinations of proteins as large as 40-50 kDa (1).

Meanwhile, the major role of NMR spectroscopy in structural biology has been gradually shifting from structure determination to the characterization of dynamics with various time-scales of biologically interesting targets, such as membrane proteins and extremely large protein complexes, for which the three-dimensional structures are often determined by alternate methods, including cryo-electron microscopy. In this lecture, I will describe our recent results on the isotope-aided methods for obtaining structural and dynamic information for larger proteins, using customized labeled amino acids (1-3). Finally, I will provide my personal views on the next generation of isotope-aided methods in the coming era of ultra-high field NMR spectroscopy over 1 GHz, in light of the historical background (3).

REFERENCES:

S43 Roman LIGHTENECKER

Late metabolic precursors – advantages in selectivity and efficiency concerning cell-based protein isotope labeling

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ABSTRACT

The structure and interplay of macromolecules determines the cell’s proliferation, development, function and fate. A deep understanding of their complex conformational properties and interaction networks represents the key issue to unravel the principles of life at a molecular level. NMR investigation of large macromolecules is limited by sensitivity and resolution issues. Moreover, data acquisition and interpretation are still time consuming processes and sample preparation is often associated with high costs. In the last two decades, protein NMR underwent an impressive evolution to overcome some of these drawbacks. This process was driven by novel isotope labeling techniques in order to produce the essential protein samples to perform state-of-the-art NMR analysis.

During the past years, we established novel techniques of selective Val-, Leu-, Phe-, Tyr-, Trp- and His-residue labeling.¹⁻⁵ The resulting isotope patterns are tailored to the needs of diverse NMR experiments and are currently applied by our cooperation partners to elucidate the structures of large protein complexes, investigate sparsely-populated high energy conformations or map protein interaction sites. Our concept of using multistep organic synthesis to prepare late metabolic precursors in combination with cell-based overexpression systems promises to solve cost issues, which have impeded protein NMR to be used as a routinely applied method in drug development processes so far. Currently, we are extending our toolbox of labeling approaches towards novel precursors, alternative isotope sources, additional target residues, as well as innovative applications.

REFERENCES:

Selective unlabeling strategies of a 120 kDa membrane protein for solid-state NMR spectroscopy

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ABSTRACT

ATP-binding cassette (ABC) transporters use the energy of ATP binding and hydrolysis to translocate a variety of molecules across cell membranes. Some exporters, like the protein BmrA, are involved in multidrug resistance phenotypes, thereby participating to antimicrobial resistance in yeasts and bacteria, or drug resistance in human anti-cancer chemotherapies. During a pump catalytic cycle, the membrane domains of drug transporters alternate between inward-facing (IF) and outward-facing (OF) conformations, during which they translocate drugs across the membrane. 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.

In order to decipher the mechanistic events of the ABC transporters, we investigate using solid-state NMR the ABC transporter BmrA. However, due to its lipids environment and its size (120 kDa) giving rise to a large number of resonances and signal-to-noise-ratio limitations, NMR investigations on BmrA are a challenge. Indeed, even though there is no size limit in solid-state NMR, large proteins are a challenge due to heavy signal overlap and a full sequence-specific assignment is very demanding.

Using unlabelling strategies in combination with paramagnetic relaxation enhancement, we report on conformational differences identified between two states of the protein adopted during the drug-export cycle: the inward-facing and outward-facing states. These strategies allow to analyse the different fingerprints of different states of BmrA and initiate sequential assignment using 2D DARR experiments with long mixing time showing inter-residue correlations.

Using the fingerprints and the partial assignment, we highlight the important role of an ABC exporters specific motif: the X-loop. Using X-loop mutant together with functional data, we show that while (basal) ATP hydrolysis is largely retained, its transport activity is abrogated. This feature clearly shows that ATP hydrolysis is uncoupled from the drug-exportation parts in this mutant and so the crucial role of the X-loop motif.
Intrinsic paramagnetic $\chi$-tensors of lanthanide tags by isotopic labelling

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Pseudocontact shift (PCS) NMR is a powerful technique to study interactions, dynamics and structures of protein – protein and protein – ligand complexes in solution. Trivalent lanthanide ions are the most useful class of paramagnetic centres for PCS NMR and allow to determine the position of nuclei over distances larger than 80 Å from the metal. One option to attach lanthanides to proteins with extremely high affinity and in a rigid and non-fluctuating manner are lanthanide chelating tags (LCTs) based on the DOTA-M4 scaffold.$^{[1]}$ However, even subtle motions within the LCT or relative to the protein severely diminish the achievable anisotropy of the magnetic susceptibility tensor and, hence, the PCS. Even for the most rigid LCTs, the mobility of the linker between chelator and protein is a crucial, but difficult to determine parameter.

We present here for the first time an experimental assignment of strongly paramagnetic LCTs which allow to derive the intrinsic tensor parameters of these LCTs and, thus an upper limit of the achievable PCSs. The extremely short T2 times of these LCTs in connection with vast PCS, leading to proton chemical shift ranges of up to 1400 ppm, render conventional 2D-NMR assignment strategies impossible. We therefore applied a combination of different selective $^2$H and $^{13}$C labelling schemes, as well as double resonance techniques.

REFERENCES:

S46 Alvar Diego Gossert

NMR studies of proteins produced in eukaryotic cells – challenges in expression and NMR spectroscopy.

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ABSTRACT

Our aim is to study drug target proteins by NMR and to understand how their functions are modulated by drugs. The target proteins are often difficult to produce because they are part of larger signaling complexes or because they are membrane proteins. Often they can only be produced in eukaryotic expression hosts.

We have therefore developed economic protocols for isotope labeling in insect cells, the most widely used eukaryotic expression host in structural biology. The labeling techniques are based on using labeled algal amino acid extracts and allow production of a large variety of labeling patterns, including partial (60%) deuteration, which is essential for studying large proteins. [1]

In order to also be able to produce isotope labeled proteins in mammalian cell lines, we have started to develop labeling protocols for HEK cells based on the learnings from insect cells. The proteins produced in mammalian cells with the current protocols are however not deuterated. We therefore developed optimized pulse sequences that enable studying large (>100 kDa) protonated biomolecules.

This combination of labeling techniques and NMR technology finally allows us to characterize signaling complexes consisting of GPCRs and effector proteins.

REFERENCES

S47 Ichio SHIMADA

Function-related Dynamics of High Molecular Proteins

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ABSTRACT

High molecular proteins, such as membrane proteins, play fundamental roles in many physiological processes and are target proteins for drug development. For better understanding of the functions of the 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 (nuclear magnetic resonance spectroscopy) provides us information about proteins dynamics, including conformation equilibrium related to functions. However, it is frequently difficult to obtain information about the 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 stable isotope labelled 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 high molecular proteins.
P01 Beate BERSCH

Structural Basis of Membrane Protein Translocation Through the Mitochondrial Intermembrane Space

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ABSTRACT

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. 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. After translocation through the TOM complex, the precursors are taken over by ATP-independent complexes of small Tim chaperones 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 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 or TIM8/13 chaperones. We employed an integrated structural biology approach, combining NMR, SAXS, in-vivo experiments and various biophysical methods with data-guided molecular dynamics simulations to determine the structural ensemble and dynamics of the chaperone-preprotein complexes. Our findings decipher how the small TIM hexameric complexes transport both α-helical and β-barrel proteins across the mitochondrial intermembrane space. The precursor protein binds to a conserved cleft on the chaperone in a highly dynamic manner, ensuring high overall affinity through avidity of multiple binding sites. The stoichiometry of TIM9/10 or TIM8/13 in complex with different preproteins depends on the substrate-protein length, revealing thus a modular architecture of this versatile chaperone unique in the mitochondrial intermembrane space.

REFERENCES

P02 Takanori KIGAWA

Cell-free protein synthesis for advanced isotopic labeling

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ABSTRACT

We have been developing *E. coli* cell-extract based cell-free protein synthesis system (CF) for protein production, which has been used not only for a small-scale expression in microgram quantity on 96-well plates but for a large-scale expression in milligram quantity even with the fully automated expression system. It is especially useful for preparing sophisticatedly stable isotope (SI)-labeled proteins for the NMR analysis of complex biomacromolecules. Recently, we have proposed a combinatorial selective labeling (CSL) method named Stable isotope encoding (SiCode), which is based on the idea that amino-acid residue information encoded in the SI-labeling pattern of the residue can be decoded by observing the NMR spectrum [1]. SiCode requires accurate and precise SI labelling, which can only be achieved through CF with metabolic inhibitors to fully suppress isotopic scrambling and dilution [2]. SiCode enables us to combine NMR analysis of protein with information techniques such as Bayesian inference to achieve high tolerance to noisy NMR spectra [3,4]. We have also introduced a new approach called “Stable-isotope-assisted Parameter extraction (SiPex)”, integrating the NMR signal “assignment” step with the “characterization” step, such as $^{15}$N relaxation analysis, by combining SiCode and tensor factorization [5]. This enables the decomposition of overlapped signals, and the direct analysis of sparsely sampled time-domain NMR data without Fourier transform. These SI-labeling strategies achieved with CF would be particularly useful for analyzing difficult targets under challenging conditions, such as higher molecular size, lower solubility, lower signal intensity, and molecules in living cells (in-cell NMR).

REFERENCES


**P03 Francois-Xavier THEILLET**

**α-Deuteration makes kinases more amenable to NMR studies**

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**ABSTRACT**

Kinases are effectors of cell signaling and drive cell fate to a large extent, from cell cycle progression, proliferation, differentiation etc. They are important drug targets with no doubts. However, they are not always easily amenable to structural investigations because of their dynamic, multiconformational behavior. Regarding NMR studies, they are relatively large proteins of ~35-40kDa, which calls for advanced labeling strategies. In this context, we have explored the potential benefits of α-deuterated amino acid labeling. α-protons are responsible for about half of dipolar relaxation. Homogeneous deuteration is beneficial but generates longer $T_1$-relaxation, hence long interscan delays and low signal-to-noise per experimental time. Selective α-deuteration would thus be attractive, but is subject to enzymatic back protonation during recombinant expression in living organisms. Using $^{15}$N-amino acids α-deuterated¹ and an *E.coli* cell-free expression system, we have produced ²Da/$^{15}$N-Ala/Ser labeled 40kDa MAP-kinases. These samples generates exploitable $^{1}H$-$^{15}$N SOFAST-HMQC spectra (with ~30% slower $T_2(1H)$) that require 10 times less recording time than standard TROSY-HSQC approaches. This permits to distinguish kinases conformations and to detect drug interactions, which should be helpful for pharmacological investigations.

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P04 Galia DEBELOUCHINA

A Molecular Engineering Toolbox for Integrated Chromatin Structural Biology

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ABSTRACT

Histone post-translational modifications and the proteins that install, erase and interact with them are key players in chromatin biology and DNA transactions such as replication, transcription and repair. Elucidating their role in biological processes and their mechanism of action, however, require access to biochemical substrates that reproduce the chromatin environment and the multitude of DNA and protein interactions that characterize this complex polymer. Here, I will describe strategies to produce synthetic chromatin polymers that contain multiple nucleosomes and post-translationally modified histones that are ideally suited for integrated structural biology investigations. To produce and study these polymers, we utilize state-of-the-art protein engineering tools such as unnatural amino acid incorporation, segmental isotopic labeling, native chemical ligation, cysteine alkylation and cross-linking. These techniques have allowed us to produce 2.4 MDa modified chromatin polymers suitable for solution and solid-state NMR, hydrogen-deuterium exchange and mass spectrometry. I will illustrate the use of these synthetic polymers in the study of histone ubiquitylation by NMR spectroscopy. Our work has illuminated key ubiquitin-chromatin interactions that play a role in chromatin higher order structural organization and dynamics.

REFERENCES

P05 Sigrid MILLES

The Measles virus phosphoprotein – an intrinsically disordered chaperone that regulates nucleocapsid assembly

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ABSTRACT

Measles virus genome encapsidation is essential for viral replication and is controlled by the phosphoprotein (P) maintaining the nucleoprotein (N) in a monomeric form before nucleocapsid assembly. All paramyxoviruses harbor highly disordered amino-terminal domains of P (PNTD) that are hundreds of amino acids in length and whose function remains unknown. We previously demonstrated how the presence of a short 50 residue peptide of PNTD can prevent premature assembly of N during expression by forming a monomeric N0P construct that self-assembles into NC-like particles upon addition of RNA in vitro [1]. We now describe the structure and dynamics of the 90-kDa N0P complex comprising the full N and PNTD with a total of 450 disordered amino acids, at atomic resolution [2]. NMR relaxation dispersion reveals the existence of an ultraweak N-interaction motif, hidden within the highly disordered PNTD, that allows PNTD to rapidly associate and dissociate from a specific site on N while tightly bound at the amino terminus, thereby hindering access to the surface of N. Mutation of this linear motif quenches the long-range dynamic coupling between the two interaction sites and completely abolishes viral transcription/replication in cell-based minigenome assays comprising integral viral replication machinery. This description transforms our understanding of intrinsic conformational disorder in paramyxoviral replication. The essential mechanism appears to be conserved across Paramyxoviridae, opening unique new perspectives for drug development against this family of pathogens.

REFERENCES


Looking into chaperone assisted protein folding using NMR spectroscopy, X-ray crystallography and Molecular Dynamic Simulations

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ABSTRACT

Chaperones are essential elements in the regulation of protein folding, aggregation prevention and cellular stress response. Despite their critical biological function, their mechanisms of action remain elusive, in part due to the important role played by conformational disorder¹.

Here, we describe the interaction between the small ATP-independent periplasmic chaperone Spy and Im7, an in vivo substrate². A possible role of Spy conformational dynamics in its activity was suggested as Spy mutants with higher flexibility present improved chaperone activity³.

To understand how Spy and Im7 interacts, we developed two parallel approaches, combining coarse grain modelling with either NMR spectroscopy or X-ray crystallography. In the first approach, NMR data were used to create NMR-informed molecular models that were applied to characterize at the residue-specific level the two partners either individually or in interaction⁴. In the second approach, classical crystallography revealed only Spy structure due to the high degree of Im7 dynamics in the complex. To overcome this problem, we implemented a strategy based on multiple site-specific iodine labelling⁵,⁶, that allowed us to obtain key information about Im7 behaviour while bound to Spy. The two approaches provide a coherent and convergent picture of a chaperone-substrate interaction allowing for an unprecedented description of the mechanism by which chaperone proteins act.

REFERENCES

Observation of Intermolecular Transferred-NOE interactions in a 40 kDa Complex: MAP Kinase p38α interactions with its recognition motif

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ABSTRACT

NMR is a very powerful tool to study weak protein-ligand interactions exhibiting dissociation constants in the 1 µM – 1 mM range. This weak binding is usually associated with fast ligand off-rates. One of the most widely used NMR technique to study such complexes is the measurement of chemical shift perturbation upon binding. This is being used to map the residues in the binding protein affected by ligand binding. However, this method is limited as it only provides information on which residues are possibly in the binding site but not on specific interactions with the ligand. It is our claim that transferred NOE measurements can be implemented to study intermolecular interactions for such complexes to obtain more informative structural data that can be used for the structure determination of these complexes. The isotope-edited/isotope-filtered technique is a powerful NMR method used to study intermolecular interactions. However, due to the duration of the editing and filtering steps, which are of the order of 10 ms each, the signal-to-noise ratio drops rapidly for complexes with a molecular mass exceeding 35 kDa and exhibiting tight binding. Uniform deuteration of the proteins labeled with protonated $^{13}$C-methyl groups, considerably enhances the signal-to-noise ratio in such spectra. A further improvement in signal-to-noise ratio can be obtained for protein complexes with small ligands like peptides by using transferred-NOE, TRNOE, when the ligand is in large excess in comparison to the protein. When the ligand is in fast exchange and in large excess the average T2s of the peptide protons are dominated by that of the free peptide thus mitigating the relaxation loses in the filtering step.

Here we demonstrate the applicability of the intermolecular transferred NOE effect for studying intermolecular interactions between the MAP-Kinase p38α and a kinase interaction motif (KIM) from the STEP phosphatase. The isotope-edited/isotope-filtered experiment was optimized to eliminate artifacts. At a 1:1 protein:peptide molar ratio the observed NOEs were weak and the signal-to-noise ratio was low. By increasing the molar ratio to 1:3.5 the signal-to-noise ratio improved 3.5-fold and many intermolecular NOEs could be clearly observed. Only a small improvement was achieved by further raising the molar ratio to 1:5. Numerous interactions were observed between the methyl groups of Ile, Leu and Val of p38α and protons of the STEP peptide. Additional intermolecular NOEs were detected using a uniformly $^{13}$C/$^{15}$N labeled p38α sample and an unlabeled STEP KIM peptide, that together provide restraints to generate structures of the complex.
Selective Edition of Intra- vs. Inter- Subunit NOEs: A New Tool to Study Large Protein Complexes

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ABSTRACT

Specific labeling of methyl probes, combined with optimized NMR spectroscopy is a powerful method to study large protein assemblies. However, the sequence specific assignment of methyl groups in high molecular weight protein complexes remains a major bottleneck. Recently, computational methods, based on analysis of inter methyl NOEs and known structures, were proposed. Yet, their application to protein assemblies remains limited, due to the presence of inter-chain NOEs detected simultaneously with intra-chain NOEs. Misanalysis of these intermolecular connectivities can affect significantly the robustness of the assignment approaches, precluding accurate assignment.

To speed up assignment of homo-oligomeric complexes, we have developed a new combinatorial labeling approach, allowing to edit only intra-chain methyl NOEs. It relies on the labeling of half of the subunits with $^{13}$CH$_3$ isotopomers in an otherwise ($^2$H, $^{12}$C)-uniformly protein background, while the other subunits have both methyl group and first covalently bound carbon $^{13}$C-labeled. Combined with 3D-NOESY edited in constant time in both $^{13}$C dimensions, this strategy allows to filter out inter-chain NOEs, while preserving intra-chain NOEs edited in a high-resolution 3D spectrum.

The distinction between intra- and inter-chain NOES, obtained from comparison with conventional 3D NOESY spectra, serve as an important tool to cross-validate the assignment. Additionally, this approach can be used in hetero-oligomeric complexes to edit simultaneously both intra-chain and inter-chain NOEs with an opposite sign, allowing immediate and unambiguous identification of precious intermolecular distance restraints.

Principle and application of this new optimized labeling strategy will be demonstrated on prefoldin, an 86 kDa heterohexameric complex, composed of two α and four β subunits. This strategy improved the assignment process and enabled the study of interaction between prefoldin and amyloidogenic proteins.

REFERENCES

Cholesterol Deuteration and Exploitation for the Study of HDL/LDL Exchange Phenomena in Atherosclerosis

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ABSTRACT

Atherosclerosis, the main killer of the west (1,2), is directly associated with the plasma levels of low and high-density lipoproteins (LDL and HDL) (3,4). These particles have been traditionally considered as bad and good cholesterol, respectively, as they either deposit or remove lipids from the vessel wall. These water-soluble aggregates stabilise and transport cholesterol and other fats in the blood. The ratio of LDL to HDL is of greater importance in the development of atherosclerosis than the absolute blood lipoprotein concentrations of the LDL and HDL separately (5). Therefore, lipid exchange between lipoproteins and the cell membrane is of crucial significance for an understanding of cardiovascular disease. It has been shown previously that neutron reflection excels at following lipid exchange processes between human fractions of LDL and HDL. The exchange is dependent on lipid charge and lipoprotein type (6,7). As cholesterol is of such importance in cell membranes for modulating the properties of lipids and further interactions with proteins, understanding the position of cholesterol in bilayers (8,9) and its role in these systems is also crucial to understand.

We have carried out studies on the surface interaction of human HDL and LDL particles with supported lipid bilayers via neutron reflection. In order to follow exchange of lipids, deuterated materials of varying composition with differing levels of deuteration can be used. Results show kinetics of lipid exchange to be dependent on the bilayer composition and the lipid type present; slower exchange was seen in the presence of cholesterol and when using an unsaturated lipid compared to a faster exchange seen when using a fully saturated lipid. These results highlight the effect the lipid environment has on the interaction with lipoprotein particles - notably the level of saturation of the lipids and incorporation of cholesterol on the degree of exchange.

Initial results from small angle neutron scattering and neutron reflectometry structural studies will be discussed alongside technical developments, for
example of the production of deuterated cholesterol, that have been undertaken to provide a clear biomarker within the lipoprotein systems.

REFERENCES
P10 Vinardas KELPŠAS

Development of *Escherichia coli* Strains for Protein Perdeuteration

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**ABSTRACT**

Neutron macromolecular crystallography (NMX) offers a unique approach for locating individual atoms by leveraging the neutron scattering properties of the hydrogen isotope deuterium (D). However, production of deuterated proteins and growing large crystals of proteins is a bottleneck in neutron crystallography. While recombinant production of perdeuterated proteins is relatively straightforward, improvements are needed. Bacteria in heavy water-based growth medium experience severely reduced growth rate and biomass yield. We provide examples of improving protein perdeuteration by evolving *Escherichia coli* in deuterated conditions, pinpointing genetical adaptations and developing such strains for production of recombinant perdeuterated proteins. Developed strains have higher growth rate in deuterated conditions, with recombinant protein yields comparable to the parental strain. This is the first step in developing better strains for perdeuterated protein production as these strains are suitable for further genetical improvements.

The improved strains were used to perdeuterate *Leishmania mexicana* Triosephosphate Isomerase (TIM) for NMX. TIM is a key enzyme in glycolysis and it catalyzes the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate via proton transfer. Thus, NMX could provide additional information on protonation state of active site. We have crystalized perdeuterated TIM and collected high-resolution neutron diffraction data. In conclusion, we have developed faster growing *E. coli* strains for production of perdeuterated recombinant proteins and provided proof of usability by perdeutering TIM and collecting neutron diffraction data.
P11 Olga VITSYNA

Structural studies of the 5’-UTR of HCV RNA by solid-state NMR based structural biology

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Abstract

Hepatitis C virus (HCV) is a single-stranded plus strand RNA virus that belongs to the Flaviviridae family. Liver abundant human microRNA miR-122 acts in a non-typical fashion by stimulating HCV replication and translation upon interaction with the two binding sites within the highly organized 5’ end (domain I) upstream of the IRES (Internal ribosome entry site) of HCV genome and leads to changes in architecture of the HCV 5’ UTR.¹ Extensive functional and structural studies have been performed on the IRES region of the HCV RNA, however precise structural information and exact mechanism of action of mir122 is missing.²,³,⁴ In this project we aim to decipher the interaction of miR-122 with the 5’ UTR using a broad range of structural biology techniques.

Large RNAs are challenging objects for structural biology. While solution-state NMR has an intrinsic molecular weight limit, X-ray crystallography and cryo-EM are not applicable due to high flexibility of the RNA. Solid-state NMR is an emerging technique that can provide structural information for large biomolecules at atomic resolution. Recently we have developed a protocol for structural characterization of RNA by ssNMR and obtained the first de novo structure of RNA.⁵ In the current study we propose a combined approach that utilizes small-angle scattering methods and ssNMR together with segmental labelling of RNA that allows to study a more than 100 kDa large 5’ UTR RNA. While ssNMR provides us with local structural information, SAXS allowed us to study overall structure of the 5’ UTR in the apo state and in complex with miR-122 at low resolution, while SANS on segmentally deuterated samples can provide with a selective view of single domains. Taken together such integrative approach should provide in depth structural information about a large and flexible complex of the HCV 5’UTR and miR-122.

References

Deciphering the Molecular Regulatory Mechanisms of FUS Phase Separation

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ABSTRACT

Cytoplasmic aggregates of the RNA-binding protein FUS are characteristic for a subset of patients with frontotemporal dementia (FTD) or amyotrophic lateral sclerosis (ALS). A key step that is disrupted in these patients is nuclear import of FUS mediated by the import receptor Transportin 1 (TNPO1). In patients with ALS, this is caused by weakened binding of FUS to TNPO1 due to mutations in the nuclear localization signal (NLS). In FTD caused by FUS, post-translational arginine methylation of FUS is lost, which regulates the FUS – TNPO1 interaction, causing aggregation of TNPO1.

Recently we described the crucial function of TNPO1 and arginine methylation to suppress RGG/RG-driven phase separation and stress granule formation of FUS. The C-terminal RGG3-PY domain and arginines in RGG motifs of FUS are essential for phase separation of FUS, in consequence of RNA-binding. Using NMR-based studies on isotope labeled FUS RGG3-PY we observed the liquid-liquid phase separation upon addition of RNA and show that TNPO1 directly interacts with the PY NLS and arginines in the RGG3-PY domain, thereby displacing RNA from phase separated FUS droplets and dissolving them. TNPO1 acts as a FUS chaperone, suppressing the phase separation and stress granule association of FUS.

Arginine methylation of FUS reduces phase separation and stress granule association of FUS, as seen in FTD-FUS patients, and directly promotes FUS aggregation. Mutation of the FUS-NLS P525L, is associated with ALS, comprises the FUS-TNPO1 interaction, thereby preventing nuclear import and enhancing phase separation and stress granule accumulation of mutant FUS.

Our findings reveal two novel regulatory mechanisms of liquid phase homeostasis that are disrupted in FUS-associated ALS and FTD. This supports the view that phase separation and stress granule accumulation of FUS are crucially involved in ALS/FTD pathogenesis.

REFERENCES

Integrative Structural Biology of bacterial nano-machines

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ABSTRACT

Bacterial pili are thin flexible filaments that extend and retract on the cell surface to promote adherence, motility and transport of macromolecules. Pili are membrane anchored polymers of protein subunits assembled in helical fibers. These nano-machines are major virulence determinants of many plant, animal and human pathogens. Medical, environmental and fundamental relevance of these molecular machines can explain the growing interest in their study. Due to their dynamic, polymeric and membrane nature, their structural study is only possible by an integrative approach. Here, by combining NMR, cryo-EM and sophisticated modeling, we determined the structure of two bacterial pili at 5 and 8 Å resolution (1,2). The structural data together with mutagenesis and functional assays allowed us to identify residues crucial for assembly and function of these nano-machines.

REFERENCES

P14 Hermann HEUMANN

**In vivo-stable isotopic labeling of animal models such as mice, zebrafish for quantitative proteomics and metabolomics.**

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**ABSTRACT**

**Stable Isotopic Labeling of Amino Acids** (such as lysine and arginine) in Cell culture (SILAC) and **Stable Isotopic Labelling of Mammalians** (SILAM) uniformly with $^{13}$C and $^{15}$N are approaches facilitating data collection for quantitative proteomic and metabolomics. It will be shown the essentials of SILAC- and SILAM-approaches respectively, their strength and weaknesses. We will present the SILAC-food chain concept, a method for isotopic labeling of higher organisms as mass reference (EP 2 786 153).
Making RNA at maximum purity and yield with minimal effort and cost

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ABSTRACT

Structural biology, and especially NMR spectroscopy puts some harsh requirements at its RNA samples, that go further than the needs of classical molecular biology. Many techniques require large amounts, often isotope labels and do not tolerate even small heterogeneity in construct length.

The standard method for in-house production of RNA is in vitro transcription with T7 RNA polymerase (T7RNAP) from a DNA template, which can lead to problems for many RNAs. T7RNAP tends to produce transcripts inhomogeneous 5' and 3' ends from certain sequences. The desired full-length construct requires laborious purification from those transcripts that are too long or too short, which further lowers the overall yield of the RNA obtained.

In the need of large amounts of pure RNA, we developed a protocol to improve T7 transcription reaction at reduced cost and time. It is based on transcription of a repetitive template of the target sequence and successive cleavage with E. Coli RNase H guided by a DNA-based chimeric splint. The robustness of RNase H allows the transcription and cleavage reactions to happen in the same tube, only separated by annealing of the cleavage guide. Simple purification with HPLC or an analogous method gives the full-length product without detectable side products as well as the cleavage guide for reuse.

Alternative template routes can be pursued to reduce time and cost for template design for a specific application. For NMR samples with high demand, we used a plasmid with a repetitive insert, while other routes are based on linear synthetic templates with few repeats or rolling circle transcription.

Even though the initial cost is slightly higher due to design of template and cleavage guide, this method makes RNA sample preparation for structural biology simpler, easier and cheaper than with traditional in vitro transcription.

REFERENCES

P16 Roman LIGHTENECKER

Late metabolic precursors – advantages in selectivity and efficiency concerning cell-based protein isotope labeling

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ABSTRACT

The structure and interplay of macromolecules determines the cell’s proliferation, development, function and fate. A deep understanding of their complex conformational properties and interaction networks represents the key issue to unravel the principles of life at a molecular level. NMR investigation of large macromolecules is limited by sensitivity and resolution issues. Moreover, data acquisition and interpretation are still time consuming processes and sample preparation is often associated with high costs. In the last two decades, protein NMR underwent an impressive evolution to overcome some of these drawbacks. This process was driven by novel isotope labeling techniques in order to produce the essential protein samples to perform state-of-the-art NMR analysis.

During the past years, we established novel techniques of selective Val-, Leu-, Phe-, Tyr-, Trp- and His-residue labeling.¹⁻¹ The resulting isotope patterns are tailored to the needs of diverse NMR experiments and are currently applied by our cooperation partners to elucidate the structures of large protein complexes, investigate sparsely-populated high energy conformations or map protein interaction sites. Our concept of using multistep organic synthesis to prepare late metabolic precursors in combination with cell-based overexpression systems promises to solve cost issues, which have impeded protein NMR to be used as a routinely applied method in drug development processes so far. Currently, we are extending our toolbox of labeling approaches towards novel precursors, alternative isotope sources, additional target residues, as well as innovative applications.

REFERENCES

P17 Denis LACABANNE

Selective unlabeling strategies of a 120 kDa membrane protein for solid-state NMR spectroscopy

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ABSTRACT

ATP-binding cassette (ABC) transporters use the energy of ATP binding and hydrolysis to translocate a variety of molecules across cell membranes. Some exporters, like the protein BmrA, are involved in multidrug resistance phenotypes, thereby participating to antimicrobial resistance in yeasts and bacteria, or drug resistance in human anti-cancer chemotherapies. During a pump catalytic cycle, the membrane domains of drug transporters alternate between inward-facing (IF) and outward-facing (OF) conformations, during which they translocate drugs across the membrane. 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.

In order to decipher the mechanistic events of the ABC transporters, we investigate using solid-state NMR the ABC transporter BmrA. However, due to its lipids environment and its size (120 kDa) giving rise to a large number of resonances and signal-to-noise-ratio limitations, NMR investigations on BmrA are a challenge. Indeed, even though there is no size limit in solid-state NMR, large proteins are a challenge due to heavy signal overlap and a full sequence-specific assignment is very demanding.

Using unlabelling strategies in combination with paramagnetic relaxation enhancement, we report on conformational differences identified between two states of the protein adopted during the drug-export cycle: the inward-facing and outward-facing states. These strategies allow to analyse the different fingerprints of different states of BmrA and initiate sequential assignment using 2D DARR experiments with long mixing time showing inter-residue correlations.

Using the fingerprints and the partial assignment, we highlight the important role of an ABC exporters specific motif: the X-loop. Using X-loop mutant together with functional data, we show that while (basal) ATP hydrolysis is largely retained, its transport activity is abrogated. This feature clearly shows that ATP hydrolysis is uncoupled from the drug-exportation parts in this mutant and so the crucial role of the X-loop motif.
Pseudocontact shift (PCS) NMR is a powerful technique to study interactions, dynamics and structures of protein – protein and protein – ligand complexes in solution. Trivalent lanthanide ions are the most useful class of paramagnetic centres for PCS NMR and allow to determine the position of nuclei over distances larger than 80 Å from the metal. One option to attach lanthanides to proteins with extremely high affinity and in a rigid and non-fluctuating manner are lanthanide chelating tags (LCTs) based on the DOTA-M4 scaffold.[1] However, even subtle motions within the LCT or relative to the protein severely diminish the achievable anisotropy of the magnetic susceptibility tensor and, hence, the PCS. Even for the most rigid LCTs, the mobility of the linker between chelator and protein is a crucial, but difficult to determine parameter.

We present here for the first time an experimental assignment of strongly paramagnetic LCTs which allow to derive the intrinsic tensor parameters of these LCTs and, thus an upper limit of the achievable PCSs. The extremely short T2 times of these LCTs in connection with vast PCS, leading to proton chemical shift ranges of up to 1400 ppm, render conventional 2D-NMR assignment strategies impossible. We therefore applied a combination of different selective $^2$H and $^{13}$C labelling schemes, as well as double resonance techniques.

REFERENCES

Site-specific description of disordered protein dynamics from NMR relaxation modelling

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ABSTRACT

While intrinsically disordered proteins (IDPs) constitute about half of the human proteome, a complete understanding of their functional behavior remains enigmatic. As proteins of this class do not possess a stable, three-dimensional structure, but rather sample flat energy landscapes, information about their function is inherently linked to their dynamics occurring on multiple timescales. Herein, we present an application and further development of a recently introduced approach [1] based on model-free analysis of ¹⁵N relaxation rates in combination with an Arrhenius type analysis to characterize temperature-dependent ps to ns dynamics of the intrinsically disordered domain of the cancer-associated mitogen-activated protein kinase kinase (MKK4), which was recently characterized structurally using chemical shifts and residual dipolar couplings [2]. In agreement with our previous observations, the obtained results point towards existence of three dominant dynamic modes on relaxation active timescales associated with fast librational motion, conformational sampling of backbone dihedral angles and slower chain-like segmental dynamics [1]. In addition, the temperature-dependent analysis in a range from 273.1 to 288.1 K allowed us to derive activation energies of the two latter modes at single-residue resolution revealing the existence of sequence dependent features of the dynamics in the unfolded chain. Moreover, we are exploring the use of macromolecular crowding to induce changes in viscosity and, consequently, in spin relaxation rates to obtain mechanistic insight into the dynamics of IDPs in native environment, allowing for direct comparison with ¹⁵N relaxation rates measured in-cell. Altogether, the study presents a novel method for understanding the dynamic behaviour of IDPs underlying their function and/or malfunction in a myriad of diseases.

REFERENCES


P20 Stefi BENJAMIN

Selective labelling of IgE-Fc to study allostERIC communication upon ligand binding using NMR

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ABSTRACT

Immunoglobulin E (IgE) is the central effector molecule of allergic reactions. IgE has two different cellular receptors, FcεRI and CD23. An allergic reaction is initiated by allergen-mediated crosslinking of IgE-bound FcεRI, leading to degranulation of mast cells and release of inflammatory mediators. Structural studies have shown that IgE is a highly dynamic molecule. Crystal structures have shown that the Fc region of IgE exists in bent and extended conformations when bound to different ligands. Previous studies also provide evidence to suggest that the Fc region of IgE undergoes allostERIC changes upon ligand binding. IgE-Fc is a 75kDa homodimer making it a challenging candidate for NMR studies. This challenge, therefore, demands the use of creative approaches that overcome the size issue and allow us to determine the molecular mechanisms of IgE’s allostERIC processes. We are currently using selective isotope labelling strategies to allow NMR-based studies of ligand-mediated structural changes in IgE-Fc.

REFERENCES

P21 Florent BERNAUDAT

The Partnership for Structural Biology (PSB)

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ABSTRACT

The Partnership for Structural Biology (PSB) was established by a Memorandum of Understanding in 2002 by the European Molecular Biology Laboratory (EMBL), the European Synchrotron Radiation Facility (ESRF), the Institut Laue Langevin (ILL) and the Institut de Biologie Structurale (IBS) to provide a unique environment for state-of-the-art integrated structural biology, and comprises about 300 active scientists (staff scientists, students, post-docs and technicians).

This presentation will introduce the activities of the partnership and the wide range of technological platforms available in the PSB.

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P22 Mathilde BERTONI

Production and assembly of isotopically labeled a-synuclein amyloid fibrils

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ABSTRACT

We will present the production, purification and assembly of Parkinson's disease associated a-synuclein fibrils isotopically labeled for solid-state NMR study.
Novel Routes to Deuterated Lipids for Neutron Studies

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ABSTRACT

Analysis of hydrogen-containing molecules via neutron scattering analyses often benefits from the substitution of hydrogen with deuterium atoms, since hydrogen (protium) and deuterium have different scattering length densities (SLDs). Current methodologies for the production of deuterated molecules fall broadly under the categories of ‘chemical’ – using H/D exchange reactions, or deuterated reagents, to exchange or install deuterons – or ‘biological’ – growing organisms in D₂O, often with a deuterated carbon source, followed by extraction and purification of the molecules of interest.

The deuteration platform at ESS is aiming to establish a combined chemical-biochemical approach to deuterated molecules using chemical and enzymatic modification of biologically-produced materials such as lipids. Enzymatic catalysis is advantageous because it is safer than and operates under milder conditions than conventional chemical synthesis and because it shows excellent chemo-, regio- and enantioselectivity, greatly increasing efficiency.

Figure 1. Tail-deuterated POPC, a useful membrane component mimic for neutron scattering studies.
Interaction between HIV Maturation Inhibitors and HIV-1 GAG precursor

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ABSTRACT

Maturation of HIV-1 particle is a key step for viral infectivity. During this process¹,² the polyprotein Gag is cleaved by the viral protease into four proteins MA, CA, NC and p6 and 2 spacer peptides SP1 and SP2. The last cleavage between CA and SP1 could be blocked by maturation inhibitors³. We identified EP39, a derivative of bevirimat, as a new maturation inhibitor that interferes with the protease for this last cleavage⁴. The mechanism of interaction between CA-SP1 and maturation inhibitors is still unknown.

To decipher this mechanism, we determined, by NMR, the structure of the domain spanning CA_Cter, SP1 and NC (CA_Cter-SP1-NC) without and with EP39. The structure of the protein without EP39 showed a stable CA_Cter containing four α-helices, a flexible domain (82-109) comprising the last seven amino acids of CA, SP1 and the first seven amino acids of NC and finally the NC with its two stable zinc fingers. The CA-SP1 junction in the flexible domain is in a dynamic equilibrium of coil-helix⁵. In the presence of EP39, CA_Cter and NC retain the same conformation while the domain (82-109) folds into a stable α-helix. The relaxation studies of the protein alone and in complex with EP39 showed that there was no obvious change in the dynamics of CA_Cter and NC proteins after binding of EP39 while a net decrease of the dynamics of domain (82-109) confirms EP39 binding.

Our results suggest that EP39 inhibits the maturation of HIV particles by interfering with the dynamics equilibrium of coil-helix in CA-SP1 junction and driving this domain to fold into a helix conformation. Further studies to identify the precise interaction site of EP39 with CA-SP1 within Gag need to be performed to develop and optimize new HIV maturation inhibitors.

REFERENCES

P25 Attia BOUCHRA

Structural characterization of the GltJ protein and its interactions with the cytoplasmic platform during the adventurous motility of Myxococcus xanthus

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ABSTRACT

The motility of bacterial cells promotes a range of physiological phenomena such as nutrient detection, predation, biofilm formation and pathogenesis. Myxococcus xanthus uses the so-called adventurous motility (A-motility) to explore new places, change direction and leave the cell swarms. All the studies available revealed that this motility is controlled by cytoplasmic proteins¹⁻³, MglA and AglZ, coupled to the bacterial cytoskeleton mreb and a molecular motor agl, as well as a multiprotein complex⁴ (Glt) crossing the entire bacterial membrane. This assembly forms focal adhesion complexes (FACs) which propel the cell in a certain direction beforehand defined by the cytoplasmic protein, MglA. Within the Glt multiprotein complex, GltJ is a protein who plays a critical role in FACs assembly and regulation. GltJ is located in the inner membrane with a cytoplasmic n-terminus composed of a "zinc ribbon" (ZnR) domain and a "glycine-tyrosine-phenylalanine "(GYF) domain. In order to understand at the molecular and structural level the underlying mechanism behind the A-motility, we used NMR spectroscopy to solve 3D structures of GltJ domains and decipher how the contacts between GltJ domains and cytoplasmic proteins regulate the assembly of FACs. Our results not only represent the first high resolution structural data concerning the ZnR and GYF domains of the Glt complex but also highlight a direct contact between a cytoplasmic effector of the A-motility machinery and the complex Glt. These data allowed us to propose a role of molecular switch for the ZnR and GYF domains of GltJ regulating the assembly and disassembly of FACs.

REFERENCES

Proton-detected NMR spectroscopy at 100 kHz MAS frequency on uniformly-labeled intact bacterial peptidoglycan

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ABSTRACT

Peptidoglycan is one of the main components of the bacterial cell wall. This large polymer is essential in maintaining the integrity of the prokaryotic organism. Due to its multi-gigadalton size, heterogeneity and intrinsic dynamics, atomic-resolution studies of this polymer alone or in complex with constitutive extra-cytoplasmic constitutive enzymes are inherently complex. In this context, solid-state NMR¹ is an important technique to gain insight into its structure, dynamics and interactions. Here, we explore the possibilities to study the PG with ultra-fast (100 kHz) magic-angle spinning NMR on uniformly labeled samples. We demonstrate² that highly resolved spectra can be obtained, and show strategies to obtain site-specific resonance assignments and distance information. We also explore the use of proton-proton correlation experiments,² thus opening the way for NMR studies of intact cell walls or entire cells without the need for isotope labeling.

REFERENCES


Resolving the DNA interaction of the MexR antibiotics resistance regulatory protein
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ABSTRACT

Acquired multidrug resistance (MDR) in pathogenic microbes is a worldwide threat to human health [1]. Several MarR–like proteins regulate the expression of efflux pumps [2]. Since incapacitating the repressor protein leads to continuously high production of the efflux proteins and thus increased survival for the bacteria, there is high mutational pressure for acquired MDR [3]. MexR is a key member of the MarR family and is also mutated in MDR. Analysing the structure-function relationships of MarR family proteins, both in their native and mutated forms, is fundamental to learn how to overcome innate and acquired MDR in future drug development. Previously, the Sunnerhagen group has shown how MDR mutations of MexR retain their original fold but loose DNA binding by shifting the conformational ensemble to exclude the DNA-binding state [4,5]. To prove this hypothesis and to advance structural knowledge on the biologically active repressed state, we need to experimentally assay the DNA-bound conformation of MexR. Unfortunately, however, there is yet no crystal structure of the DNA bound complex of MexR, possibly due to dynamics in the bound state(s).

The DNA binding site of MexR, which controls the repressor of efflux pumps, contains two regions: P1 and P2. We have characterized the MexR complex with P2 and FL (P1+P2) DNA by Isothermal Titration Calorimetry (ITC), Fourier Transform Infrared spectroscopy (FTIR), Dynamic Light Scattering (DLS), Small Angle X-ray Scattering (SAXS) and by Small Angle Neutron Scattering (SANS). By ITC, the MexR-P2 and for MexR-FL affinity and the stochiometry ware been measured. By FTIR we have determined the secondary structure of variously deuterated MexR and found the same amount of secondary structure as in hMexR, indicating maintained structure. By DLS we were able to determine hydrodynamic diameters of the complexes, that is tantamount with their dimension. Structural characterisation of MexR and DNA complexes has been obtained by SAXS. A Guinier analysis showed that MexR alone is aggregation-prone, while the presence of the DNA fragment stabilises a monodisperse complex. Furthermore, we have also performed neutron scattering experiments by recording SANS small and large angle data. We established the respective inter-component distances and topology, exploiting the intrinsic DNA-protein scattering contrast by adjusting the D2O content in an H2O buffer to the DNA (58% D2O) and the d73MexR (97% D2O) matching points. A set of complex models at an atomic level consistent with the scattering data is refined by ensemble modelling of bound states as well as comparative principal component analysis of motions in bound and free states.

REFERENCES
P28 Nina-Eleni CHRISTOU

Light-induced changes in the conformational dynamics of a reversibly photo-switchable fluorescent protein revealed by NMR spectroscopy

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ABSTRACT

Reversibly photo-switchable fluorescent proteins are excellent tools, routinely used for Near-Field Super Resolution Microscopy techniques, e.g RESOLFT. Their characteristic switching between a fluorescent “on” state and a non-fluorescent “off” state, combined with signal processing algorithms has allowed for image resolution of cells in the range of a few nanometers¹. Crystallographic studies of such RSFPs of the GFP family have provided crucial insights on their structure, that have guided the field of fluorescent protein engineering in the search for better tags. However, the crystal form of such proteins as studied at cryogenic temperatures does not provide a realistic picture of the conformational dynamics, and how they influence the photophysics of the RSFP. Here, we present the first, to our knowledge, extensive multi-dimensional NMR study on a RSFP (a green negative RSFP, rsFolder) studied in solution at 40 °C in the fluorosecent “on” and “off” states using an in-situ laser illumination device. Significant changes in the dynamic behaviour of rsFolder are observed between the “on” and the “off” states, especially in the region where the phenol ring of the endogenous chromophore is pointing towards the β-barrel (strands 7-11). We hope our work not only provides scientists with the necessary tools for studying RSFPs using multidimensional NMR, but also contributes to the general advancement of photoactivatable fluorescent proteins.

REFERENCES

Innovative Labeling Techniques to Tackle Challenging Therapeutic Targets by NMR Spectroscopy

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ABSTRACT

Biomolecular Nuclear Magnetic Resonance (NMR) is routinely used to study biological processes. This technique allows the atomic characterization of the 3D structure of biological macromolecules, their dynamics and interactions with physiological partners. NMR is an established tool for hit identification and validation of ligand interaction with therapeutic targets, which is of a great interest for the pharmaceutical industry. However, the standard NMR technology is a low sensitive technique dedicated only to small size proteins (c.a. 30 kDa). This limitation is critical since numerous proteins, which are physiologically relevant, present higher sizes.

In this context NMR-Bio develops new solutions and strategies for advanced isotopic labeling to facilitate the NMR characterization of protein ligand interactions and to enable protein NMR spectroscopy beyond the small protein limits. Recent R&D data of NMR-Bio will be presented illustrating the pertinence of the methyl based-technology to study, at the atomic level, valuable therapeutic targets and agents, expressed in various expression systems.
P30 Guillaume DAVID

A combination of wheat germ cell-free expression and solid state NMR for structural studies of challenging membrane proteins

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ABSTRACT

Despite their utmost importance in many biological processes, membrane proteins have always been a great challenge for structural biology because of the notorious difficulty of producing them in their native form and in enough quantity for structural studies with classical methods, like crystallography and NMR. Indeed, it is usually not possible to produce them in high amount in regular cell-based systems because of the lack of a native-like lipidic environment, of their complexity and because of toxicity at high concentrations. In vitro protein production systems called “cell-free” have been developed to circumvent these issues as their open nature solves any toxicity problem and allows for potential addition of a whole range of molecules to help producing the protein in the right conditions (detergent, lipids, chaperones…).

Amongst all cell-free systems available, the one derived from wheat germ extracts is especially interesting for the most complex proteins, as it is eukaryotic and provides a good yield, which allows for production of a few milligrams of protein1. This yield was still incompatible with solid-state NMR until very recently, with the development of 0.7 mm NMR rotors operating at 110 kHz MAS that need less than a milligram of protein. The combination of the wheat germ cell-free system and solid-state NMR is also very promising on the NMR side, since the protein production happens without any cell thus without any metabolism, minimizing drastically isotope scrambling. This means an effective and specific incorporation of chosen labeled amino acids can be performed, opening up a lot of possible selective labeling strategies.

To illustrate this, we show2 here that milligram amounts of the small envelope protein (DHBs S) of the duck hepatitis B virus (DHBV) can be produced using wheat germ cell-free expression, and that the protein self-assembles into 30 nm subviral particles. 2D proton-detected NMR spectra recorded at 110 kHz magic angle spinning on less than 500 μg of protein show a number of isolated peaks with linewidths between 120 and 190 Hz which is comparable to model membrane proteins. Spectra obtained on numerous samples display great consistency in terms of resolution and signal-to-noise, with potential improvement for the latter by means of further purification of the sample.

REFERENCES

TRBP and PACT pose stoichiometric questions for Dicer complex assembly

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ABSTRACT

Double-stranded RNA-binding domains (dsRBDS) are commonly found in modular proteins that interact with RNA. 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. In humans, Dicer associates with HIV TAR RNA-binding protein (TRBP) or protein activator of PKR (PACT), while in Drosophila, Dicer-1 associates with Loquacious (Loqs). The Type B dsRBD in each of these proteins interacts with the RNA helicase domain of Dicer. All three dsRBPs are also reported to homodimerize, while yeast-two-hybrid studies suggest that TRBP and PACT form heterodimers.

We will present data that show that the Type B dsRBDS of Loqs, PACT and TRBP self associate to form homodimers that have significant structural asymmetry. We have elucidated the 3D structures of the Type B dsRBDS of TRBP and PACT, which are consistent with a previous structure of Loqs. Together, these structures 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. However, 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.

We have also determined that the Type-B dsRBDS of TRBP and PACT preferentially form a heterodimer. Unlike the two modes of interaction that are observed in self-association, heterodimerisation of these Type B dsRBDS involves only a single mode of interaction. Finally, these data show that dsRBD-dsRBD interactions involving Loqs, PACT and TRBP utilize the same surface that is required for binding Dicer, which suggests that dissociation of dsRBD-dsRBD interactions may be a key step in the assembly of a functional Dicer complex.
Characterizing Lectin-Glycan Interactions using Perdeuteration and Neutron Diffraction experiments

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ABSTRACT

Lectins are carbohydrate-binding proteins that play important roles in cell recognition and host-pathogen interactions. The project is focused on lectins from pathogenic bacteria *Photorhabdus luminescens* and *Pseudomonas aeruginosa*. Both bacteria produce several lectins that are specific for the host glycoconjugates and potentially have a role in pathogenicity1,2. X-ray structures of the proteins have been solved. To elucidate the complex interactions between lectins and glycans in more details, we will be using neutron protein crystallography (NMX)

Perdeuterated lectin from *Photorhabdus luminescens* (PLL) have been produced in the deuteration laboratory at the Institut Laue-Langevin (ILL). Both hydrogenated and fully-deuterated PLL have been successfully purified. Medium-size to large crystals of both these proteins (hydrogenated and perdeuterated) have been grown and diffraction data have been collected using x-rays at the ESRF, Grenoble, France. X-ray diffraction data of these H- and D- proteins in the presence of a monosaccharide ligand (L-fucose in the presented work) have been measured. NMX data on a large H/D exchanged PLL crystal have also been collected at the ILL. All these x-ray and neutron diffraction data are being analysed.

A unique part of this project is the *in vivo* production of a perdeuterated monosaccharide, L-fucose, using genetically modified strains of *E. coli* bacteria designed by Dr. Eric Samain at CERMAV. Deuterated fucose has already been successfully produced and purified and is being used as a ligand in further crystallization trials. Future perspectives include the use of slightly longer oligosaccharides, if possible in their deuterated form, to carry out similar studies. The structural information will be of importance to understand the molecular basis of the interaction but also for the design of glycomimetic compounds with anti-infectious properties.

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ABSTRACT

In soft matter and life science neutron scattering experiments, access to full or partially deuterated materials is critical. The molecules that are of most interest include proteins, lipids, fatty acids, small molecules, membranes and so on. For small angle neutron scattering (SANS), neutron reflectometry (NR), and neutron protein crystallography (NPX), using deuterated samples has numerous benefits. For SANS, NR deuteration is most commonly used to enable contrast variation, allowing scientist to selectively “match out” components of complexes. In NPX deuteration is used to boost weak signal-to-noise ratios, reduce the incoherent background due to hydrogen, improve neutron scattering length maps, and enable direct visualization of hydrogen bonds and solvent networks.

DEMAX is the Deuteration and Macromolecular Crystallization support lab for soft matter and life science users of the European Spallation Source (ESS). DEMAX has three support pillars and will be available to all users of ESS instrument: chemical deuteration, biological deuteration, and large crystal growth. We have fully equipped chemistry and life science labs and during operations will offer service for specific classes of deuterated inorganic or organic small molecules (e.g. lactic acid), deuterated biomass/crude lipids/proteins, and access to our crystallization labs for large crystal growth. For biological deuteration and protein crystallization we have established a partnership with Lund University’s Lund Protein Production Platform (LP3), a cross-faculty support lab for the production and crystallization of proteins, both unlabeled and labeled ($^{13}$C, $^{15}$N, $^2$H) using bacteria, insect cells, and yeast. DEMAX and LP3 are co-located in the Biology Department of LU. Access to DEMAX will be granted on a proposal, peer-reviewed basis. We aim to solicit requests for support services and expressions of interest to help grow our competence and develop methods in early 2019. ESS is also the organizing node for DEUNET, a network of deuteration facilities around Europe, more information and news can be found at: http://www.deuteration.net.
In drug design, a detailed characterization of structural changes in proteins upon ligand binding can help to further optimize compounds towards therapeutic interventions. In many cases, these structural alterations are distant from the small molecule binding site, providing a potential to achieve functional implications through allosteric effects. Such dynamic changes are often difficult to observe by static methods, i.e. X-ray crystallography, but can be monitored by NMR spectroscopy [1]. The latter, however, has size-limitations when investigating the protein backbone structure in solution-state. To overcome this, we herein establish an innovative approach employing 100 kHz magic angle spinning (MAS) NMR on the soluble extra-cellular domain of the neonatal Fc receptor (FcRnECD), a crucial protein in regulation of Immunoglobulin G (IgG) and serum albumin catabolism. It is a clinically validated drug target for the treatment of autoimmune diseases caused by pathogenic antibodies, via the inhibition of its interaction with IgG [2]. In combination with computational methods, fragment screening, X-ray crystallography and other biophysical tools, we present the discovery of a small molecule that binds into an evolutionarily conserved cavity of FcRnECD. We provide a detailed structural characterization to explore possibilities for refining the compound as an allosteric modulator. Proton-detected NMR experiments at 100 kHz MAS on fully protonated [$^{13}$C,$^{15}$N]-labeled FcRnECD enabled us to observe ligand-induced structural changes for amino acid residues in the binding pocket and in remote regions of the protein. Labeling with $^2$H was not necessary to record triple-resonance experiments for resonance assignments. The introduced MAS NMR approach can be applied to a large variety of proteins to support structure-based drug discovery campaigns facilitating the detection of allosteric effects.

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P35 Darren HART

User access to high-level research platforms through EU Instruct and French FRISBI programs

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ABSTRACT

In recent years, European structural biology has benefited from large investments in scientific instrumentation. National and European centres have been established with a mission to provide supported user access to their state-of-the-art research platforms. I will present the Instruct-ERIC and French FRISBI infrastructures that provide fully or partially funded user access to their platforms.
Selective High-resolution DNP-enhanced NMR of Biomolecular Binding Sites

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Abstract
Locating binding sites in biomolecular assemblies and solving their structures is of the utmost importance to unravel functional aspects of the system and provide experimental data that can be used for structure-based drug design. This often still remains a challenge, both in terms of selectivity and sensitivity for X-ray crystallography, cryo-electron microscopy and NMR. Here we present a novel approach, dubbed Sel-DNP that allows selectively highlighting and identifying residues present in the binding site of proteins using DNP-enhanced solid-state NMR. DNP has rapidly developed in the last years to increase the overall sensitivity of NMR and has been demonstrated in various applications. Its use for biomolecular systems is however often limited by the necessity to run experiments at cryogenic temperatures, which can induce line broadening and loss of resolution. In this context, Sel-DNP is a powerful site-directed approach, which relies on the use of localized paramagnetic relaxation enhancement, induced by a ligand-functionalized paramagnetic construct, combined with difference spectroscopy to recover high-resolution multidimensional spectra for the binding site. This approach is demonstrated on the galactophilic lectin LecA. The well resolved Sel-DNP spectra enabled the de novo assignment of the binding interface residues. Since this approach produces clean and resolved difference spectra containing resonances from a limited number of residues only, it can be applied for the study of binding sites without any size limitation of the system, and does not require any selective isotopic labelling.
Role of Protein Dynamics in Residence Time of HSP90 Ligands

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ABSTRACT

It is well recognized that proteins are flexible entities susceptible to adopt different conformations and that this protein conformational dynamic is crucial for their functions.\textsuperscript{1} Proteins plasticity plays a role in their ability to bind to their physiological partners including to their drug-like ligands. However, current rational methods to design small molecules ligands are typically based on rigid protein structures. Nonetheless, protein dynamics plays a role in both kinetics and thermodynamics of ligand binding. NMR spectroscopy is an excellent method to investigate protein-ligand interactions but also structure and dynamics over a broad range of functionally relevant timescales.\textsuperscript{2} The Human Heat Shock Protein 90 (HSP90), that stabilizes many signaling proteins such as kinases and nuclear hormone receptors, was identified as a therapeutic target for cancer.\textsuperscript{3} Several ligand chemotypes were found to inhibit HSP90 in an ATP competitive manner including the resorcinol-based ligands.\textsuperscript{4}

In this poster, we will present that using NMR spectroscopy combined with methyl specific labelling in perdeuterated background we were able to investigate the changes in N-HSP90 dynamics upon binding of resorcinol-based ligands. Compared to standard labeling of backbone amides, the advanced methyl labeling improves sensitivity of NMR experiments and lowers peak overlap, while covering all protein structure. To monitor the N-HSP90/ligands dynamics at atomic scale in the $\mu$s-ms timescale we employed the Carr Purcell Meiboom Gill (CPMG) relaxation dispersion method. We show that ligand binding modulates the dynamics of target protein residues and we were able to establish a relationship between the residence time of the ligand on the protein and the N-HSP90 dynamics.

REFERENCES

BRCA2 breast cancer variants: protein phosphorylation kinetics

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ABSTRACT

BReast CAncer susceptibility 2 (BRCA2) is a DNA repair protein that exhibits a large intrinsically disordered N-terminal region of about 1000 residues. Phosphorylation of this BRCA2 region by PLK1 regulates mitotic spindle assembly checkpoint and cytokinesis. Here we used liquid-state NMR spectroscopy to follow BRCA2 phosphorylation by PLK1 (Figure 1, left). We showed that the highly conserved region between aa 190 and 210 is rapidly phosphorylated by PLK1 at S193 and T207, and that phosphorylation at T207 creates a further docking site for PLK1. We tested the impact of several BRCA2 mutations detected in breast cancer patients and found that BRCA2 is significantly less phosphorylated in the case of variants S193A, S206C and T207A2. In parallel, we developed a new method based on the CaCo correlation (Figure 1, right) to follow phosphorylation kinetics by NMR in physiological conditions and provide a more accurate analysis of phosphorylation at BRCA2 residues S193 and T207 in different contexts.

Figure 1. BRCA2 phosphorylation by Plk1 is followed by recording either 1H15N HSQC (left) or CaCO (right) NMR experiments.

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2. Ehlén A., Martin C., Miron S., Julien M., Theillet FX., Boucherit V., Ropars V., Duchampon P., El Marjou A., Zinn-Justin S., Carreira A. Proper chromosome alignment depends on BRCA2 phosphorylation by PLK1 (in revision)
α-PET - new probes in proteins for proton detected solid-state NMR

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ABSTRACT

α-Proton exchange by transamination (α-PET) is presented as a labeling method to introduce $^1$H, while keeping a high deuteration level within the protein.

Over the past decade, proton detected solid state NMR has become a leading techniques to carry out structural and dynamical studies on membrane proteins in near native conditions. However, for fully-protonated samples, fast magic-angle spinning (MAS) of ~55 kHz is insufficient to average proton dipolar couplings, contributing to line broadening. Even at ultra fast MAS of 111 kHz, these couplings are not completely removed.1 Perdeuteration has proved useful to reduce proton dipolar couplings, but only exchangeable amide protons are accessible. In the past, different methods have been proposed to overcome these limitations, for example, by introduction of methyl groups at particular residue types, most commonly ILV, or through more random approaches such as RAP.2

The α-PET works as intended for hydrophobic residues, with full deuteration in the side chain and above 90% protonation at the alpha position. Hydrophilic amino acids demonstrated higher levels of protonation in the side-chain. Residues Arg, Lys and His show less than 10% incorporation. The α-PET samples is limited by inhomogeneous contributions. The method can be used with 1.3 mm rotors to obtain information from amides, as well as additional $^1$H probes, which results in higher sensitivity as compared to 0.7 mm rotors.

REFERENCES


The yeast C/D box snoRNA U14 adopts a K-turn conformation in solution

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ABSTRACT

Non-coding RNAs associate with proteins to form ribonucleoproteins (RNPs), such as ribosome, box C/D snoRNPs, H/ACA snoRNPs, ribonuclease P, telomerase and spliceosome to ensure cell viability. The assembly of these RNA-protein complexes relies on the ability of the RNA to adopt the correct bound conformation. The kink-turn or K-turn motifs represent ubiquitous binding platform for proteins found in several cellular environment. This structural motif has an internal three-nucleotide bulge flanked on its 3’ side by a G●A/A●G tandem pairs followed by one or two non-Watson-Crick pairs, and on its 5’ side by a classical RNA helix (1). This peculiar arrangement induces a strong curvature of the phosphodiester backbone, which makes it conducive to multiple tertiary interactions. SNU13/Snu13p (Human/Yeast) binds specifically the C/D box snoRNA K-turn sequence motif (2,3). This event is the prerequisite to promote the assembly of the RNP, which contains NOP58/Nop58 and NOP56/Nop56 core proteins and the 2’-O-methyl-transferase, Fibrillarin/Nop1p (4). The U14 small nucleolar RNA is a conserved non-coding RNA found in yeast and vertebrates required for the pre-rRNA maturation and ribose methylation (5). Here, we report the solution structure of the free U14 snoRNA K-turn motif (kt-U14) as determined by Nuclear Magnetic Resonance. We demonstrate that a major fraction of free kt-U14 adopts a pre-folded conformation similar to protein bound K-turn, even in the absence of divalent ions. In contrast to the kt-U4 or tyrS RNA, kt-U14 displays a sharp bent in the phosphodiester backbone. The U●U and G●A tandem base pairs are formed through weak hydrogen bonds. Finally, we show that the structure of kt-U14 is stabilized upon Snu13p binding. The structure of the free U14 RNA is the first reference example for the canonical motifs of the C/D box snoRNA family.

REFERENCES

P41 Antoine LOQUET

Raising the sensitivity limits of biological solid-state NMR by combining spin dilution, 1H-detection, very-fast MAS and DNP

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ABSTRACT
We present an approach that uses both dynamic nuclear polarization (DNP) and proton-detection combined with strategic isotopic labelling and fast magic-angle spinning in order to enable an unprecedented sensitivity improvement of solid-state NMR experiments on proteins, opening a new avenue for breaking the sensitivity boundaries in solid-state NMR-based structural biology. Our approach is demonstrated on the HET-s prion domain in its fibrillar state.
P42 Agathe MARCELOT

Phosphorylation of BAF, a small protein at the interface between the nucleoskeleton and DNA

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ABSTRACT

Barrier-to-auto integration factor (BAF) is a 10-kDa abundant DNA binding protein essential for development in metazoans. It is involved in mitosis, nuclear assembly, viral infection and epigenetic regulation [1]. BAF’s ability to bridge distant DNA sites is essential for guiding membranes to form a single nucleus at the end of mitosis [2]. Moreover, BAF is able to interact simultaneously with LEM-domain proteins anchored at the nuclear membrane and lamins that form the nucleoskeleton [3]. BAF function is regulated by phosphorylation at Ser4 by vaccinia-related kinases [4]. BAF phosphorylation by VRK1 abrogates its interaction with DNA in vitro and reduces its association with the nuclear chromatin / matrix in cells. Deletion of VRK1 leads to BAF retention on mitotic chromosomes and abnormal nuclear assembly [5].

We here present new data that highlight how BAF and several variants interact with double-stranded DNA. We reveal, using SEC-MALLS, ITC, fluorescence and NMR, that phosphomimetic BAF mutant S4E and phosphorylated BAF weakly bind to DNA, but exhibit significant differences in their conformations and capacities to bind to the LEM-domain protein emerin and lamin A/C. On the basis of these results, we discuss the mechanisms of BAF function regulation by vaccinia-related kinases.

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NMR solution studies of the WBSCR27 protein
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ABSTRACT

WBSCR27 is an 27 kDa protein, associated with the Williams syndrome, rare human genetic disorder characterized by mental and physiological problems including severe cardiovascular abnormalities. The function of WBSCR27 is unknown, but the bioinformatics predicts that this is a methyltransferase from the S-adenosyl-L-methionine (SAM) dependent methyltransferase superfamily. This assumption is confirmed by the observation of strong interaction of WBSCR27 and SAM using NMR methods. The main goal of our research is determination of 3D NMR structure of WBSCR27 in soluiton. This task is complicated by the presence of multiple resonances for each amide group in ¹⁵N-¹H HSQC spectra of WBSCR27, indicating existence of several protein forms. Being expressed in E.coli cells, it is co-purified as a mixture of complexes with two different ligands, which can not be removed by dialysis. Moreover, it was found that many resonances in ¹⁵N-HSQC spectra change their intensity and position with time. Such changes during long measurement of 3D spectra complicate their analysis.

We found that many such changes are due to the ability of WBSCR27 protein to catalyze reaction of SAM decomposition, which results in the formation of the apo-form.

This reaction is slow. Therefore it is possible to stabilize protein in one of the ligand-bound forms by adding large excess of the ligand. Using this approach the NMR assignments of WBSCR27 in complex with SAM were obtained (1). The determination of 3D protein structure is in progress.

REFERENCES

P44 Laura MARIÑO

Shedding light on the effects of glycation on the protein structure

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ABSTRACT

Protein glycation (PG) is a non-enzymatic process that involves the reaction between reducing sugars and protein amino groups. It ends with the formation of advanced glycation end products (AGEs), which are able to modify the physicochemical features of proteins and therefore, induce their loss of function and the development of diabetes-related diseases¹. For many years, it has been assumed that PG had a chaotropic effect on the protein structure, which would facilitate the protein aggregation. However, recent studies have proven that PG might induce aggregation under the protein native structure²⁻⁴.

To better understand the glycation effect on the protein structure, we have used NMR spectroscopy to analyze at residue level the effect of the AGEs formation on the protein fold. Firstly, we studied a fifteen-residue model peptide (EKE-peptide), which holds a single glycation hot-spot (K) and undergoes a pH-induced coil-helix transition during the pH exchange (from pH ~7.0 to pH ~3.0). We also analyzed the glycation effect on the structure of hen egg white lysozyme (HEWL), which was taken as a model of a globular protein since it has a low aggregation tendency under physiological conditions, and its sequence include several Lys and Arg as potential glycation targets.

The obtained data proved that the glycation of the EKE-peptide mediated by ribose or methylglyoxal (two powerful glycating agents in vivo) is not able to induce any folding event on the disordered structure of the EKE-peptide (at pH 7.4). In addition, we observed that its α-helical structure acquired at pH ~3.0 was not disrupted as a result of glycation.⁵ In addition, we observed that glycation was not able to modify the native structure of HEWL but induced its aggregation through the formation of small oligomers whose mechanism of formation was dependent on the chemical nature of the glycating agent³⁻⁴. Altogether, our data prove that PG scarcely affects the protein structure, but it is able to directs the protein aggregation mechanism.

REFERENCES

P45 Sophie McKENNA

Structural and dynamic characterisation of Streptococcus pyogenes cell-envelope proteinase (SpyCEP)

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ABSTRACT

Streptococcus pyogenes (Group A streptococcus; GAS) is an important human pathogen, responsible for a significant global disease burden from mild to life-threatening conditions such as acute rheumatic fever, pneumonia and necrotising fasciitis, with no effective vaccine and increasing reports of antibiotic resistance. SpyCEP (S. pyogenes cell-envelope proteinase) is a 180 kDa surface-exposed serine protease with a unique structural architecture that inactivates interleukin-8, impairing neutrophil recruitment and host bacterial clearance¹. A promising vaccine candidate and novel target for anti-bacterial virulence therapy, SpyCEP is a modular protease comprised of nine domains derived from 2 non-covalently linked fragments generated by autocatalytic processing². Crystallographic and NMR analysis revealed significant intrinsically disordered regions (IDRs) at the extreme N- and C-termini of SpyCEP (>100 residues). HN, N, Cα and Cβ chemical shift analysis exposed secondary structure propensity in the N-terminal IDR, while the C-terminal region was devoid of structural features. Structural propensity hints at a functional role of the N-terminal IDR with its removal destabilising the SpyCEP heterodimer. To probe the dynamics of the SpyCEP core, selective isotope labelling of isoleucine, leucine, methionine and valine methyl groups was carried out. These complementary samples have been used to decipher the mechanism of interleukin-8 inactivation and determine the functional role of disorder, essential for driving inhibitor design and vaccine development.

REFERENCES

Production of perdeuterated lipids for structural and interactions studies

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ABSTRACT

Scientific interest in the structure and behaviour of lipids in biology and soft matter has developed enormously in the recent years. Lipids are vital to life and are present in all living organisms as an energy reserve and as functional molecules in animals and plants, as well as in fungi to bacteria. Lipids are divided in different groups including fatty acids, cholesterol, triglycerides and phospholipids.

Perdeuterated lipids are extremely useful for neutron scattering studies of biological membranes (or model membranes) as well as membrane proteins. Chemical synthesis of unsaturated perdeuterated lipids is challenging and rarely used. In the ILL Life Sciences group, new protocols have been developed by the Deuteration Laboratory (D-Lab) for the perdeuteration of recombinantly expressed cholesterol\textsuperscript{1}, cardiolipins and matchout labelled phosphatidylcholine for nano-discs\textsuperscript{2,3,4}.

Further developments are on going for oleaginous organisms such as yeasts (\textit{Yarrowia lipolytica}) and marine algae (\textit{Cryptothecodinium cohnii})\textsuperscript{5} which can produce either triglycerides and fatty acids or omega 3 for algae.

The production of perdeuterated lipids for structural and interaction studies will offer new insights to membranes structures and lead to an improved understanding of their important interactions.

REFERENCES

P47 William Brad O’DELL

Deuterium-Induced Limitations to Pro(aox1)-regulated protein overexpression in K. phaffii.

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ABSTRACT

Neutron scattering studies of biomolecules and biological systems typically benefit from some degree of covalent deuterium (D) labeling either to manipulate contrast in the low-q regime or to enhance the scattering contribution from a specific component due to the differences in H/D coherent scattering lengths and incoherent scattering cross sections. Currently most fully, partially and specifically D-labeled proteins used in neutron scattering studies are produced by heterologous expression in Escherichia coli growing in D-enriched media. However, bacteria like E. coli lack pathways for protein post-translational modifications vital to the structures and functions of many eukaryotic proteins. The yeast Komagataella phaffii (Pichia pastoris) is a strong candidate host for expressing large quantities of D-labeled heterologous proteins as it can produce high protein yields (> g L⁻¹) in bioreactor cultures, tolerates growth in highly D-enriched media and can utilize less-expensive D-labeled methanol as sole carbon source and expression inducer. While abundant D-labeled biomass has been obtained from methanol-based D-enriched cultures of K. phaffii, heterologous protein expression under control of the methanol-induced alcohol oxidase 1 (aox1) promoter typically results in yields diminished by several fold. We will present our approaches to and current progress in comparing D-induced effects using different expression promoters in order to obtain abundant D-labeled heterologous proteins from K. phaffii.
Iza OGRIS

Isotopic labelling of Muramyl ligase D for NMR studies of ligand-protein binding

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ABSTRACT
Muramyl ligase D (MurD) is a 47.7 kDa enzyme, belonging to the family of ATP-driven bacterial enzymes Mur ligases, which are involved in the biosynthesis of the bacterial peptidoglycan. Therefore, Mur ligases are an attractive target for the development of novel antibacterial agents.

An important aspect in the design of effective Mur ligases inhibitors appears to be the dynamic nature of ligand-MurD complexes, as revealed by our studies. We investigated the binding mode of MurD inhibitors with various molecular scaffolds in aqueous environment, using both ligand-based and protein-based NMR methods in combination with extensive molecular dynamic (MD) simulations.

In order to carry out the protein-based NMR studies of ligand-MurD binding we have prepared two types of isotopic labelled Escherichia coli MurD:
- selectively $^{13}$C labelled MurD on the methyl groups of Ile ($\delta 1$), Leu and Val to determine the ligand binding sites,
- perdeuterated $^{15}$N labelled MurD for $^{15}$N relaxation studies of MurD domains motions in relation to ligand binding.

With this aim, MurD was heterologously expressed in E. coli strain BL21 (DE3). We optimised the expression and isolation protocol to increase the yield and purity of the labelled protein. The crucial step towards a higher yield of perdeuterated MurD was the optimisation of the unfolding/refolding process at the end of the purification process, in order to exchange $^2$H at the amide group into $^1$H. In these conditions aggregation and misfolding of a protein should be minimised.

Our studies have provided a solid basis for the effective characterization of a site specific MurD ligand binding, as demonstrated here for the various types of MurD inhibitors.

This work was supported by the Slovenian Research Agency (Grant numbers J1-8145 and P1-0010) and EN-FIST Centre of Excellence.

REFERENCES
P49 Lukasz OLEGINSKI

Selective Isotope Labeling to Facilitate RNA Structure and Dynamics Studies by NMR Spectroscopy

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ABSTRACT

Ribonucleic acids (RNAs) are involved in numerous cellular functions, such as gene regulation, catalysis, signaling, and retroviral infection.¹ RNA owes this functional diversity, in part, to its structural plasticity and dynamics.² Unlike X-ray crystallography and cryo-electron microscopy, nuclear magnetic resonance (NMR) spectroscopy is the only high-resolution structural technique capable of probing RNA dynamics on functionally relevant timescales in solution. Yet NMR, as a necessary tool to study RNA functions, faces two challenges for large (> 60 nucleotides, nt) RNAs: spectral crowding and signal loss. Uniform isotopic (¹³C/¹⁵N) labeling minimizes these obstacles for small RNAs, but NMR spectra for large RNAs remain crowded and exhibit signal decay.³ This problem arises from poor chemical shift dispersion and strong dipolar and scalar couplings of adjacent ¹³C-atoms. As a result, NMR experiments run on uniform labeled RNAs show decreased sensitivity and resolution.³ To overcome these obstacles, we combine chemical and enzymatic syntheses to make selective isotope labeled ribonucleotide triphosphates (rNTPs) and ribonucleosides. These rNTPs are used to make RNAs with atom specific labels by T7 RNA polymerase based in vitro transcription.⁴ In parallel, the selective labeled ribonucleosides are used to also make commercially unavailable phosphoramidites to synthesize large RNAs with position specific labels by solid phase synthesis.⁵ Our selective isotope labels reduce spectral crowding and signal decay and improve methods for resonance assignment. Moreover, solid phase synthesis enables structure and dynamic measurements to be made at nucleotide resolution, when used with customized NMR experiments.⁴⁻⁷ Examples for RNAs ranging in size from 63 to 103 nt will be featured.

REFERENCES

P50 Thibaut ORAND

Structure and Dynamics of the Intrinsically Disordered Scaffold Protein JIP1 and Its Interaction With the MAP Kinase JNK

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ABSTRACT

Scaffold proteins play essential roles in mediating signaling specificity in the mitogen-activated protein kinase (MAPK) cell signaling pathways.¹,² They assemble multiple kinases into large signaling complexes thereby leading to sequential and specific activation of the associated kinases. Key to this assembly is their intrinsic disorder often spanning over several hundred residues. Here we use a divide-and-conquer approach to assign several overlapping constructs of the scaffold protein JIP1 leading to a complete assignment of its 450-amino acid intrinsically disordered domain. We report the secondary structure propensities and nuclear relaxation rates of this domain. In a first step towards elucidating the mechanism of action of this scaffold protein, we study the interaction with the JNK kinase using chemical shift titrations and ¹⁵N nuclear relaxation rates. Our results demonstrate an extended binding site compared to what is observed in the co-crystal structure of JNK with a peptide of JIP1.³

REFERENCES

Investigation of the structure and function of Hsp40 (Sis1) by NMR

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ABSTRACT
Protein folding in the cell is usually aided by molecular chaperones, from which the Hsp70 (Hsp=heat shock protein) family has many important roles, such as aiding nascent folding and participating in translocation. Hsp70 has ATPase activity which is stimulated by binding to the J-domain present in co-chaperones from the Hsp40 family. Hsp40s have many functions, as for instance the binding to partially folded proteins to be delivered to Hsp70. However, the presence of the J-domain characterizes Hsp40s or, by this reason, as J-proteins. The J-domain can be isolated maintaining it function of Hsp70 ATPase stimulator. Several lines of investigation, including high-resolution approaches, have been used to understand this complex formed by Hsp70/Hsp40 and a client protein, however, due to the presence of highly flexible domains, the transient interaction between Hsp70 and Hsp40, a detailed full-length of an Hsp40 structure and a detailed interaction mechanism of client folding is still missing. In this work, we present the high-resolution structure of the J-domain of Sis1, the Class B J-domain protein from Saccharomyces cerevisiae, which was solved by NMR. Then, chemical-shift perturbation approaches were used to further study the structure/function relationship of the Sis1/Hsp70 interaction. First, the isolated J-domain was compared to the full-length protein, residues 1-352, and to a G/M domain deletion mutant, and the perturbed residues were identified. The second investigation identified the residues that were affected by the presence of Hsp70. Since two of the residues, T39 and F52, as well as two regions, 26-39 and 67-73, appeared to be equally affected, the results suggest that the J-domain in the full-length Sis1 may be in an intermediate conformation, favorable of binding Hsp70 and facilitating the interaction between the two proteins. Finally, the work also confirmed that the addition of ATP is followed by the disruption of the J-domain/Hsp70 complex, a necessary step for aiding the folding of the client protein.

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Advancements in the efficient production of isotopically labelled proteins in mammalian cells for NMR studies of high-molecular weight complexes

Philip Rößler¹, Daniel Mayer¹, Ching-Ju Tsai², Fred F. Damberger¹, Dmitry Veprintsev², Gebhard F.X. Schertler² and Alvar D. Gossert¹

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ABSTRACT

Isotopic labelled in *E. coli* has become a standard procedure for the production of proteins for NMR spectroscopy. However, despite being a powerful tool capable of producing a variety of different labelling schemes, bacterial expression hosts often struggle to express correctly folded proteins for challenging targets and cannot perform posttranslational modifications. More advanced expression hosts like mammalian cells can overcome these problems, but isotopic labelling is not yet economically feasible due to the need to supplement the growth medium with labelled amino acids. Furthermore, deuteration can presently not be achieved in these cells, thereby complicating the applications to high-molecular weight assemblies.

Here, we present strategies for the production of isotopically labelled membrane proteins in HEK293 cells. For this purpose, a stabilised GPCR mutant (turkey β1 adrenergic receptor) serves as a test protein. Furthermore, we will present a modified version of the ALSOFAST-HMQC experiment [1], which can be more suitable than the standard SOFAST [2] approach for protonated high-molecular weight complexes.

In conclusion, we can show that isotopically labelled proteins from mammalian cell culture can be used in combination with our ALSOFAST approach to characterise large complexes by NMR spectroscopy.

REFERENCES

P53 Jack ROWBOTHAM

A novel biocatalytic platform for selective isotopic labelling of small molecules: applications in the NMR analysis of large proteins


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ABSTRACT

Biocatalysts offer many promising advantages for the introduction of deuterium atoms at specific points on molecules, most notably their excellent chemo-, regio-, and stereo-selectivity. Applications of biocatalytic deuteration systems have been restricted by their requirement for a supply of super-stoichiometric quantities of a specifically labelled $^2$H-pre-cursor, which can be both costly to purchase and complex to prepare. Overcoming this hurdle, we have demonstrated a novel and easy to-use platform for the incorporation of $^2$H-atoms across a number of molecular functional groups. This talk will focus particularly on the use of our technology for the preparation of a suite of isotopically $\{^2\text{H}, \ ^{13}\text{C}, \ ^{15}\text{N}\}$ labelled amino acids, and their subsequent incorporation into large proteins for investigation by NMR spectroscopy. As an example, results will be presented from the $^{13}\text{C}-^1\text{H}$ HMBC analysis of a 400 kDa protein prepared with multiply-labelled alanine. The talk will also discuss the future scope of this versatile labelling technique for applications in structural biology and beyond.

Figure 1 Biocatalytic approach to the synthesis of isotopically labelled compounds for studying large proteins by NMR spectroscopy
Selective isotopic unlabelling of proteins to aid protein structure elucidation by solution NMR spectoscopy

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ABSTRACT

Selective isotopic labelling of specific amino acid types is a useful tool for the study of proteins by NMR spectroscopy. This approach has proven particularly useful for larger or intrinsically disordered proteins where spectral complexity due to signal overlap can be problematic. In ideal cases, specific labelling of amino acid types can be achieved by supplementing minimal growth media with [¹⁵N]- or [¹³C, ¹⁵N]-labelled amino acids. However, for many types of amino acid, this approach leads to isotopic scrambling. For certain residues, it is possible to reduce the effects of metabolic scrambling by supplementing the medium with a biosynthetic precursor of the target amino acid. A combination of these strategies allows specific labelling of a wide range of amino acid types with minimal scrambling.

The application of residue-specific isotope labelling in protein NMR spectroscopy is often prohibited by the high cost of the labelled reagents. Selective isotopic unlabelling of an amino acid type offers a cheaper alternative to specific labelling. In this approach, E. coli are grown in [¹⁵N]- or [¹³C, ¹⁵N]-labelled growth media supplemented with an unlabelled (i.e. natural abundance) amino acid or a metabolic precursor. The resulting protein is uniformly [¹⁵N]- or [¹³C, ¹⁵N]-labeled with the selected residue type at natural abundance and therefore undetectable by NMR spectroscopy. Here, we combine selective residue-type unlabelling of [¹³C, ¹⁵N]-labelled proteins and 2D filter/edit (¹H, ¹H) NOESY experiments to detect NOEs between differentially-labelled sites within the same protein. The combination of selective unlabelling with filter/edit NOESY experiments provides a novel way to detect intramolecular NOEs in otherwise crowded NMR spectra without the need for expensive reagents or selective labelling.

REFERENCES

P55 Johannes SALOMONSSON

Structural Basis for Selective Targeting of Proteasome Deubiquitinases by Enone-containing Compounds

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ABSTRACT

The ubiquitin proteasome system (UPS) is a promising area for drug development where targets such as E1/E2 ubiquitin ligases and deubiquitinases (DUBs) contain functional cysteines. Cysteines can be targeted by compounds containing α,β-unsaturated ketones (enones) by hetero-Michael addition but the extent of selectivity that can be achieved is controversial. In collaboration with our pharmacology collaborators, we addressed this question by screening a library of ~5000 enone-containing compounds for inhibition of proteasome processing. By a range of biophysical, biochemical and cellular methods, we characterized 10 different active drugs and found evidence of selective inhibition of USP14 and proteasome DUB activity. The results suggested that all compounds can bind to a USP14 crevice close to the active site and some compounds also bind covalently, presumably to the active cysteine. We finally demonstrate limited developmental toxicity and significant antineoplastic activity of enone DUB inhibitors in zebrafish embryos. In this presentation, we describe our most recent advances in interpreting the structural and functional properties of USP14 by a combination of NMR and small-angle X-ray scattering.

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P56 Germán Gustavo SGRO

Structural insights into the bacteria-killing Type IV Secretion System from Xanthomonas citri

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ABSTRACT

Type IV Secretion Systems (T4SSs) are multiprotein complexes involved in the transport of DNA and proteins from bacterial and archaeal cells. Recently, our group described a new function for T4SSs from the Xanthomonadaceae family of bacteria, the secretion of antibacterial effectors that target and kill competing bacteria. T4SSs are typically composed of 12 components that form two major assemblies: the inner membrane complex embedded in the inner membrane and the core complex embedded in both the inner and outer membranes. Although progress is steadily being made to decipher the structural and molecular basis of T4SS function, it has been hampered by the lack of high-resolution structures. We heterogously expressed and purified from the membrane fraction the intact T4SS core complex of Xanthomonas citri (Xac), a member of this family of bacteria. The sample was submitted to cryo-electron microscopy single-particle reconstruction, from which a 3.3-Å-resolution model could be built. We also made use of solution NMR to validate and further study a region of this structure that presents some level of flexibility. The vast network of protein-protein interactions in this 1.13-MDa assembly was functionally probed in an exhaustive mutational investigation of interface residues. This unprecedented structure significantly expands our knowledge of the molecular details of T4SS organization and assembly, allowed the identification of specific interactions that could potentially be used as rational drug design targets, and helps us understand how these systems have evolved to inject toxins into target cells. Here we are also presenting the preliminary structural insights into a conserved Xanthomonadaceae-T4SS associated protein with unknown function by NMR studies. This protein displays a three dimensional structure similar to nucleic acids-interacting proteins, suggesting a possible regulatory function.

REFERENCES

Linking opiates-selective signaling with distinct µ-opioid receptor conformations

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ABSTRACT

Recent developments in the field of pharmacology revealed the importance of transmembrane signaling efficacy and functional selectivity for the design of safer drugs acting through the G protein-coupled receptors (GPCRs). It is now well recognized that ligands are able to orchestrate GPCR functional selectivity by modulating the receptor conformational landscape. Here we have combined advanced molecular pharmacology experiments in living cells and solution NMR spectroscopy to analyze the relationship between signaling and conformation of the G protein-coupled µ-opioid receptor (µOR) in complex with either functionally non-selective (DAMGO, BU72) or selective (buprenorphine, TRV130 and PZM21) opioids drugs. For those five compounds, we analyzed the receptor conformational rearrangements around several molecular switches previously shown to be important for the allosteric coupling between extracellular and intracellular receptor domains. Interestingly, we found that selective ligands (i.e. those only activating G proteins) stabilize a different active state conformation. Our results provide a mechanistic description on how opioids drugs modulate the µ-opioid receptor conformations to control transmembrane signaling efficacy and selectivity.
P58 Tsutomu TERAUCHI

Preparation of residue- and site-selectively protonated large proteins using cell-free protein synthesis

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ABSTRACT

The observation of isolated 1H-12C 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 direct proton NMR approach for selectively protonated proteins was not widely used and became 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 1D and other low-dimensional NMR spectroscopy will be revitalized in the coming era, featuring ultrahigh-field spectrometers, for observing the isolated 1H-12C pairs in quite large proteins. Cell-free protein synthesis is a robust protein synthesis technique that enables proteins to be labeled by not only selective protonation but also any type of labeling patterns. In this presentation, we especially focus on the practical protocols for preparing selectively protonated proteins using cell-free protein synthesis. Some of our latest sample preparation techniques will also be presented.

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ABSTRACT

Peroxiredoxins (PRDXs) are thiol active peroxidases responsible of the regulation of peroxides in the cell thanks to a cysteine within their active site. Upon high peroxides rates, they are subjected to catalytic inactivation by hyperoxidation involving structural modifications which leads to the exhibition of a chaperone function. Recently, PRDXs were found to play a role as modulators of the rate of aging in yeasts and act in lifespan extension. In a follow-up study, the anti-aging effect of Tsa1, the major cytosolic PRDX in S. cerevisiae, was shown to be linked to the molecular chaperone Ssa1, member of the Hsp70 family. Tsa1 recruits Ssa1 to aggregated proteins through a H₂O₂-specific redox switch. More specifically, the sulfinylation of Tsa1 supports Ssa1 binding to damaged proteins accumulating in the aging process and its subsequent reduction is required for the disaggregation of misfolded and aggregated proteins. However, besides the fact that Tsa1 and Ssa1 physically interact, the structural and dynamical details of this interaction at the molecular level remain elusive. Therefore, by using high-resolution NMR-spectroscopy, we are studying the mechanism of Tsa1-Ssa1 interaction, in order to characterize this complex in detail and to decipher the functional consequences for both enzymes. As classical solution-state NMR-approaches are limited regarding to the size of the system, two approaches are used: the characterization of smaller Tsa1-variants using sire directed mutagenesis, and the application of methyl-TROSY NMR experiments in combination with methyl-specific isotopic labeling for the study of the larger wild-type protein complex. For the characterization of Ssa1, a divide-and-conquer approach is implemented, consisting of the study of Ssa1 sub-domains separately. Characterization of Tsa1-WT and Tsa1-S78D variant correlated with first NMR experiments on specific-methyl-labeled Tsa1 will be presented showing the feasibility of the approach, as well as preliminary results on Ssa1-NBD subdomain.

REFERENCES

P60 Alicia VALLET

NMRlib 2.1: User-friendly liquid and solid pulse sequence tools for Bruker NMR spectrometers

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ABSTRACT

We present an updated version (2.1) of our pulse-sequence-tool software NMRlib that allows easy setup, running, and sharing of complex NMR experiments on Bruker spectrometers. Each experiment consists in a combination of python setup scripts and a Bruker pulse-sequence program. In particular, shaped pulse parameters are computed on the fly within the pulse sequence from user defined input values (in ppm) for the desired excitation band, that makes the experimental setup independent from the magnetic field strength. The different experiments are accessible via a graphical user interface (GUI) powered by Java swing classes included in the Bruker acquisition software TopSpin. The GUI provides a convenient way of personalizing and classifying the experiment library. A new experiment is set-up by simply clicking on the corresponding button of the NMRlib GUI. In some cases, pop-up windows will open that allow the user to define the most important parameters for the experiment, or choose among different options, e.g. constant time and conventional frequency editing.

This new release provides a set of solid state MAS experiments combined with tools performing automatic calibration of hard pulse lengths and cross-polarization transfer parameters. Interestingly, NMRlib also provides a tool that allows to save any interesting experiment as a NMRlib-type python script, and add a corresponding button in the NMRlib GUI. NMRlib is compatible with TopSpin versions 3.2 and 3.5. The software is freely available for academic users from the IBS web page (http://www.ibs.fr/science-213/scientific-output/software/pulse-sequence-tools/)

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Isotopic labelling for the determination of the Intrinsic paramagnetic $\chi$-tensors of lanthanides

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\textbf{ABSTRACT}

Pseudocontact Shift (PCS) NMR-spectroscopy is widely used to study the dynamics, interactions and structures of proteins. PCS are observed on a protein if a paramagnetic center is brought into close proximity of the protein. Lanthanides are most often used as paramagnetic centers due to their unique paramagnetic properties. The size of the observed PCSs is proportional to the anisotropic part of the magnetic susceptibility the $\chi$-tensor. Movement of the lanthanide relative to the protein reduces the size of the $\chi$-tensor. Lanthanide chelating tags (LCTs) have been developed in order to attach lanthanides in a stereo-chemically pure and rigid manner to the protein.\textsuperscript{[1]} So far the anisotropic components of the magnetic susceptibility ($\Delta\chi_{ax.}$ & $\Delta\chi_{rh.}$) have been determined only on the protein where they are reduced by motional averaging. The intrinsic tensor a lanthanide exhibits within its chelator is unknown. This is due to strong PRE induced by the lanthanide, preventing measurement of 2D-NMR in most cases, resulting in only very spares spectroscopic data. In this work, we used different labelling schemes to obtain chemical shifts directly from 1D-NMR and double resonance spectra. Herein we present a synthetic route to DOTA-M7FPy, which allows for the synthesis of different labelling schemes as well as the fitting process necessary in order to obtain the $\Delta\chi$-parameters. Fitting also revealed the presence of significant contact shifts.

\textbf{REFERENCES}

ABSTRACT

NMR is a powerful tool in early stage drug discovery. Its most prominent roles in the pharmaceutical industry are as a sensitive screening method for finding de novo hit matter or as an orthogonal biophysical method for confirming hits. Primary hit finding, and validation is most efficiently performed using so-called 'ligand observed' experiments. The corollary of this approach is 'protein observed' NMR, where chemical shift changes in a protein are measured upon addition of a hit molecule and which can be used to fully validate a protein-ligand interaction as far NMR is concerned. Ligand titration and structure determination of the protein-ligand complex can further characterize the complex in terms of affinity and binding mode.

While ligand observed methods are generally and broadly applicable, protein observed methods suffer from the usual size limitations encountered in NMR. The advent of TROSY NMR in conjunction with high levels of protein deuteration has pushed the molecular weight (MW) limit of the applicability of $^{15}$N-$^1$H-correlation spectra to ~50 kDa$^{1)}$. Exploiting the TROSY effect in selective methyl protonated and methyl-$^{13}$C-labelled proteins extends the MW limit for observing $^{13}$C-$^1$H-correlations to well above 100 kDa$^{2)}$. While the full benefits of the $^{15}$N-$^1$H TROSY effect can only be achieved at high magnetic fields, the $^{13}$C-$^1$H TROSY effect is field independent$^{3,4)}$. This makes it of particular interest to labs with routine access only to lower field spectrometers. We show here a couple of examples where $^{13}$C,${}^1$H-methyl labelling (with or without background deuteration) has enabled us to characterize proteins and protein-ligand interactions in systems with MWs greater than 50 kDa on our 500MHz spectrometer.

REFERENCES

P63 Anna WEHLIN

Structural and functional characterization of PLA2G16

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ABSTRACT

The Picornavirus family is a large group of non-enveloped RNA viruses, which can cause a wide range of biomedically important human and animal diseases, such as polio or the common cold. During infection, the picornaviruses depend upon cellular proteins, so called host factors, in order to replicate and complete their viral life cycle. Due to the high mutation rates and rapid evolution of viral proteins, host factors, which are non-essential for the cell, are attractive alternative targets in the development of novel antiviral therapies. PLA2G16 is a human phospholipase which recently was identified as a host factor required for the viral infection of certain enteroviruses. PLA2G16 is involved early in the virus entry and its role as a host factor is utterly reliant on its catalytic activity.¹ However, the actual mechanism of action is not yet known. PLA2G16 is composed of an N-terminal catalytic domain, followed by a C-terminal hydrophobic region. Prior structural studies have revealed that the N-terminal globular domain houses a conserved catalytic triad in an arrangement compatible with catalysis.²,³,⁴ However, it appears that the C-terminal hydrophobic region is vital for the proper function of the enzyme.² To clarify the role of the C-terminal and to gain a better understanding of its mechanism of action, we aim to elucidate the full-length protein structure by NMR. Alongside, we will also probe the dynamics of the protein as well as its interactions with lipids and model membranes.

REFERENCES

P64 Eiso AB

A Complete Pipeline for Enabling Efficient and Timely NMR Structural Biology on Challenging Pharmaceutical Targets

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ABSTRACT

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.
P65 Adrien FAVIER

Integrated NMR and cryo-EM atomic-resolution structure determination of a half-megadalton enzyme complex

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ABSTRACT

Atomic-resolution structure determination is the key requirement for understanding protein function. Cryo-EM and NMR spectroscopy both provide structural information, but currently cryo-EM does not routinely give access to atomic-level structural data, and, generally, NMR structure determination is restricted to small (<30 kDa) proteins. We introduce an integrated structure determination approach¹ that simultaneously uses NMR and EM data to overcome the limits of each of these methods. The approach enabled determination of the high-resolution structure of the 468 kDa large dodecameric aminopeptidase TET2 to a precision and accuracy below 1 Angstrom by combining secondary-structure information obtained from near-complete magic-angle-spinning NMR assignments of the 39 kDa large subunits, distance restraints from backbone amides and specifically labelled methyl groups, and a 4.1 Angstrom resolution EM map. The resulting structure exceeds current standards of NMR and EM structure determination in terms of molecular weight and precision. Importantly, the approach is successful even in cases where only medium-resolution (up to 8 Angstrom) cryo-EM data are available, thus paving avenues for the structure determination of challenging biological assemblies.

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