AILM 2015

Advanced Isotopic Labelling Methods for Integrated Structural Biology

February 2nd to 5th 2015
GRENOBLE - France

The past few years have seen many significant advances in the stable isotopic labelling technologies that are used for studying biological systems. These tremendous developments underpin numerous experimental techniques, which interrogate the structure, dynamics and function of biomolecular systems across a wide range of molecular sizes and sample conditions, including inside living cells. The experimental possibilities offered by these methods is just starting to be explored.

The AILM 2015 workshop will survey recent developments in isotopic labelling techniques and their applications to the study of biomolecular structure, dynamics and function. This interdisciplinary meeting will bring together researchers from solution and solid-state NMR spectroscopy, mass spectrometry, neutron scattering and diffraction. The goal is to promote scientific exchange about the latest techniques for producing and analyzing isotopically labelled biological samples. The number of attendees as well as the very high quality of the communications are rewarding signs that the biophysical research community has a keen interest in this subject area.

A number of sponsors have provided travel grants to invited scientists and helped us to gather a large and international panel of experts in isotopic labelling. The contribution of sponsors to specific sessions is indicated in the detailed program. We gratefully acknowledged their generosity. Several companies will be in attendance at AILM 2015. Company exhibits will be situated in the main entrance hall of the IBS. We invite you to visit them and discuss solutions they may have for your research. Lastly, we are indebted to both the National and European institutional sponsors listed below. Their contributions have made this meeting possible.

We hope that this workshop, situated in the heart of the French Alps, will meet your expectations and will inspire future development and exciting uses of isotopic labelling technologies in integrated structural biology

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

The organizing committee

Jerome Boisbouvier (IBS-Grenoble)

Bruno Kieffer (IGBMC- Strasbourg)

Michael Plevin (University of York)

Carine Tisné (LCRB-Paris)

GENERAL INFORMATION

Registration and Venue

The AILM2015 Conference takes place at IBS new building 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.** Badges that will allow access to the conference site as well as to the site restaurant will be given at the Registration Desk.

Registration:

Registration takes place at the IBS Central Building on Monday, February 2nd from 10 am to 2 pm. Late registrations will be possible at Help Desk situated in the IBS Entrance Hall.

Venue:

- from Lyon Airport: take the shuttle bus to Grenoble and stop at “Presqu’île – Résistance” (you may ask the driver to stop there). The bus stop is within walking distance to the EPN Campus (see access map on next page).

- 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 next page).

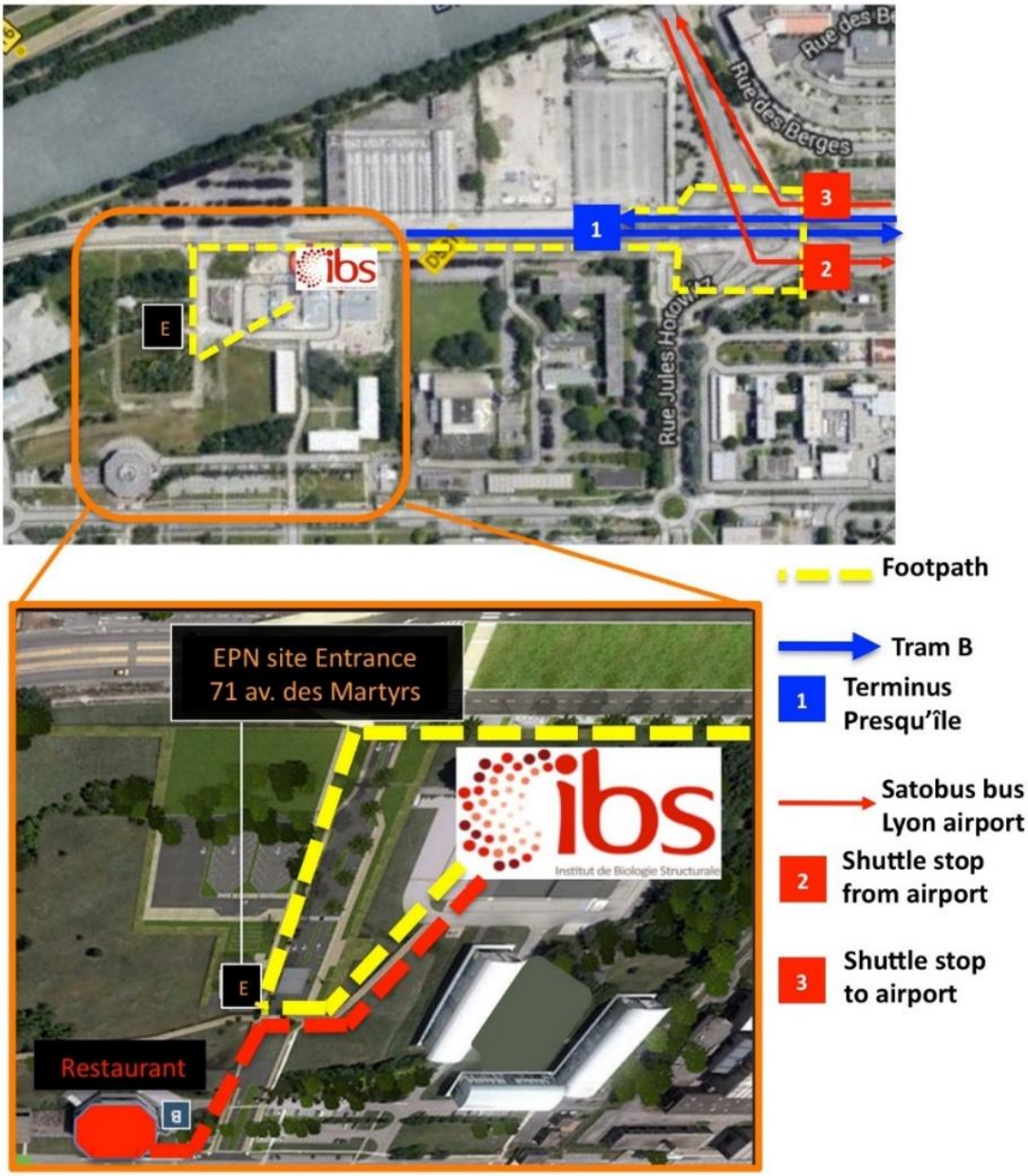
- from Lyon or Valence by car: stay on the A48 when approaching Grenoble, following signs for "Grenoble Bastille-Gares-Europole". Leave the A48 at exit N°16 ("Polygone Scientifique") and cross the suspension bridge over the Isère. At the end of the bridge filter right and turn right, heading north along 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.

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. Tram stop is only 400 m from the new EPN site entrance, and correspond to the terminus of tram B called 'Presqu'île'. Tickets are available from a machine. A four days ticket pass will be given to IBS visitors at registration desk. Tickets have to be stamped to access to the tram (on the platform) or bus (in the bus).



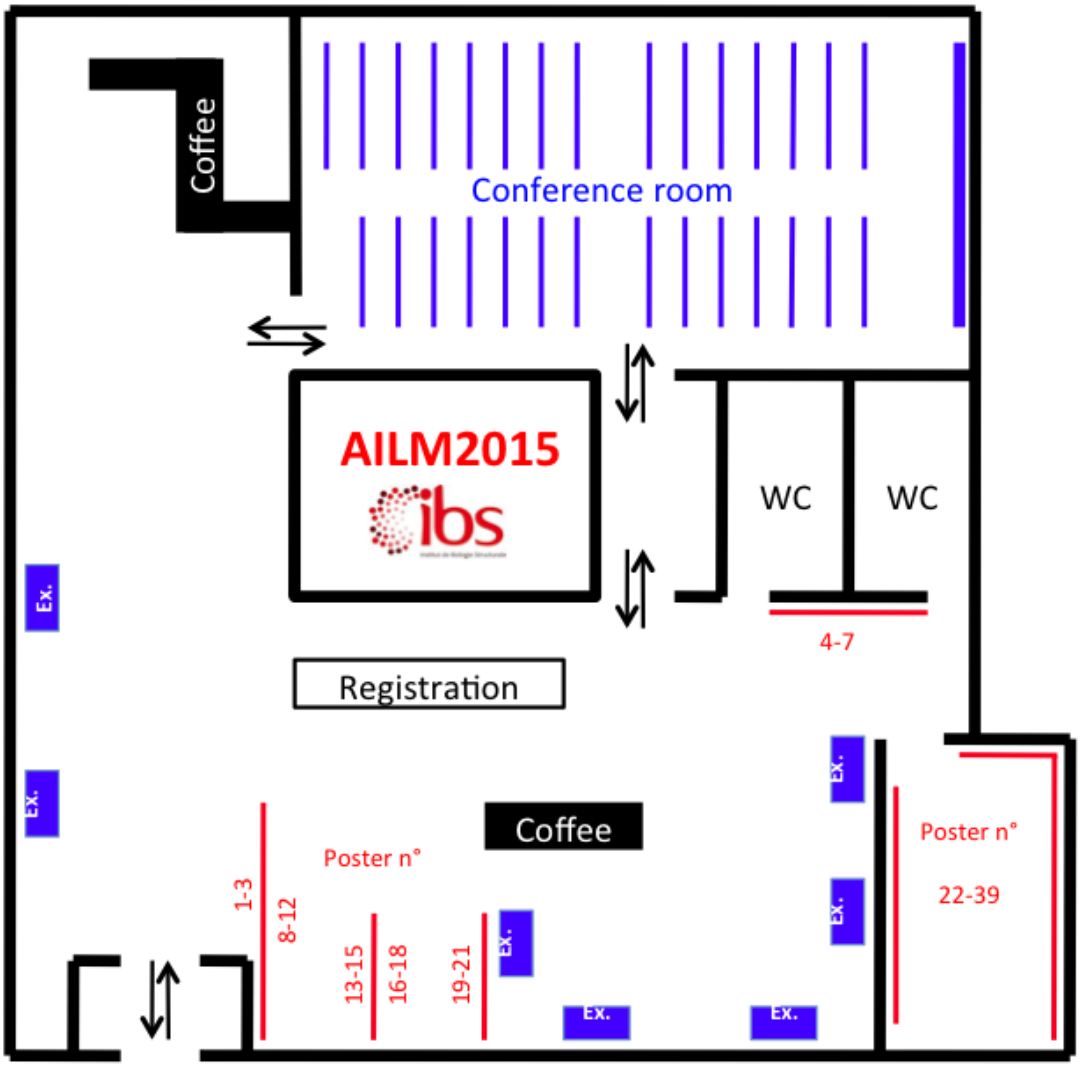
Conference Organization

Lectures:

Lectures will be held in the IBS seminar room.

Poster Sessions and Exhibition:

Stands will be in the IBS Entrance Hall. Posters with odd numbers will be presented on Tuesday, those with even numbers on Wednesday (*see map below for the location of the posters*).



Coffee Breaks:

will be served in the IBS Entrance Hall.

Lunch:

Monday and Thursday: lunch will be taken at the Site Restaurant. Two self-services are available, menu and plates are shown at the entrance. Payment will be done using the conference badge at the cash point at the self-service exit. AILM2015 attendees **must register monday 02/02 by 1 pm** in order to access to self-service of EPN restaurant. Tuesday and Wednesday: a lunch will be served on table at the EPN site restaurant.

WiFi Access:

Free access, login will be communicated upon registration

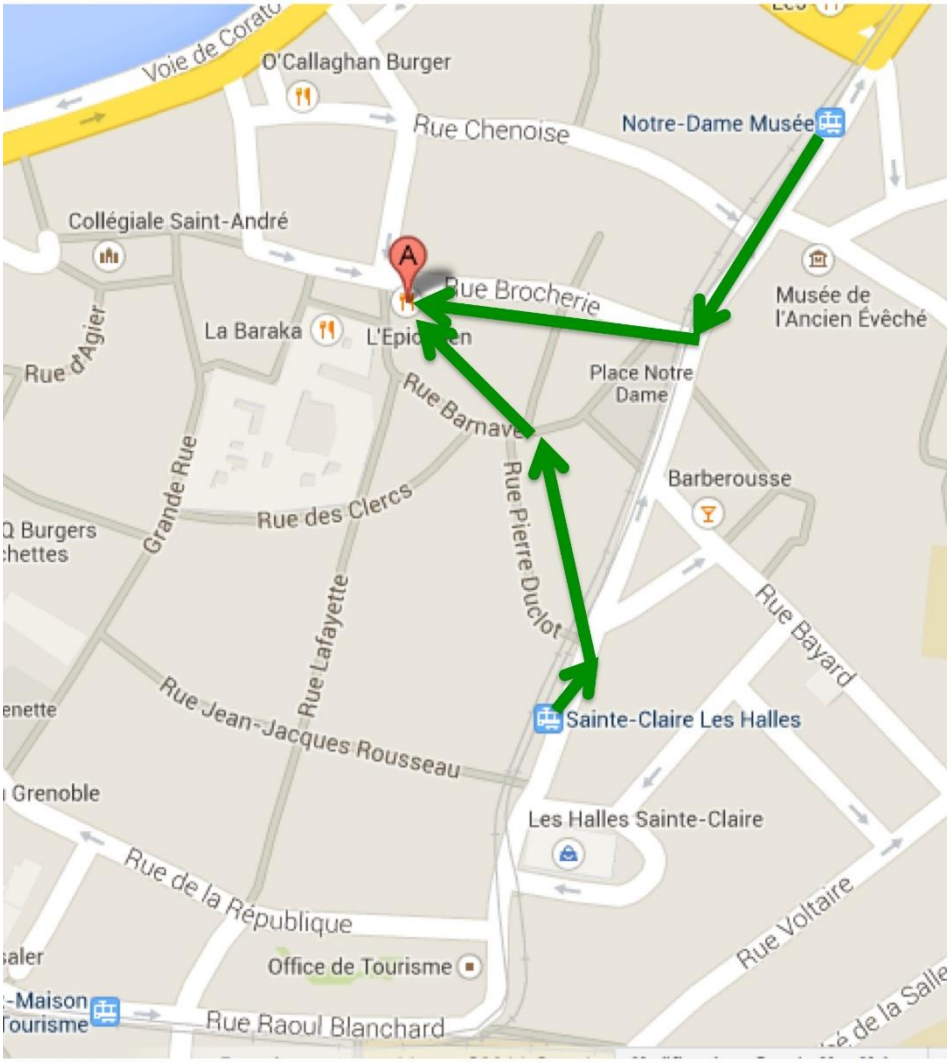
The Conference Dinner

will take place on wednesday evening at the restaurant "L'Epicurien" for participants who registered for the conference dinner. This restaurant is located in Grenoble down town, 1 place aux Herbes, and can be .reached by Tram B. You should stop at station "*Sainte-Claire les Halles*" or "*Notre Dame Musée*". The restaurant is at about 250 m from these two Tram stations (see map below).

The ski outgoing

is programmed thursday 5th February afternoon from noon to 7 pm. Transportation, lunch box, ski pass are included in ski option fees, and you will have to rent your skiing equipment at the ski resort. Only participants who registered for the ski outgoing can attend it.

The ski outing will take place at 7 Laux - Prapoutel. The departure is scheduled at 11:45 am at EPN site entrance and the return by bus to the train station at 18:45 and to the EPN site entrance at 19:00. You will find below the piste map: http://www.les7laux.com/media/files/H15-PlanPistesHiverBD.pdf.



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Exhibitors

BioSilta

Cambridge Isotope Laboratories, Inc. (CIL)

euriso-top — Your partner for labelled compounds (^{13}C, ^{15}N, D, ^{18}O)

Silantes — Stable Isotope Labeled Biomolecules

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Organization

Université Paris Descartes

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FRISBI — Integrated Structural Biology

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PROGRAM

Monday, February 2, 2015

10:00-13:00 Registration

11:30-13:00 Lunch at EPN self service

13:00 **OPENING AILM 2015:** J. Neyton (IBS Deputy Head) & J. Boisbouvier (IBS)

High Molecular Weight Proteins (Chair: Teresa Carlomagno)

13:30 ***B. Kalodimos*** (Rutgers Univ.- USA): Determining structures of large dynamic protein complexes by NMR. *(NMR-Bio sponsored talk)*

14:15 ***B. Reif*** (München Univ.- DE): Amyloid aggregates and large soluble protein complexes.

14:50 ***R. Sprangers*** (MPI Tuebingen - DE): Obtaining high quality spectra of large asymmetric protein complexes: challenges and solutions.

15:25 ***G. Mas*** (IBS – Grenoble – Fr): Methyl specific Labelling, a tool to study a 1 MDa chaperonin in action.

15:45 ***R. Walser*** (AstraZeneca - UK): Elucidating Structure and Function of a Nucleosome-Chromatin-Reader Complex.

16:05 ***R. Lichtenecker*** (University of Vienna - Austria): Isotope Labelled Precursors to be used in E. coli Protein Overexpression.

16:25 ***Y. MONNEAU*** (Rutgers Univ.- USA/ IBS – Grenoble – Fr.): Development of non-stochastic and locally exhaustive search-based software to automatically assign $^{13}C^{1}H$ resonances of methyl-labelled protein from 3D NOESY spectra and crystal structure.

16:45-17:15 **Coffee Break**

Dynamics Studies of Large Poteins (Chair: Ichio Shimada)

17:15 ***V. Tugarinov*** (NIH - USA): Methyl Isotope Labelling in NMR Studies of Protein Dynamics, Allostery and Recognition

17:50 ***P. Schanda*** (IBS - Grenoble - Fr): - Seeing "invisible states" of proteins by MAS-NMR: new methods provide functional insight in a half-megadalton enzymatic assembly.

18:25 ***K. Gardner*** (CUNY - New York - USA): NMR-based studies of bacterial signalling pathways: Insights into nature's view of the environment

Tuesday, February 3, 2015

Isotopic Labelling in Eukaryotic Cells (Chair: Michaël PLEVIN)

08:30 ***I. Shimada*** (Tokyo Univ.- Jap): Functional dynamics of GPCR in micelles and lipid bilayers. *(NMR-Bio sponsored talk)*

09:15 ***A. Gossert*** (Novartis, Basel - CH): Affordable uniform isotope labelling with ^{2}H, ^{13}C and ^{15}N in insect cells.

09:50 ***F. Bontems*** (ICSN- Gif-sur-Yvette - Fr.): Cost effective production of labelled proteins in insect cells for NMR studies. Application to class II viral fusion proteins.

10:10 ***R. Sounier*** (IGF Montpellier – Fr.): Towards a mechanistic understanding of opioid receptors activation by liquid-state NMR spectroscopy.

10:30-11:00 **Coffee Break**

11:00 ***R. Trouillard*** (Rouen Univ. - Fr): Production of uniformly $^{15}N/^{13}C$ labelled glycosylated proteins by hairy roots in view to NMR structural studies.

11:20 ***C. Laguri*** (IBS – Grenoble - Fr.): Glycoconjugates isotopic labelling as tools to study cell surfaces and interactions of cells with their environment.

11:40 ***R. Kerfah*** (NMR-Bio, Grenoble - Fr) Advanded Solutions for the Investigation of Challenging Biological Systems using NMR Spectroscopy *(Presentation from sponsor).*

12:00-13:45 **Lunch at EPN restaurant**

13:45-15:15 **Poster: Session**

Isotopic Labelling for NMR Studies of Proteins (Chair: Beate Bersch)

15:15 ***J. Markley*** (Madison Univ. - USA): Labelling strategies for NMR studies of interactions between nuclei and unpaired electrons in iron-sulfur proteins.

15:50 ***L. Elantak*** (CNRS - Marseille - Fr.): Galectin-1 dependent pre-B cell receptor activation.

16:10 ***M. Casiraghi*** (IBPC -Paris - Fr) : Towards the detection of transiently formed G Protein–Coupled Receptor conformers in nanometric lipid bilayers by NMR spectroscopy.

16:30 ***R.A. Byrd*** (NIH - USA): Bio-orthogonal ^{19}F Labelling – New Approaches to ^{19}F Methyl Labelling.

16:50-17:20 **Coffee Break**

Isotopic Labelling for Mass Spectrometry (Chair: Jérôme Garin)

17:20 ***J. Williamson*** (Scripps Institute - USA): Dynamics of Ribosome Assembly in Bacteria.

17:55 ***V. Brun*** (iRTSV - Grenoble - Fr): Protein isotope-labelling and mass spectrometry analysis: Selected biomedical applications.

18:30 ***J. Benesch*** (Oxford Univ. - UK): Weighing the quaternary dynamics of proteins.

Wednesday, February 4, 2015

In vitro and Segmental labelling (Chair: Bruno Kieffer)

08:30 ***M. Kainosho*** (Tokyo Univ. - Jap): Perspectives of the SAIL Method for Studying Structures and Dynamics of Larger Proteins. *(IUPAB sponsored talk)*

09:15 ***V. Dötsch*** (Franckfurt Univ. - DE): New labelling approaches for the structure determination of membrane proteins.

09:50 ***B. Odaert*** (CBMN – Bordeaux - Fr.): Initial steps in describing the F1Fo ATP synthase dimer interface and modelling the small hydrophobic subunits of the Fo region with solution state NMR.

10:10 ***E. Lescop*** (ICSN - Gif-sur-Yvette - Fr): A well-balanced pre-existing equilibrium governs electron flux efficiency in a 70kDa diflavin reductase from NMR/SAXS combined techniques.

10:30-11:00 **Coffee Break**

11:00 ***T. Schubeis*** (Giotto Biotech - Florenze - It) : Segmental Isotope labelling of insoluble proteins for solid state NMR analysis.

11:20 ***E. Michel*** (Zurich Univ. - Switzerland - CH): Amino acid-selective segmental isotope labelling of multidomain proteins.

11:40 ***D. Hart*** (ISBG – Grenoble - Fr) User access to high-level research platforms through EU Instruct and French FRISBI programs. (*Presentation from sponsor).*

12:00-13:45 **Lunch at EPN restaurant**

13:45 **Poster: Session**

Isotopic Labelling for Neutron Studies (Chair: Trevor Forsyth)

15:15 ***M. Weik*** (IBS - Grenoble - Fr): Combining deuterium labelling and neutron spectroscopy to study protein and hydratation-water dynamics.

15:50 ***A. Podjarny*** (IGBMC - Strasbourg - Fr): Solving biological puzzles using “tiny” perdeuterated crystals for subatomic resolution X-Ray and neutron diffraction.

16:25 ***A. Duff*** (National Deuteration lab. – Australia): A robust and reliable method for high yield deuterated recombinant protein production using Escherichia coli BL21.

16:45-17:15 **Coffee Break**

Isotopic Labelling for Integration Structural Biology (Chair: Jérôme Boisbouvier)

17:15 ***P. Macek*** (IBS – Grenoble – Fr. /AstraZeneca UK): Unraveling Self-Assembly Pathways of Large Protein Machinery by Combining Time-Resolved EM, Native MS on Isotopically Hybridized Particle and Methyl-TROSY NMR.

17:35 ***P. Rajagopal*** (Washington Univ. – Seattle - USA): Hybrid Methodologies for Elucidating Structure and Function of a Chaperone.

17:55 ***T. Carlomagno*** (EMBL - Ger.): RNA-protein complexes in RNA metabolism: an integrative structural biology approach. *(Biochemical Society sponsored talk).*

19:45 **Conference Dinner at Epicurien**

Thursday, February 4, 2015

Isotopic Labelling of Nucleic Acids (Chair: Carine Tisné)

08:30 ***C. Kreutz*** (Innsbruck Univ.- AT): Advanced stable isotope labelling methods for RNA NMR spectroscopy.

09:05 ***C. Wunderlich*** (Innsbruck Univ.- AT): Dynamics of naturally occurring methylated RNA building blocks probed by NMR spectroscopy.

09:25 ***J. Chugh*** (IISER - Pune - India): Visualizing Transient Structures in A-site RNA of the Ribosome: New Structures of Known Molecules for Drug Target.

09:45 ***I. Lebars*** (IGBMC - Strasbourg - Fr): A fully enzymatic method for site-directed spin labelling of long RNA.

10:05-10:35 Coffee Break

10:35 ***S. Keane*** (HHMI - USA): Structure of the HIV-1 RNA Packaging Signal.

11:10 ***L. Ponchon*** (Univ. Paris V - Fr.): In vivo production of uniformally labelled RNA in Escherichia coli using a tRNA scaffold.

11:20 Closing AILM 2015

11:45 Lunch at EPN self-service / Ski Outgoing

SPEAKERS

High Molecular Weight Proteins

S 01 Babis KALODIMOS

Determining structures of large dynamic protein complexes by NMR

Charalampos (Babis) Kalodimos

Department of Chemistry & Chemical Biology, Rutgers University, Piscataway, NJ USA

ABSTRACT

I will discuss how NMR can be used to determine the structure of large, dynamic complexes that are refractory to crystallography. I will primarily discuss how the optimization of labelling schemes that includes methyl-bearing and aromatic residues can provide the first ever high-resolution structures of complexes between molecular chaperones and unfolded proteins.

REFERENCES:

Saio T, Guan X, Rossi P, Economou A, Kalodimos CG (2014). Structural basis for protein anti-aggregation activity of the Trigger Factor chaperone. **Science** 344:1250494.

High Molecular Weight Proteins

S 02 Bernd REIF

Amyloid aggregates and large soluble protein complexes

Reif B

Technische Universität München (TUM), Department Chemie, Lichtenbergstr. 4, 85747 Garching, Germany; Helmholtz-Zentrum München (HMGU), Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

ABSTRACT

Perdeuteration and back-substitution of exchangeable protons in microcrystalline proteins in combination with recrystallization from D_2O containing buffers reduces ^1H, ^1H dipolar interactions such that amide proton line widths on the order of 20 Hz are obtained (Chevelkov et al., 2006). Aliphatic protons are either accessible via specifically protonated precursors or by using low amounts of H_2O in the bacterial growth medium (Asami et al., 2010). This labeling scheme is applied to amyloid aggregates like fibrils formed by the Alzheimer's disease ß-amyloid peptide (Aß) (Linser et al., 2011). We present data on solid-state NMR studies of drug induced Aβ aggregates focussing in particular on the interactions between Aβ and the polyphenolic green tea compound epigallocatechin-gallate (EGCG). We show that MAS solid-state NMR techniques are applicable for the structural characterization of large soluble protein complexes (Mainz et al., 2009; Mainz et al., 2013), in case the tumbling correlation time exceeds the rotor period. Experimental results are presented for the small heat shock protein αB crystallin (600 kDa) as well as for the 20S proteasome core particle in complex with its 11S activator (1.1 MDa).

REFERENCES:

Asami S, Schmieder P, Reif B *(2010).* High resolution 1H-detected solid-state NMR spectroscopy of protein aliphatic resonances: Access to tertiary structure information. **J Am Chem Soc** 132: 15133–15135.

Chevelkov V, Rehbein K, Diehl A, Reif B *(2006).* Ultra-high resolution in proton solid-state NMR at high levels of deuteration. **Angewandte Chemie Int Edt** 45: 3878-3881.

Linser R, Dasari M, Hiller M, Higman V, Fink U, Lopez del Amo J-M, Handel L, Kessler B, Schmieder P, Oesterhelt D, Oschkinat H & Reif B *(2011)*. Proton detected solid-state NMR of fibrillar and membrane proteins. *Angewandte Chemie Int Edt* 50: 4508-4512.

Mainz A, Jehle S, van Rossum BJ, Oschkinat H, Reif, B. *(2009)*. Large Protein Complexes with Extreme Rotational Correlation Times Investigated in Solution by Magic-Angle-Spinning NMR Spectroscopy. **J Am Chem Soc** 131, 15968–15969.

Mainz A, Religa T, Sprangers R, Linser R, Kay LE, Reif B (2013). NMR Spectroscopy of Soluble Protein Complexes at One Mega-Dalton and Beyond. **Angewandte Chemie Int. Edt.** 52, 8746–8751.

High Molecular Weight Proteins

S 03 Remco SPRANGERS

Obtaining high quality spectra of large asymmetric protein complexes: challenges and solutions.

Sprangers, R

Max Planck Institute for Developmental Biology, Spemannstrasse 35, 72076 Tübingen, Germany

ABSTRACT

NMR spectroscopy is ideally suited to study protein dynamics and transient interactions. Traditional NMR methods are, however, only applicable to systems that are up to 30 kDa in molecular weight. Recent advances, including methyl TROSY techniques and methyl labeling methods have pushed this limit to far over 100 kDa, Applications have often been restricted to highly symmetric complexes where all subunits provide the same NMR spectrum. This is mainly due to the fact that these complexes are relatively easy to prepare recombinantly and that they results in simplified NMR spectra with a higher signal to noise ratio.

Eukaryotic protein complexes, as opposed to their bacterial or archaea counterparts, are often highly asymmetric. This is illustrated nicely for the 9-component exosome complex that in archaea contains 3 copies of each 3 different subunits. In eukaryotes, the exosome complex lost all symmetry and contains 9 different protein chains. This lower symmetry of the eukaryotic system makes NMR spectra of the 450 kDa exosome complex highly complicated. Even for methyl labeled samples spectral overlap is significant, as can be appreciated from the fact that the yeast exosome complex contains more than 200 unique isoleucine residues.

Labeling specific subunits of a large protein complex would solve the spectral overlap problem. However, based on our experience, highly asymmetric complexes cannot be reconstituted efficiently in vitro from purified NMR active and NMR inactive components. This is mainly due to poor solubility of isolated subunits and to reconstitution protocols that have incorrectly or incomplete assemblies as side-products. Here, we discuss a method to label, express and generate oligomers (LEGO) for NMR spectroscopy. This approach yields protein complexes that can be flexibly labeled with NMR active subunits and where the assembly of the complex takes efficiently place within the e-coli expression system. We show that this method results in significantly simplified NMR spectra that can be interpreted in a straightforward manner.

REFERENCES:

Mund M, Overbeck JH, Ullmann J, Sprangers R (2013). LEGO-NMR spectroscopy: a method to visualize individual subunits in large heteromeric complexes. Angew Chem Int Ed Engl. 18;52(43):11401-5

High Molecular Weight Proteins

S 04 Guillaume MAS

Methyl specific labelling a tool to study a 1 MDa chaperonin in action

Mas G, Macek P, Crublet E, Moriscot C, Schanda P, Schoehn G, Boisbouvier J

Institut de Biologie Structurale, CNRS/ CEA /UJF, 71 avenue des Martyrs, 38044 Grenoble

ABSTRACT

The study of the assembling, structural and functional properties of biomolecular nanomachines remains a considerable practical challenge. The sheer size of these nanoparticles and the complexity of the structural rearrangements involved present an array of logistical problems. Even if X-ray crystallography and cryo-EM methods can provide static pictures of the system, kinetic data are necessary for a full, atomic resolution understanding of the mechanism of action.

NMR spectroscopy offers an unique ability to monitor structural and dynamic changes in real-time and at atomic resolution. However, the NMR studies of large proteins and complexes has been a real challenge for a long time. Recent developments in specific isotope labelling of methyl groups in a perdeuterated protein has significantly extended the frontier of liquid state NMR[1]. In recent years, we have exploited metabolic pathways in *E. coli* and synthesized new isotope-labelled precursors to allow the labelling of any combination of methyl groups in proteins reporting directly on the structure and dynamics of both the protein backbone and the side chains extremities[2,3].

We have established, with a combination of specific isotope labelling schemes of methyl groups and optimized NMR spectroscopy, that NMR studies can also be applied to macromolecular assemblies with a size up to 1 MDa.

In this communication, I will present that combination of methyl specific labelling, NMR and EM can be used to probe different functional states and refolding cycle of a 1 MDa active chaperonin. To decipher this mechanism, we have reconstituted the functionnal assembly deuterated and specifically labeled on methionine methyl groups. An approach based on mutagenesis of methyl group residues[4,5] has been used to link NMR signals to the methionine residues. Thereby methionine residues have been use as a probe of the chaperonin structure allowing the identification of NMR spectra corresponding to the intermediate state and active species of the functional cycle. The combination of NMR and EM allowed us to simultaneously investigate in an atomic- and time-resolved manner the structural rearrangement processes occurring in a large biological machinery at work.

REFERENCES:

1. Gardner and Kay. (1997). Production and Incorporation of 15N, 13C, 2H (1H-δ1 Methyl) Isoleucine into Proteins for Multidimensional NMR Studies. **Journal of the American Chemical Society.**

2. Ayala et al. (2009). An efficient protocol for the complete incorporation of methyl-protonated alanine in perdeuterated protein. **Journal of Biomolecular NMR.**

3. Mas et al. (2013). Specific labelling and assignment strategies of valine methyl groups for NMR studies of high molecular weight proteins. **Journal of Biomolecular NMR.**

4. Amero et al. (2011). A systematic mutagenesis-driven strategy for site-resolved NMR studies of supramolecular assemblies. **Journal of Biomolecular NMR.**

5. Crublet et al. (2014). A cost-effective protocol for the parallel production of libraries of 13CH3-specifically labeled mutants for NMR studies of high molecular weight proteins. **Methods in Molecular Biology.**

High Molecular Weight Proteins

S 05 Reto WALSER

Elucidating Structure and Function of a Nucleosome-Chromatin-Reader Complex

Walser R, Milbradt A

AstraZeneca Discovery Sciences, Structure & Biophysics, Mereside, Alderley Park, Macclesfield, SK10 4TG, UK

ABSTRACT

The nucleosome is a the basic building block of chromatin, the packaged DNA in the nucleus. Crystal structrues of mono- and higher-order nucleosomes have shed light onto the molecular interactions underlying DNA packaging (1,2). Four histones, each of which occurs twice, form the octameric protein core around which the DNA is wound in two turns. The histone tails have long been known to be the subject of heavy post-translational modifications (PTMs). Among other things these PTMs control the condensation state of the DNA and concomittantly the transcriptional activity of a stretch of DNA. Histone reader proteins "read" the PTM pattern of a given histone tail and translate this into an appropriate response.

High resolution crystal structures of histone reader proteins in complex with the nucleosome are difficult to obtain, mainly due to the inherent flexibility of the histone tails. Therefore alternative sources for structural information are highly desired. NMR has been shown to be able to provide such information (3). Other techniques such as SAXS/SANS and cryo-EM in combination with molecular modelling are anticipated to be ideally suited to complement the primarily local information obtained from NMR.

We have incorporated a PTM into the nucleosome core particle (NCP) and are characterizing the interaction of this modfied NCP with bromodomains from different histone reader proteins. The focus of our work is on NMR spectrosocpy, but we are employing other biophysical and biochemical techniques to characterise the NCP-reader complex.

REFERENCES:

1) Luger, 1997, Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature*, 389, 251-260

2) Schalch, 2005, X-ray structure of a tetranucleosome and its implications for the chromatin fibre. *Nature* 436, 138-141

3) Kato, 2011, Architecture of the high mobility group nucleosomal protein 2-nucleosome complex as revealed by methyl-based NMR. *Proc Natl Acad Sci U S A.*, 108, 377-381

High Molecular Weight Proteins

S 06 Roman LICHTENECKER

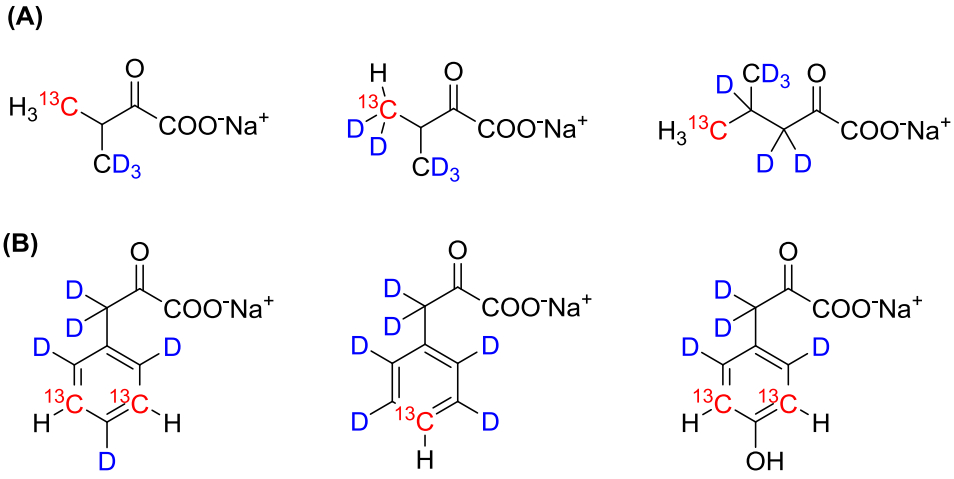
Isotope Labeled Precursors to be used in *E. coli* Protein Overexpression

Schörghuber J, Weinhäupl K, Sara T, Konrat R, Lichtenecker RJ

Institute of Organic Chemistry and Department of Structural and Computational Biology, University of Vienna, Währingerstr. 38, A-1090 Vienna, Austria.

ABSTRACT

The rapid development of protein NMR spectroscopy in the last decade(s) provided new insight not only into the structure of proteins, but also into the conformational dynamics involved in enzyme catalysis, ligand binding and molecular recognition. Most of the applied NMR experiments require selective patterns of 13C, 15N and 2H in the target proteins to reduce signal overlap and/or optimize magnetization transfer pathways. The labeling techniques must be tailored to the requirements of the corresponding NMR application used. We develop synthetic routes to access isotope labeled amino acid precursors, which serve as additional nutrients in the growth medium of an overexpressing microorganism. Our synthetic approaches feature robust reaction sequences in combination with low-cost isotope sources (e.g. 13C-acetone, 13CH3I, 2H2O, 13C-glycine). By now our precursor toolbox features compounds for selective labeling of Ile-, Val-, Leu-, Met-, Phe-, Tyr-, and Trp-residues (a selection of aliphatic (A) and aromatic precursor compounds (B) is given below). The synthesis of these molecules, as well as their use in protein overexpression and examples of protein NMR applications will be reported.



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[1] Lichtenecker RJ, Coudevylle N, Konrat R., Schmid W (2013) Selective Isotope Labelling of Leucine Residues by Using α-Ketoacid Precursor Compounds. **ChemBioChem** 14:818-821.

[2] Lichtenecker RJ, Weinhäupl K, Reuther L, Schörghuber J, Schmid W, Konrat R (2013) Independent valine and leucine isotope labeling in Escherichia coli protein overexpression systems. **J. Biomol. NMR** 57:205-209.

[3] Lichtenecker RJ, Weinhäupl K, Schmid W, Konrat R (2013) α-Ketoacids as precursors for phenylalanine and tyrosine labelling in cell-based protein overexpression. **J. Biomol. NMR** 57:327-331.

[4] Lichtenecker RJ (2014) Synthesis of Aromatic 13C/2H-alpha-Ketoacid Precursors to be Used in Selective Phenylalanine and Tyrosine Protein Labelling. **Org. Biomol. Chem.** 12:7551-7560.

[5] Schörghuber J, Sara T, Bisacchia M, Konrat R, Schmid W, Lichtenecker RJ (2014) Novel approaches in selective tryptophan isotope labeling using Escherichia coli overexpression media.

High Molecular Weight Proteins

S 07 Yoann MONNEAU

Development of non-stochastic and locally exhaustive search-based software to automatically assign $^{13}C^{1}H$ resonances of methyl-labelled protein from 3D NOESY spectra and crystal structure.

Monneau Y 1, Rossi P 2, Kalodimos C G 2

1, Institut de Biologie Structurale, SAGAG group, Grenoble, France;
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ABSTRACT

Deuteration and selective methyl labelling have expanded the scope and applicability of liquid state NMR to large protein assembles up to 1 MDa. The labelling methods and related reagent precursors are now well established. All methyl-containing aminoacids can be introduced to serve as probes to study protein structure, interactions and dynamics. However, the methyl assignment step is still complicated and time-consuming, especially for large systems in which backbone assignment is unavailable. The strategy here is to i) mutate every single methyl-containing residue and observe the peak disappearance or ii) use existing crystal structures as template and correlate the experimental methyl NOESY spectrum to a simulated one generated from the 3D coordinates. The fact that many proteins can now be homology modelled is particularly advantageous and often methyl assignment can be conducted by a combination of mutagenesis and simulation. Even so, assignment is still expert driven, time consuming and difficulties arise when mismatch exists between the structure in solution and in the crystal conditions. Toward this goal, two algorithms designed to automatically assign methyl-labelling protein based on crystal structure and 3D NOESY have been developed (Chao et al. 2012; Xu et al. 2013). The basic approach is to iteratively assign methyls by swapping assignment in a Monte Carlo procedure until the convergence is achieved between the simulated vs. experimental spectrum. As a result, both software packages converge to a unique solution, which often contains significant amount of incorrectly assigned peaks even in well-folded parts of the proteins where the NOE network is very extensive and should, in theory, yield the most accurate results. Also, the assignments are not sufficiently annotated as to provide hints on accuracy or possible issues.

In order to provide more accurate and reliable methyl assignment, we designed a new computing approach based on challenging all possible complete assignments by comparison to the simulated one. To overcome the combinatorial problems of such an approach (the number of complete assignment follows the factorial law), the exhaustive search is performed locally, i.e. for just few peaks connected by NOE as defined by the actual data. Then only the best local assignments are merged together to provide the final output. The latter is an assignment table including complete assignments sorted by score. All assignments that match the data with equivalent scores are present. Thus, ambiguous peak assignments are easy to identify, to check manually and the information can be used for design point mutation with the highest resolving outcome. As a proof of concept, the assignments of several methyl-labelled proteins are presented. The input is the methyl residue type, the crystal structure and the 3D HMQC-NOESY-HMQC spectra (CCH-NOESY). The result is compared to those of the two major already reported softwares, named MAPX2 and Flamengo. Both robustness of the software and reliability of the output are then discussed.

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Dynamics Studies of Large Proteins

S 08 Vitali TUGARINOV

Methyl Isotope Labeling in NMR Studies of Protein Dynamics, Allostery and Recognition

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ABSTRACT

The utility of methyl isotope labelling on the deuterated background for NMR investigations of dynamics and recognition in proteins will be described on a number of examples drawn primarily from the recent studies of large scale inter-domain rearrangements in Enzyme I (EI) and binding of GroEL chaperonin to destabilized mutants of FynSH3 domain.

EI, the first component of the bacterial phosphotransfer signal transduction system, undergoes large substrate-induced interdomain rearrangements. Relaxation dispersion NMR study of ILV methyl groups and amides in combination with small-angle X-ray scattering (SAXS) of EI in complexes with the natural substrate phosphoenolpyruvate (PEP) and the inhibitor α-ketoglutarate (αKG), showed that the open-to-closed conformational switch of EI is triggered by complete suppression of micro- to milli-second dynamics within the C-terminal domain of the enzyme.[1] In particular, the ligand-induced transition from a dynamic to a more ordered conformational state of the C-terminal domain stabilizes the interface between the N- and C-terminal domains observed in the structure of the closed state, thereby promoting the resulting conformational switch.[1]

Emerging approaches based on combined analysis of relaxation-based NMR experiments - lifetime line-broadening, dark-state exchange saturation transfer (DEST), and CPMG relaxation dispersions – allow probing the "dark" NMR-invisible states of proteins. These experiments are dependent in different ways on the overall exchange rates and populations of free and bound states of the substrate. The practical aspects of the extension of this methodology to selectively labeled methyl groups[2] in deuterated proteins is described on the example of a NMR study of dynamics of GroEL binding to mutants of FynSH3 domain.

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Dynamics Studies of Large Proteins

S 09 Paul SCHANDA

Seeing “invisible states” of proteins by MAS-NMR: new methods provide functional insight in a half-megadalton enzymatic assembly

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ABSTRACT

A dynamic balance between different conformational states governs the function of proteins. Microsecond-to-millisecond exchange processes are particularly relevant for biology, as many molecular events, such as allostery, gating and binding occur on this time scale.

In this presentation I will describe our recent advances to study such dynamic events at atomic resolution using solid-state NMR. Based on novel methodologies, we show that one can obtain detailed insight into conformational exchange dynamics, in terms of thermodynamics and kinetics, and also in terms of structural properties of the short-lived exchanging states, even though these conformations are too transient and low-populated to be directly seen. In a first application, we investigate conformational dynamics in a microcrystalline protein, and investigate the effect of the crystalline packing on these motions. We then demonstrate that the methodology can be equally applied to very large systems, that have so far resisted detailed dynamic characterization. We investigate motion in a ~half-megadalton enzymatic complex. We find evidence for functionally important motions in gating regions of this enzyme, and discuss the relevance of motion for the function.

Dynamics Studies of Large Proteins

S 10 Kevin GARDNER

NMR-based studies of bacterial signalling pathways: Insights into nature's view of the environment

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ABSTRACT

Environmental cues regulate many biological processes, controlling cellular pathways used to detect and respond to changing conditions. Such regulation is often initiated by sensory protein domains that use ligands to convert environmentally-triggered changes into altered interactions with downstream catalytic and signaling partner proteins. Several families of these domains have evolved with remarkable diversity in the stimuli they sense and outputs that they control. Changes in the structure and dynamics of these proteins are essential to their roles as signalling proteins, making solution NMR spectroscopy an ideal element of a multidisciplinary study of their properties.

Here I will discuss the embodiment of these principles in several bacterial signalling pathways united by their involvement in responses to blue light stimulation. Within the initial sensory proteins themselves, photochemical events – bond formation within photosensory Light-Oxygen-Voltage (LOV) domains – subsequently generate allosteric structural and dynamic changes that release previously-inhibited catalytic and non-catalytic domains. Despite differences in these downstream effectors, similarities in these variable signaling proteins are readily clear as identified by isotope-assisted NMR studies, providing information of use in basic and applied settings.

Once activated, many of these signaling proteins lead to phosphorylation events in downstream partners. Here, activation-induced phosphorylations substantially alter protein structure and dynamics, including examples that I will present that highlight effects on protein folding and domain reorientation.

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Isotopic Labelling in Enkaryotic Cells

S 11 Ichio SHIMADA

Functional dynamics of GPCR in micelles and lipid bilayers

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ABSTRACT

G-protein-coupled receptors (GPCRs) exist in conformational equilibrium between active and inactive states, and the former population determines the efficacy of signaling[1)]. However, the conformational equilibrium of GPCRs in lipid bilayers is unknown, due to the low sensitivities of their NMR signals. To increase the signal intensities, we developed a deuteration method for GPCRs expressed by an insect cell-baculovirus expression system. The NMR sensitivities of the methionine methyl resonances from β_2-adrenergic receptor (β_2AR) in lipid bilayers of reconstituted high density lipoprotein (rHDL) were increased by ~5-fold upon deuteration. Our NMR analyses revealed that the exchange rates in the conformational equilibrium of β_2AR in rHDLs were remarkably different from those in detergents. The timescales of the GPCR signaling, calculated from their exchange rates, were faster than those of receptor tyrosine kinases, and thus enable rapid neurotransmission and sensory perception[2)].

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Isotopic Labelling in Enkaryotic Cells

S 12 Alvar GOSSERT

Affordable uniform isotope labeling with 2H, 13C and 15N in insect cells

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ABSTRACT

Many biological questions can be addressed by NMR using proteins with selective isotope labeling of one or two amino acid types. We have established refined protocols for such labeling in insect cells with reproducibly high protein yields and >90% incorporation of ^{15}N or ^{13}C isotope labels, for a wide variety of amino acids. In the recent years $^{13}C^{e}$ Methionine labeling has become the most popular label in our labs, as it allows obtaining excellent NMR spectra of proteins up to 100 kDa without the need for deuteration.

However, for several experiments, it is desirable to obtain uniformly labeled protein samples. Especially for larger and complex proteins high-level deuteration is a key factor for spectral quality. An approach will be presented to obtain proteins in insect cells uniformly labeled with ^{2}H, ^{13}C and ^{15}N. ^{15}N incorporation of 80% is achieved; for ^{2}H,^{15}N; ^{13}C,^{15}N and ^{2}H,^{13}C,^{15}N labeling efficiency is slightly lower (70-75%) due to metabolic reasons. The media are based on isotope labeled algal extracts, which lowers the cost about 10-fold when compared to commercially available media.

Examples of applications with different labeling patterns are shown. The first example reveals that a deuteration degree of 75% already dramatically improves the quality of spectra of a 34 kDa protein. In this case, deuteration is not random, but individual amino acids are labeled to 98%. In an additional example it is shown how methyl group labeling can be improved in order to obtain a complete spectrum of all methyl groups in a protein. These protocols enable all common uniform isotope labeling patterns, allowing studies of proteins, which were not accessible to NMR before.

Isotopic Labelling in Enkaryotic Cells

S 13 François BONTEMS

Cost effective production of labeled proteins in insect cells for NMR studies. Application to class II viral fusion proteins.

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ABSTRACT

Most structural biology studies by NMR rely on more or less sophisticated stable isotopic labeling of the samples. In the case of proteins, labeling are in general simple and not too expensive when the proteins can be over-expressed in Escherichia coli, but there is no really satisfying solution (for cost reasons) to label the protein expressed in eukaryotic cells.

We developed a simple and cost effective procedure to produce uniform ^{15}N-labeled proteins in S2 or Sf9 (with baculovirus) cells (Meola et al. 2014) based on the use of an optimized commercial medium depleted in all amino acids and supplemented with 10 g.l^{-1} of ^{15}N-labeled yeast autolysate and 5 mM of $^{15}NH_4Cl$. Yeast autolysates are obtained by enzymatic proteolysis ensuring the preservation of all amino acids. The adjunction of $^{15}NH_4Cl$ compensates the low content of the medium in glutamine. We showed that it is possible to amplify the cells in a regular unlabeled culture medium and to transfer them in our labeled medium just prior to the induction. We reached production yields similar to those obtained in standard commercial media with a final ^{15}N incorporation ratio of 60-65%. We found that this incorporation ratio is due to the non-labeled amino acids present in the cell at the moment of the transfer and that it is possible to increase it up to 80 % by incubating the cells in a medium depleted in all amino acids prior their transfer in the labeling medium.

We applied this procedure to the production of recombinant actin in Sf9 cells and of domains III of « class II » viral fusion proteins. Using the new opportunities opened by this tool, we have undertaken to study by NMR the transition between the pre- and post-fusion forms of these proteins.

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Isotopic Labelling in Enkaryotic Cells

S 14 Rémy SOUNIER

Towards a mechanistic understanding of opioid receptors activation by liquid-state NMR spectroscopy

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ABSTRACT

Opioid receptors (OR), members of the G protein-coupled receptor (GPCR) superfamily, constitute the major and the most effective target for the treatment of pain[1]. The use of opioid drugs acting at these receptors is however a leading cause of death by overdose in Europe and North America. Both beneficial and adverse effects of illicit opioid drugs (opium, heroin) as well as approved therapeutics (morphine and codeine) are mediated by the activation of the mu-opioid receptor (µOR).

We recently described the structure of an inactive conformation of the µOR[2]. It provided important information regarding the binding site of small morphinan antagonists, revealed a largely exposed binding pocket, and demonstrated key molecular determinants for antagonist binding preferences for OR. However, much remains to be learned about the mechanisms by which different agonists can induce distinct levels of Gi protein activation and/or arrestin recruitment upon activation of µOR. Pharmacological and biophysical studies suggest that this versatility can be achieved through the structural plasticity of GPCRs[3-5].

In this study, we propose to analyse the conformational landscape of the µOR in distinct pharmacological conditions using liquid-state NMR spectroscopy. In particular, we have developed a double isotope-labelling scheme to monitor signals from distinct methyl probes sampling the conformational changes of all µOR subdomains. This double labelling approach consists in first expressing the receptor in Sf9 cells grown in media in which methionine is replaced by 13CH3-ε-Methionine as described recently[6-7]. The second labelling reaction is performed using a Lysine reductive methylation of purified µOR in the presence of 13C-formaldehyde and a mild reducing agent. With this double labelling approach, we are able to probe the conformational landscape of µOR both in the transmembrane core and in solvent accessible domains. Our goal is to provide a mechanistic understanding of opioid receptor activation upon binding of ligands presenting distinct efficacy and/or biased signalling properties. A better knowledge of the structural basis for opioid drug efficacy may lead to new therapeutic approaches with limited side effects.

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Isotopic Labelling in Enkaryotic Cells

S 15 Romain TROUILLARD

Production of uniformly $^{15}N/^{13}C$ labeled glycosylated proteins by hairy roots in view to NMR structural studies.

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ABSTRACT

Almost half of eukaryote proteins are glycosylated[1]. However, glycosylation impact on the protein structures remains little studied. Actually, heterogeneous glycan chains of glycoproteins often hinder the crystal growth, preventing the use of crystallography, the most widespread method for structural biology.[2] Nuclear magnetic resonance (NMR) spectroscopy is an appealing alternative technique, but it requires proteins exhibiting ^{15}N or $^{15}N/^{13}C$ isotopic labeling for overcoming resonance overlap and assignment difficulties. Hairy roots may constitute a good candidate for the production of uniformly labeled glycoproteins in view to NMR structural studies. Indeed, these transformed roots can be cultivated in vitro in a confined and controlled medium allowing easy supply of labeled molecules.[3] They can perform glycosylation of their proteins and can produce exogenous proteins in high amount (about 120 mg L^{-1}). Furthermore, they are able to secrete the protein of interest thanks to the fusion of a signal peptide, making protein purification easier to achieve.

In this context, we have initiated a program aiming at exploring the possibility to produce, into hairy roots, labeled glycoproteins. In a first step, we focused on the ^{15}N isotopic incorporation in the enhanced green fluorescent protein (eGFP), a non glycosylated model protein already produced in high amount by the system.[3] Heterogeneous isotopic distributions constituted of different labeled protein populations were obtained. Thus, we developed a new strategy for the isotopic measurement, from mass spectrometry analyses, able to calculate the proportion of these different labeled populations. Such isotopic measurement allowed us to demonstrate that hairy roots were able to produce ^{15}N labeled proteins with a relatively high labeling ratio.

Following these first encouraging results, the protocol was extended to ^{13}C labeling. Results indicated a very efficient labeling enrichment for ^{13}C, near 95%. Thus, we initiated a key step of the project, the labeling protocol enhancement for the $^{15}N/^{13}C$ eGFP production. We also started the production of an artificial glycosylated eGFP in order to investigate the abilities of hairy roots to produce a glycosylated $^{15}N/^{13}C$ labeled protein. These results would allow validating hairy roots as good candidates for uniformly labeled glycosylated protein production in view to NMR structural studies.

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Isotopic Labelling in Enkaryotic Cells

S 16 Cédric LAGURI

Glycoconjugates isotopic labeling as tools to study cell surfaces and interactions of cells with their environment

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ABSTRACT

Heparan Sulfate (HS) are linear polysaccharides covalently linked to a protein to form proteoglycans. In eukaryotes these complex polysaccharides are present at all cell surfaces and in the extracellular matrices. They assume critical roles in mediating interactions with a great number of proteins that regulate in turn cell growth, development, inflammatory responses, host pathogens interactions... HS consists of a backbone formed of 20-200 repeating disaccharide units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) that are variably modified by several enzymes ensuring epimerization and sulfation of the polymer. These modifications give rise to specific molecular designs devoted to protein recognition, in which highly sulfated/epimerized domains alternate with unmodified regions of the polymer. While these molecules endorse important functions, their inherent heterogeneity and flexibility seriously hinders their structural study.

We have developed a chemo-enzymatic approach to produce 13C/15N labeled HS molecules for structural studies by NMR spectroscopy. The HS backbone, naturally produced by E. coli K5 bacteria, is produced by fermentation in 15N/13C labeled culture media and purified. Two human biosynthetic enzymes C5-epimerase and 2-O sulfotransferase are expressed and used, in conjunction to chemical reactions, to reproduce the modifications found in natural Heparan sulfates. Isotopic labeling of HS molecules permits the rapid, quantitative and non-destructive identification of the sugar composition by recording 13C-1H HSQC experiments.

This enabled to follow, for the first time, in real time by NMR, the activity of the two biosynthetic enzymes C5-epimerase and 2-O sulfotransferase. The analysis of the enzyme products also demonstrated that the two enzymes collaborate to generate extended modified domains inside HS polysaccharides and shed an important light on this crucial step of HS biosynthesis. Chemo-enzymatically generated polysaccharides can also be fragmented into smaller species, oligosaccharides, which represent the optimal binding sites of proteins. Libraries of 13C/15N HS oligosaccharides were generated in order to examine the structural determinants of protein-HS interactions. The interaction of oligosaccharides with proteins can be followed by NMR on both partners and these informations, taken together can help determine high resolution structure of protein-HS complexes1.Gains in sensitivity and resolution provided by isotopic labeling permits also the NMR characterization of HS structures directly issued from human HeLa cells strains as well as changes in HS during differentiation processes in Caco cells2.

Isotopic labeling has also been applied to the prokaryotic glycoconjugate, peptidoglycan (PG), a highly crosslinked polymer essential for maintaining bacteria shape and integrity. Its structure and dynamics, even in highly polymeric form, have been studied alone or in interaction with proteins by combinations of liquid and solid-state NMR providing atomic informations about these molecules.

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Isotopic Labelling in Enkaryotic Cells

S 17 Rime KERFAH

NMR-Bio Technologies: Advanced Solutions for the Investigation of Challenging Biological Systems using NMR Spectroscopy

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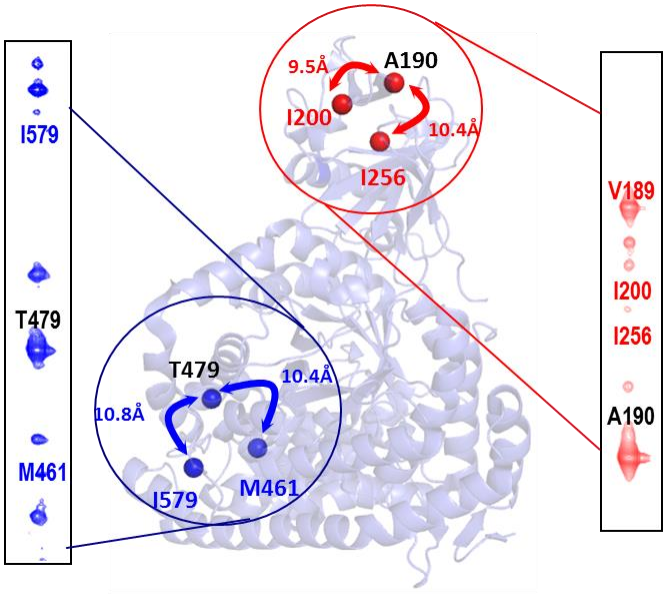
ABSTRACT

Biomolecular Nuclear Magnetic Resonance (NMR) is a powerful method to study protein structure dynamics and interactions in solution. The recent development of innovative isotopic labelling approaches and particularly the selective protonation of methyl groups in perdeuterated proteins [1-6] have made possible the NMR study of protein assemblies as large as 1MDa.

NMR-Bio, a new company hosted by CEA/IBS, makes available a unique set of user-friendly labelling kits for NMR studies. With these tools in hand, the labelling of any combination of methyl groups in proteins with the appropriate isotopic pattern (e.g. 13CH3, 13CHD2, with linearized 13C chains …etc.) and optimized incorporation rates is achievable, offering the possibility to study challenging protein systems.

In this communication, we will present some applications to illustrate the pertinence of methyl based-technology including the possibility to extract long-range NOEs distance between remote probes separated by more than 10 Å in large proteins [6].



Extraction of long-range nOes in U-[^{2}H], Ala-[CH_3]$^{\beta}$, Ile-[CH_3]$^{\delta 1}$, Leu-[CH_3]^{proS}, Val-[CH_3]^{proS} MSG (82KDa) prepared using NMR-Bio QLAM-A$^{\beta}$I$^{\delta 1}$LV^{proS} kit.

For proteins whose expression is critical or not possible in M9/D2O culture medium, NMR-Bio has developed new enriched culture media designed for the specific labelling of methyl-containing amino acids. Their use boosts the protein expression yield and enhances its solubility.

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Isotopic Labelling or NMR Studies of Proteins

S 18 John MARKLEY

Labeling strategies for NMR studies of interactions between nuclei and unpaired electrons in iron-sulfur proteins

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ABSTRACT

Members of the ancient class of proteins that contain iron ligated to sulfur atoms carry out a wide range of essential functions in modern life forms. We are interested in establishing the mechanism of their biosynthesis, the ways in which these proteins are regulated, and the mechanism by which the sequence of the protein controls the physical properties and reactivity of the iron center [1-4]. The metal centers of iron-sulfur proteins are paramagnetic in all accessible oxidation centers. Regions of iron-sulfur (Fe-S) proteins that are removed from the paramagnetic center can be studied by conventional proton-detected, multinuclear NMR methods with samples labeled uniformly with 15N and/or 13C. However, NMR investigations of the highly interesting region near the paramagnetic center require specialized approaches involving direct detection of nuclei having lower magnetogyric ratios (γ), (2H, 13C, 15N) and selective labeling to enable assignments [5]. Because paramagnetic relaxation effects on NMR signals increase as $\gamma 2$, 1H signals usually are too broad to be detected. Interactions of unpaired electrons with nuclei yield a wealth of information about local structure and the existence and strengths of hydrogen bonds. We have used a variety of labeling strategies to make assignments of hyperfine-shifted signals from Fe-S proteins, including residue-selective labeling, dual labeling, and chemical synthesis. Two studies carried out in our laboratory illustrate approaches to isotope-assisted NMR studies of the electron-nuclear interactions in Fe-S proteins: (i) the role of hydrogen bonds in controlling the redox potential in rubredoxin, and (ii) evidence for concerted proton- and electron-transfer and conformational changes linked to quinone binding in the Rieske Fe-S protein involved in the electron transport chain. NMR studies provide detailed information about Fe-S centers that cannot be obtained by X-ray crystallography. In certain cases, it has proved possible to model the electron-nuclear interactions by quantum-chemical methods and replicate the observed NMR parameters.

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Isotopic Labelling or NMR Studies of Proteins

S 19 Latifa ELANTAK

Galectin-1 dependent pre-B cell receptor activation

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ABSTRACT

Galectins are glycan-binding proteins involved in various biological processes including cell/cell interactions. During B cell development, bone marrow stromal cells secreting galectin-1 (GAL1) constitute a specific niche for pre-BII cells. Besides GAL1/glycan interactions, GAL1 is also a pre-B cell receptor (pre-BCR) ligand that induces receptor clustering, first checkpoint of B cell differentiation. The pre-BCR constitutes an immunoglobulin heavy chain and a surrogate light chain composed of the invariant λ5 and VpreB proteins. GAL1 interacts with the pre-BCR via the unique region of λ5. The GAL1/pre-BCR interaction is the first example of a GAL1/unglycosylated protein interaction in the extracellular compartment. Here, we investigated the solution structure of a minimal λ5-UR motif that interacts with GAL1. This motif adopts a stable helical conformation that docks onto a GAL1 hydrophobic surface adjacent to its carbohydrate binding site. We identified key hydrophobic residues from the λ5-UR as crucial for the interaction with GAL1 and for pre-BCR clustering (Elantak et al. 2012). Moreover, we evidenced that GAL1/pre-BCR interaction modifies GAL1/glycan affinity and particularly inhibits binding to LacNAc containing epitopes. GAL1/pre-BCR interaction induces local conformational changes in the GAL1 carbohydrate-binding site generating a decrease in GAL1/glycan affinity. This fine tuning of GAL1/glycan interactions may be a strategic mechanism for allowing pre-BCR clustering and pre-BII cells departure from their niche. Altogether, our data suggest an original way for a cell to modify the equilibria of the GAL1/glycan lattice involving GAL1/unglycosylated protein intdereractions (Bonzi et al. 2014).

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Isotopic Labelling or NMR Studies of Proteins

S 20 Marina CASIRAGHI

Towards the detection of transiently formed G Protein–Coupled Receptor conformers in nanometric lipid bilayers by NMR spectroscopy

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Abstract

G protein-coupled receptors (GPCRs) are a large family of integral membrane proteins present in most eukaryotic cell membranes, taking part in many biological processes. They are also involved in many diseases making them one of the main targets of the available clinical drugs on the market. Signal transduction through GPCRs occurs by a cascade of biological events, starting with the interaction of an extracellular signaling molecule, triggering a cellular response. Understanding how the signal crosses the cell membrane thanks to GPCRs represents one of the main issues in biology. Although many crystal structures of GPCRs have been described at the atomic scale during the past few years, the molecular mechanism governing the function of these membrane proteins still remains unclear. Additional investigations are needed to assess the energy landscape of these complex allosteric machines. In such a context, NMR spectroscopy is a powerful technique to explore the kinetic barriers, in particular with the ability to detect low and transient populated states [1]. To do so, one of the best isotope-labeling scheme dedicated to the study of large proteins or protein complexes is to immerse $^{13}CH_3$ probes in a deuterated environment [2]. To that purpose Escherischia coli is the most appropriate host by far as it allows a homogeneous and perdeuterated environment for designed protonated and ^{13}C-labeled methyl probes. Importantly, a homogeneous ^{2}H environment is mandatory to correctly interpret future NMR relaxation data.

For few years, we developed a recombinant approach [3] where we target on purpose the GPCR expression to inclusion bodies. Thanks to amphiphilic polymers named amphipols [4], we were able to obtain high yields of active and stable GPCRs in vitro [5]. Now, we developed a new protocol where amphipol-trapped GPCRs can be transferred to nanometric lipid bilayers (NLBs) [6] where the protein is 100%-active and stable in the conditions of the NMR experiments. Associated to efficient isotope-labeling strategies using neat precursors (from NMRBio, Grenoble, France), our protocol gives rise to 2 mg/liter of culture of pure $^{13}CH_3$-u-^{2}H-GPCRs. Preliminary NMR investigations indicate a perfect incorporation of these precursors which give rise to the best resolution ever obtained in a lipid environment, opening an avenue to explore kinetic barriers in these receptors.

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Isotopic Labelling or NMR Studies of Proteins

S 21 Andrew BYRD

Bio-orthogonal 19F Labeling - New Approaches to 19F Methyl Labeling

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Abstract

We are exploring the extension and combination of two powerful methodologies in biomolecular NMR: 1) the selective labeling of methyl groups using metabolic precursors, and 2) the use of ^{19}F NMR to study interactions and dynamics in complex biological systems. The pioneering work from the Kay laboratory (1) has made biosynthetic methyl labeling in proteins a common and tremendously valuable methodology for studying structure and dynamics in proteins. The combination of $^{13}CH_3$ labeling in a deuterated background combined with methyl-TROSY NMR methods has enabled the study of very large molecular systems. These methods have been extended to enable methyl labeling of Thr, Ala, and Met residues in addition to the original Ile, Leu, and Val residues. Further important work from Boisbouvier et al. (2) has enabled biosynthetic stereospecific labeling of methyl groups to reduce the number of signals, and spectral complexity, without losing structural information. These tools now enable structural and dynamic information to be obtained in very complex systems. In a separate area, the use of ^{19}F NMR has been regaining popularity (4). In particular, work from Wüthrich et al. (3) and Prosser et al. (4,5) have shown that tagging GPCRs with fluorinated methyl groups provide probes that are exquisitely sensitive to conformational and dynamic affects, which may be initiated by the binding of small molecule agonists. It is also known that ^{19}F probes can be sensitive reporters of protein:protein interactions, due to the large chemical shift range of ^{19}F and the sensitivity to local environment. The ^{19}F NMR literature illustrates that only methyl probes are likely to be effective at higher magnetic field strengths (due to complications from CSA relaxation effects) and, since it is important to utilize field dependence for extensive analysis of dynamics data (e.g. CPMG experiments), there is a need for a facile method to incorporate fluorinated methyl probes ($C^{19}F_3$) into proteins. Furthermore, it is valuable to achieve CF_3 labeling orthogonal to other labeling schemes, such as deuteration, ^{15}N, and different types of $^{13}CH_3$ labeling. We will report on our experiences in achieving biosynthetic incorporation of CF_3 groups into proteins, as well as the type of spectral properties (chemical shifts, relaxation, PRE, PCS) that can be exploited for structural, binding, and dynamics studies.

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Isotopic Labelling for Mass Spectrometry

S 22 James WILLIAMSON

Dynamics of Ribosome Assembly in Bacteria

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ABSTRACT

The assembly of the bacterial ribosome involves association of over 50 ribosomal proteins with three large RNA molecules, facilitated by several dozen assembly cofactors. The process is highly efficient, and accounds for ~one third of the energy budget of a rapidly growing bacterium. We have developed stable isotope pulse labelling methods using quantitative mass spectrometry, to monitor the composition and dynamics of assembly intermediates.

Under normal conditions, few intermediates are populated, but it is possible to perturb the biogenesis process by deletion of an assembly cofactor, or by limiting expression of a ribosomal protein. We have characterized a number of such perturbation, which reveals particular parallel or sequential assembly events. In conjunction with the pulse labeling, we are performing electron microscopy studies to look a the structure and distribution of intermediates that occur when perturbations are introduced. Finally, we have used isotope labeling to monitor the modification of the ribosomal RNA, which provide assembly information complementar to the protein binding data. The overall goal is to provide a mechanistic framework for understanding ribosome assembly in bacteria.

Isotopic Labelling for Mass Spectrometry

S 23 Virginie BRUN

Protein isotope-labelling and mass spectrometry analysis: Selected biomedical applications

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ABSTRACT

Quantitative and dynamic analysis of proteomes is essential to our understanding of biological and physiopathological phenomena. Over the last two decades, protein quantification by mass spectrometry (MS) has attracted considerable interest, particularly for the study of systems biology and as part of biomarker development. This interest is mainly linked to the high multiplexing capacity of MS analysis, and to the availability of stable-isotope-labelled amino acids that allow efficient protein labelling and quantification.

In this presentation, we will first describe the interest and the use of labelled proteins (PSAQ™ standards) to evaluate biomarker candidates in biofluids. PSAQ™ (Protein Standard Absolute Quantification) standards are isotopically-labelled proteins, analogues of the proteins to be assayed. PSAQ standards are produced using cell-free, bacterial or mammalian expression systems. They are directly added to the collected samples and they can correct for protein losses during sample preparation. Because of this, MS-based quantification of target proteins is very accurate and precise [1]. The advantages of the PSAQ approach will be specifically illustrated in the monitoring of new biomarker candidates in serum samples from patients with fulminant hepatitis.

In a second part, we will present an in vivo protein labelling strategy for the determination of protein turnover in mouse brain [2]. Protein turnover determination pushes a step further the knowledge of biological systems by providing dynamic data about protein biosynthesis and degradation. In our study, stable isotope-labelled valine was administered as metabolic precursor during 40 days. A dedicated MS analysis was optimized to precisely determine the turnover values of 40 major proteins in the mouse brain. These results open the way for clinical studies investigating turnover modifications in brain tumours and neurodegenerative diseases.

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Isotopic Labelling for Mass Spectrometry

S 24 Justin BENESCH

Weighing the quaternary dynamics of proteins

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ABSTRACT

We use a combination of mass spectrometry (MS) based approaches to interrogate directly the quaternary structure and dynamics of proteins in the 100 kDa to 1 MDa range, intact in vacuum [1]. Of particular interest to us are the small heat-shock protein molecular chaperones, which are responsible for ensuring proteins reach and maintain their native fold in the cell [2]. Their study however is often hampered however due to their frequent heterogeneity and motions at equilibrium.

MS and tandem MS enable us to identify and quantify the relative abundances of different protein stoichiometries present in solution, while real-time experiments allow the extraction of quaternary fluctuations. This leads us to obtain equilibrium and rate constants for the underlying protein-protein and protein-ligand interactions. Concurrently, ion mobility (IM) MS measurements provide information as to the physical size of the proteins. These experiments therefore provide powerful restraints in modeling the structures of protein assemblies that are difficult and time-consuming to study by means of conventional structural biology approaches.

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In vitro and Segmental labelling

S 25 Masatsune KAINOSHO

Perspectives of the SAIL Method for Studying Structures and Dynamics of Larger Proteins

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ABSTRACT

The stereo-array isotope labeling (SAIL) method has been successfully utilized for accurate determinations of proteins as large as 50 kDa, for which conventional NMR methods are difficult to apply.[1)] However, the relatively high cost of synthesizing a protein exclusively composed of SAIL amino acids, which can only be prepared by the cell-free protein expression system, obviously hampers the dissemination of the SAIL method throughout the NMR community. Over the past several years, we have been trying to improve this situation by overcoming these hurdles. A possible option is to apply the SAIL method for studying the structures and dynamics of particular residues with important biological roles in a targeted protein. For such investigations, proteins selectively labeled with limited types of SAIL amino acids, which can be prepared by conventional cellular expression systems, should be sufficient. This type of approach; i.e., the selective SAIL method, is economically feasible and also practical for NMR laboratories, which often lack access to the cell-free expression systems. The selective SAIL method now has favorable momentum due to recent trends in integrative structural biology, in which the major expected role of NMR spectroscopy is to elucidate the dynamics of larger protein complexes in biologically relevant environments, while their static structures are mainly determined by X-ray crystallography, electron microscopy, and other methods.

In this lecture, we describe our results over the past few years with the selective SAIL method using new types of SAIL amino acids, which have been redesigned to implement improved relaxation properties. These relaxation optimized SAIL amino acids allow us to observe NMR signals for any atoms belonging to the aromatic ring, methylene, and methine groups, even for proteins larger than 80 kDa. We also developed a robust protocol to prepare fully deuterated proteins selectively labeled with the relaxation optimized SAIL amino acids, using E. coli cellular expression.[2)] Therefore, this approach will be widely applicable for filling the gap between the methyl only approach and the conventional NMR methods, and also for facilitating a variety of experimental possibilities to investigate protein dynamics with wider amplitude and time-scale ranges for larger proteins.

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In vitro and Segmental labelling

S 26 Volker DöTSCH

New labeling approaches for the structure determination of membrane proteins

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ABSTRACT

Cell-free expression systems are ideal for the production of proteins that cannot be overexpressed in E. coli cells. In particular for NMR spectroscopy, cell-free expression provides major advantages since it allows for amino acid type selective labeling with only a minimum of metabolic scrambling. We have developed several labeling protocols that allow us to obtain the backbone assignment of membrane proteins in an efficient way. The TMS-labeling (Transmembrane segment enhanced labeling) is based on the fact that 60% of the amino acids of the transmembrane helices consist of only six different amino acid types. Double labeling membrane proteins with these six amino acids produces large consecutive stretches of labeled amino acids that can be analyzed with standard triple resonance experiments with a significantly reduced peak overlap. In addition, we have developed a combinatorial labeling scheme that allows us to assign specific amino acids. Using these methods we have obtained the backbone assignment for the C-terminal fragment of presenilin, the catalytic component of the g-secretase which is responsible for cleaving the amyloid precursor protein and have determined its three-dimensional structure. In addition, we have used these labeling schemes to determine the structure of proteorhodopsin, a retinal binding protein with seven transmembrane helices.

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In vitro and Segmental labelling

S 27 Benoit ODAERT

Initial steps in describing the F_1F_o ATP synthase dimer interface and modelling the small hydrophobic subunits of the F_o region with solution state NMR.

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ABSTRACT

The multi-subunit complex of F_1F_o ATP synthase is the principle source of cellular ATP throughout the biological kingdom. Although variability exists in the precise F_1F_o composition between species, the most studied protein is from Saccharomyces cerevisiae where the complex is comprised of 17 core subunits. Two of the Yeast F_1F_o subunits (e and g) and the N-terminal membrane domain of subunit 4, which all locate to the mitochondrial membrane F_o region, have recently been shown [1] to be absolutely required for the dimerisation of ATP synthase which facilitates the formation of the mitochondrial cristae [2]. Currently, no high-resolution structural information for the interface of dimeric ATP synthase exists and as a result, the interactions which stabilise the ATP dimer are unknown; with prior investigations typically hampered by poor yields of the membrane domain subunits.

To this end, we have optimised a home-made **cell-free expression system** for the production and isotopic enrichment of these subunits - with final yields >1 mg which allows for investigation with **nuclear magnetic resonance** (NMR) spectroscopy. Initial studies, using unlabelled and ^{15}N-Ala samples, focussed on validating the incorporation of samples into LMPG micelles and optimising the quality of NMR spectra for an 83 residue (8.7 kDa) construct comprising the N-terminal region of subunit 4 from the Yeast F_1F_o ATP synthase (S4T). From synthesising single (^{15}N) and double (^{13}C, ^{15}N) labelled samples of S4T, two and three-dimensional NMR spectra (^{15}N and ^{13}C HSQC, HNCACB, CBCACONH, HNCA, HNCOCA and , HCACO) were acquired at 800 MHz enabling us to assign 95% of the S4T backbone nuclei. In silico predictions from primary sequence alone suggested that S4T would have a predominantly alpha helical conformation and this agrees with both chemical shift analysis and a preliminary 3D model from CS-ROSETTA [3] which describes the structure of S4T as a bundle of 3 trans-membrane helices. Furthermore, recent Hydrogen: Deuterium exchange experiments on the S4T construct have given insight into the residues which are shielded from amide exchange.

Future work will focus on optimising the acquisition of TOCSY and NOESY-based NMR experiments for classical distance-based structure determinations of S4T before beginning NMR investigations into subunits e and g. Studies into the interactions which stabilise the dimeric state of ATP synthase will then be undertaken through a number of biophysical techniques to investigate the different affinities and sites of interaction amongst the three subunits.

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In vitro and Segmental labelling

S 28 Ewen LESCOP

A well-balanced pre-existing equilibrium governs electron flux efficiency in a 70kDa diflavin reductase from NMR/SAXS combined techniques.

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ABSTRACT

Multidomain proteins are predominant (>80%) in higher organisms and often result from gene fusion of globular domains that exist in lower organisms. However evolutionary forces driving domain association are still not fully understood. Structural and dynamic characterization of multidomain proteins and their link to function are therefore a prerequisite to better understand how domain communication in a single polypeptide may provide novel functions or improved regulatory mechanisms. To address this question, we studied domain dynamics in the NADPH cytochrome P450 reductase (CPR), a diflavin bi-domain protein composed of FAD- and FMN-binding domains covalently bound by a short flexible linker. In the CPR, electrons flow from the substrate NADPH, to FAD, then to FMN and to the final transfer to acceptors, such as cytochrome P450. To achieve the successive transfers the shuttling of the FMN-domain between the FAD-domain and the final acceptor during the catalytic cycle has long been hypothesized, albeit yet not fully demonstrated.

Crystal structures provide a wealth of detailed structural information, however crystal packing is prone to perturb domain organization in multidomain proteins. Solution-state techniques are then crucial to provide minimally-biased experimental data for studying domain organization and motion in such proteins. In this work, we introduce a new method combining of NMR and SAXS techniques to map the domain conformational landscape of CPR under near physiological conditions. Despite the large molecular size of the protein (70kDa), state-of-the-art isotope labeling allowed successful residue-specific resonance assignment, and the collection of high quality residual dipolar couplings and relaxation parameters to probe domain mobility and exchange rates for ionic strengths ranging from 0 to 1M. SAXS was used to determine structural and thermodynamic parameters under various ionic strength and pH conditions. Briefly, CPR exists in solution as a salt- and pH-dependent equilibrium between a closed and compact conformation and a highly flexible conformational state with the two domains almost freely tumbling. We further demonstrated that maximal enzyme efficiency is governed by an optimal stability of interdomain interface, which could be rationalized by a kinetic mechanism coupling rapid domain conformational changes and slow chemical reactions. We will discuss how these results improve our understanding of the role of domain dynamics in catalysis and provide novel ideas about potential evolutionary mechanisms of multidomain proteins.

In vitro and Segmental labelling

S 29 Tobias SCHUBEIS

Segmental Isotope labelling of insoluble proteins for solid state NMR analysis

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ABSTRACT

Magic Angle Spinning (MAS) solid-state NMR spectroscopy has become a powerful tool for the characterization and high-resolution structure determination of supra-molecular protein assemblies such as fibrillar aggregates, lipid-embedded membrane proteins or microcrystalline proteins. However, sensitivity, spectral complexity, and the differentiation between intra- and intermolecular contacts remain crucial issues. The successful assignment of NMR resonances is often hindered by spectral crowding and resonance overlap, which increase rapidly with the number of residues. In cases of low sequence diversity or in the presence of a dominant secondary structure element, these challenges become even harder to solve. Therefore a combination of spectroscopic techniques and specific isotope labelling schemes are frequently used to overcome those challenges.

Segmental isotope labelling has the potential of opening access to resonance assignments of very large systems and highly repetitive sequences. It has already been successfully employed to reduce spectral complexity in solution NMR experiments. We present an efficient method for the reduction of spectral complexity in solid-state NMR spectra of insoluble protein assemblies without loss of signal intensity. The approach is based on segmental isotope labeling using the split intein DnaE from Nostoc punctiforme, exploiting its unique features such as high tolerance of the junction sequence and high activity in the presence of chaotropic agents. We show that segmentally $^{13}C^{15}N$ labeling of protein fibrils significantly reduces spectral overlap, while retaining wild-type structure and spectral quality. The protocol presented can be utilized for soluble as well as insoluble but refoldable proteins. By combining segmental with sparse labelling the scope of investigated samples can be broadened even further

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In vitro and Segmental labelling

S 30 Erich MICHEL

Amino acid-selective segmental isotope labeling of multidomain proteins

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ABSTRACT

Current solution NMR techniques enable structural investigations of proteins in molecular particles with sizes up to several hundred kDa. However, the large molecular weight of such proteins results in increased numbers of NMR signals, which typically imposes limitations due to spectral overlap. For multi-domain proteins, segmental isotope labeling of individual domains facilitates the spectral interpretation by reducing the number of signals, but signal overlap may persist in domains with small signal dispersion. To overcome limitations arising from spectral overlap, we developed a strategy that combines cell-free expression and ligation of the expressed proteins to produce multi-domain proteins with selective amino acid-type labeling in individual domains. Initially low cell-free expression yields of precursor molecules were overcome by introducing new fusion constructs that allowed milligram production of ligation-competent domains labeled in one or multiple amino acid types. Ligation-competent unlabeled partner domains were produced in-vivo and subsequent domain ligation was achieved using an on-column strategy. We illustrate the development and show applications of this approach for the preparation of amino acid-selective segmentally labeled multi-domain proteins.

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In vitro and Segmental labelling

S 31 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 national investments in scientific instrumentation. National and European centres have been defined with a mission to provide supported user access to their state-of-the-art research platforms. I will present the EU ESFRI project Instruct and French ANR project FRISBI that provide fully or partially funded user access to these platforms.

Isotopic Labelling for Neutron Studies

S 32 Martin WEIK

Combining deuterium labelling and neutron spectroscopy to study protein and hydration-water dynamics

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ABSTRACT

The combination of neutron spectroscopy and specific deuterium labelling is a powerful tool to study the dynamics of biological macromolecules and their hydration water on the nano- to picosecond time scales. The incoherent neutron scattering signal from biological samples is dominated by the one from hydrogen atoms that scatter neutrons about two orders of magnitude more strongly than other atoms, including deuterium atoms. Therefore, the deuteration of parts of a biological sample masks their contribution to the neutron scattering signal and allows to selectively probe the dynamics of the unlabeled parts. For instance, neutron scattering from a perdeuterated protein hydrated in H_2O puts the focus on hydration water dynamics and an unlabelled protein in D_2O provides access to protein dynamics (Wood et al., 2007; Wood et al., 2008). By conducting neutron scattering experiments on soluble, membrane and intrinsically disordered proteins, in deuterated and natural-abundance versions, we showed that there exists a gradient of coupling with hydration-water motions across different protein classes (Gallat et al., 2012). Neutron scattering from perdeuterated biological cells hydrated in H_2O allowed water dynamics to be monitored in a variety of cells and compared (Tehei et al., 2007; Jasnin et al., 2008). Specific hydrogen labelling of certain types of amino acids in a deuterated protein hydrated in D_2O (reverse labelling) allowed exploring the dynamics of certain regions of interest, such as the core (Reat et al., 1998) or the surface of a protein (Wood et al., 2013) and permitted the contribution of methyl groups to the average protein dynamics to be determined (Wood et al., 2010; Wood et al., 2013).

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Isotopic Labelling for Neutron Studies

S 33 Alberto PODJARNY

Solving biological puzzles using “tiny” perdeuterated crystals for subatomic resolution X-Ray and neutron diffraction.

Podjarny A, Blakeley MP, Haertlein M, Moulin M, Guillot B, Petit-Haertlein I, Hazeman I, Mitschler A, Cousido-Siah A, Fisher SJ, Salvay AG, Muller-Dieckmann C , Popov A, Afonine P, Ventura O, Cachau R, Ginell S, Joachimiak A, Meilleur F, Petrova T, Myles D and Howard EI.

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ABSTRACT

Large crystal volumes (> 1mm^3) have been a difficult requirement for Neutron Protein Crystallography. The use of full deuteration (i.e. perdeuteration) has allowed to diminish this requirement by one order of magnitude, as shown by the neutron diffraction studies of human Aldose Reductase (h-AR(D), 36 kDa) [1], Antifreeze protein (AFP(D), 7 kDa) [2] and Heart Fatty Acid Binding Protein (H-FABP, 14 kDa).

Human Aldose Reductase (h-AR(D)) neutron quasi-Laue diffraction data complexed with the inhibitor IDD594 and NADP$^+$ were collected to a resolution of 2.2 Å at room temperature at the ILL on LADI-I from a “radically small” (V = 0.15 mm^3) crystal. To complement the neutron data in a joint X+N refinement, X-ray room temperature data were collected to 1.8 Å at the SLS Synchrotron. The structure was also determined at 100K with X-ray data collected up to 0.8 Å resolution at the APS Synchrotron. The analysis of both the high resolution X-ray maps and the neutron maps suggested the mobility of catalytic protons in the system Asp43-Lys77-Tyr48. These observations allowed the validation of a MD-QM model of the proton donation mechanism, which showed that the residue donating the proton is Tyr 48, and that this donation is activated by the movement of neutral Lys 77.

Antifreeze proteins (AFPs) bind to ice through an ice-binding surface (IBS), thus inhibiting ice growth in-vivo at sub-zero temperatures. We have determined the structure at 293K of a fully perdeuterated type-III AFP(D) by joint X-ray and neutron diffraction, providing a very detailed description of the structure of the protein and its surrounding solvent. X-ray data to 1.05 Å were collected at the ESRF Synchrotron and neutron quasi-Laue data to 1.85 Å were collected at ILL on LADI-III, from a “radically small” (V=0.13 mm^3) crystal. The identification of a tetrahedral water cluster both in neutron and X-ray maps has allowed the reconstruction of the ice crystal primary prismatic face bound to the IBS. The analysis of the corresponding interactions reveals the role of the hydrophobic residues. They bind inside the holes of the ice surface, thus explaining the specificity of AFPs binding to ice versus water.

Fatty acid binding proteins (FABPs) have a large internal cavity with an ordered water cluster mediating the interaction between the protein and the fatty acid. We have obtained very detailed information about the internal water molecules in this cavity, in the presence of a bound fatty acid (FA), by Ultra High Resolution X-ray Crystallography (UHR) to 0.98 Å and Neutron Protein Crystallography (NPC) to 1.9 Å using a “radically small” (V=0.05 mm^3) crystal. These waters form a very well ordered cluster of 12 molecules, positioned between the hydrophilic internal wall of the cavity and the fatty acid molecule. This cluster is very dense, and most of the water molecules have a tetrahedral environment. This information has been used for a detailed electrostatic analysis based on the charge distribution description modeled in the multipole formalism and on the ‘Atoms in Molecules’ theory. The experiment has been done with oleic acid, which is produced with the protein expressed in E. Coli. The results have been analyzed in order to understand the interactions between the FA, the internal water and the protein, and in particular the role played by the water molecules in determining the potency and specificity of FA binding to FABPs.

These examples show how neutron diffraction of fully (per) deuterated crystals, combined with high-resolution X-ray diffraction, are able to reveal the structural details necessary for the understanding of complex biological mechanisms.

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Isotopic Labelling for Neutron Studies

S 34 Anthony DUFF

A robust and reliable method for high yield deuterated recombinant protein production using *Escherichia coli* BL21.

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ABSTRACT

We have developed a method that has proven highly reliable for the deuteration of a broad range of proteins by recombinant expression in Escherichia coli BL21. Typical biomass yields are 40-80 g/L wet weight, yielding 50-400 mg/L purified protein. This method uses a simple, relatively inexpensive defined medium, and routinely results in a high yield expression without need for optimisation. The key elements are: very tight control of expression, careful starter culture adaption steps, and strict maintenance of aerobic conditions ensuring exponential growth. Temperature is reduced as required to prevent biological oxygen demand exceeding maximum aeration capacity. Glycerol is the sole carbon source. We have not encountered an upper limit for the size of proteins that can be expressed, achieving excellent expression for proteins from 7-112kda and the quantity produced at 1L scale ensures that no SANS, NMR or neutron crystallography experiment is limited by the amount of deuterated material. Where difficulties remain, these tend to be cases of protein solubility exacerbated by high protein concentration and slightly increased stickiness of proteins in D2O. There are some very few cases in which we have been unable to express a protein by our method despite unlabelled expression being reliable in rich media using induction at low OD. Few proteins tested have not expressed in deuterated medium despite unlabelled expression being reliable.

Isotopic Labelling for Integrated Structural Biology

S 36 Pavel MACEK

Unraveling Self-Assembly Pathways of Large Protein Machinery by Combining Time-Resolved EM, Native MS on Isotopically Hybridized Particle and Methyl-TROSY NMR

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ABSTRACT

The spontaneous formation of higher order structures from smaller building blocks, i.e. self-assembly, is a fundamental attribute of life. Although self-assembly is a time-dependent process that occurs at the molecular level, the current understanding of this process originates from static structures, low-resolution techniques and modelling. Nuclear magnetic resonance (NMR) spectroscopy possesses the unique ability to monitor structural changes at the atomic level in real-time; however, its size and the constraints of its time resolution remain a practical challenge in studies of self-assembly. In this work, we overcome the main limitations of by the unique application of methyl specific labeling, in an otherwise deuterated protein (Ayala et al. 2008), combined with relaxation optimized, fast acquisition real-time NMR (Amero et al. 2009) to overcome both size and time scale limits, respectively. In addition, the NMR data are complemented and corroborated with data from time-resolved isotopically hybridized assembly reaction analyzed by native mass spectrometry and electron microscopy.

We report the study of the self-assembly of a 468kDa megadalton protein complex, dodecameric tetrahedral aminopeptidase 2 from Pyrococcus horikoshii (TET2). Starting from the stabilized monomeric solution of the protein, we used stopped flow mixing device to apply an in situ pH jump and initiate the oligomerization directly during the NMR data acquisition. We observed that after an initial burst-phase, the assembly evolves towards the stable native dodecameric nanomachine. During the self-assembly we were able to detect signals of two different intermediates. Using native MS on isotopically hybridized TET2 we conclude that the first transient intermediate reflect a flexible monomeric state in the assembly pathway. This flexible monomer is transformed into the ensemble of assembly intermediates at various oligomerization states and topologies, slowly evolving towards native dodecamer particles. For the first time the self-assembly process of a nearly half mega Dalton biological particle was monitored at structural level, including the characterization of intermediate states, by integrating real-time NMR approach with time-resolved electron microscopy and native mass spectrometry.

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Isotopic Labelling for Integrated Structural Biology

S 37 Ponni RAJAGOPAL

Hybrid Methodologies for Elucidating Structure and Function of a Chaperone.

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ABSTRACT

Cells combat stress by up regulating ATP-independent chaperones called small Heat Shock Proteins (sHSPs) that act as protective reservoirs for proteins prone to undergo misfolding or aggregation due to changes in the cellular environment. It has been hypothesized that stressed cellular conditions induce a conformational change in sHSPs to facilitate efficient client binding. It has long been a challenge to elucidate the structure and function of these polydisperse, macromolecular sHSPs. We are investigating human HSPB5, a ~560 kDa archetypal sHSP containing a distribution of multimers ranging from 10-mers to 36-mers with a 20 kDa subunit as the repeating entity. We have used a combination of MAS solid-state NMR, EM, SAXS and computational modeling to determine a structural model of HSPB5 (Jehle et al., 2011).

Next, we addressed the functional question by investigating an 89-residue domain in HSPB5 (HSPB5-ACD) that is conserved in all sHSPs. Solution state NMR relaxation dispersion experiments show that HSPB5-ACD samples both a major and a minor conformational state. The population of the minor conformation in HSPB5-ACD increases when pH is changed from pH 7.5 to 6.6. It has been shown that the pH of brain ischemic cells drops to pH 6.6 as measured by CEST-NMR (McVicar et al., 2014). HSPB5 is overexpressed in the brain during ischemia and we hypothesized that the minor conformation becomes more populated in HSPB5 for efficient client binding. A His-to-Lys mutation, H104K, stabilizes the minor conformation and activity assays show that H104K-HSPB5 (~800 kDa) is indeed more effective as a chaperone than the wild type. SEC-MALS experiments show that H104K-HSPB5 forms longer-lived complexes with a client protein that are < 400 kDa in size.

We have determined the structures of the major and minor conformations of HSPB5-ACD using solution state NMR methods by performing investigations at pH 7.5 and 6.6. The conformation at pH 6.6 (minor conformation) is a challenge to investigate by traditional methods and so we have used ILV-methyl protonation and RDCs to get distance restraints. We are also investigating H104K-HSPB5 and a disease-causing mutant, R120G-HSPB5 (~ 900 kDa) with MAS solid-state NMR techniques using specific methyl probes to understand how activity is enhanced in the former and decreased in the latter. We show that the assignments of the methyl probes can be transferred from the smaller ACDs to the oligomers obviating the need for assignment by mutational analysis.

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Isotopic Labelling for Integrated Structural Biology

S 38 Térésa CARLOMAGNO

RNA-protein complexes in RNA metabolism: an integrative structure biology approach

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Abstract

During the biosynthesis and processing of the pre-rRNA and mRNA transcripts post-transcriptional modifications of ribonucleotides occur in functionally relevant regions. 2'-OH ribose methylation, was shown to protect RNA from ribonucleolytic cleavage, stabilize single base pairs, serve as chaperone, and impact the folding of RNAs at high temperatures. In eukaryotes and archaea this modification is carried out by the Box C/D small nucleolar RNA-protein complex (s(no)RNP). The archaeal Box C/D sRNP complex consists of three core proteins (L7Ae, Nop5 and Fibrillarin) assembled around the methylation guide sRNA containing two similar conserved motifs: box C/D and box C'/D'. The guide sRNA in the complex base pairs with two complementary substrate RNAs (10-21bp) and selects the methylation site, namely the 5th nucleotide upstream to the canonical box D (box D').

In the first part of the talk I will present the structure of the catalytically active Box C/D sRNP complex in solution (390 kDa) assembled around a physiological sRNA construct. The structure is obtained by a powerful combination of solution state NMR and small angle neutron scattering (SANS). We show that the active sRNP is a pseudo-tetrameric complex: by solving the structure of both the apo- and the holo-complex we are able to decipher the mechanisms of methylation and to explain the specificity of the enzyme. Furthermore, with an NMR detected activity assay we reveal that the methylation at different rRNA sites is regulated, which in turns offer implication for rRNA folding.

In the second part of the talk, I will present a novel strategy to assign RNA resonances in solid-state NMR. Solid-state NMR (ssNMR) is becoming a very important instrument for the elucidation of structure-function relationships in large biomolecular complexes. To date, substantial progresses have been made in the structure determination of membrane proteins and amyloid fibrils, while significantly fewer studies have addressed the structure of RNA or protein-RNA complexes (RNP) by ssNMR. Nevertheless, the application of ssNMR to study large RNP complexes holds excellent promises, due to the independence of the ssNMR line widths from the molecular size. Here, I present a comprehensive ssNMR-based resonance assignment of the 26mer box C/D RNA in complex with L7Ae.

Isotopic Labelling of Nucleic Acids

S 39 Christoph KREUTZ

Advanced stable isotope labeling methods for RNA NMR spectroscopy

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ABSTRACT

Our research is focused on the development of advanced stable isotope labelling protocols for nucleic acids. Using chemo-enzymatic transformations site-specifically 13C/15N/2H-modified building blocks are amenable that can be either used for the solid phase chemical synthesis of RNA (i.e. synthesis of phosphoramidite building blocks) or for the enzymatic production of RNA by in vitro transcription (i.e. synthesis of ribonucleotide triphosphate building blocks). The building blocks currently available in our laboratory will be presented along with selected applications including CPMG relaxation dispersion experiments1,2, ZZ-exchange NMR1 and paramagnetic relaxation enhancements studies3.

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Isotopic Labelling of Nucleic Acids

S 40 Christoph WUNDERLICH

Dynamics of naturally occurring methylated RNA building blocks probed by NMR spectroscopy

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ABSTRACT

The most frequent chemical modification is the methylation of functional groups of the RNA building blocks. For example, the 2'-O-methylation of the standard RNA building blocks, adenosine, cytidine, guanosine and uridine, is found in many ribosomal and transfer RNAs. The presumed main function for this modification is the stabilization of the A-form helix by favoring the C3'-endo conformation of the ribose unit. Other helix-stabilizing modified RNA nucleotides are pseudouridine, ribotyhmidine and 5-methylcytidine

Here, we report on the synthesis of 13CH3-modified RNA phosphoramidites bearing the methyl group at either the 2'-O-position or at the 5-position in uracil and cytosine. The stable isotope labeling pattern allows the application of the Carr Purcell Meiboom Gill (CPMG) relaxation dispersion experiment, which is suitable to address conformational dynamics in the µs- to ms time regime. The 13CH3 labels were incorporated into the full-length yeast phenylalanine transfer RNA by the solid phase synthesis approach in combination with a T4 RNA ligase ligation strategy illustrating the potential of solid phase RNA synthesis in segmental isotope labeling. In more detail, we addressed the conformational dynamics of the anticodon stem loop (ASL) using a shorter hairpin construct of this very transfer RNA comprising the methyl-modified nucleotides along with a pseudouridine residue. We incorporated the m5C-13C-lable also into a ribosomal RNA motif, where this modification naturally occurs. The label was introduced in the bacterial decoding region of the 16S ribosomal RNA comprising A1492 and A1493, residues involved in the discrimination of cognate and near-cognate tRNAs. We then compared the influence of the m5C label at this position by comparing relaxation dispersion experiment data in the presence and absence of the methyl modification.

Isotopic Labelling of Nucleic Acids

S 41 Luc PONCHON

In vivo production of uniformally labelled RNA in Escherichia coli using a tRNA scaffold

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ABSTRACT

RNA has emerged as a major player in many cellular processes. Understanding these processes at the molecular level requires homogeneous RNA samples for structural, biochemical and pharmacological studies. We devised a generic approach that allows efficient in vivo expression of recombinant RNA in Escherichia coli.

By growing cells in labeled medium, it is possible to enrich the recombinant RNA with stable isotopes, such as ^{15}N and ^{13}C and to confirm by NMR that the RNA is correctly folded: the ^{1}H-^{15}N TROSY spectrum of the chimeric RNA shows a number of well resolved peaks, indicative of a defined, folded structure. In a second time, we release of the RNA insert from the tRNA scaffold by RNase H cleavage and obtained doubly labeled RNA.

Finally, we have extended this method to RNA/protein co-expression. We have engineered several plasmids that allow overexpression of RNA-protein complexes in E. coli. We have investigated the potential of these tools in many applications, including the incorporation of a post-transcriptional tRNA modification by co-production with the appropriate modifying enzyme TrmI. We overexpressed the $tRNA^{Lys}_3$ and TrmI in E. coli. After purification, the methylation state of the $tRNA^{Lys}_3$ was probed by NMR spectroscopy. The success of the co-production in vivo with TrmI was evidenced by the comparison of the NMR spectra of $tRNA^{Lys}_3$ with those of the tRNA overproduced alone in E. coli.

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Isotopic Labelling of Nucleic Acids

S 42 Isabelle LEBARS

A fully enzymatic method for site-directed spin labeling of long RNA

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ABSTRACT

RNAs and their complexes are involved in many steps of cellular functions, genetics or microbial infections. The key to understand the biological functions of such molecules is to investigate their structure, their plasticity and their dynamics. Site-directed spin labeling is emerging as an essential tool to investigate the structural and dynamical features of RNA [(1-3)]. During recent years, several spin labeling techniques of RNA relying on partial or complete solid-phase chemical synthesis methods were developed [(4-9)].

We propose here an enzymatic method, which allows the insertion of a paramagnetic center at a specific position within an RNA molecule. This method is based on a segmental approach using a ligation protocol with T4 RNA ligase 2. One transcribed acceptor RNA is ligated to a donor RNA in which a thio-modified nucleotide was introduced at its 5'-end by in vitro transcription with T7 RNA polymerase. The paramagnetic thiol-specific reagent is subsequently attached to the RNA ligation product. This novel strategy was demonstrated by introducing a paramagnetic probe into the 55 nucleotides long RNA corresponding to K-turn and Specifier Loop domains from the Bacillus subtilis tyrS T-Box leader RNA. The efficiency of the coupling reaction and the quality of the resulting spin-labeled RNA were assessed by Mass Spectrometry, EPR and NMR. This method enables various combinations of isotopic segmental labeling and spin labeling schemes, a strategy that will be of particular interest to investigate the structural and dynamical properties of large RNA complexes by NMR and EPR spectroscopies.

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Isotopic Labelling of Nucleic Acids

S 43 Sarah KEANE

Structure of the HIV-1 RNA Packaging Signal

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ABSTRACT

The 5´-leader of the HIV-1 genome contains conserved residues that promote selective packaging of the unspliced, dimeric viral RNA into assembling particles. Using a ^{2}H-edited NMR approach, we determined the structure of a 155-nucleotide portion of the leader that is independently capable of directing packaging (Core Encapsidation Signal; Ψ^{CES}). The RNA adopts an unexpected tandem three-way junction structure, in which residues of the major splice donor and translation initiation sites are sequestered by long-range base pairing and unpaired guanosines are exposed in helical junctions. The structure reveals how unspliced genomes are selected, translation attenuated, and binding to cognate Gag proteins promoted, by the RNA conformer that directs packaging.

Isotopic Labelling of Nucleic Acids

S 44 Jeetender CHUGH

Visualizing Transient Structures in A-site RNA of the Ribosome: New Structures of Known Molecules for Drug Target

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Abstract

Dynamic changes in RNA structure drive many essential processes in living cells. Studies of RNA dynamics have focused on fluctuations about the dominant ground state at sub-microsecond timescales or large-scale transformations in secondary structure occurring at timescales slower than seconds. By using NMR relaxation dispersion and mutagenesis, we show that non-canonical regions of A-site Ribosomal RNA undergo transient excursions away from the ground state towards short-lived (ms lifetimes) and low populated (2%) excited states that feature local rearrangements in secondary structure and base-pair alignment in regions rich in non-canonical residues. A-site ribosomal RNA contains two highly conserved internal loop adenines A1492 and A1493, which serve to decode the mRNA message by looping out and stabilizing a codon-anticodon mini-helix when it is formed between mRNA and its cognate aa-tRNA. A-site is also known to bind to many antibiotics where drug binds the internal loop, flips the two adenines out and the adenines are forced to bind the codon-anticodon minihelix irrespective of correctness of tRNA. The excited state conformation we proposed is highly conserved and defines a new type of RNA switching that can be integrated into biological circuits. The A-site ES sequesters the A92 and A93 into base-pairs, such that they are no longer available for interacting with incoming tRNAs. Indeed, the C1407U mutation, which stabilizes the A-site ES has previously been shown to significantly increase the stop-codon readthrough and frame shifting, suggesting that the mutation weakens codon-anticodon interactions in the A-site and decreases the fidelity of elongating ribosomes.

POSTERS

P 01 GELEV V.

High purity deuterated lipids and detergents for NMR of membrane proteins

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ABSTRACT

NMR investigations of the structure and dynamics of membrane proteins can greatly benefit from deuteration of the solubilizing lipid or detergent medium. Improvements in the protein spectra, especially for the methyl $^{13}C/^{1}H$ resonances, although not as pronounced as the effect of protein deuteration, can be critical for observing the signals of interest. We have produced deuterated versions of a broad range of lipids and detergents useful for membrane protein NMR, including DMPC and DPPC (and other phosphatidylcholines,) DMPG and DPPG (and other phosphatidylglycerols), LMPG and LPPG (and other lyso-phospholipids), DDM (and other alkyl maltosides), LDAO (and other amine oxide analogs), DPC (and other fos-cholines). Considering the sensitivity of membrane protein samples, we have paid special attention to the final purification of the lipids and detergents, resulting in equal or better quality than the commercially available natural abundance analogs. The compounds have been used successfully in a number of NMR spectroscopic studies [1-4].

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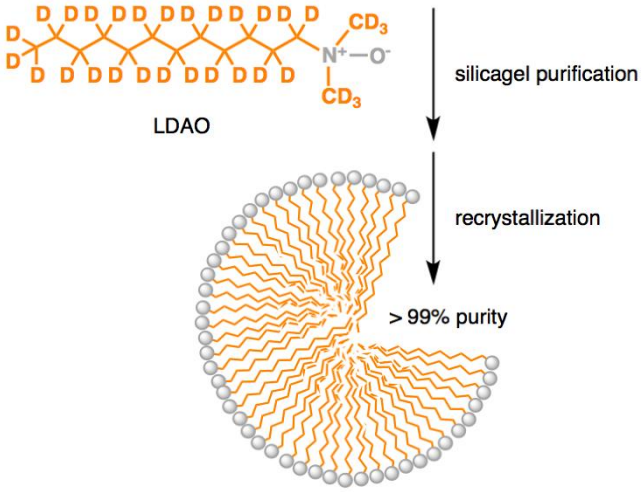

P 02 Tatiana KOZYREVA

Development of the isotopically enriched growth media

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ABSTRACT

The most widely used method to obtain proteins labelled with stable isotopes for NMR experiments is through the expression by bacterial cells grown on minimal media containing ^{13}C-enriched glucose and ^{15}N-enriched salts. Although this method has proven successful in the determination of many protein solution structures by NMR, there are limitations associated with using minimal media for protein expression. Growth rates and protein expression can be increased with the use of “rich media” products that contain more complex substrates rather than simple sugars and nitrogen salts. Rich media that contain mixtures of amino acids, peptides, nuclear acids, and complex carbohydrates can decrease doubling times and increase total protein expression. As a result, these media improve efficiency of the total labelling process and increase the yields of the proteins of interest. We focused our work on the development of a method for producing a nutrient rich medium isotopically labelled in at least one of ^{13}C or ^{15}N elements for growing bacterial and mammalian cells in the culture.

The developed medium is a rich growth medium optimized to ensure reproducible growth rates, cell density and high yields of heterologous expressed proteins. This rich growth medium is composed of a bacterial cell hydrolysate and contains primarily amino acids, some low MW oligopeptides and small amounts of carbohydrates. This complex medium facilitates cell growth and significantly increases protein expression levels compared to M9. Overall yield of protein expressed by the method of this study was estimated to be at least three times greater than that using M9 medium. These media have the same characteristics as conventional LB media: high expression rates and short fermentation times and are competitive for quality with other similar commercially available media.

P 03 Tsutomu TERAUCHI

Escherichia coli Expression System Aimed at Improving the Versatility of Stereo-Array Isotope Labeling

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ABSTRACT

The stereo-array isotope labeling (SAIL) method, currently recognized as the most promising isotope-aided NMR technique, enables the accurate determination of the structures of large proteins and protein complexes in solution, for which conventional NMR methods are insufficient [Kainosho et al., 2006]. However, the adoption of the SAIL method has been hampered, for the following two major reasons. The most frequently mentioned criticism is its inherently high cost, since it requires chemically and/or enzymatically synthesized regio- and stereo-selectively [2H, 13C, 15N]-labeled amino acids for the 20 amino acids constituting proteins. The other problem is that a cell-free protein synthesis system must be used, in lieu of the common cellular expression systems, to prepare proteins exclusively composed of the SAIL amino acids. Although the cell-free expression systems drastically reduce the cumbersome problems associated with isotopic scrambling/dilution during protein expression and also require only small amounts of expensive SAIL amino acids (only a few milligrams of each amino acid per NMR sample), most NMR groups still use the familiar cellular expression systems.

We now present a practical and robust protocol for preparing proteins selectively labeled with SAIL amino acids by using the conventional E. coli expression system. To obtain labeling rates equal to or greater than 90%, the required quantity of each amino acid was optimized. Thirteen amino acid residues (Ala, Arg, His, Ile, Leu, Lys, Phe, Pro, Met, Thr, Tyr, Trp, and Val) were found to be simultaneously or individually incorporated into the targeted proteins by using relatively small amounts of labeled amino acids. With this protocol, we successfully prepared “mini-SAIL” proteins that were labeled with all thirteen of the SAIL amino acids listed above, in an otherwise fully deuterated background. The “mini-SAIL” proteins, prepared at a relatively low cost by conventional cellular expression systems, were quite useful for structure determinations. If local structural and dynamic information for selected amino acid residues is needed, then the cost to prepare such “selectively SAIL” proteins by E. coli expression can be significantly reduced. Therefore, we hope that the results presented here will facilitate expanded applications of the SAIL method within the biological NMR community.

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P 04 Isabel AYALA

IBS Isotopic Labelling Platform

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ABSTRACT

The isotopic labelling platform of the IBS is devoted to the large-scale production of proteins uniformly or specifically enriched in stable isotopes (^{15}N, ^{13}C and ^{2}H) for biomolecular NMR spectroscopy studies. The platform offers access to innovative specific methyl labelling schemes to study high molecular weight proteins and complexes. The platform benefits from extensive scientific contacts with the IBS high field NMR Spectroscopy and Cell-Free platforms.

Users have access to the facility for large-scale expression of labelled proteins in E.coli, under the supervision of a qualified platform engineer. Dedicated benches, optimized protocols and adequate isotopically labelled chemicals are available for the following services:

- Training in large scale production of isotopically labelled proteins (practical experience in protein biochemistry is required).
- Feasibility studies of protein over-expression in perdeuterated media.
- Large scale over-expression of [15N,13C,2H]-proteins, and [13C1H3]-methyl
- specifically labelled proteins in highly (>98%) perdeuterated protein background.
- Development of innovative labelling scheme (collaboration only).

Application form is available online and should be sent to isabel.ayala@ibs.fr. Users can apply to INSTRUCT (http://www.structuralbiology.eu/) to obtain supports covering travel, accommodation and Platform fees (consumables, isotopes). Further information on isotopic labelling platform is available on IBS website:

http://www.ibs.fr/platforms/structure-determination/isotopic-labelling/?lang=en

The isotopic labelling Platform is part of Grenoble Partnership for Structural Biology and Instruct organization. The Platform is open to all national and international users from academia and industry.

The isotopic labelling Platform obtained an ISO 9001/v2008 quality management certification in July 2011, a key label and a warranty for all users of well-managed activities.

P 05 Lionel IMBERT

IBS Cell Free expression Platform for NMR applications

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ABSTRACT:

The cell-free technology is a fast and economical method for production of protein or RNA samples labeled with stable isotope for NMR studies. Cell-free expression allows to reduce the time of experimentation, simplifies purification and enables the production of the soluble and membrane proteins. The reaction may be adapted to each target sequence by the addition of additives such as cofactors, inhibitors, redox systems, chaperones, detergents, lipids, nanodisks, surfactants to optimize the expression of toxic, membrane or intrinsically disordered proteins. The cell-free technology allows the production in large scale of RNAs, macromolecular assemblies and membrane proteins, with an average yield of 2 mg/ml (and up to 6 mg/ml), compatible with structural studies by NMR, X-ray crystallography or electron microscopy.

The main advantages of cell free expression for NMR studies are:

- Simplification of expression compared to in vivo expression
- Small volumes of expression, with an optimal incorporation of labelled compounds
- Only the target protein is synthesized during the reaction and incorporates labelled amino acids
- Uniform or specific labelling available for NMR with low scrambling level compared to in vivo expression

The platform is part of UMS Integrated Structural Biology Grenoble (ISBG), an INSTRUCT research center. The platform is open to local, national and international academic users and proposes:

- Feasibility tests, to optimize the expression and the solubility of the proteins/RNAs for structural studies. These tests are performed by the platform engineer, before the arrival of PF's users.
- Training and support for new users.
- Large scale production of RNAs and Proteins: trained users benefit from a dedicated RNAse free workspace, home made E. coli S30 extracts and T7 RNA polymerase, optimized protocols and advices from Cell-free PF engineer, consumables and reagents for the production of their targets RNA, soluble or membrane proteins.

Application form is available online and should be sent to lionel.imbert@ibs.fr. The users can apply to INSTRUCT (http://www.structuralbiology.eu/) to obtain support covering travel, accomodation and platform fees (consumables, isotopes, detergents). Further information on cell-free platform are available on ISBG and Instruct web sites:

http://www.isbg.fr/samples-preparation/cell-free-666/

http://www.structuralbiology.eu/resources/platforms/instruct-centre-france-2/vitro-production-rna-proteins-ibs-grenoble

P 06 Adrien FAVIER

High field NMR research infrastructure: the IBS NMR platform.

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ABSTRACT

Since 2008 France has established a national network of flagship NMR facilities providing High-Resolution and High-Field capabilities, making available the latest cutting edge developments to a broad community of national and international users.

This multi-sited structure features research teams of international visibility providing a unique combinations of high-cost and high-performance instruments and associated skills in development of methods and application to biology, chemistry, physics, geology, or materials sciences.

Each of the sites is committed to provide one third of its experimental time and associated expertise (100 days per year) to the community, through a constantly open call for proposals.

The NMR plateform of IBS provides access to a 950MHz spectrometer equipped with a cryoprobe (liquid state, high sensitivity) and solid state probes (Magic Angle Spinning).

The typical studies that are carried out on this machine are:

- N°High resolution structures of peptides, oligosaccharides, and biological macromolecules (proteins, nucleic acids)
- Interaction studies proteins/ligands
- Site specific dynamic studies

The latest methodological developments designed in the laboratory are available to users of the platform.

REFERENCES:

http://www.ir-rmn.fr

P 07 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 national investments in scientific instrumentation. National and European centres have been defined with a mission to provide supported user access to their state-of-the-art research platforms. I will present the EU ESFRI project Instruct and French ANR project FRISBI that provide fully or partially funded user access to these platforms.

P 08 Maria STRAVROPOULOU

NMR Spectroscopic Investigations of alphaB-crystallin Substrate Complexes

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ABSTRACT

The a-crystallins are molecular chaperones, which belong to the small heat shock protein (sHsp) family [1]. sHsps maintain the cellular protein homeostasis by binding non-native, aggregation-prone polypeptides and assisting their folding and assembly [2]. There are two a-crystallin genes. However, while the expression of aA-crystallin is essentially restricted to the eye lens, aB-crystallin is ubiquitously expressed and thus associated to several human diseases, like neurodegenerative disease, myopathies and multiple sclerosis [3]. aB-crystallin forms highly dynamic oligomers with variable number of subunits [4] and it exhibits a tripartite organization with a central α-crystallin domain (ACD) flanked by an N-terminal domain (NTD) and a short C-terminal extension (CTE) [5]. The ACD forms stable dimers [6] that further assemble into higher-order oligomers via interactions mediated by the NTD and CTE [7]. aB does not possess ATPase activity and its chaperone function is regulated by phosphorylation, in response to various kinds of stress [8], which leads to a reduced mean oligomer size compared to aB-WT and increased chaperone activity and substrate binding [9]. For aB, the three major phosphorylation sites are Ser19, Ser45 and Ser59 (aB-3E mutant), all located within the NTD, which as revealed recently, gains flexibility and solvent accessibility in this state, and this promotes its cooperation with the Hsp70 system. Here, we exploit a phosphorylation mimetic of aB-WT, the aB-6E mutant, where Ser19, 21, 43, 45, 53 and 59, are replaced by glutamates. As a tool to study the structure and function of aB in a lower oligomeric state, we employed Electron Microscopy and Solution- and Solid- state NMR Spectroscopy, next to other biophysical techniques.

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P 09 Ruslan NEDIELKOV

NMR studies of the 216 kDa transmembrane enzyme complex Na^+-NQR from V. cholerae

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ABSTRACT

The Na+-translocating NADH:quinone oxidoreductase (Na+-NQR) plays a crucial role in V. cholerae's energy metabolism and Na+-homeostasis. The Na+-NQR is a respiratory transmembrane enzyme complex comprising of six subunits and at least five redox-active cofactors utilizing the free energy liberated during oxidation of NADH with quinone to pump sodium ions across the cytoplasmic membrane. The sodium gradient in turn drives a number of other cellular processes like H+/Na+ antiporters and the flagellum. How redox chemistry is coupled to ion translocation as well as the molecular details of the final electron transfer steps are still unclear.

STD NMR spectroscopy in combination with other methods revealed that the NqrA subunit (50 kDa) harbors the catalytic quinone binding site. Furthermore, STD-NMR, SPR experiments, and Trp fluorescence quenching titrations indicated that NqrA can bind two quinone ligands. We could show by recording interligand NOEs between ubiquinone-1 and the inhibitors DBMIB and HQNO that NqrA simultaneously interacts with two quinone-type molecules in an extended binding site providing, for the first time, direct experimental evidence for the long-standing double occupancy hypothesis. The NqrA subunit was prepared in 2H, 13C, 15N labelled form for backbone assignment of the protein and the quinone binding site was located by chemical shift perturbation mapping. This result was further corroborated by an analogous experiment monitoring chemical shift changes of ε-13C-methionin labelled NqrA.

To transfer and extend our results obtained with isolated NqrA to the holo-complex, Na+-NQR (216 kDa) will be expressed in V. cholerae in perdeuterated and specifically labelled form to allow for recording meaningful spectra (ε-13C-methionin-, ILV-labelling etc.). First promising results with uniformly 2H, 15N labelled Na+-NQR have already been obtained.

P 10 Olivier HAMELIN

Specific Isotopic Labelling of Methyl Group: A Tool Box for the Dynamics and Structural Studies of Large Proteins and Complexes

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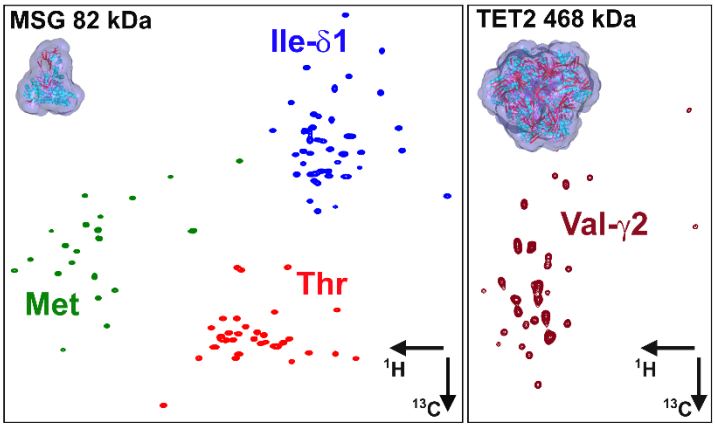
ABSTRACT

The NMR study of large proteins and complexes has been a real challenge for a long time. Recent developments in specific isotope labelling of methyl groups in a perdeuterated protein has significantly extended the frontier of liquid state NMR. In recent years, we have exploited metabolic pathways in E. coli and synthesized new isotope-labelled precursors to allow regio- or stereo-specific labelling of the methyl groups of Isoleucine (Ayala et al. Chem Comm 2012 ; Kerfah et al. J. Biomol NMR 2015), Leucine and Valine (Gans et al. Angew. Chem. 2010), including the specific labelling of methyl groups of Valine without Leucine in order to reduce overlaps in very large protein assemblies (Mas et al. J. Biomol NMR 2013).

Additionally, new protocols have been developed to extend and optimize specific labelling approaches to methyl groups of Methionine and Threonine residues (Hamelin et al. in preparation), as well as Alanine residues (Ayala et al. J. Biomol NMR 2009). With these new tools in hand, we can now label any combination of methyl groups in proteins (Kerfah et al. Curr. Opin. Struct. Biol. submitted) reporting directly on the structure and dynamics of both the protein backbone and side chains extremities. These advances extend the available labelling tools offering the possibility to study more and more challenging protein systems. Applications to the assignment and structural studies of proteins of 82 kDa and 468 kDa will be presented.



P 11 Jérome BOIBOUVIER

Specific Edition of Long-range Intermolecular NOEs using Advanced Isotopic Labeling of Methyl Groups

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ABSTRACT

Large protein complexes are involved in many of the key processes in the cell but their study by NMR has been a real challenge for a long time. Nowadays, it is unequivocally recognized that the strategy of specific isotope labeling of methyl groups in a perdeuterated protein has significantly extended the frontier of liquid state NMR. Production of perdeuterated protein with selective protonation of the methyl groups can be carried out by adding the specifically labeled metabolic precursors in the fully deuterated culture medium before induction of protein expression.

In the last years, we have exploited metabolic pathways in E. coli and synthesized new isotope-labeled precursors to extend the library of methyl labeling methods. These new strategies allow the stereospecific labeling of the prochiral methyl groups of Leucine and Valine as well as the regioselective labeling of Isoleucine methyl probes[1,2]. To further improve quality of spectra for protein system with molecular size of several hundred of kilodalton, we have demonstrated that the specific labeling of methyl of Valine groups without Leucine enhances significantly the resolution of spectra[3]. Additionally, new protocols have been developed to extend and optimize specific labeling approaches to methyl groups of Methionine and Threonine residues, as well as Alanine residues[4,5]. Using these new tools, we have setup robust and scrambling-free protocols to label in targeted proteins any combination of methyl groups.

These specific labeling strategies are particularly adapted to extract precise long-range NOE restraints between remote probes separated by up to 10 Å in large proteins[6,7]. In this communication we will show applications of these combinatorial labeling strategies in order to edit separately structurally meaningful intermolecular NOEs from the large set of detected NOEs. Application to the structural study of a 500 kDa homododecameric protein will be presented.

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P 12 Elodie CRUBLET

SeSAM: A cost-effective protocol for Sequence-Specific Assignment of methyl groups by Mutagenesis for NMR studies of high molecular weight proteins

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ABSTRACT

There is increasing interest in applying NMR spectroscopy to the study of large protein assemblies. Development of methyl-specific labeling protocols combined with improved NMR spectroscopy enable nowadays studies of proteins complexes up to 1 MDa. For such large complexes, the major interest lies in obtaining structural, dynamic and interaction information in solution, which requires sequence-specific resonance assignment of NMR signals. While such analysis is quite standard for small proteins, it remains one of the major bottlenecks when the size of the protein increases.

Here, we describe implementation and latest improvements of SeSAM, a fast and user-friendly approach for assignment of methyl resonances in large proteins using mutagenesis. We have improved culture medium to boost the production of methyl specifically labeled proteins, allowing us to perform small-scale parallel production and purification of a library of 13CH3-specifically labeled mutants. This optimized protocol is illustrated by assignment of Alanine, Isoleucine and Valine methyl groups of the homododecameric aminopeptidase PhTET2. We estimated that this improved method allows assignment of ca. 100 methyl cross-peaks in 2 weeks, including 4 days of NMR time and less than 2 k€ of isotopic materials.

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P 13 Halina MIKOLAJEK

Structural basis of eEF2K activation by Calcium/Calmodulin

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ABSTRACT

Eukaryotic elongation factor 2 kinase (eEF2K) is a calcium/calmodulin (Ca^{2+}/CaM)-dependent protein kinase that phosphorylates and inactivates the translation elongation factor eEF2, thereby slowing down the rate of translation elongation.

The protein consists of an unstructured N-terminal region containing a Ca^{2+}/CaM binding region, a highly atypical α-kinase domain, an unstructured central region with regulatory phosphorylation sites and a structured C-terminal domain containing four Sel1-like repeats. We have taken a dissect and conquer approach to study the structure and regulation of this kinase (1,2). However, the structure and molecular mechanism of regulation of this potential cancer target are not known.

Using SAXS and molecular modelling we have determined a solution structure of the kinase domain in complex with the C-terminal Sel1 repeats. We also determined the solution structure of the tripartite complex of CAM, the kinase domain and C-terminal Sel1 repeats using SAXS and NMR. The functional implications were further tested by mutational studies. The data we present here demonstrate the first insight into the molecular mechanism of regulation of eEF2K by CaM and Ca^{2+}.

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P 14 Michael JUEN

Site-specific ^{13}C labeling of RNA via reverse direction RNA synthesis

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ABSTRACT

We report on the chemical synthesis of site-specifically ^{13}C-modified pyrimdine reverse phosphoramidites. In detail, we synthesized 6-^{13}C-modified uridine and cytidine reverse phosphoramidites with a purity greater than 99% as analyzed by ^{31}P-NMR spectroscopy. These compounds proved to be fully compatible with the RNA solid phase synthesis approach. Using this methodology oligonucleotides can be assembled in the 5'- to 3'- direction in analogy to the biological RNA synthesis via polymerases.[1] The reverse approach competes with or even surpasses the standard solid phase RNA synthesis, where the ribonucleotide is assembled in the 3'- to 5'-direction, in terms of coupling efficiency.

We obtained several high quality RNA products with the desired 13C labeling patterns. These stable isotope labeled ribonucleotides will be used in NMR spectroscopic investigations on structural and dynamic features of biological relevant RNAs. More recently, we focused on the dynamic features of an in vitro selected RNA aptamer responsive to biotin (Vitamin H).[2] Preliminary NMR spectroscopic data shedding light on the ligand recognition mechanism will be presented.

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P 15 Nadine ASSRIR

Cost effective production of labeled proteins in eukaryotic cells for NMR studies.

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ABSTRACT

Many eukaryotic (human in particular) proteins cannot be produced in *E. coli* because they need either specific chaperones to be correctly folded or specific post-translational modifications to be functional. It is sometime possible to over-express them to eukaryotic cells. In an other hand, it will be particularly interesting to follow protein modifications during physiological process on cellular context using *in cell* NMR. This emerging application of the NMR offers a promising prospect to studies on structural modifications of proteins in a cellular context, ie post-translational modifications, interactions...

We developed a simple and cost effective procedure to produce uniform ^{15}N-labeled proteins in S2 or Sf9 (with baculovirus) cells (Meola et al. 2014) based on the use of an optimized commercial medium depleted in all amino acids and supplemented with 10 g.l^{-1} of 15N-labeled yeast autolysate and 5 mM of $^{15}NH_4Cl$. We applied this procedure to the production in Sf9 cells of recombinant actin and of domains III of “class II “ viral fusion proteins. Now, we attempt to transfer this process to mammalian cells.

The labeling method will be used to develop a NMR-based approach concerning the dynamics of the transitions and the caraterization of the intermedates between the pre- and post fusion forms of “class II” viral proteins. We will applied to the study of actin polymerization regulation by small disorder proteins and to the perturbation of this process induced by mycolactone, a toxin produced by *Mycobacter ulcerans*, responsible of buruli ulcer.

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P 16 Anne DIEHL

Towards Structural Investigation of ChannelRhodopsin expressed in Pichia pastoris and Insect cells

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ABSTRACT

In 2010 OPTOGENETICS became the “Method of the Year” awarded by Nature Methods. Powerful optogenetic tools are light gated ion channels like ChannelRhodopsin (ChR) from green algae. The dark state crystal structure of a hybrid of ChR1 and 2 was solved recently (Kato et.al 2012). By solid state NMR we want to investigate structural dynamics of ChR (ca. 40kDa) linked to the opening and closing of that membrane protein, around the chromophore and along the channel. Since the first de-novo structure of a natively folded protein was determined in our group (Castellani, 2002) by solid state NMR, other groups and we have developed that method further on to target more complex systems now

The expression of functional ChR in E.coli (the easiest host for NMR labelling) failed untill now.

Therefore we applied different expression methods with insect cells and yeasts as hosts.

Stable isotope labelling as needed for NMR is very expensive with these systems in contrast to E.coli. So we started with the incorporation of synthesised ^{13}C labelled retinal into unlabelled apo-protein from these sources. Then we tried amino acid specific labelling in Pichia.

Furthermore the expression of ChR in Pichia on D_2O based media was established. As we want to monitor proton movements during the photocycle, we should provide a deuterated NMR invisible protein background.

The current work is part of a research collaboration center SFB 1078 “Proton Dynamics in Protein Function” coordinated at the Free University Berlin.

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P 17 James TOLCHARD

Initial steps in describing the F_1F_o ATP synthase dimer interface and modelling the small hydrophobic subunits of the F_o region with solution state NMR.

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ABSTRACT

The multi-subunit complex of F_1F_o ATP synthase is the principle source of cellular ATP throughout the biological kingdom. Although variability exists in the precise F_1F_o composition between species, the most studied protein is from Saccharomyces cerevisiae where the complex is comprised of 17 core subunits. Two of the Yeast F_1F_o subunits (e and g) and the N-terminal membrane domain of subunit 4, which all locate to the mitochondrial membrane F_o region, have recently been shown [1] to be absolutely required for the dimerisation of ATP synthase which facilitates the formation of the mitochondrial cristae [2]. Currently, no high-resolution structural information for the interface of dimeric ATP synthase exists and as a result, the interactions which stabilise the ATP dimer are unknown; with prior investigations typically hampered by poor yields of the membrane domain subunits.

To this end, we have optimised a home-made cell-free expression system for the production and isotopic enrichment of these subunits - with final yields >1 mg which allows for investigation with nuclear magnetic resonance (NMR) spectroscopy. Initial studies, using unlabelled and ^{15}N-Ala samples, focussed on validating the incorporation of samples into LMPG micelles and optimising the quality of NMR spectra for an 83 residue (8.7 kDa) construct comprising the N-terminal region of subunit 4 from the Yeast F_1F_o ATP synthase (S4T). From synthesising single (^{15}N) and double (^{13}C, ^{15}N) labelled samples of S4T, two and three-dimensional NMR spectra (^{15}N and ^{13}C HSQC, HNCACB, CBCACONH, HNCA, HNCOCA and, HCACO) were acquired at 800 MHz enabling us to assign 95% of the S4T backbone nuclei. In silico predictions from primary sequence alone suggested that S4T would have a predominantly alpha helical conformation and this agrees with both chemical shift analysis and a preliminary 3D model from CS-ROSETTA [3] which describes the structure of S4T as a bundle of 3 trans-membrane helices. Furthermore, recent Hydrogen:Deuterium exchange experiments on the S4T construct have given insight into the residues which are shielded from amide exchange.

Future work will focus on optimising the acquisition of TOCSY and NOESY-based NMR experiments for classical distance-based structure determinations of S4T before beginning NMR investigations into subunits e and g. Studies into the interactions which stabilise the dimeric state of ATP synthase will then be undertaken through a number of biophysical techniques to investigate the different affinities and sites of interaction amongst the three subunits.

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P 18 Ronnie FREDERICK

Production and stable isotope labeling of proteins for NMR investigations of the biosynthesis of iron-sulfur proteins

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ABSTRACT

The Mitochondrial Protein Partnership (MPP) investigates structure-function relationships of mitochondrial proteins. One area of interest focuses on the mechanism and control of the biosynthesis of iron-sulfur proteins. This has led us to clone and produce a number of proteins involved in the iron-sulfur cluster (ISC) assembly system of mitochondria and their homologs from Escherichia coli (E. coli). This work utilizes a research platform developed at the Center for Eukaryotic Structural Genomics (CESG) over the past 14 years, which has enabled us to rapidly express and efficiently purify numerous proteins with high rates of success. The platform combines the use of E. coli cell-based, small-scale protein production and optimization, various N-terminal solubility tags including small ubiquitin-like modifier proteins (SUMO) and maltose binding protein (MBP), and different affinity-purification-based vectors, together with large-scale cell growths (using PET 2L bottles) to lower costs for production and to maximize protein yields. The SUMO and Flexi®_vector systems use His-tag and IMAC based systems for purification. In addition, we express and purify TEV and SUMO proteases locally which are used to cleave the N-terminal tags from the protein targets fused to MBP and SUMO, respectively. We use this platform to produce proteins with a variety of stable isotope labeling patterns. In this poster we present the successful application and optimization of this platform to a group of difficult proteins and results from investigations of their structure and function. The studies have revealed the functions of some of the proteins, the effects of mutations on structure and function, and information about the tangled web of protein-protein interactions that lead to Fe-S cluster assembly and transfer to acceptor proteins.

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P 19 Frank LÖHR

Combinatorial ^{15}N, $^{13}C'$ and $^{13}C^{\alpha}$ labelling for backbone assignment of membrane proteins

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ABSTRACT

Resonance assignment of a-helical membrane proteins by solution NMR is often hampered by their inherently low chemical shift dispersion and insufficient sensitivity of crucial triple-resonance experiments that involve 13C-13C magnetization transfer steps, such as HNCACB and HN(CA)CO. In this situation, combinatorial 15N (Wu et al., 2006) or dual 15N/13C' labelling (Parker et al., 2004; Trbovic et al., 2005; Maslennikov et al., 2010) can provide useful complementary amino-acid type as well as sequential information. Recently, we have introduced combinatorial triple-selective labelling (Löhr et al., 2012) in an attempt to improve the coverage of residues in a protein sequence for which such information is available, while restricting the number of samples in order to minimize sample preparation efforts and expenses. The method involves incorporation of 15N-, 1-13C- and 13C/15N-labelled amino acids via cell-free expression and requires acquisition of a set of BEST-TROSY type (Farjon et al., 2009) 2D HN(CX) triple-resonance experiments for distinction of the various isotopomeric dipeptides.

Here we present a refined combinatorial protocol which additionally includes 2-^{13}C selectively labelled amino acids, significantly increasing the number of sequential pairs that can be identified. If such pairs are unique in a protein's amino acid sequence their sequence specific NH assignment is established and can be used as an anchor point in the subsequent assignment process based on ^{13}C chemical shift matching in 3D spectra. Experimentally, the only amendment to be made is a supplementary filtered 2D spectrum which is required to distinguish $^{13}C^{\alpha}_{i-1}$-$^{12}C'_{i-1}$-$^{15}N_i$-$^{12}C^{\alpha}_{i}$ from $^{12}C^{\alpha}_{i-1}$-$^{12}C'_{i-1}$-$^{15}N_i$-$^{13}C^{\alpha}_{I}$ moieties. The new combinatorial labelling method has been applied to two α-helical membrane proteins, the dimeric 142-residue protein TMD0 in DHPC micelles and the 194-residue protein LspA expressed into nanodiscs. In both cases virtually complete backbone assignments could be obtained using a single complementary 3D HNCA on uniformly $^{13}C/^{15}N$-labelled samples to fill the gaps between the selectively labelled residue pairs.

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P 20 Jiafei MAO

Protein Perdeuteration and Synthetic Ligand Labeling for MAS NMR and DNP-Enhanced NMR Studies of Color Tuning in Membrane Protein Proteorhodopsin

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ABSTRACT

Membrane proteins (MPs), especially those of large molecular weight, poor chemical shift dispersion and conformational dynamics/heterogeneity, are often difficult targets for high resolution SSNMR studies. In this abstract, we will present that the combination of tailored isotope labeling schemes with high resolution MAS SSNMR and DNP-enhanced MAS NMR permits the study of the functional aspect – color tuning – in a 7TM membrane protein proteorhodopsin. First, the perdeuteration of both the protein and its chemical environment (lipid and water) cleans up the 1H background and permits the selective observation of protein-ligand contacts without isotope labeling of the ligand. Furthermore, the selective labeling of key sites on ligand through chemical synthesis provides an opportunity on observing the protein-regulated ligand structural changes by DNP-enhanced MAS SSNMR. In summary isotope labeling could be a powerful and necessary tool in such SSNMR studies on membrane protein targets.

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P 21 Daniel HÄUSSINGER

Selective 15N-Leu labelling of human Carbonic Anhydrase type II for Pseudo Contact Shift NMR Spectroscopy

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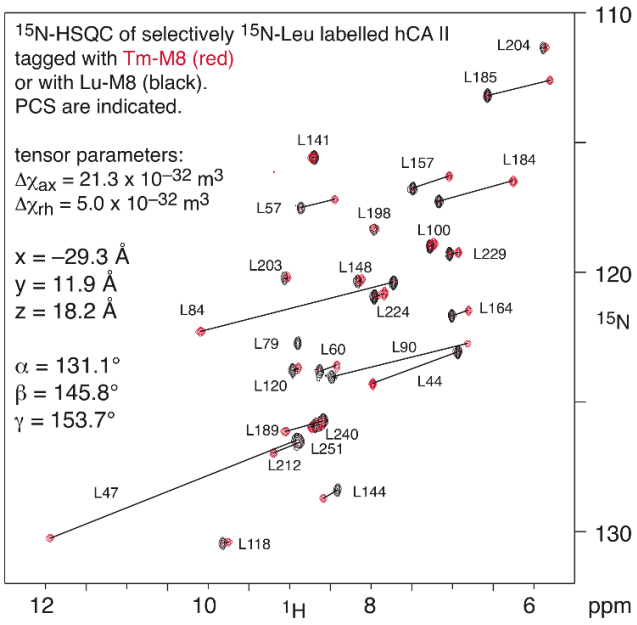
ABSTRACT

Pseudo Contact Shift (PCS) NMR Spectroscopy is a unique and powerful method to characterise protein-protein and protein-ligand complexes [1] in solution. Specifically its unusual long-range radius of information of up to 70Å in combination with the applicability to protein complexes in the 100 kDa range make it a versatile tool for structural biology.

We are interested in human Carbonic Anhydrase type II (hCA II) and its complexes with ligands that bind to the active Zn atom in the catalytic activity. Our collaborator, Th. Ward in Basel, uses such systems as artificial metallo-enzymes to perform homogeneous transition metal catalysis inside the scaffold of a protein in order to obtain highly stereospecific reactions like hydrogenation, oxidation and metathesis.

Here we present the determination of the position of a fluorinated ligand inside the catalytic cavity of hCA II, solely based on PCS NMR by tagging the protein with the lanthanide chelating tag DOTA-M8 [2]. To achieve the assignment of the paramagnetically shifted resonances, a selectively 15N-Leu labelled hCA II sample was required and successfully prepared without significant scrambling of the 15N label.



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P 22 Raquel GARCIA-CASTELLANOS

In-vivo overexpression and purification of labelled peptides for NMR studies

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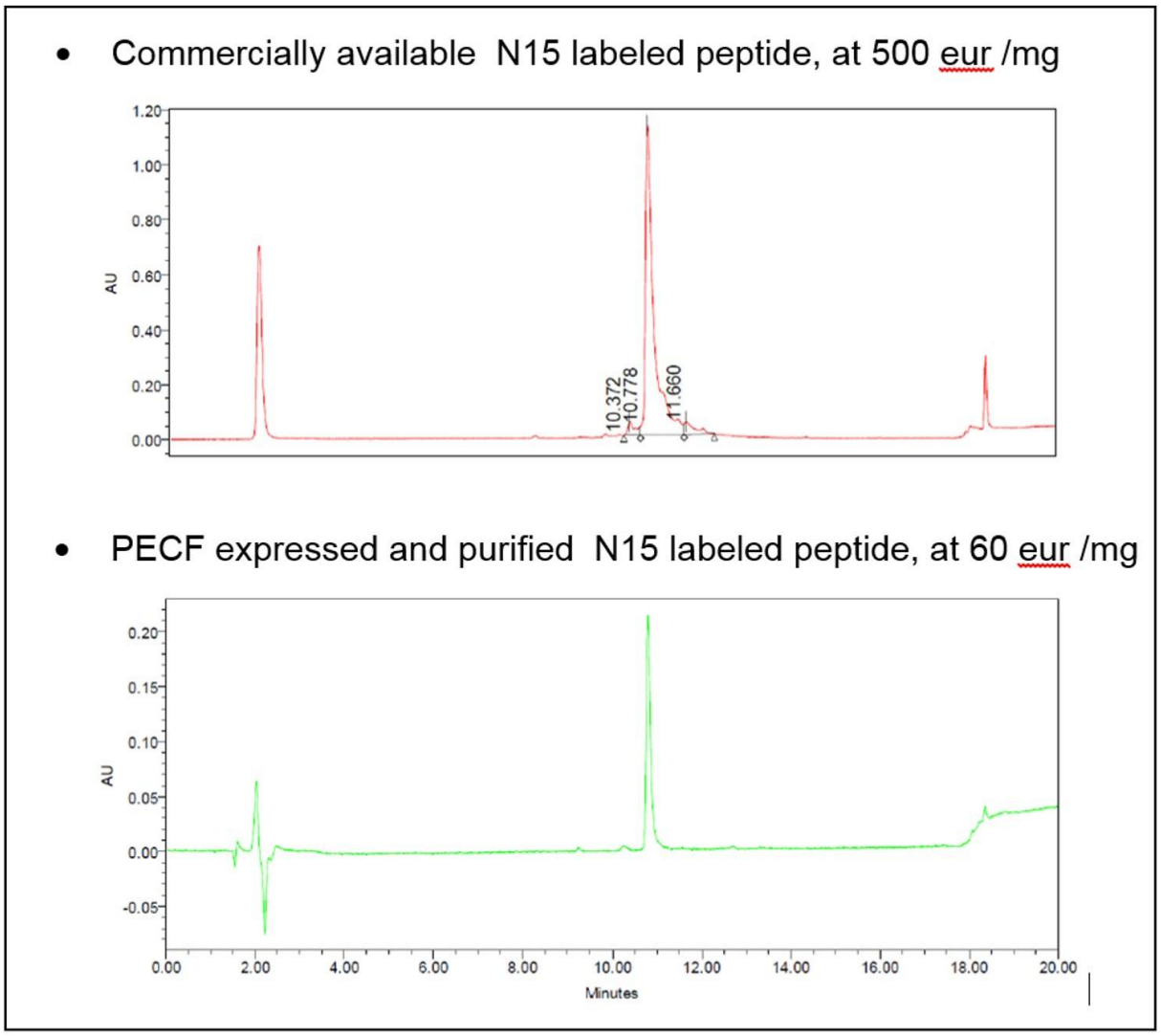
ABSTRACT

At our core facility, we are currently overexpressing different labelled peptides in E.coli. This has resulted in cheaper and better quality material for the NMR experiments of the researchers of our institute.

In this poster we show the results obtained with two peptides in terms of purity and efficiency of N15 and H2 incorporation.



Example of the reduction of the costs and increase of the quality of a substrate for NMR experiments.

P 23 Caroline MAS

Structural Insight in the recognition of human eIF3b by HCV IRES-III domain

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ABSTRACT

In order to initiate the translation of *hepatitis C virus (*HCV) polyprotein, an Internal Ribosome Entry Site (IRES) RNA motif recruits a specific set of host cell factors, from which the *eukaryotic translation initiation factor 3 (*eIF3) is a key element. Indeed, a transient binding of eIF3 in the 48S ribosomal translation initiation complex is required before formation of a productive translation particle. The IRES IIId domain of the *HCV is responsible for the* recruitment of the N-terminal RNA recognition motif (RRM) of the eukaryotic Initiation Factor 3b to form independent binding unit involved in hijacking of eIF3 particle by HCV RNA (Perard, J. et al., FEBS Lett 2009). The shape of eIF3 particle was previously characterized by cryo-EM (Siridechadilo et al., Science 2005), but few high resolution structural information are available on eIF3 subunits

Here, we demonstrate that the IIId sub-domain of HCV IRES mediates the interaction with eIF3. We solved the solution structure of this independent eIF3/IRES binding unit using a combination of SAXS and NMR spectroscopy and integrate the model in the envelope of the 48S ribosome/eIF3/IRES particle.

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P 24 Jose Luis ORTEGA-ROLDAN

Topology and Interactions of the Human Sigma-1 Receptor Chaperone

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ABSTRACT

The Sigma-1 Receptor (S1R) is a ligand-regulated membrane protein chaperone involved in the ER stress response. S1R activity is implicated in diseases of the central nervous system including amnesia, schizophrenia, depression, Alzheimer's disease, and addiction. S1R has been shown previously to regulate the Hsp70 BiP and the IP3R calcium channel through a C-terminal domain. It also binds and is regulated by a large number of small molecules such us psychostimulants (cocaine, methamphetamine and DMT), antidepressants, antipsychotics and steroids. We have developed methods for bacterial expression and reconstitution of the chaperone domain (Ortega-Roldan et al, 2013) and a truncation construct lacking the first transmembrane domain (TM) of human S1R into DPC:DPPC mixed micelles that enable its study by solution NMR spectroscopy. S1R is found to contain a first TM, two helices in the cytosolic loop, followed by the second TM and a juxtaposed helix, a largely dynamic region and a structured, helical C-terminal region that encompasses a membrane associated domain containing four helices. The helical region at residues ~198-206 is strongly amphipathic and proposed to anchor the chaperone domain to micelles and membranes. Three of the helices in the C- terminal region closely correspond to previously identified cholesterol and drug recognition sites. Moreover, we show experimentally using chemical shifts and spin relaxation that the second TM, whose position hasn't been predicted accurately, spans the residues 90 to 107 and includes the entire Sterol Binding Domain Like 1 (SBD1), partially responsible for drug binding. In addition, we show that the chaperone domain interacts with full- length BiP in a Calcium dependent manner, as it has been observed in vivo. Independent titrations of both nucleotide binding domain (NBD) and substrate binding domain (SBD) of BiP, show only binding by the NBD, suggesting that the nucleotide binding domain is sufficient for S1R interactions. These results, that constitute the first structural information obtained for this protein and its interactions, advance our understanding of S1R and are likely to be useful in refining models of the S1R drug binding sites.

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P 25 Felipe OSSA

Oligomeric States of the Human Sigma-1 Receptor

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ABSTRACT

The Sigma-1 Receptor is a 26.5 kDa ER resident receptor chaperone. It is known to bind to and mediate the effects of a wide range of pharmaceutical compounds, many of which are in current clinical use. The Sigma-1 Receptor exerts its effects through the regulation of a wide range of ion channels such as the IP3 receptors and voltage gated sodium, potassium and calcium channels among others.

Recent reports have shown that the functional receptor may exist in an Oligomeric state. This work demonstrates that the functional purified receptor exists predominantly as a dimeric species that can be transiently observed in a construct missing the first TM of the receptor (D35), as well as examining the impact of D126A and E173A two previously reported drug-binding knockout mutations on the dimeric state and functionality of the protein.

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N+

P 26 Katharina WEINHAUPL

Atomic-resolution studies of structure, dynamics and interactions in chaperone assemblies by NMR spectroscopy

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ABSTRACT

Mitochondria perform a wide range of key cellular functions, such as the generation of energy in the form of ATP or degradation of metabolites via the urea cycle and beta-oxidation. A requirement for these processes is the import and export of metabolites through the mitochondrial membranes. This transport is performed by membrane proteins in the inner and outer membrane of mitochondria. Only 1 % of all mitochondrial proteins are encoded by mitochondrial DNA. As a consequence, the predominant part of mitochondrial proteins needs to be imported. For the import of mitochondrial membrane protein precursors a sophisticated transport machinery is necessary, leading the protein precursors from the cytosol, through the mitochondrial outer membrane to the intermembrane space for eventual insertion into the membrane. In this process chaperones need to protect membrane protein precursors from the aqueous environment of the cytosol and the mitochondrial inter-membrane space. The 70 kDa chaperone TIM10 is dedicated to leading membrane protein precursors through the intermembrane space to their location of insertion into either the inner or the outer membrane. TIM10 is responsible for the transport of membrane protein precursors intended for insertion as b-barrel proteins as well as a-helical mitochondrial carriers. Well-known substrates of TIM10 are the most abundant protein in the mitochondrial outer membrane VDAC, the central channel protein of the TOM complex Tom40 and the mitochondrial carriers AAC, GGC or ORC.

The mechanism by which chaperones transport their substrate proteins is currently poorly understood. The challenge in studying chaperone-substrate complexes relates to the fact that substrates are generally unfolded and highly dynamic, which poses a severe problem for most structural biology techniques. In the case of TIM10, a crystal structure of the apo form, without membrane protein precursor, has been reported. However, the mechanisms by which TIM10 carries its different substrates remain unclear. Interestingly, TIM10 is able to act as a chaperone for a-helical inner membrane proteins, like the mitochondrial carriers, as well as the outer membrane b-barrel proteins VDAC and Tom40. This is remarkable, as these classes of proteins possess markedly different physico-chemical properties.

NMR spectroscopy is a powerful method to obtain atomic resolution information, even in highly dynamic and unstructured systems, as is expected of TIM10-substrate complexes. We will use state of the art NMR methods and isotope labeling techniques to provide information about the structural propensities and position of the substrate, dynamics in the complex, specific interactions between the substrate and TIM10 and also characterize transient low-populated states.

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P 27 Jolyon CLARIDGE

M2 interactions involved in influenza replication

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ABSTRACT

The protein M2 from influenza is a tetrameric membrane protein with several roles in the influenza life-cycle. The transmembrane helix (TMH) of M2 has proton channel activity that is required for unpacking the viral genome. Additionally a C-terminal juxtamembrane region includes an amphipathic helix (APH) important for virus budding and scission and that has been previously shown to be important for stability of the M2 tetramer. The C-terminal region of M2 also contains motifs that interact with other viral proteins and the host protein LC3. One protein that has been suggested to interact with M2 is matrix protein 1 (M1). M1 is essential for the structural integrity of the viral capsid and is also involved in interactions with several viral proteins. Neuraminidase (NA) and hemagglutinin (HA) are viral raft associated proteins, which initiate the formation of the viral budzone. Both HA and NA have been shown to interact with M1 through their cytoplasmic tails. M1 then binds to the viral nucleoprotein and thus connects the nucleoprotein core and the viral membrane.

Although it is known that the presence of M2 is essential for the successful exit of the nascent virus from the cell membrane, little is known about how M2 is recruited to the 'budzone' where membrane scission takes place. Here we confirm that M1 and M2 interact in vitro. We also show using solution-state NMR that beta strand residual structure is present in the otherwise unfolded C-terminal tail of M2. It is likely that the interaction with M1 provides a mechanism for the recruitment of M2 to the viral budzone, thus enabling the release of the virus from the cell.

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P 28 Florent DELHOMMEL

Structural study of Whirlin, a crucial PDZ containing protein involved in the mechanotransduction of auditory hair cells.

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ABSTRACT

Mammals perceive sound thanks to mechanosensory hair cells. The sound-induced vibration displaces cilia that are bound together by a network of cadherins and scaffolding proteins. The stretching of the network is directly responsible for the opening of an ion channel that translates the vibration into an electric signal transmissible to the brain. Nearly all proteins involved in the cilia-associated network contain a PDZ binding motif (PBM). PBM are short C-terminal motifs recognized by PDZ domains. PDZ are the most common protein-interaction domains that maintain scaffolding complexes by binding PBM to their target proteins. Only two proteins of the cilia-associated network contain PDZ: Harmonin and Whirlin. They are central for the link between membrane proteins and the cytoskeleton. We study Whirlin, a protein composed of three PDZ domains. The N-terminal part of the protein encompasses two PDZ domains and two HHD domains (Harmonin Homology Domain). HHD is known to interact with the PDZ domain in a highly homologous protein, modulating the PDZ affinity and specificity. Using sequence alignment, we recently identified the second domain HHD downstream to Whirlin second PDZ domain, creating a symmetric organization: HHD1-PDZ1-PDZ2-HHD2. We show that PDZ2 and HHD2 domains are in cis-interaction, likely to modulate the binding of the PDZ domain. We cloned, expressed and purified constructions of each four domains, along with the three tandem constructions to decipher Whirlin interactome and the function of Whirlin modular organization.

P 29 Diego Fernando GAUTO

Structural propensity analysis of an intrinsically disordered RNA binding domain

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ABSTRACT

For a subset of IDPs, function is linked to the acquisition of a folded structure upon partner recognition. A challenging issue in this subset of IDPs is whether the sampling of conformational space is in a way linked to their ability to recognize different partners. Therefore, in the present work we investigate the exploration of the conformational space by the first dsRBD of DCL1 from A. thaliana. This domain acquires a folded conformation with a complex topology upon binding dsRNA.

We make use of NMR observables to characterize conformational sampling at residue level. The protein shows a narrow distribution of chemical shifts, indicating its disordered nature. However titration with urea leads to further narrowing, showing that the native state is not fully unfolded. We find that different regions of the protein show a varying degree of unfolding with urea, suggesting that partial structuring is not homogeneous along the protein. Finally, with the help of secondary chemical shifts and residual dipolar couplings we achieve a physical description of the disordered state in terms of a ensemble of structures.

The present work adds new information to the relatively unexplored field of plant IDPs, reveling the dynamics and structural connection between free DCL1 and DCL1-RNA complex, and its link with molecular recognition.

P 30 Daniel GRIFFITHS

Interactions of carrier proteins with partner enzymes during antibiotic assembly

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ABSTRACT

Polyketide synthases (PKS) are comprised of multi-enzymatic domains that facilitate the biosynthesis of vital natural products such as antibiotics, antifungal agents and anti-tumour drugs. Investigations of novel biosynthetic pathways are providing routes to new therapeutic natural products. However, creating novel molecules by engineering PKSs often leads to reduced yield of product and incompatibility between protein domains. A detailed structural understanding of the mechanism of chain assembly, and protein-protein interactions thereby, will provide a basis for rational modification of existing biosynthetic pathways to yield new drugs.

A core component of PKSs is the acyl carrier protein (ACP), which communicates transiently between partner enzymes whilst tethered to a growing natural product. ACPs are responsible for the transport of substrates between subsequent stages of natural product assembly, and display inherent conformational mobility. Using chemical shift perturbations, and surface plasmon resonance, we have identified specific protein-protein interactions between ACPs and partner domains involved in a novel method for antibiotic chain release in enacyloxin PKS.

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P 31 Sarina GRUTSCH

Structural and functional analysis of the major birch pollen allergen Bet v 1

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ABSTRACT

The major birch pollen allergen Bet v 1 represents one of the best-characterized model allergens in immunology and it is one of the main causes of Type I allergic reactions with an estimated 100 million people affected. [1] Previous studies have suggested that Bet v 1 probably functions as ssch Steroid storage protein or as transport protein that can bind different types of ligands. However, the exact physiological function of Bet v 1 remains elusive. [2] [3]

The goal of our study is to gain insight into the mechanism of Bet v 1 ligand binding and therefore we examine how binding partners are captured by this allergen and what kind of structural changes occur. [4] Any structural rearrangements of Bet v 1 probably influence its activity. [1] We use a combination of NMR spectroscopic methods (i.e. HSQC titrations, PFG experiments, relaxation (CPMG - dispersion, R_1, $R_{1\rho}$) and triple resonance experiments) and other techniques such as FT-ICR MS analysis, ITC measurements and computational studies to provide a comprehensive structural picture of the ligand capturing mechanism of Bet v 1.

An additional goal of this study is to characterize the structure, the stability and the dynamics of Bet v 1 variants. For this purpose we analyze and compare different isoforms and mutants of Bet v 1 by CPMG relaxation dispersion experiments.

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P 32 Bruno KIEFFER

Molecular basis of gene expression regulation by Retinoic Acid nuclear receptor phosphorylation

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ABSTRACT

Nuclear Retinoic Acid Receptors (RARs) are ligand-dependent transcriptional regulators, which mediate the effects of RA, the major active metabolite of vitamin A. They regulate a multitude of key biological functions such as embryonic development, organogenesis, homeostasis, energy balance, vision, immune functions and reproduction. RAR share a common architecture with all other nuclear receptors that includes a ligand binding domain (LBD), responsible of the ligand dependent activity, a DNA binding domain (DBD) involved in the recognition of hormone specific response elements and an N-terminal intrinsically disordered region (NTD). Recent studies highlighted the importance of the topology of RARs DNA cognate sequences and kinase signalling pathways for fine spacio-temporal regulation of RAR-target genes expression. We have been studying the molecular mechanism by which the phosphorylation of a conserved serine within the intrinsically disordered region modulates the gene activation of RARγ. This mechanism involves the phosphorylation-dependent modulation of the affinity between a proline-rich region within the NTD and a SH3 domain contained within the vinexinβ, a RARγ co-regulator protein. Our results highlight a complex allosteric mechanism linking RARγ DNA binding properties with its phosphorylation state.

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P 33 Desmond LAU

Auto-Inhibition: a mechanism for regulating DNA binding by the PEA3 transcription factor

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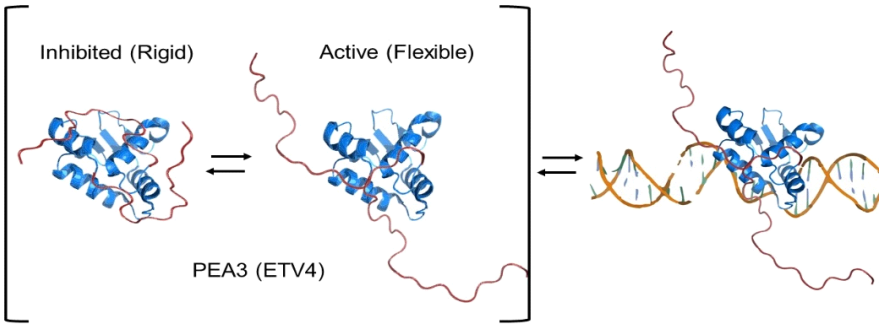
ABSTRACT

ETS (E26 transformation specific) transcription factors play critical roles in regulating cellular growth, development and differentiation[1]. All of the 28 ETS factors identified in humans share a conserved ETS domain that interacts with specific DNA sequences in the promoter and enhancer regions of target genes. Our research focuses on three members of the ETS family called the PEA3 sub-group: Er81, Erm, and PEA3 (polyoma enhancer activator 3). These PEA3 factors are of great medical interest given their frequent involvement in prostate cancer[2]. Although the regulatory mechanisms governing PEA3 factors remain unclear, their ability to bind DNA is auto-inhibited by intrinsically disordered sequences flanking their ETS domains[3,4]. This is reminiscent of the well characterized cases of Ets-1[5] and Tel[6], except that the inhibitory sequences of these factors attenuate DNA-binding by forming α-helices appended on their ETS domains. We hypothesize that the flexible auto-inhibitory sequences provide distinct routes for the specific regulation of the PEA3 factors.

An extensive set of PEA3 deletion fragments encompassing the ETS domain were created and the boundaries of the sequences responsible for auto-inhibition of DNA binding identified. These sequences lie both N- and C-terminal to the ETS domains and appear to function additively. By isotopically $^{15}N/^{13}C$-labeling the deletion fragments and assigning the corresponding spectra, we confirmed that the inhibitory sequences are indeed predominantly disordered. Furthermore, based on chemical shift perturbation mapping and paramagnetic relaxation enhancements studies. we found that the inhibitory sequences transiently interact with a coarsely defined surface on the ETS domain. By analogy to Ets-1 and Tel, the inhibitory sequences may act by shifting a conformational equilibrium of the PEA3 ETS domain from flexible/active to rigid/inactive states. Indeed, the DNA recognition helix of the uninhibited PEA3 ETS domain is relatively flexible as evidenced by hydrogen exchange and relaxation dispersion measurements. Similar studies of the inhibited fragments are underway. Overall, our studies will help define the molecular mechanisms underlying PEA3 factor auto-inhibition, and may inspire new anti-cancer strategies. For example, small molecules that reinforce PEA3 auto-inhibition should counteract their over-expression in prostate cancers.



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P 34 Assia MOUHAUD

The C-terminal domain of Gag : multiple interactions and role for the assembly and the budding of HIV-1

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ABSTRACT

During HIV assembly, $Pr55^{Gag}$ proteins interact with the genomic RNA (gRNA), lipids of the plasma membrane, host proteins through the ESCRT complex, viral proteins (Vif, Vpr) and are involved in intermolecular interactions with other $Pr55^{Gag}$ proteins. This network of interactions is responsible for the formation of the viral particle, the selection of gRNA and the packaging of Vpr. The C-terminal domain of $Pr55^{Gag}$ that is encompassed in NCp15 is involved in the majority of these interactions, either by the nucleocapsid (NC) or by the p6 domains.

Our aim is to study the NCp15 protein as a model of the C-terminal domain of $Pr55^{Gag}$ to better understand the role of this domain in the assembly and budding of HIV. The interactions between NCp15 and its various partners are investigated by Nuclear Magnetic Resonance (NMR) and Isothermal Titration Calorimetry (ITC). Recent results will be presented.

P 35 Zsofia SOLYOM

NMR study of transient structure and dynamics and their modulation by phosphorylation and interaction in the intrinsically disordered region of HCV protein NS5A

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ABSTRACT

Intrinsically disordered proteins are characterized by a lack of a stable, 3D structure. Viral regulatory proteins often contain disordered regions as flexibility is advantageous for their multiple interactions with various partners. NMR spectroscopy is the method of choice for their atomic resolution studies, as X-ray crystallography is not amenable to them because of their highly dynamic character.

NS5A protein of hepatitis C virus (HCV) – a protein crucial for viral replication and capsid assembly – contains two intrinsically disordered domains. Here we present our results on the NMR characterization of structure and dynamics in the entire intrinsically disordered region of the protein using BEST-TROSY-type experiments optimized for IDPs.

Our NMR study revealed the presence of significant amount of residual secondary structure, that could be important for recognition of its binding partners. We show that long-range interactions in this NS5A contruct are dominated by electrostatic interactions. These interactions are perturbed by phosphorylation of NS5A with CK2, a biologically relevant kinase of NS5A during HCV infection. These long range features of the conformational ensemble are also modulated by interaction of NS5A with one of its host interaction partners, the SH3 domain of Bin1 tumor suppressor protein. This interaction may play a role in development of hepatocellular carcinoma in HCV-infected cells. Understanding of the structural implications of this interaction at atomic resolution may contribute to a better understading of the malignant transformations during HCV infection.

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P 36 Shyam SUNDER MALL

Conformational flexibility of transthyretin leads to amyloid fibril formation through non-native intermediate state population

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ABSTRACT

Transthyretin (TTR) is a thyroxine transporter protein. It is involved in several kinds of polyneuropathies and cardiomyopathy, resulting from protein aggregation. Crystallographic studies for wild type TTR (WTTR) and several mutants suggest that the tertiary structures of most of the TTR variants are similar. 1 In spite of the similar structures, differences in the aggregation propensities suggest an involvement of protein dynamics in fibril formation.

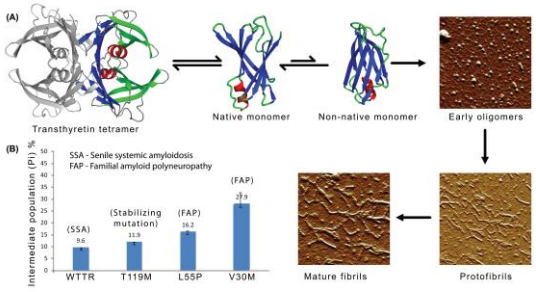

Figure 1 (A) Schematic representation of TTR dissociation, destabilization and fibril formation. (B) Population of non-native Intermediates for different mutants as obtained by NMR.

To understand the relation of conformational flexibility of TTR with aggregation behavior, we probed its backbone dynamics (from picosecond to millisecond time scales), using solution NMR spectroscopy. Three pathogenic mutants (L55P, V30M and V122I) and a trans-suppressor mutant T119M, known to rescue V30M from amyloidosis, along with WTTR were chosen for this study. Mutations were made by site directed mutagenesis using WTTR plasmid as template. The protein expression and purification was optimized from previously described method. 2 The final yield of uniformly labeled 2H, 13C and 15N protein was 40 mg/L in minimal media culture. In addition, the concentration, pH and temperature were optimized for long term stability for all the protein samples, before relaxation studies using NMR spectroscopy. For all the NMR experiments ~99% uniformly 2H, 13C and 15N labeled protein samples with concentration between 0.5 to 3.0 mM were used since lower 2H incorporation caused significant line broadening. The overall dynamics and conformational flexibility obtained from NMR experiments suggests the presence of non-native intermediate state population between ~10% (WTTR) to ~28% (V30M). Formation of these intermediates through conformational changes in native structure was found energetically more favorable in pathogenic mutants, compared to WTTR. 3 These non-native entities may be the initiation point in transthyretin amyloidosis. In addition we have optimized fibril forming conditions that will be used to obtain the core of amyloid fibrils by 1H/2H exchange using NMR.

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P 37 Sabu VARGHESE

Structural and dynamical characterisation of immobilised enzymes using solid-state NMR

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ABSTRACT

Enzymes are macromolecular biological catalysts, responsible for regulating the rates of chemical reactions without themselves being altered in the chemical reactions. The term immobilised enzyme refers to an enzyme physically confined or localized in a certain defined region of space with retention of its catalytic activities and which can be used repeatedly and continuously for industrial applications.[1] Although immobilised enzymes have been extensively characterized by various biochemical methods, rational design of the catalytic system still remains a considerable challenge as very little is known about the state of the protein upon immobilisation. Very recently, solid-state NMR has been successfully employed to characterise an immobilised enzyme consisting of a model enzyme α-Chymotrypsin, mesoporous silica as the matrix, and (3-glycidyloxypropyl) trimethoxysilane (GOPS) as the covalent linker.[2] However, the state of the enzyme and its dynamics upon immobilisation are not well understood. The relatively large size of the Chymotrypsin (25 kDa) and the difficulty of isotopically labelling the protein in its active state make it a daunting task to gain any structural and dynamical information using solid-state NMR.

To gain atomic-level information about the structure and dynamics of immobilized enzymes, a relatively small enzyme Ribonuclease A (RNase A) was chosen as a model system and the characterisation of the isotopically labelled (^{13}C,^{15}N) protein using solid-state NMR is envisaged as a future work. RNase is a relatively low molecular weight (~13.7 kDa, 124 residues) enzyme suitable for catalysing the cleavage of single-stranded RNA and the structure of the enzyme has been solved by NMR[3] and X-ray crystallography.[4] Prior to the study of the isotopically labelled (^{13}C,^{15}N) immobilised enzyme, we have successfully carried out the immobilisation of RNase A in the unlabelled state on epoxy functionalised silica. The silica, linker and the enzyme were then characterised by ^{1}H, ^{13}C, ^{29}Si and ^{15}N in natural abundance using solid-state NMR. Here we report our preliminary results for RNase A immobilized on silica support (100 Å pore size) using GOPS as the covalent linker. Our results are found to be in very good agreement with similar, previously reported studies.[2]

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P 38 Sergio GIL-CABALLERO

HNCA+, HNCO+ and HNCACB+ experiments: Improved performance by simultaneous detection of orthogonal coherence transfer pathways

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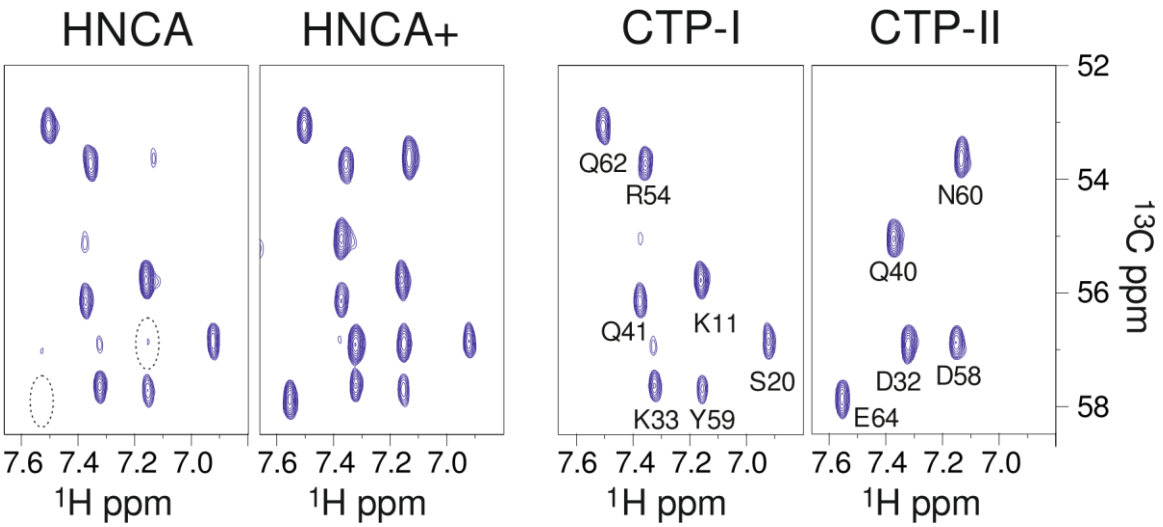
ABSTRACT

Sequential resonance assignment of ^{13}C, ^{15}N labelled proteins is usually achieved by a combination of 3D HNC-type correlation experiments. Three experiments, BEST-TROSY HNCA+, HNCO+ and HNCACB+ are presented for sequential backbone resonance assignment of ^{13}C, ^{15}N labelled proteins. The novelty of these experiments with respect to conventional pulse sequences is the detection of additional orthogonal coherence transfer pathways that results in enhanced sensitivity for sequential correlations without much compromising the intensity of intra-residue correlation peaks.

In addition, a 2-step phase cycle allows separating orthogonal coherence transfer pathways for the discrimination of sequential and intra-residue peaks, similar to so-called time-shared techniques were 2 complementary correlation spectra are acquired simultaneously by the same pulse sequence.



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Pico- to nanosecond motion in protein sidechains explored with a combination of high-field NMR and high-resolution relaxometry

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ABSTRACT

Understanding protein function requires the characterization of both structure and dynamics at atomic resolution. NMR relaxation is well-known to be an efficient technique to obtain quantitative information about protein dynamics. Here, we use a combination of high-field relaxation measurements and high-resolution relaxometry to probe pico- and nanosecond motions in the protein ubiquitin.

Over the past two decades, methyl groups have been shown to be a valuable probe of protein sidechain dynamics through the measurement of nuclear spin relaxation parameters. Deuterium, proton and carbon-13 relaxation rates can be used to probe the spectral density function describing the motions of the methyl group.

The full characterization of the spectral density function is usually prevented by the need for the resolution and sensitivity provided by high magnetic fields. Combining the advantages of relaxometry and high-field NMR on proteins is possible with the use of a sample shuttling apparatus that allows for relaxation to take place at low field while polarization and detection are carried out at low high field. In collaboration with Bruker Biospin, we are developing a shuttle device to measure relaxation at low magnetic fields on a 600 MHz spectrometer.

The selective labelling of isoleucine {13C2H21H} in an otherwise fully deuterated protein should provide simpler relaxation in the 13C1H pair that is almost isolated than observed in a fully protonated methyl group. We have first carried out a theoretical study of all the relaxation pathways as a function of the magnetic field. These calculations have been performed using Mathematica software with I. Kuprov formalism and take into account the theoretical 839808 relaxation pathways.

We have measured both high-field and low-field relaxation rates in the protein Ubiquitin uniformly deuterated, nitrogen-15 labelled and selectively labelled on Isoleucine methyl groups {13C2H21H} 15N relaxation rates were used to characterize the overall diffusion tensor, while 13C relaxation rates were used to quantify local motions.

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