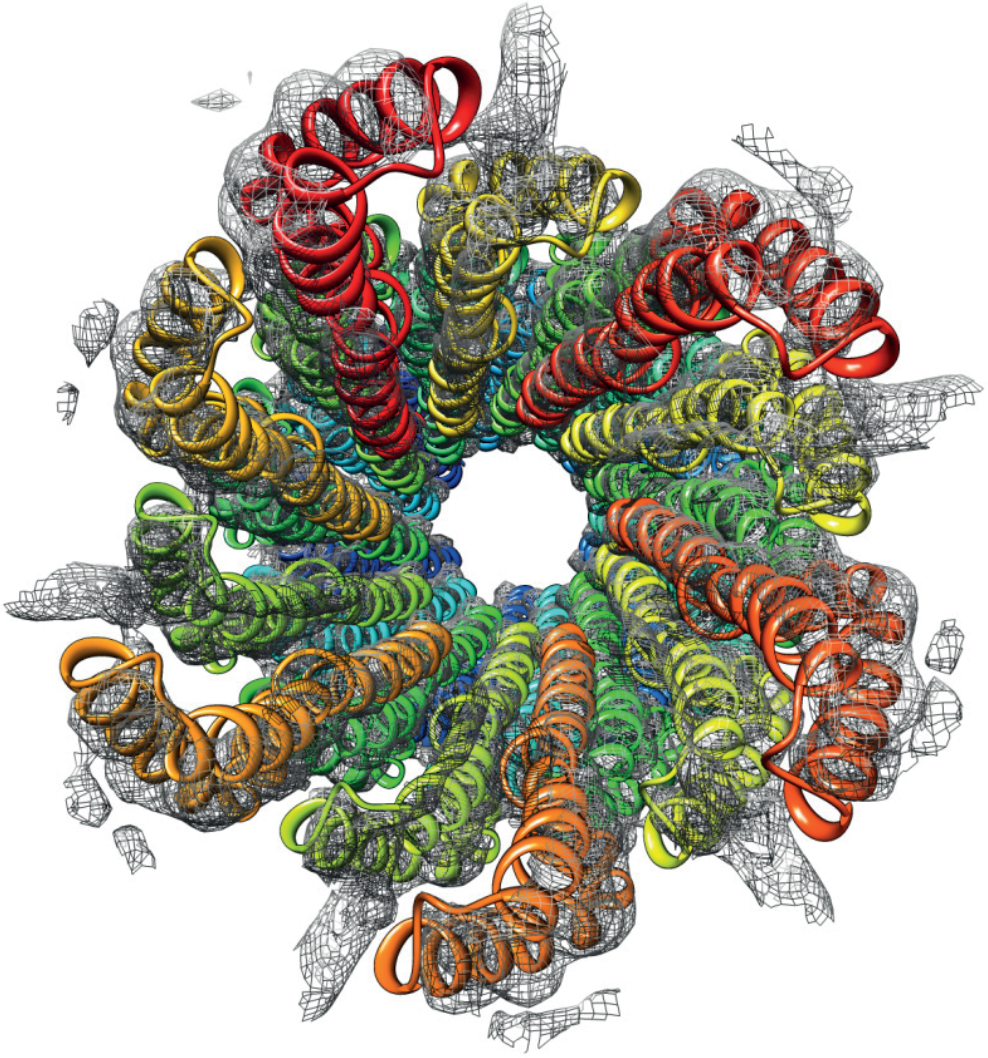
ROYAL SOCIETY OF CHEMISTRY

NMR DG

UK Nuclear Magnetic Resonance Discussion Group

Royal Society of Chemistry NMR Discussion Group

NMR in Structural Biology



Cambridge, April 10 – 11 2014

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Local arrangements

Emergencies

The conference organisers can be contacted on 07590 503753 throughout the meeting.

Registration

The registration desk will be in the foyer outside Bristol Meyers Squibb lecture theatre and will be manned from 10:00 on Thursday until the start of the scientific sessions, and thereafter during tea and coffee breaks.

Scientific sessions

The lectures will take place in the Bristol Meyers Squibb lecture theatre. Please sit in the front section. The poster session will take place in in the Wolfson teaching Laboratory.

Posters

Please mount your posters as soon as possible on Thursday. Velcro pads will be provided.

Tea and coffee breaks

Refreshments will be served in the Wolfson Teaching Laboratories

Exhibitors

Exhibitors will have their displays in the Wolfson Teaching Laboratories. Please make a point of visiting them.

Web access

There is a wireless network in the lecture theatre and the teaching laboratories. If you have EDUROAM you will be able to connect straightforwardly, otherwise you will need a temporary *Lapwing ticket*, available from the registration desk on request.

Meals at Downing College (only available if pre-booked)

Dinner on Thursday evening is a cafeteria service between 19:30 and 20:30. Lunch on Friday is a sit-down meal at 12:15. *Please move promptly over to Downing so that lunch can be served in good time*. You will be directed to the Paddock Gate which gives direct access to Downing from just opposite the Department on Lensfield Rd. This gate will be manned at the start of the dinner break, and throughout the lunch break. It is locked at other times; access is then via the Porters' Lodge on Regent St.

Eating and drinking

There is an outlet serving coffees and sandwiches in the foyer outside Bristol Meyers Squibb lecture theatre. There are several sandwich bars and cafes along Hills Rd, and numerous restaurants along Regent St, Hills Rd and in the central area. A range of more 'ethnic' restaurants are to be found along Mill Rd. There are pubs on Panton St, Lensfield Rd, Hills Rd and Regent St.

NMR Discussion Group Spring Meeting. Cambridge 10th and 11th April 2014

Wednesday 9th April 2014

14:00 Check in at Downing College and St Catharine's College from 14:00 (Porters' Lodge)

Thursday 10th April 2014

08:00 09:00 Breakfast for those staying at Downing or St Catharine's
10:00 13:15 Registration at Chemistry from 10:00 (bag drop available at Chemistry)
14:00 Check in at Downing College and St Catharine's College from 14:00 (Porters' Lodge)

Scientific session 1: Bristol Meyers Squibb Lecture Theatre, Department of Chemistry

13:25	13:30	**Welcome**	
13:30	13:35	**Jennifer Potts**	*Iain Campbell remembered*
13:35	14:15	**Gerhard Wagner**	*NMR structural studies of miochondrial membrane proteins*
14:20	14:50	**Adam Lange**	*3D structures of bacterial supramolecular assemblies by solid-state NMR*
14:55	15:25	**Claudio Dalvit**	*Recent developments in ^{19}F NMR spectroscopy for fragment screening and drug design*
15:30	16:00	Tea (Wolfson teaching laboratory)	
16:00	16:20	**Roberto Buratto**	*Beyond the millimolar range: measuring ultra-weak ligand-protein affinities using NMR of long-lived states*
16:20	16:40	**Wing Ying Chow**	*Applying a solid-state NMR approach to probe atomic changes in collagen matrices in health and disease*
16:40	17:10	**Jennifer Potts**	*Molecular interactions in staphylococcal infections*
17:15	17:45	**Thomas Prisner**	*Advances in high field DNP and EPR*
17:50	19:30	Wine reception and posters (Wolfson teaching laboratory)	
19:30	20:30	Cafeteria Dinner at Downing College (if booked)	

Friday 11th April 2014

08:00 09:00 Breakfast for those staying at Downing or St Catharine's
09:30 Checkout: r**ooms MUST be vacated by 09:30** (bag drop available at Chemistry)

Scientific session 2: Bristol Meyers Squibb Lecture Lecture Theatre, Department of Chemistry

09:00	09:30	**Sjors Scheres**	*Recent advances in high-resolution cryo-EM structure determination*
09:35	10:05	**Torsten Herrmann**	*Turning failure into success: New perspectives for unsupervised NMR studies of proteins*
10:10	10:30	**Justin Lecher**	*Analysis of the ion channel gating mechanism in solution by NMR spectroscopy*
10:30	11:00	Coffee (Wolfson teaching laboratory)	
11:00	11:30	**Elisabetta Chiarparin**	*Fragment based drug discovery: challenges and opportunities*
11:35	11:55	**Hiroki Takahashi**	*Dynamic nuclear polarisation enhanced solid-state NMR spectroscopy and magnetic resonance force microscopy for structural biology*
11:55	12:15	**Peter Schmidt**	*The Structure of Neuropeptide Y bound to its G protein-coupled Y2 receptor*
12:15	13:45	Lunch at Downing College	- this is a served meal so please move swiftly over to Downing
13:45	14:25	**Ad Bax**	*Probing motions and structural rearrangements by RDCs*
14:30	15:00	**Mario Schubert**	*Insights into protein–carbohydrate recognition by NMR spectroscopy*
15:05	15:25	**Christopher Waudby**	*Mapping the co-translational folding energy landscape of the ddFln5 immunoglobulin domain*
15:25	15:55	**Bernd Reif**	*Amyloid Aggregates and Large Soluble Protein Complexes*
16:00		Tea & Close (Wolfson teaching laboratory)	

Organising Committee

James Keeler (Department of Chemistry), David Neuhaus (MRC LMB), and Daniel Nietlispach (Department of Biochemistry).

Acknowledgements

Special thanks are due to **Bethan Lewis** (Department of Chemistry) who has borne the brunt of the administrative and organisational work for the meeting. It simply would not have been possible to arrange the meeting without her, and we are extremely grateful for all she has done.

The organising committee would like to express their sincere thanks to, and appreciation of, the following people who have all been crucial in the organisation of this meeting.

Accounting and financial support

Jaci Agarwala and Tanya Radic (Department of Chemistry); Dr John Parkinson (NMR DG)

Stewarding

Mona Bassuni, Sandra Berndt, Dr Mark Bostock, Duncan Crick, Luisa Moretto, Dr Helen Mott, Lira Puebla, Steven Vance and Rowina Westermeier (Department of Biochemistry)

Harriet Crawley-Snowdon, Laura Easton, Katy Hedgethorne and Wilfred Wu (MRC LMB)

Technical services

Mykola Karabyn (lecture theatre); Christopher Chalk and David Pratt (wireless network)

Teaching Laboratory

Sylvia Gonzalez Calera, Mark Hudson and Ivan Williams

Reprographics

Gabriella Bocchetti

Meals and accommodation

Jacqui Cressey (Downing College), Alicja Duma (St Catharine's College), John Lucas-Phillips (tea and coffee breaks)

Abstracts of invited lectures and promoted posters

NMR Studies of Integral Membrane Proteins

Gerhard Wagner, Manuel Etzkorn, Thomas Raschle, Franz Hagn
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston MA

Structural studies of membrane proteins are still hampered by problems in finding appropriate membrane mimetics that maintain structure and function of the embedded systems. While most studies of membrane proteins use detergent micelles as membrane mimetics these may have destabilizing effects for extra-membrane moieties of receptors. More seriously, detergent micelles may be unsuitable for interaction studies of integral membrane proteins with soluble binding partners. To address this problem we have investigated the use of amphipoles and phospholipid nanodiscs and studied both β-barrel and helical membrane proteins. This included the voltage-dependent anion channel VDAC, OmpX, a helical inner mitochondrial membrane protein and a GPCR/G-protein system.

To enhance performance of NMR on these large systems we optimized methods for NMR data acquisition and processing. A selection of these methods will be presented.

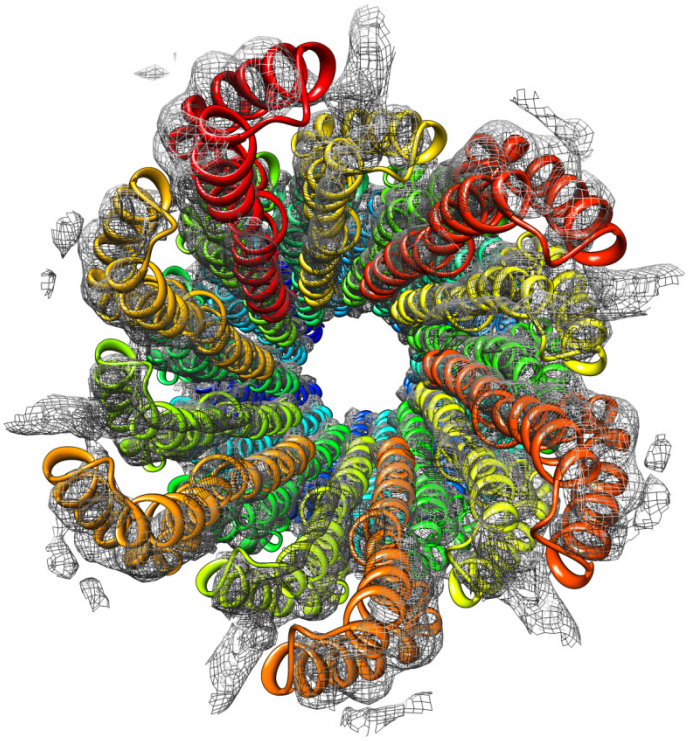
Type III secretion needles studied by solid-state NMR

Jean-Philippe Demers[1], Antoine Loquet[1], Veniamin Chevelkov[1], Birgit Habenstein[1], Pascal Fricke[1], Chaowei Shi[1], Suresh Vasa[1], Karin Giller[1], Stefan Becker[1], **Adam Lange**[1]

[1]Department of NMR-based Structural Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany

EMAIL alange1@gwdg.de

Gram-negative bacteria use a molecular machine called the type three secretion system (T3SS) to deliver effector proteins to host cells. Our research group has recently solved an atomic model of the extracellular T3SS needle of *Salmonella typhimurium* (Loquet et al., *Nature*, 2012). Concurrently, a high-resolution cryo-electron microscopy density map of the T3SS needle of *Shigella flexneri* was obtained by Fujii et al. (*PNAS*, 2012). Modeling of the *Shigella* needle subunit protein to fit the EM density produced a model incompatible with the atomic model of the *Salmonella* needle in terms of secondary structure and subunit orientation. We then determined directly the secondary structure of the *Shigella* needle subunit using solid-state NMR, and its orientation using *in vitro* and *in vivo* immunogold labeling in functional needles. We found that *Shigella* subunits adopt the same secondary structure and orientation as in the atomic model of *Salmonella*, and we generated a homology model of the *Shigella* needle consistent with the EM density (Demers et al., *PLOS Pathogens*, 2013). Here, we will discuss our recent efforts in obtaining higher resolution structures of *Shigella* and *Salmonella* needles. We will also present proton-detected solid-state NMR experiments on perdeuterated T3SS needles. A set of five 3D correlation experiments allowed for the unambiguous assignment of the backbone resonances. Finally, we will show first results of DNP experiments on uniformly ^{13}C-labeled needles.



Recent developments in ^{19}F NMR spectroscopy for fragment screening and drug design

Claudio Dalvit*and Anna Vulpetti†

*Faculty of Science, University of Neuchatel, Avenue de Bellevaux 51, Neuchatel, 2000, Switzerland

†Global Discovery Chemistry, Novartis Institutes for Biomedical Research, Basel, 4002, Switzerland

EMAIL claudio.dalvit@unine.ch

Over the last few years, fluorine NMR spectroscopy has gained significant attention in the pharmaceutical industry and academic institutions as an efficient and reliable method for performing functional and binding assays.[1] The high relative sensitivity of the binding experiments to protein interactions results in a large dynamic range, thus allowing the detection of very weak-affinity chemical fragments. This is due to two physical mechanisms that simultaneously contribute to the line width of the observed ^{19}F NMR signals: the ^{19}F chemical shift anisotropy (CSA) in the bound state and the exchange originating from the ^{19}F difference in chemical shift between free and bound state. In the binding assay a library of fluorinated molecules is first screened, and the identified binders are then used as *spy molecules* for subsequent screening experiments and for measuring the binding constant of the molecules interacting with the receptor. Different types of fluorinated libraries can be generated by using different selection criteria.[2] The choice of fluorinated motifs present in the library is fundamental in order to ensure a large coverage of chemical space and local environment of fluorine (*LEF*).[3] Complex mixtures of highly diverse fluorine motifs can be rapidly screened using optimized pulse sequences and deconvoluted in the same NMR tube with a novel combined procedure for the identification of the active molecule(s).[4] This allows for high throughput and fast data analysis.

The *LEF* determines the ^{19}F NMR chemical shift and the interactions of fluorine with the receptor. A correlation between the ^{19}F NMR isotropic chemical shifts and the type of close intermolecular contacts to the fluorine atoms was derived. Based on all these findings the *rule of shielding* was proposed for providing some insight into and guidelines for the judicious selection of appropriate fluorinated moieties to be inserted into a molecule for making the most favorable interactions with the receptor.[5] Novel chemical scaffolds, based on the *rule of shielding*, have been designed for recognizing distinct structural motifs present in proteins.

References

[1] C. Dalvit, *Progress in Nuclear Magnetic Resonance Spectroscopy* **2007**, *51*, 243-271.

[2] A. Vulpetti, C. Dalvit, *Drug Discov Today* **2012**, *17*, 890-897.

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[4] a) C. Dalvit, A. Vulpetti, *Magnetic Resonance in Chemistry* **2012**, *50*, 592-597; b) A. Vulpetti, C. Dalvit, *ChemMedChem* **2013**, *8*, 2057-2069.

[5] C. Dalvit, A. Vulpetti, *ChemMedChem* **2011**, *6*, 104-114.

Beyond the millimolar range: measuring ultra-weak ligand-protein affinities using NMR of Long-Lived States

R. Buratto §, D. Mammoli §, E. Chiarparin *, Glyn Williams *, and G. Bodenhausen §¶

§ Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland.
¶ Ecole Normale Supérieure, 24 Rue Lhomond, 75231 Paris Cedex 05, France.
* Astex Pharmaceuticals Ltd, 436 Cambridge Science Park, Cambridge, CB4 0QA, UK

Recently, a new powerful NMR technique (1), based on Long-Lived States (LLS) (2), has emerged that can investigate the strength of protein-ligand interactions over an exceptionally wide range of affinities.

$[L]_{tot}/[P]_{tot}$	Contrast (%)	X^{bound} (%)
56	72	1
125	63	0.6
202	54	0.4
272	45	0.3
366	41	0.25
548	29	0.17
707	23	0.13

Contrast and molar fractions of bound ligand, for different ligand/protein ratios.

Provided that the exchange is fast compared to the difference in their resonance frequencies, the relaxation rate is a weighted sum of the two contributions:

$$R_{LLS}^{obs} = X^{bound} R_{LLS}^{bound} + X^{free} R_{LLS}^{free}$$

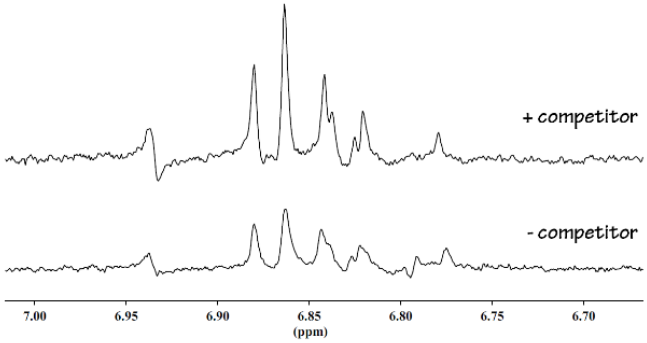
where X^{bound} and X^{free} are the molar fractions of the bound and free forms of the weak ligand.

Since $R_{LLS}^{bound} \gg R_1^{bound}$ and $R_{LLS}^{free} \ll R_1^{free}$, the contrast between R_{LLS}^{free} and R_{LLS}^{bound} can be much larger than for R_1 or $R_{1\rho}$, boosting the sensitivity of the technique.

These virtues make it an attractive fragment screening method, where the binding of molecular fragments to a target protein must be detected and their weak dissociation constants quantified.

In this work, we have studied the interaction between the heat shock protein 90 (Hsp90) and a variety of ligands (3).

In the first step, we have identified a spy molecule (vanillic acid diethylamide) that is suitable in competition experiments (4): we proved that a favorable contrast could be achieved between free and bound ligand, even at ligand/protein ratios greater than 700.



Competition screening experiments by using LLS of the spy molecule in the absence (bottom) or in the presence of a weak competitor (k_D=2.3±0.3 mM) .

By observing changes in the signal of the spy molecule during competition experiments we could efficiently detect and measure the dissociation constants of three weak fragments: we show that our approach allows accurate measurement of K_D's ranging from relatively strong (8±3 μM for ADP) to very weak, but specific, binders (K_D=24±5 mM for 2-amino-pyrimidine).

This method extends the dynamic range of ^{1}H NMR experiments for screening and determination of dissociation constants K_D beyond the current low millimolar range, using small concentrations of unlabeled proteins.

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Applying a Solid-state NMR Approach to Probe Atomic Changes in Collagen Matrices in Health and Disease

W Ying Chow*, David G Reid*, Wai Ching Wong*, Rakesh Rajan†, Roger A Brooks†, Richard W Farndale‡, Dominique Bihan‡, Melinda J Duer*

*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK

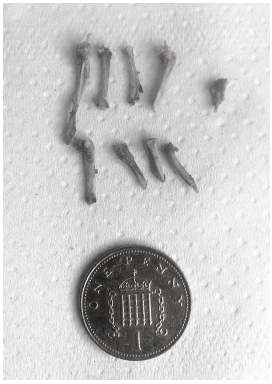
†Orthopaedic Research Unit, Addenbrooke's Hospital, Cambridge , CB2 0QQ, UK

‡Department of Biochemistry, University of Cambridge, Building O, Downing Site, Cambridge CB2 1QW, UK

EMAIL wyc25@cam.ac.uk

Collagen proteins form the bulk of many structural tissues, such as skin, tendons, cartilage, the wall of arteries, and bone. In structural tissues, the collagen proteins form large, heterogeneous and insoluble matrices which house and interact with the cells, while contributing to mechanical integrity and function. Recent developments in solid-state NMR (ssNMR) provide a means of elucidating atomic structures of insoluble proteins and macromolecules. Therefore, instead of working on isolated and purified proteins, our study tackled tissue samples that contain a high proportion of collagen, such as bone, or extracellular matrix (ECM) generated in vitro.

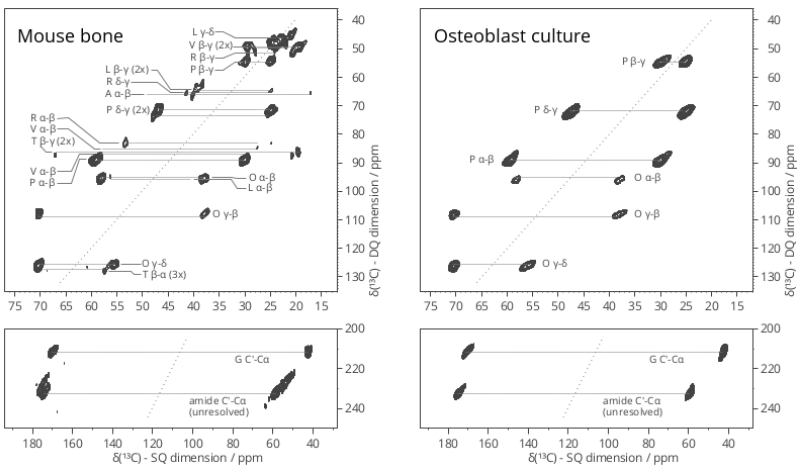
By feeding a mouse on an isotopically-enriched diet, we obtained ^{13}C and ^{15}N-enriched bone tissues that facilitated 2D ssNMR experiments. From the increased resolution of these 2D spectra, we can make assignments to many more residue types for collagen proteins in native tissue than was possible prior to this study. These assignments serve as parameters against which we can compare synthetic attempts at generating collagen matrices.



We can compare the ssNMR spectrum of synthetic collagen-like peptides directly to bone. From our spectra, it is clear that the model peptides only mimic a subset of structural variations that exist along the collagen triple helix in bone. Using these peptides, for which atomic structures are already available, we can identify regions which correspond to proline-rich and likely more ordered regions in bone collagen.

Mammalian osteoblast cell cultures which lay down collagenous ECM enable us to probe a biologically-generated source of collagen proteins with more targeted isotopic-enrichment schemes. After fine-tuning the cell culture conditions so that we can generate isotopically-enriched ECM that yield ssNMR spectra that closely resemble bone, we can go on to test different models for disease. In aging and diabetes, collagen proteins are known to undergo irreversible changes, such as increased crosslinking and change in material properties. By isotopically enriching the sugar component of the cell culture samples, we can probe the atomic structures formed in both healthy glycosylation and unhealthy glycation of collagen proteins. From these spectra, we identify signals which correspond to sugar phosphates, which may act as a facilitator in bone tissue mineralisation, or an aggressive glycating species if produced in other tissues.

In conclusion, we now have a method of validating atomic-level structure of collagen matrices, which can be used by tissue engineers looking to improve their collagen-based implants. We can also begin to elucidate the atomic-level mechanisms by which age-related diseases and processes affect the structure and function of collagen proteins in our tissues.



Repetitive and disordered proteins in Gram-positive infections

Dominika Gruszka*, Fiona Whelan, Gemma Harris, Kate Atkin, Andrew Brentnall and **Jennifer Potts**

Department of Biology, University of York, Wentworth Way, York, YO10 5DD

*Present address: Dept of Chemistry, University of Cambridge, Cambridge, CB2 1EW

EMAIL Jennifer.potts@york.ac.uk

Staphylococci and streptococci cause human infections associated with significant morbidity and mortality. Our lab has studied repetitive and/or disordered bacterial proteins that are attached to the bacterial cell wall and facilitate host cell invasion and/or formation of medical-device associated biofilm infections. These proteins have posed particular challenges that have been addressed using NMR spectroscopy, isotope-labelling strategies, X-ray crystallography and a range of biophysical techniques. Our studies have revealed novel mechanisms of protein-protein recognition and novel protein structures (Fig. 1).

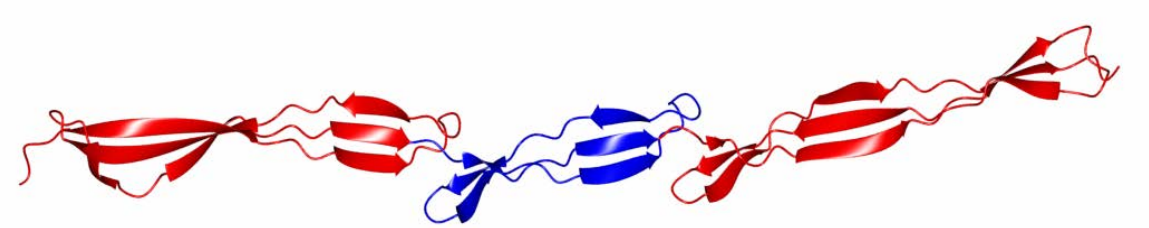


Fig. 1 Structure of $G5^1$-E-$G5^2$ from SasG, an *S. aureus* surface protein involved in formation of biofilms. Gruszka et al. *Proc. Natl. Acad. Sci. (USA)* 109, E1011-1018 (2012)

Advances in High-Field DNP and EPR

Vasyl Denysenkov, Petr Neugebauer, Jan Krummenacker, Dmitry Akhmetzyanov and **Thomas Prisner**

Institute of Physical and Theoretical Chemistry and Center of Biomolecular Magnetic Resoanance, Goethe University Frankfurt

Prisner@Chemie.Uni-Frankfurt.de

In recent years several hyperpolarization methods boosting NMR sensitivity attained much attention. Dynamic nuclear polarization (DNP) is one of these methods, transferring the much higher Zeeman polarization of unpaired electron spins onto nuclear spin systems by resonant microwave excitation of paramagnetic DNP agents. This method, already discovered in the 1950's, attracted new attention after it has been demonstrated that significantly enhanced NMR signals could also be achieved at high magnetic fields as used nowadays for most NMR applications[1]. Encouraged of this work performed on solid state samples we started to investigate the potential of DNP on liquid state samples at high magnetic fields[2]. Unexpected large polarization transfer from organic radicals to solvent protons could also be detected in liquids[3], which can be rationalized by fast local dynamics between the DNP agent (radical) and target (solvent) molecule[4]. Experimental requirements, polarization transfer mechanism and potential applications of this method will be illustrated and discussed.

Pulsed EPR methods allowing to measure distances in the 1-8 nm range with very high precision become more and more popular to obtain long range restraints in macromolecular biological systems[5]. So far mostly nitroxide spin labels covalently attached to the biomolecule are used as paramagnetic spin probes. Here we will show the performance of the PELDOR (Pulsed Electron Electron Double Resonance) experiment on a manganese-nitroxide model compound, demonstrating that at high magnetic fields such experiments can be successful performed and quantitatively interpreted. This is especially interesting for biological applications, where naturally occurring magnesium ions can be replaced by manganese.

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Recent advances in high-resolution cryo-EM structure determination

Sjors HW Scheres*

*MRC Laboratory of Molecular Biology, Cambridge Biomedical Campus, CB2 0QH, Cambridge, UK

EMAIL scheres@mrc-lmb.cam.ac.uk

Radiation damage is the main limiting factor in imaging biologically relevant macro-molecular complexes with electrons. Still, it has been estimated that 3D reconstructions should be possible to near-atomic resolution, i.e. ~3 Å, by combining projection images of a few thousand particles with a molecular mass of ~100 kDa [1]. In practice, resolutions obtained have been much lower, even when combining more and/or larger particles. However, this field is undergoing rapid changes, and near-atomic resolution reconstructions have now been reported for data sets comprising only tens of thousands of particles [2,3]. I will discuss two advances that underlie this sudden progress: direct-electron detectors; and statistical refinement algorithms.

Newly developed direct-electron detectors have a significantly improved signal-to-noise performance compared to conventional detection media like photographic film and charged-coupled device (CCD) detectors. The improved signal-to-noise performance allows for better reconstructions to be calculated from smaller amounts of particles. Moreover, these detectors can be read at high speeds, with frame rates in the range of 17-400 s-1. As frozen macro-molecular complexes typically move upon irradiation with electrons, recording movies while the sample is being imaged allows correcting for beam-induced movements, which further improves resolution [2,3].

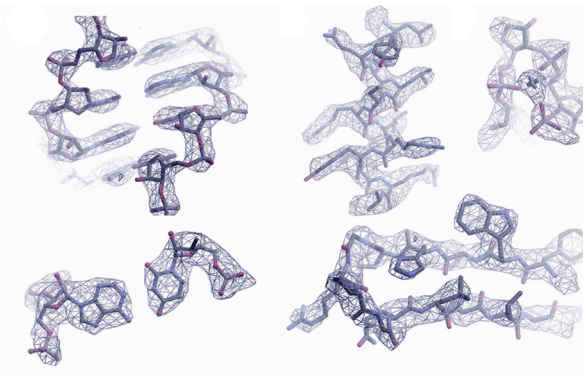

Figure 1. *Reconstructed cryo-EM density for parts of the large subunit of the yeast mitoribosome.*

The development of a regularised likelihood optimisation algorithm for single-particle reconstruction [4], in combination with a strict prevention of overfitting [5], has further improved the quality of cryo-EM density maps. Optimisation of a regularised likelihood target provides an elegant derivation of the optimal (i.e. Wiener) filter for the reconstruction task. The resulting algorithm infers many parameters from the data, so that expert user decisions may largely be avoided. Moreover, for macro-molecular complexes that adopt multiple three-dimensional structural states, in silico classification may be used to identify structurally homogeneous subsets of the data.

As an illustration of the combined power of the new detectors and the statistical image processing approach, I will discuss the structure determination of the large subunit from the yeast mitochondrial ribosome, or mitoribosome. Using less than fifty thousand individual particles, we obtained a 3.2 Å resolution density map (Fig. 1). This map allowed us to propose a nearly complete atomic model, for which approximately 600 kDa of protein and RNA molecules was built de novo. This work was funded by the Medical Research Council under grant MC_UP_A025_1013.

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Turning failure into success: New perspectives for unsupervised NMR studies of proteins

Torsten Herrmann

Institut des Sciences Analytiques, Centre de RMN à très Hauts Champs, Université de Lyon, UMR 5280 CNRS, ENS Lyon, UCB Lyon 1, 5 rue de la Doua, France

EMAIL torsten.herrmann@ens-lyon.fr

In the first part of the presentation, the J-UNIO protocol for structure determination of small globular proteins by solution NMR will be introduced. The J-UNIO protocol requires only a minimal set of NMR spectra and allows efficient interactive validation of intermediate and final results. Application of the J-UNIO protocol to more than 40 de novo protein structures with sizes up to 180 residues shows that the procedure is highly robust and efficient, and well road-tested.

In the second part, new concepts for homology modeling–driven NMR structure determination of complex biological systems will be described. We will show how database knowledge can be effectively incorporated into backbone, side-chain and NOE assignment in order to enable structural and dynamical studies of large biological systems. Applications of homology-driven NMR structure determination to proton-detected solid-state NMR studies will conclude the presentation.

Analysis of the Ion Channel Gating Mechanism in Solution by Nuclear Magnetic Resonance (NMR) Spectroscopy

Claudia Börger*,†, **Justin Lecher***,†, Sven Schünke†, Matthias Stoldt*,†, Dieter Willbold*,†

*Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany;

†Institute of Complex Systems, Structural Biochemistry (ICS-6), Research Center Jülich, Jülich, Germany.

EMAIL j.lecher@fz-juelich.de

Ion channels, which can be activated by binding of cyclic nucleotides, play a crucial role in the regulation of the excitation of cardiac muscle cells, and they are of importance in signal transduction of olfactory and visual neurons. All of these channels belong to the class of voltage-gated cation channels, which can be sub-classified in cyclic nucleotide-gated ion channels (CNG) and hyperpolarization-activated and cyclic nucleotide-gated ion channels (HCN) (Schünke and Stoldt 2013).

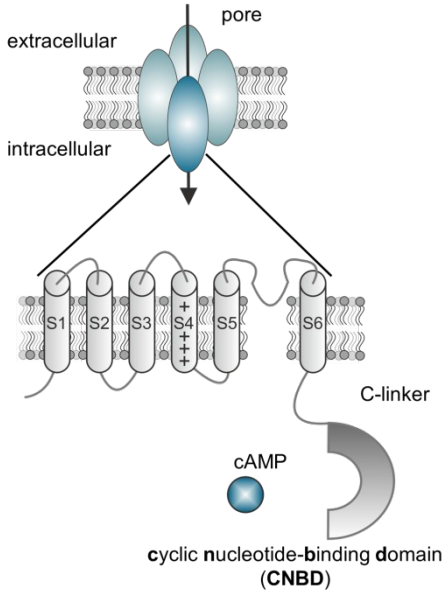

Figure 1: Subunit topology of cyclic nucleotide-activated ion channels. HCN and CNG channels consist of four subunits. Each subunit contains a six trans-membrane segment (S1-S6), an intra-cellular cyclic nucleotide-binding domain and a connecting C-linker.

For CNG channels the binding of cyclic nucleotides is important for the opening of the membrane pore whereas they are only weakly dependent on membrane depolarization. Cyclic nucleotides, bound to the cyclic nucleotide-binding domain of HCN channels, solely modulate the opening behaviour. In this case, the voltage, necessary for the pore opening, is shifted to more positive values (Schünke and Stoldt 2013).

Upon binding of the cyclic nucleotide to the CNBD, the signal is transferred to the membrane domain, in order to open the pore or to change the opening behaviour. To analyse the underlying gating mechanism three-dimensional structures at atomic resolution are indispensable.

Up to now, several crystal structures of eukaryotic HCN CNBDs are available. However, comparison of cyclic nucleotide free and bound structures did not reveal significant differences (Zagotta et al. 2003 and Taraska et al. 2009). In contrast, structures of the CNBD from the prokaryotic organism Mesorhizobium loti, solved by liquid state NMR spectroscopy, did show substantial rearrangements upon binding of a cyclic nucleotide (Schünke et al. 2009 and Schünke et al. 2011).

Here, we show the first structural analysis of an eukaryotic CNBD using liquid state NMR spectroscopy. We could demonstrate that binding of cyclic nucleotides to the CNBD results in significant conformational changes yielding new insights into the ion channel gating mechanism.

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Fragment Based Drug Discovery: Challenges and Opportunities

Elisabetta Chiarparin and Glyn Williams

Astex Pharmaceuticals
436 Cambridge Science Park, Milton Road, Cambridge, CB4 0QA, UK
EMAIL elisabetta.chiarparin@astx.com

Over the past decade, fragment-based drug discovery (FBDD) has been successfully applied in industry and in academia to discover drug candidates with improved physicochemical properties for a wide variety of targets [1]. At Astex, we now have experience of over 30 high-throughput crystallographic and biophysical fragment screens against a broad range of protein classes, and our results in terms of hit rates, hit solubility and affinity contributed to the design of the third generation of Ro3 fragment library. Here we recount our experiences of fragment-based drug discovery with challenging protein-protein interaction targets, such as Inhibitors of Apoptosis Protein (IAP) family, key regulators of anti-apoptotic and pro-survival signalling pathways. Emphasis will be given on how at Astex structural biology and biophysical methods, such as X-ray crystallography, NMR spectroscopy and mass spectrometry and isothermal titration calorimetry (ITC) are fully integrated to identify protein constructs that are suitable for screening purposes, and subsequently to screen drug fragments and optimise them into lead and candidate drugs using structure-guided design. Highlights will be given on how NMR spectroscopy is exploited as a physical chemistry, biophysical and structural biology technique at different stages of a drug discovery programme.

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Dynamic nuclear polarisation enhanced solid-state NMR spectroscopy and magnetic resonance force microscopy for structural biology

Hiroki Takahashi*, Daniel Lee†, Jean-Pierre Simorre‡, Michel Bardet†, Sabine Hediger†, Gaël De Paëpe†, Romana Schirhagl*, Brad Moores* and Christian Degen*

*Department of Physics, ETH Zurich, Otto-Stern-Weg 1, Zurich, 8093, Switzerland

†Laboratoire de Chimie Inorganique et Biologique, UMR-E3 (CEA/UJF) and CNRS, INAC, CEA, 17 rue des Martyrs, Grenoble, 38054, France

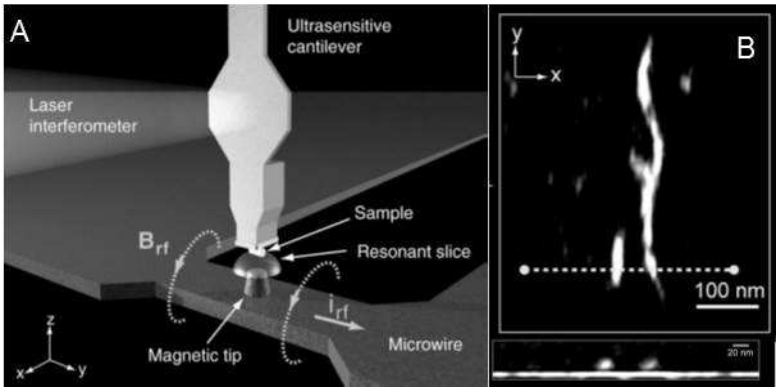
‡Institut de Biologie Structurale, UMR 5075, 6 rue Jules Horowitz, Grenoble, 38027, France

EMAIL: thiroki@phys.ethz.ch

Low sensitivity is always a central concern in the NMR community and a number of techniques have been developed to improve the sensitivity. In particular, high-field magic-angle-spinning dynamic nuclear polarisation (MAS-DNP) has been proven to be a powerful technique to enhance the sensitivity of solid-state NMR in many different types of systems. We have recently developed a new technique to selectively enhance surface signals using MAS-DNP and demonstrated “on-cell” NMR on bacterial cells, which will be a great investigation tool for “on-cell” studies [1].

However, application of MAS-DNP is not always straightforward mainly due to the use of frozen DNP-matrices which uniformly distribute polarising agents around the sample of interest at low temperature. In order to successfully perform MAS-DNP experiments, we have recently demonstrated matrix-free DNP [2, 3]. This utilises a binding affinity of polarising agents, clearly evidenced during the on-cell MAS-DNP studies above. We have obtained very encouraging results where only 20 minutes were sufficient to record natural-abundance 2D ^{13}C–^{13}C correlation experiments on cellulose [2]. Furthermore, intermolecular constraints were obtained on self-assembled peptide nanotubes at natural ^{13}C abundance, which demonstrates the feasibility of supramolecular structure determination of such nano-assemblies without isotopic labelling [4].

Though above technique will allow one to reduce the sample amount dramatically, conventional NMR still requires > 10^{10} molecules. Magnetic resonance force microscopy (MRFM) which utilises force detection instead of traditional inductive detection has been emerging as a promising technique to study structures of single nano-objects (see Figure) [5]. We will present our approach towards structural biology using MRFM, including the use of isotopic labelling for regional image contrast. We will also discuss potential target systems, such as proteins embedded in membranes.



A: Basic principle of MRFM. B: 3D images of virus particles at a spatial resolution of about 5 nm.

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The Structure of Neuropeptide Y bound to its G protein-coupled Y2 receptor

Peter Schmidt*, Anette Kaiser†, Paul Mueller*, Tristan Zellmann†, Holger A. Scheidt*, Mathias Bosse*, Rene Maier†, Jens Meiler#, Annette G. Beck-Sickinger† and Daniel Huster*

*Institute for Medical Physics and Biophysics, Medical Department, Universität Leipzig, Härtelstrasse 16-18, 04107 Leipzig, Germany

†Institute of Biochemistry, Universität Leipzig, Brüderstrasse 34, 04103 Leipzig, Germany

#Center for Structural Biology, Vanderbilt University, 465 21st Ave South, Nashville, TN 37203, USA

EMAIL peter.schmidt@medizin.uni-leipzig.de

In order to influence the numerous pharmacological important signal transduction pathways involving G protein-coupled receptors (GPCRs), agonists or antagonists with high specificity and selectivity have to be developed to avoid adverse reactions. This is accomplished by large scale screening or considerably more efficiently by structure based drug design. The latter requires detailed information about the atomistic structure and dynamics of the natural ligand in its receptor bound state. NMR spectroscopy is an excellent method to study ligand/receptor interaction in their native lipid environment.

Here, we determined the structure and binding sites of the 36 amino acids comprising Neuropeptide Y (NPY) bound to the Y2 receptor. The interaction of NPY with the Y2 receptor plays, among others, an essential role in food intake and in the regulation of the circadian rhythm. Our approach was applying solution as well as solid-state NMR spectroscopy as complimentary methods. While backbone angle information were obtained from 13C-13C correlation MAS NMR spectra, interaction sites could be identified by comparing 1H-15N chemical shifts of NPY in presence and in the absence of the receptor, acquired from HSQC solution NMR spectra.

The required milligram amounts of the Y2 receptor were obtained by recombinant expression in *E.coli* as inclusion bodies and subsequent refolding of the GPCR into lipid membranes. Full functionality of the prepared receptor was shown by ligand binding and G protein activation. Several variants of NPY with four 15N/13C labelled amino acids each for easy signal assignment were produced by solid-phase syntheses.

Using the obtained restraints from NMR measurements, the structure of receptor bound NPY was calculated and modelled into the receptor binding pocket. The result revealed a structural change for the last five C-terminal amino acids of NPY from α-helix to random-coil upon receptor binding. The known C-terminal binding site could be confirmed. Further, a second binding site was identified, that interacts with the second extracellular loop of the Y2 receptor, which was finally proven in cell culture assays.

Probing motions and structural rearrangements by RDCs

Julien Roche, Alexander Grishaev, Jinfa Ying and Ad Bax

Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

The antibiotic squalamine, originally isolated from dogfish shark, forms a lyotropic liquid crystal at very low concentrations in water (0.3-3.5% w/v), which remains stable over a wide range of temperature (1-40 °C), pH (4-8), and pressure (1-2500 bar). Squalamine is positively charged, and comparison of the alignment of ubiquitin in this medium relative to 36 previously reported alignment conditions shows that it falls closest to liquid crystalline cetyl pyridinium bromide. High precision ^{1}H-^{15}N, ^{15}N-$^{13}C'$, and $^{13}C'$-$^{13}C^{\alpha}$ and $^{13}C^{\alpha}$-$^{13}C^{\beta}$ residual dipolar couplings (RDCs) in squalamine medium fit well to the static structural model previously derived from NMR data. Inclusion of the new RDCs into the structure refinement procedure results in improved agreement between alignment-induced changes in $^{13}C'$ chemical shift (RCSA) and experimental values, thereby validating the high quality of the static structural model. Our result indicates that fitting of a single model to experimental data can provide a better description of the time- or ensemble-averaged conformation than do ensemble representations, whereas the latter potentially can capture dynamic aspects of a protein, thus making them valuable complements to one another. An accurate average structural model is particularly important for identifying subtle structural changes that precede pressure-induced unfolding.

Insights into protein–carbohydrate recognition by NMR spectroscopy

Mario Schubert*, Johannes Pröpster *, Thomas Aeschbacher*, Mirko Zierke[¶], Silvia Bleuler-Martinez†, Beat Ernst[¶], Markus Aebi†, Markus Künzler†, and Frédéric H.-T. Allain*

*Institute of Molecular Biology and Biophysics, ETH Zürich, CH-8093 Zürich, Switzerland

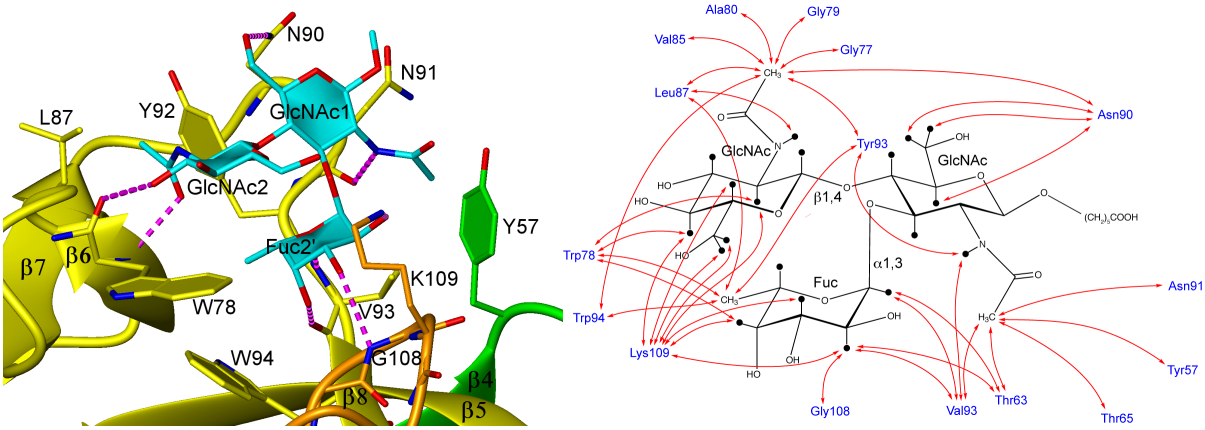
† Institute of Microbiology, ETH Zürich, CH-8093 Zürich, Switzerland

[¶] Institute of Molecular Pharmacy, University of Basel, CH-4056 Basel, Switzerland

EMAIL schubert@mol.biol.ethz.ch

Protein-carbohydrate interactions play key roles in a vast amount of biological processes like cell-cell interactions, modulation of the immune system, cancer progression and metastasis and pathogen-host recognition. An enormous variety of glycoepitopes is functionally relevant – the glycocode. We are just at the beginning to understand how glycoepitopes are recognized by carbohydrate binding proteins, also called lectins.

We demonstrate how NMR spectroscopy can play an important role in identifying the target glycoepitope, analyzing its interaction with the protein, determining three-dimensional structures of lectin-carbohydrate complexes in solution and elucidating the contribution of specific interactions to affinity and specificity [1]. In addition NMR was used to detect pre-formed bioactive conformations of some carbohydrates in solution that are stabilized by a non-conventional C-H···O hydrogen bond [2]. Examples that will be presented include a fungal defense lectin targeting N-glycans of invertebrate predators [3] and human lectin domains of immuno-modulating receptors.



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Mapping the Co-translational Folding Energy Landscape of the ddFln5 Immunoglobulin Domain

Christopher A. Waudby*, Maria-Evangelia Karyadi*, Sammy Chan*, Julian Schmidt-Engler*, Tomek Wlodarski*, Carlo Camilloni†, Michele Vendruscolo†, Lisa D. Cabrita* and John Christodoulou*

*Institute of Structural and Molecular Biology, UCL and Birkbeck College, Gower St, London, WC1E 6BT

†Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW

EMAIL c.waudby@ucl.ac.uk

Efficient protein synthesis and folding, avoiding the population of misfolded or aggregation-prone states, is critical to healthy cell function. During protein synthesis, nascent polypeptides are gradually extruded through the ribosome exit tunnel, and folding may therefore be initiated at the N-terminus of the protein and proceed in a vectorial manner before the C-terminus has fully emerged from the ribosome. This process cannot be described by a single free energy landscape or folding funnel, for the configurational space accessible to the nascent chain increases with the length of the polypeptide chain, and intermediate states may become favored and disfavored as the chain emerges. Therefore, the co-translational 'landscape' must instead be conceptualized as a nested series of landscapes spanning increasingly large conformational spaces. Developing a quantitative understanding of these surfaces, and understanding the perturbations that arise from the presence of the ribosome and associated factors, is currently a major experimental challenge that is currently being addressed by a variety of methods. Within our own group, we have pioneered NMR spectroscopy as a tool to study the length-dependent folding of stalled ribosome-nascent chain complexes [1], focussing particularly on the ddFln5 immunoglobulin domain from the *Dictyostelium discoideum* tandem repeat protein ABP-120 [2].

A complementary experimental approach to the study of stalled ribosome-nascent chain complexes is the use of N-terminal protein fragments to create a ribosome-free model of co-translational folding [3]. This has the particular advantage that the polypeptide fragments may be studied at high concentrations using high-resolution spectroscopic and biophysical methods to accurately characterize the onset of folding in structural and thermodynamical detail. Here we present such a series of N-terminal fragments of ddFln5, revealing a series of folding intermediates that we have characterized extensively using a combination of CD spectroscopy, real-time NMR, EXSY, diffusion and ^{15}N relaxation and relaxation dispersion measurements, and RDC and chemical shift based structure determination. These intermediates are related to the formation of long-range contacts within the protein, and to the isomerisation of a highly conserved native state *cis*-proline residue. These results provide an essential description of the 'ground state' co-translational landscape, against which perturbations in stalled ribosome-nascent chain complexes may be discerned and more fully understood.

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Amyloid aggregates and large soluble protein complexes

Bernd Reif

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

EMAIL: reif@tum.de

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).

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Abstracts of posters

Understanding the Aggregation of Human Lysozyme by HDX and PRE NMR

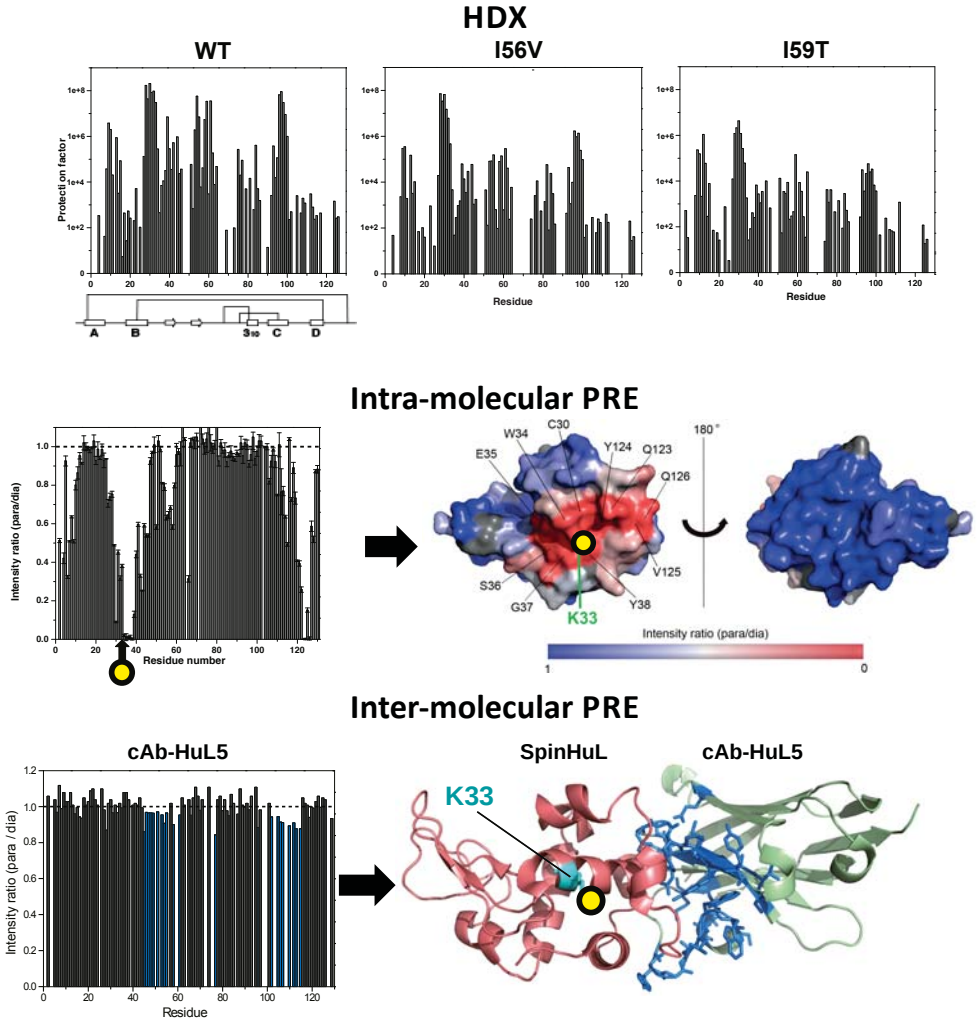
Minkoo Ahn*, Christopher A. Waudby†, Erwin De Genst*, John Christodoulou†, Christopher M. Dobson* and Janet R. Kumita*

*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK

†Institute of Structural and Molecular Biology, University College London and Birkbeck College, Gower Street, London, WC1E 6BT, UK

EMAIL ma505@cam.ac.uk

Amyloid fibril formation of human lysozyme is associated with hereditary systemic amyloidosis. Acquiring the molecular details of its aggregation process is, therefore, of crucial importance for delineating the origin and the progression of the disease, as well as other related conditions. Studies on naturally occurring disease-related variants of the protein have shown that the reduced stability and global cooperativity of the native-state of these proteins is the main reason for the aggregation into amyloid fibrils, accumulation of which are detrimental to the function of organs such as kidney, liver or spleen. We have utilised hydrogen-deuterium exchange (HDX) of amide proton of the protein in order to understand the effect of the mutations at various sites on the stability and dynamics of the native-state protein. Drastic decrease in the protection factors of b-sheet and C-helix region is observed only in the proteins with mutation on 56 and 59. This supports the importance of these residues in maintaining the stability and global cooperativity of the native-state protein. By virtue of a site-specifically introduced nitroxide spin label on human lysozyme paramagnetic relaxation enhancement (PRE) experiments were performed. The data show that spin label can be used for understanding not only the intra-molecular dynamics under various conditions, but also the inter-molecular interactions, in particular, in the initial process of the aggregation.



Contributor: David Armstrong, University of Sheffield.

Title:

Production and backbone assignment experiments of 2H, 13C, 15N-labelled protochlorophyllide oxidoreductase, a light-dependent enzyme

Abstract:

Protochlorophyllide oxidoreductase (POR) is a key regulatory enzyme in the chlorophyll biosynthesis pathway. POR catalyses the reduction of the C17-C18 double bond in protochlorophyllide (Pchlide) to produce chlorophyllide (Chlide). This is a uniquely light-dependent reaction and can only proceed once an excited state of Pchlide is formed after absorption of a photon. Although there has been much interest and many studies involving POR, little is known about the protein structure and the exact role of light in the reaction.

Previous attempts to study the protein atomic structure have been unsuccessful due to failure of extensive crystal trials. POR is a relatively large (37kDa, 322 residue) and hydrophobic protein, thus previous analysis by NMR has been problematic. The production of a highly pure, triple-labelled (2H, 13C, 15N) sample and the use of a high field magnet through the Bio-NMR infrastructure have now produced high quality spectra for analysis. Relaxation experiments have also been carried out on the deuterated protein, in order to analyse the mobility of the protein. It looks promising that this could lead to a near full NMR assignment of POR enabling the first structural and mobility data to be obtained for this unique enzyme.

NMR spectroscopy, mass spectrometry and electron microscopy elucidate the structure and dynamics of αB-crystallin oligomers

Iva Pritisanac[1], Hadi Lioe[1], Nik Sgourakis[2], Lindsay Baker[3], Simon Sharpe[3], John Rubinstein[3], David Baker[2], Justin Benesch[1], Lewis Kay[3], **Andrew Baldwin[1]**,

[1] Physical and Theoretical Chemistry, University of Oxford, UK

[2] University of Washington, Seattle, WA, USA

[3] Department of Medical Genetics, University of Toronto, ON, Canada

EMAIL andrew.baldwin@chem.ox.ac.uk

Small heat shock proteins (sHSPs) are a major class of cellular chaperone that inhibit protein aggregation and amyloid fibril formation *in vivo*. They form a front-line defence against aggregation in the brain whose failure can lead to conditions such as Alzheimer's and Parkinson's diseases. αB-crystallin is a human sHSP that naturally exists as a heterogeneous mixture of oligomers frequently comprising 10-50 monomers (average molecular weight ca. 600kDa), whose individual subunits freely exchange between the different oligomeric forms. The study of such heterogeneous mixtures has therefore proven highly challenging for conventional structural biology approaches. They are very tractable to NMR studies, however.

Here, we determine 'local' structural properties of this high molecular weight heterogeneous mixture using solution and solid NMR approaches. We combine this with 'global' information on shape and size from ion mobility mass spectrometry and tilted pair cryo-electron microscopy images. In doing so we are able to determine structures of the principally populated oligomers within the mileu. The models are refined using Rosetta and extensively cross-validated. This family of structures immediately reveal how the oligomers are able to inter-convert and explain why this protein exists as a poly-disperse ensemble.

To understand how these complexes function, we have developed and applied CPMG NMR relaxation dispersion experiments optimised for high molecular weight systems. Analysis and modelling of data allows us to determine specific conformational fluctuations that give rise to both oligomer inter-conversion, and function.

The methodology developed combines high-resolution structural information derived from NMR measurements with coarser information about shape size of individual complexes from electron microscopy and ion-mobility mass spectrometry. It is generally applicable to a wide range of heterogeneous systems that are otherwise intractable to biophysical analysis.

Solution structure and dynamics of a plant pathogen effector

James L. Tolchard*, Vicki S. Chambers*, Laurence S. Boutemy†, Mark J. Banfield†, **Tharin M. A. Blumenschein***

* School of Chemistry, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ.

† Dept. of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH.

EMAIL t.blumenschein@uea.ac.uk

Plant diseases account for at least $220 billion of crop losses worldwide every year, and have a significant impact on global food security and bio-fuel availability. Bacterial, oomycete and fungal pathogens secrete protein effectors into plant cells, where they perturb cellular processes, presumably to the benefit of the pathogen. In oomycetes, an N-terminal protein motif (RXLR) is important for targeting plant pathogen effectors into host cells (*1*, *2*). However, little is known about the molecular mechanisms by which RXLR effectors manipulate host cell pathways. We have been studying the RXLR effector AVR3a11 (from *Phytophthora capsici*, a pathogen of peppers and *Cucurbitaceae*), which shares sequence similarity with the well-studied AVR3a effector from the Irish potato famine pathogen *Phytophthora infestans* (the causative agent of late blight in potato and tomato). Using a combination of 2D and 3D nuclear magnetic resonance (NMR) spectroscopy experiments, 75% of the AVR3a11 backbone has been assigned. With additional 13C-HSQC-NOESY and 15N-HSQC-NOESY experiments, a structural ensemble model (figure 1) has been generated which is in good agreement with the X-ray structure of a shorter construct of AVR3a11 (PDB code 3ZR8, *3*), showing a four-helix bundle with a disordered N-terminal extension. Backbone amide T_1, T_2 and heteronuclear NOE relaxation experiments of the shorter construct at 500 and 800 MHz, in combination with hydrogen/deuterium exchange experiments and the presence of peaks for minor conformations in the HSQC spectra, reveal that AVR3a11 has uniform motions in the faster timescales, but a wide distribution of slower motions which may be related to its function and interactions.

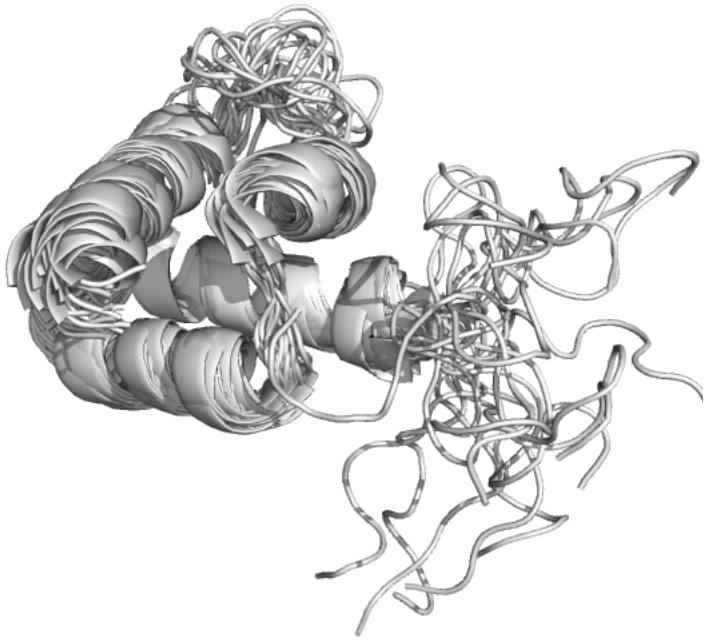


Figure 1: Final water refined ensemble of the 20 top scoring Avr3a11 structural models. Structures were calculated using the CNS 1.3 software suite (*4*) and the RECOORD (*5*) parameter set and CNS scripts.

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The structure of the type III connecting segment (IIICS) of fibronectin

Eve Blumson
Second year PhD student
Supervisors: Martin Humphries and Jon Waltho
University of Manchester

The type III connecting segment (IIICS) domain of fibronectin (Fn) is able to facilitate the adherence and spreading of leukocytes and melanoma cells, highlighting the potential for therapeutic agents based on the IIICS structure, which could be used to block melanoma metastasis and reduce inflammation. Fn is mainly composed of three repeated domains, FI, FII and FIII, with the IIICS located between the 14^{th} and 15^{th} FIII domains ($FIII_{14}$ and $FIII_{15}$). The structure of the 13kDa IIICS is unknown, with it having no sequence homology to any of the repeated domains. Interestingly, the IIICS can be divided into three subdomains, which combine via alternative splicing to give one of five variants in human Fn. The full-length IIICS contains two integrin binding sites and proteoglycan binding site, which are able to facilitate the cell binding properties.

The structure of the IIICS has been investigated by NMR using a 55kDa construct containing the full length IIICS flanked by three FIII domains ($FIII_{12-14}$) at the N-terminus and the $FIII_{15}$ domain at the C-terminus. The structure of $FIII_{15}$ is also unknown, but it is predicted to be lacking an N-terminal strand that would allow it to form a typical FIII fold of a seven-stranded beta sandwich.

A triple labelled sample of this construct was expressed and used to record a standard set of triple resonance experiments. Through these experiments almost complete backbone assignment of the IIICS and $FIII_{15}$ domain was possible. Peaks from residues in the $FIII_{12-14}$ domains were not visible in these experiments. The dynamics of the construct were investigated by recording ^{15}N relaxation experiments on a ^{2}H, ^{15}N labelled sample. This data showed the IIICS to have increased flexibility compared to the $FIII_{15}$ domain. Analysis of the chemical shift data suggests that the $FIII_{15}$ module forms a six-stranded beta sandwich fold and that the IIICS does not contribute the seventh strand to form a typical FIII fold. Further experiments will involve collecting structural restraints for $FIII_{15}$ and the IIICS by recording a combination of NOESY, PRE and RDC experiments.

Strategies for Solving Structures of Large Protein Complexes by NMR: Identification, Assignment and Use of Intermolecular Methyl-Amide NOEs to Drive Docking

Shaun Bruton[1], Philip W. Addis[1], Lorna C. Waters[1], Frederick W. Muskett[1], Gareth A. F. Hall[1], Alistair J. Henry[2], Richard J. Taylor[2], Mark D. Carr[1]

[1]Henry Wellcome Laboratories for Structural Biology, Department of Biochemistry, University of Leicester, University Road, Leicester, LE1 9HN

[2]UCB, 216 Bath Road, Slough, Berkshire, SL1 4EN

EMAIL sb351@le.ac.uk

The vast majority of proteins form functional complexes and determination of the structure of these complexes is important for understanding their mechanism of action. Solving the structure of large protein complexes by NMR is challenging and not all systems are amenable to study by X-ray crystallography.

In cases where structures of the individual components of a complex, or their homologues, are available, solving the structure of the complex by NMR restraint driven docking is an attractive approach.

Protein-protein interfaces are known to be enriched with methyl side-chains. We have developed a robust and relatively simple NOE based approach to obtain sequence-specific methyl assignments for proteins and protein complexes. This approach facilitates the identification of intermolecular methyl-to backbone amide NOEs, providing valuable restraints for use in protein-protein docking calculations.

The applicability of this approach is demonstrated by the determination of the structure of the 65 kDa complex formed by the pro-inflammatory cytokine interleukin-1β bound to a potential therapeutic antibody fragment (Fab). Highly deuterated samples of the proteins were produced by bacterial expression in 100% D_2O minimal media with either protonated or deuterated glucose as the sole carbon source. As reported previously the use of protonated glucose results in substantial selective protonation of methyl groups (55-65%) (Shekhtman et al., 2002), which we have exploited to develop a successful NOE-based assignment strategy for methyl groups. This allowed essentially complete assignment of the methyl groups for Il-1β bound to a Fab.

Intermolecular distance constraints derived from these NOEs, together with chemical shift based mapping of the interaction site on both proteins allowed reliable docking of the two proteins, with a single tightly defined family of structures obtained. The validity of the approach has been proven by independent determination of the crystal structure of the Il-1β-Fab complex.

Shekhtman, A; Ghose, R; Goger, M; Cowburn, D (2002); “NMR structure determination and investigation using a reduced proton (REDPRO) labelling strategy for proteins” FEBS LETTERS **Volume:** 524 **Issue:** 1-3 **Pages:** 177-182.

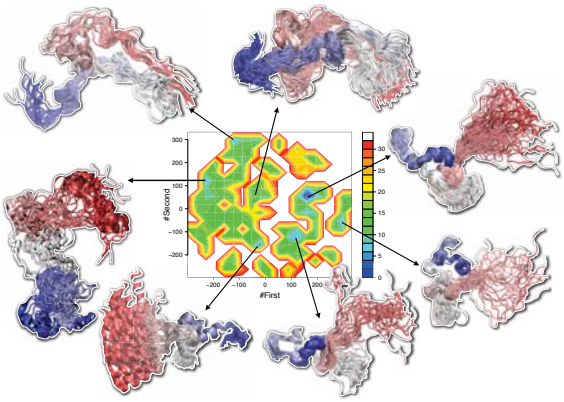
Conformational Equilibrium between the sub states of the acidic denatured state of ACBP determined by NMR Chemical Shifts Replica-Averaged Metadynamics

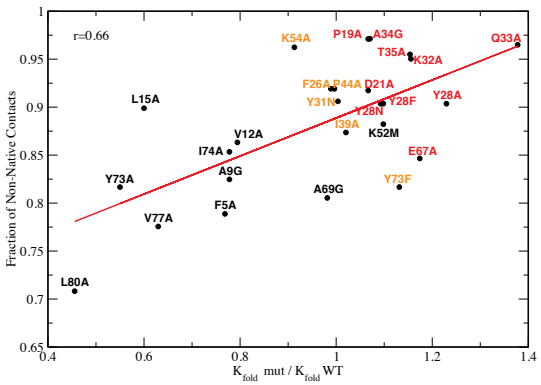
Carlo Camilloni*, and Michele Vendruscolo*

*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW

EMAIL cc536@cam.ac.uk

The structure and dynamics of the unfolded state ensembles of proteins can only be limited characterised if compared with their relative folded state[1]. Indeed the resolution of NMR observables is limited by the underlying complexity of the process under study. By means of chemical shifts replica-averaged metadynamics[2] we show that we can successfully integrate NMR derived information with advanced sampling MD techniques and so provide an ensemble of structures for the acid denatured state of ACBP[3] that is not limited neither by the quality of physical model used (the force-field) nor by the extent of the sampling of the conformational space[4]. The ensemble enable us to look for the first time with an atomistic resolution at the microstates populated in the denatured state of this protein and is used to get a quantitative insight into the role of the native and non-native residual structure in the determination of the differences in the folding rates upon mutation[5].





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Breaking Pseudo-Symmetry in Multiantennary Complex N-Glycans using Lanthanide-Binding Tags and NMR Pseudo-Contact Shifts

Angeles Canales,* Alvaro Mallagaray,† Javier Pérez-Castells,‡ Irene Boos,ǂ Carlo Unverzagt,ǂ Sadine André,Ť Hans-Joachim Gabius,Ť Francisco Javier Cañada,† and Jesús Jiménez-Barbero†

* Dep. of Organic Chemistry I, Fac. Chemistry, Univ. Complutense de Madrid, Avd. Complutense s/n 28040, Madrid, Spain.

† Dep. of Chemical and Physical Biology, Centro de Investigaciones Biológicas, CSIC, C/Ramiro de Maeztu 9, CP. 28040, Madrid, Spain.

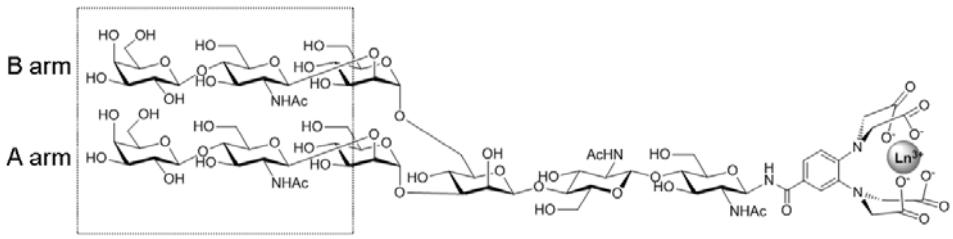
‡ Dep. of Chemistry, Fac. Pharmacia, Univ. San Pablo CEU, Boadilla del Monte, 28668, Madrid, Spain.

ǂ Dep. Bioorganische Chemie, Gebäude NWI, Universität Bayreuth, 95444 Bayreuth, Germany.

Ť Institute of Physiological Chemistry, Fac. of Veterinary Medicine, Univ. Ludwig-Maximilians, 80539 Munich, Germany.

EMAIL ma.canales@quim.ucm.es

Molecular recognition is of vital significance for life. In terms of biological coding and translating signals into cellular effects, glycans have gained a particula status, owing to their unsurpassed coding capacity and widespread presence of receptors (lectins) to read the encoded information. [1] In this context, we herein present a novel NMR approach to individually monitor the behavior of each arm, A and B, of N-glycans (Scheme 1) and thereby provide a global perspective of their conformational and interaction features in solution.



Scheme 1. Nonasaccharide derivative studied in this work. The 1–3 and 1–6 arms attached to the β-mannose unit are labeled as A and B, respectively.

The use of the lanthanide tag has permitted to break the inherent pseudo-symmetry of the NMR spectra of the identical branches, revealing that the T-shaped gg rotamer at the Manα(1-6)Man junction is the major one in solution, with minor contributions of other backfolded geometries.[2]

In addition, the recognition of this nonasaccharide by human galectin-3 has been studied. In this line, the novel methodology employed has permitted the characterization of the binding epitopes of the symmetrical N-glycan, showing that both arms are involved in the recognition of human galectin-3. [2]

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NMR observation of the vectorial folding of an immunoglobulin domain on translationally-arrested ribosomes

Anaïs M. E. Cassaignau*, Lisa D. Cabrita*, Christopher A. Waudby*, Hélène M. M. Launay*, Xiaolin Wang* and John Christodoulou*

*Institute of Structural and Molecular Biology, University College London, Gower Street, London, WC1E 6BT

EMAIL anais.cassaignau.09@ucl.ac.uk

At least some proteins have been shown to acquire native-like structure co-translationally during synthesis on their parent ribosome particle. Despite the large molecular weight of the ribosome, we have shown that solution-state NMR spectroscopy can uniquely enable a structural understanding of co-translational protein folding via stalled ribosome-bound nascent chain complexes (RNCs) [1].

Here I describe a study of the progressive biosynthesis of the model immunoglobulin domain ddFLN5 (the fifth domain of ABP-120, a multidomain filamin protein from *Dictyostelium discoideum*) using translationally-arrested RNCs in which the length of the ddFLN6 linker tethering the domain to the ribosome was varied. A two-pronged isotopic labelling strategy was developed and applied to observe the folding process by NMR spectroscopy, as a function of the polypeptide chain length. Stalled RNC 'snapshots' were probed for disordered states by measurement of ^{1}H-^{15}N correlation spectra, while specific methyl labelling against a perdeuterated background was employed to report in a very sensitive manner on the co-translational population and structure of folded states. In this way, the chain length at which the ddFLN5 domain populates folded and unfolded states equally was determined on the ribosome. A comparison with high-resolution studies of C-terminal ddFLN5 fragments will also be described that shows that the ribosome has a major role in perturbing the folding process.

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Increasing the Sensitivity of NMR Diffusion Measurements by Paramagnetic Longitudinal Relaxation Enhancement, with Application to the Study of Ribosome-Nascent Chain Complexes

Sammy HS Chan, Christopher Waudby, Anaïs Cassaignau, Lisa D Cabrita and John Christodoulou

Institute of Structural and Molecular Biology, University College London, London WC1 6BT
s.chan.12@ucl.ac.uk

NMR diffusion measurements are a powerful probe of biomolecular structure, and can be an important means of monitoring the integrity of large macromolecular assemblies such as ribosome-nascent chain complexes (RNCs) [1]. NMR studies of RNCs are severely constrained by the low solubility of the ribosome (having a maximum concentration of 10 μM), together with the limited lifetime of the nascent chain (typically just a few hours), and therefore improving experimental sensitivity is of central importance. Here we explore the use of the paramagnetic longitudinal relaxation enhancement agent NiDO2A (previously and usefully applied to 2D SOFAST-HMQC measurements [2]) to improve the sensitivity of ^{15}N heteronuclear diffusion measurements. Exploiting the dependence of paramagnetic relaxation on the gyromagnetic ratio, and using the longitudinal relaxation-optimized SORDID pulse sequence [3], we obtain typically 100–400% increases in sensitivity compared with XSTE measurements of undoped samples. When applied to *E. coli* ribosomes and RNCs we observe a two-fold improvement in SORDID sensitivity, alongside an 85% increase in SOFAST-HMQC sensitivity, without compromising sample stability. We expect these enhancements will greatly facilitate future NMR investigations of such large, dilute and unstable macromolecular machines.

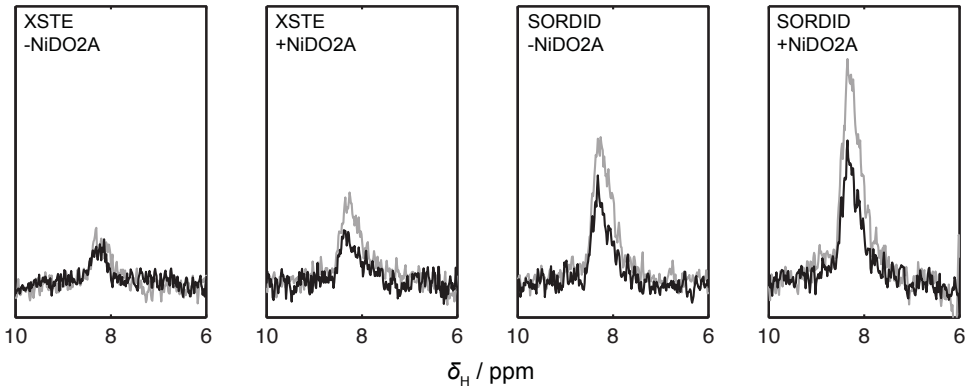

Figure 1. The effect of NiDO2A on the sensitivity of XSTE and SORDID diffusion measurements of an RNC sample (shown at high (black) and low (grey) gradient strengths).

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Applying a Solid-state NMR Approach to Probe Atomic Changes in Collagen Matrices in Health and Disease

W Ying Chow*, David G Reid*, Wai Ching Wong*, Rakesh Rajan†, Roger A Brooks†, Richard W Farndale‡, Dominique Bihan‡, Melinda J Duer*

*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK

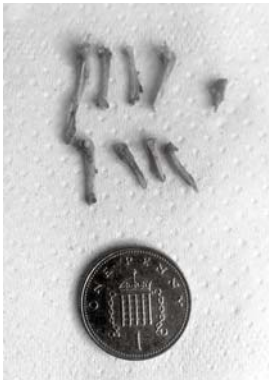
†Orthopaedic Research Unit, Addenbrooke's Hospital, Cambridge , CB2 0QQ, UK

‡Department of Biochemistry, University of Cambridge, Building O, Downing Site, Cambridge CB2 1QW, UK

EMAIL wyc25@cam.ac.uk

Collagen proteins form the bulk of many structural tissues, such as skin, tendons, cartilage, the wall of arteries, and bone. In structural tissues, the collagen proteins form large, heterogeneous and insoluble matrices which house and interact with the cells, while contributing to mechanical integrity and function. Recent developments in solid-state NMR (ssNMR) provide a means of elucidating atomic structures of insoluble proteins and macromolecules. Therefore, instead of working on isolated and purified proteins, our study tackled tissue samples that contain a high proportion of collagen, such as bone, or extracellular matrix (ECM) generated in vitro.

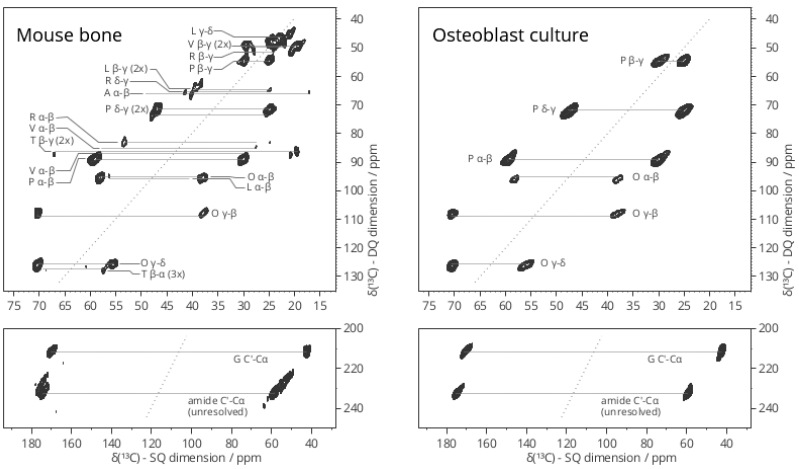
By feeding a mouse on an isotopically-enriched diet, we obtained ^{13}C and ^{15}N-enriched bone tissues that facilitated 2D ssNMR experiments. From the increased resolution of these 2D spectra, we can make assignments to many more residue types for collagen proteins in native tissue than was possible prior to this study. These assignments serve as parameters against which we can compare synthetic attempts at generating collagen matrices.



We can compare the ssNMR spectrum of synthetic collagen-like peptides directly to bone. From our spectra, it is clear that the model peptides only mimic a subset of structural variations that exist along the collagen triple helix in bone. Using these peptides, for which atomic structures are already available, we can identify regions which correspond to proline-rich and likely more ordered regions in bone collagen.

Mammalian osteoblast cell cultures which lay down collagenous ECM enable us to probe a biologically-generated source of collagen proteins with more targeted isotopic-enrichment schemes. After fine-tuning the cell culture conditions so that we can generate isotopically-enriched ECM that yield ssNMR spectra that closely resemble bone, we can go on to test different models for disease. In aging and diabetes, collagen proteins are known to undergo irreversible changes, such as increased crosslinking and change in material properties. By isotopically enriching the sugar component of the cell culture samples, we can probe the atomic structures formed in both healthy glycosylation and unhealthy glycation of collagen proteins. From these spectra, we identify signals which correspond to sugar phosphates, which may act as a facilitator in bone tissue mineralisation, or an aggressive glycating species if produced in other tissues.

In conclusion, we now have a method of validating atomic-level structure of collagen matrices, which can be used by tissue engineers looking to improve their collagen-based implants. We can also begin to elucidate the atomic-level mechanisms by which age-related diseases and processes affect the structure and function of collagen proteins in our tissues.



Combining NMR, computation and biophysics to study pathogenic malaria cytoadherence

Erin Cutts*, Leanne Slater* and Ioannis Vakonakis*

*Department of Biochemistry, University of Oxford, South Parks road, Oxford, OX13QU

EMAIL: erin.cutts@wolfson.ox.ac.uk

Plasmodium falciparum infected red blood cells (iRBC) can adhere to endothelial cells of the blood-vessel wall, causing severe side effects. This adhesion is mediated by parasite proteins from the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) family, which localise to distinct protrusions on the iRBC membrane known as knobs. *Pf*EMP1 members are transmembrane proteins with highly divergent extracellular regions that bind a range of endothelial cell receptors, such as ICAM-1 and CD36, and a predominantly flexible intracellular region known as the acidic terminal segment (ATS). NMR studies have shown that ATS consists of three distinct flexible regions separated by a small helical core [Mayer et al. (2012) J Biol Chem. 287, 7182]. Here we show that the N-terminal region of ATS interacts with erythrocyte cytoskeletal components, such as α and β spectrin, while the C-terminal region binds to members of the *P. falciparum* PHIST family, PFI1780w and PFE1605. NMR has been used in combination with X-ray crystallography, mutagenesis, binding studies and computational methods, such as CS-Rosetta structure prediction and molecular dynamics, to examine the structural stability of these proteins, investigate the interaction between them, and build a larger interaction model for the ATS within the structure of the knob.

Structure determination of intrinsically disordered p15^{PAF} bound to the 90 kDa PCNA clamp by integration of NMR and X-ray crystallography data into computational docking

Alfredo De Biasio*, Alain Ibañez de Opakua*, Gulnahar Mortuza[†], Tiago N Cordeiro[‡], Francisco Castillo[§], Maider Villate*, Nekane Merino*, Moreno Lelli[¤], Tammo Diercks*, Irene Luque[§], Pau Bernadó[‡], Guillermo Montoya[†] and Francisco J Blanco*[¶]

*Structural Biology Unit, Center for Cooperative Research in Biosciences (CIC bioGUNE), E-48160 Derio, Spain

[†]Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid (CSIC-UAM), E-28029 Madrid, Spain

‡Centre de Biochimie Structurale, Institut National de la Santé et de la Recherche Médicale (INSERM) U1054, Centre National de la Recherche Scientifique (CNRS) UMR 504, Université Montpellier 1 et 2, Montpellier, France

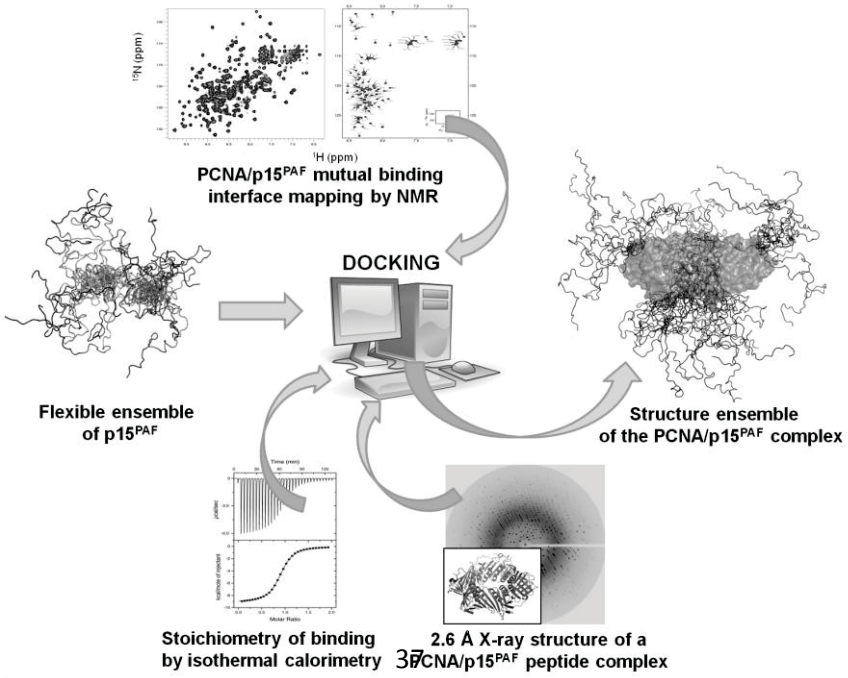
[§]Department of Physical Chemistry and Institute of Biotechnology, University of Granada, 18071 Granada, Spain

[¤]Centre de Résonance Magnétique Nucléaire à Très Hauts Champs, Institut de Sciences Analytiques (CNRS/Ecole Normale Supérieure de Lyon/Université Claude Bernard Lyon 1), Villeurbanne, France

[¶]IKERBASQUE, Basque Foundation for Science, Bilbao, Spain

EMAIL alfredo.debiasio@elettra.eu

p15^{PAF} is an intrinsically disordered nuclear protein of 12 kDa that binds the human DNA sliding clamp (Proliferating Cell Nuclear Antigen, PCNA) through its central PIP-box motif. Depletion of p15^{PAF} impairs the DNA repair process while its overexpression is observed in multiple types of human cancer. In order to shed light on the structural basis of p15^{PAF} action, we determined the structure of the PCNA/p15^{PAF} complex by molecular docking guided by NMR and X-ray crystallography-derived restraints. The structure ensemble reveals an unexpected interaction in which three p15^{PAF} chains are anchored to the canonical PIP-box binding sites, contact the inner wall and penetrate the hole of the PCNA ring in a “string on a bead” arrangement. The disordered p15^{PAF} N-termini are located at the back side of PCNA and bind different DNA substrates via electrostatic interactions. Taken together, our data suggest a functional role for p15^{PAF} as a flexible adaptor that holds PCNA and the DNA together. This work shows the power of a synergistic high-resolution structural approach to address a challenging system composed of a high molecular weight oligomeric protein and a flexible, intrinsically disordered partner.



BAZ2B bromodomain binding hotspots revealed by solution NMR

Fleur F. Fergusson*, **David M. Dias***, João P. G. L. M. Rodrigues[§], Hans Wienk[§], Alexandre M. J. J. Bonvin[§], Chris Abell* and Alessio Ciulli[†]

*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW

[§]Department of Chemistry, University of Utrecht, Padualaan 8, Utrecht, 3584 CH

[†]Division of Biological Chemistry and Drug Discovery, University of Dundee, Dow Street, Dundee, DD1 5EH

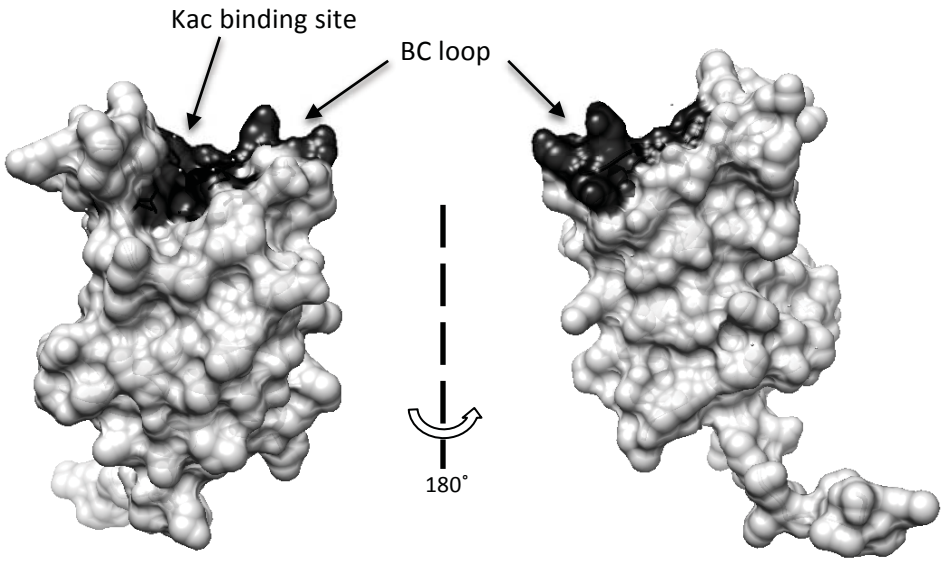
EMAIL a.ciulli@dundee.ac.uk

Bromodomains are epigenetic reader domains, which have recently come under high scrutiny both from academic and industry. Effective targeting of BAZ2B bromodomain has been recently reported but no information is yet available on its natural binding partner, acetylated lysines (Kac) in histones.

We have obtained the spectral assignment of BAZ2B bromodomain and determined its backbone dynamics. Once we have validated the backbone assignment as resource for screening small molecules, we then studied its interaction with H3 acetylated peptides by NMR.

Being an interaction mediated by water, we used both chemical shift perturbation (CSP) data and clean chemical exchange (CLEANEX) NMR experiments to characterise this interaction for the first time. We found as key hotspots for the interaction the Kac binding site and the BC loop. This information was used to create a data driven model for this interaction using HADDOCK. The models were further validated by site directed mutagenesis and isothermal titration calorimetry (ITC).

This information offers new rational to optimise current inhibitors for inspecting BAZ2B bromodomain's function.



Resonance assignment and solution structure of myristoylated Y28F/Y67F mutant of the Mason-Pfizer monkey virus matrix protein

Michal Doležal[*,†,‡], Tomáš Ruml[†], Michaela Rumlová[‡,§], Iva Pichová[‡], Richard Hrabal[*]

*Laboratory of NMR Spectroscopy, Institute of Chemical Technology, Prague, Technická 5, 16628 Prague, Czech Republic

†Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague, Technická 5, 16628 Prague, Czech Republic

‡Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., IOCB & Gilead Research Center, Flemingovo nám. 2, 16610 Prague, Czech Republic

§Department of Biotechnology, Institute of Chemical Technology, Prague, Technická 5, 16628 Prague, Czech Republic

dolezal@uochb.cas.cz

Matrix protein (MA) is the N-terminal domain of polyprotein Gag – a major structural component of all immature retroviral particles. Gag of the Mason-Pfizer monkey virus (M-PMV), a model betaretrovirus, assembles into immature particles in the cytoplasm and the particles are then transported to the cytoplasmic membrane. During the budding or shortly after, the retroviral protease cleaves Gag into individual structural domains. In immature particles, MA forms the outermost protein layer that interacts with cellular components and thus is essential for the cellular transport of the particles. In M-PMV and most other studied retroviruses, MA is N-terminally myristoylated. It was shown that the myristoylation of M-PMV MA is indispensable for the transport of immature particles to the cytoplasmic membrane. The role of MA in the budding is still unclear but it was suggested that the insertion of the myristoyl residue into the cytoplasmic membrane and the interaction of MA with a specific component of cytoplasmic membrane, phosphatidylinositol 4,5-bisphosphate, trigger the budding of viral particles from the host cell. Double-mutation Y28F/Y67F is one of mutations in M-PMV MA that impair the budding of viral particles from the host cell (Stansell et al. 2004). This mutation causes the viral particles to accumulate under the cytoplasmic membrane and considerably slows down the budding.

To investigate the changes in the structure of M-PMV MA due to the mutation and the interaction of the mutant with the cytoplasmic membrane, we prepared myristoylated Y28F/Y67F mutant of MA for NMR spectroscopy, uniformly labelled with 13C and 15N. We assigned the backbone and side-chain resonances and calculated the structure from distance restrictions derived from NOESY. We report essentially complete resonance assignment and the structure of myristoylated Y28F/Y67F mutant of the Mason-Pfizer monkey virus matrix protein.

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Solid-State NMR Studies of Immobilised Enzyme Systems

Nicole E. Fauré*, Peter Halling† and Stephen Wimperis*

*School of Chemistry and WestCHEM, University of Glasgow, Glasgow G12 8QQ, United Kingdom

†Department of Pure & Applied Chemistry and WestCHEM, University of Strathclyde, Glasgow G1 1XL, United Kingdom

EMAIL nicfau@chem.gla.ac.uk

Enzymes, when used as industrial biocatalysts, possess the astonishing virtue of leading to product formation at mild and environmentally friendly conditions with a high specificity. A core technology is the immobilisation of enzymes – the conversion of the soluble protein molecules into a solid particle form that can be easily separated from the reaction mixture. Since the advent of immobilisation of single enzymes in the 1940s, numerous methods have been developed. Despite extensive study on different systems, there is no clear approach for a given process and enzyme. One reason for this is that little is known about the state of the protein molecules in the preparation except what is deduced from the catalytic activity.

With this in mind and aiming towards a better understanding of immobilised enzymes, this contribution describes a comprehensive study of the covalent immobilisation of α-chymotrypsin on functionalised silica and alumina particles (glycidoxypropyltrimethoxysilane, GOPS, grafted onto the surface) and Eupergit® (rigid methacrylic cross-linked polymers bearing pendant epoxide groups). Using one- and two-dimensional ^{13}C, ^{29}Si (only for systems based on inorganic supports) and ^{1}H MAS NMR techniques, we have been able to characterise these bio-functionalised heterogeneous enzymatic and support systems, demonstrating the power of multinuclear solid-state NMR to provide a better understanding of immobilised enzymes at the molecular level.

Anuroctoxin and its interaction with voltage-gated K^+ ion channels Kv1.2 and Kv1.3

Krisztina Fehér[*], Kinga Rákosi[†], István Timári[‡], Ádám Bartók[#], Gábor Tóth[†], György Panyi[#], Katalin E. Kövér[‡]

[*] Department of Organic Chemistry, Ghent University, Krijgslaan 281 S4, Ghent, B- 9000, Belgium and Department of Inorganic and Analytical Chemistry, University of Debrecen, Egyetem tér 1. H-4032, Debrecen, Hungary

[†] Department of Medical Chemistry, University of Szeged, Dóm tér 8., Szeged, H-6720, Hungary

[#] Department of Inorganic and Analytical Chemistry, University of Debrecen, Egyetem tér 1., H-4032, Debrecen, Hungary

[#] Department of Biophysics and Cell Biology, University of Debrecen, Egyetem tér 1., H-4032, Debrecen, Hungary

Email: Krisztina.Feher@ugent.be

The voltage-gated Kv1.3 potassium channel plays a key role in the activation of T lymphocytes. By blocking these channels the proliferation of T cells can be inhibited thus suppressing T-cell mediated immune responses, which has a great potential in the therapy of certain autoimmune diseases. Anuroctoxin (1) is a 35-amino acid scorpion peptide characterized previously as a high affinity blocker of Kv1.3. Although with lower affinity, Anuroctoxin blocks Kv1.2 as well, which is expressed in several tissues, thus this property limits the potential clinical use of the toxin.

In order to design new mutants of Anuroctoxin with improved selectivity profile, a detailed understanding of its interaction with voltage gated K+ ion channels is needed. We have prepared synthetic analogue of Anuroctoxin with solid phase synthesis and verified its activity with Kv1.2 and Kv1.3 channels. We have determined the solution structure of Anuroctxin using homonuclear NMR techniques and molecular dynamic simulations. The determinants of its interaction with Kv1.2 and Kv1.3 channels have been investigated and explored with docking calculations. Our results provide data for the design and future therapeutic application of new, more selective peptide toxins.

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Structural basis of RNA recognition by STAR Proteins.

M. Feracci*, J. Foot*, S., Grellscheid[ϔ], D. Elliott and C. Dominguez*.

*Department of Biochemistry, University of Leicester, UK
[ǂ]Institute of Human Genetics, University of Newcastle, UK
[ϔ] School of Biological and Biomedical Sciences University of Durham, UK.

mf172@le.ac.uk

STAR (Signal Transduction and Activation of RNA) protein family plays an important role in many processes involving RNA such as the regulation of alternative splicing and RNA export. All members of this family contain a STAR domain involved in RNA binding and divided in three subdomains: the NK/QUA1 (dimerization), the KH (K-Homology) and the CK/QUA2.

Two members of this family, Sam68 and TSTAR, have an extensive sequence homology (especially for the STAR domain) and recognize similar U/A rich RNA sequences. Our aim is to define the structural basis of RNA recognition by Sam68 and TSTAR proteins and to study their structure-function relationships.

Based on SELEX (1), CLIP, and natural RNA sequences (2), we have designed a screen of RNAs bound by Sam68 and TSTAR and used NMR spectroscopy to identify the optimal protein construct and RNA sequence for structure determination (3).

Using this information, we have solved the structures of various domains of T-STAR in complex with different RNA sequences, which explain the specific RNA recognition by this family of proteins.

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Detection of salt bridges to lysines in solution in barnase

Kyle Fowler,* Andrea M. Hounslow,* Joe Ford,* Max Hebditch,* Poul Erik Hansen† and Mike P. Williamson*

*Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN

†Department of Science, Systems and Models, 18.1, Roskilde University, Universitetsvej 1, PO Box 260, DK-4000 Roskilde, Denmark

EMAIL: m.williamson@sheffield.ac.uk / kfowler2@sheffield.ac.uk

Here, we present an alternative, more direct, and less laborious method for salt bridge detection (Williamson et al., 2013). We show that salt bridges involving lysines can be detected by deuterium isotope effects on NMR chemical shifts of the sidechain amine. The deuterium isotope effect is expected to be different in salt-bridged and non-salt-bridged amines, because the formation of an N-H···O-C salt bridge makes the N-H bond longer and weaker, and therefore changes the perturbation of nuclear shielding caused by the introduction of the heavier ^{2}H nucleus. Barnase, a small bacterial ribonuclease, was used as a test system. Heteronuclear in-phase single quantum coherence spectroscopy (HISQC) (Iwahara et al., 2007) (**Figure**) and subsequent chemical shift measurements revealed that the isotope effect for K27, the sole salt bridged lysine, differs from those of all other lysines: $^{1}\Delta^{15}\Delta$ is smaller while $^{2}\Delta^{1}$H is larger (more negative). To check this hypothesis, we generated another salt bridged lysine by mutation of R69 to lysine. Mutation of R69 to lysine retains a partially buried salt bridge to D93. The pK_a of D93 was measured to be approximately 2.3, confirming that K69 is salt bridged. In the R69K mutant, the isotope effects for K69 are similar to those observed for K27, though slightly less extreme.

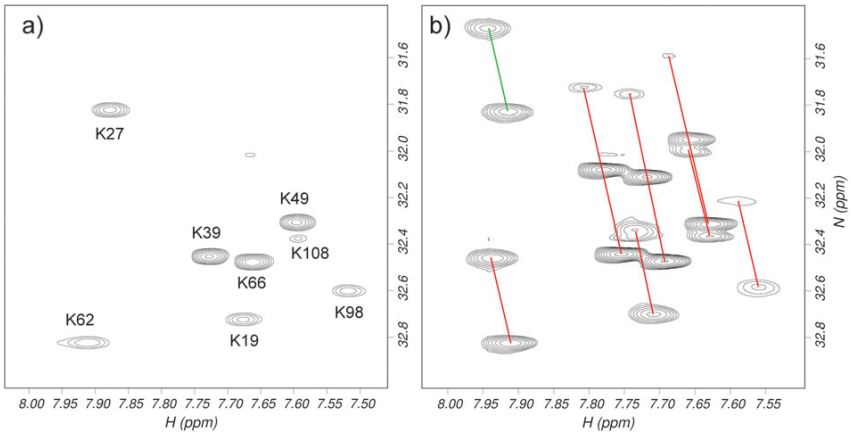

Figure. HISQC spectrum of lysine sidechains in barnase: (a) in 100% H_2O (b) in 20% D_2O - 80% H_2O. Both were measured in 10 mM acetate, pH 4.8, 3 °C. In (b), for each lysine, the lower signal (larger ^{15}N chemical shift) is from the NH_3^+ form, while the upper one is NH_2D^+. For three of the residues the NHD_2^+ peak is also visible, with the NH_3^+–NH_2D^+ spacing being equal to the NH_2D^+–NHD^{2+} spacing. The residue in the top left hand corner of the spectrum is the salt bridged K27.

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Probing the interaction between α–Synuclein and lipid membranes by NMR Spectroscopy

Giuliana Fusco*, Alfonso De Simone† , Gopinath Tata[§], Vitaly Vostricov [§], Michele Vendruscolo *, Christopher M. Dobson* , Gianluigi Veglia [§]

*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK

†Department of Life Sciences, Imperial College London, South Kensington, London, SW7 2AZ, UK

[§] Department of Chemistry & Department of Biochemistry, Biophysics & Molecular Biology, University of Minnesota, 6-155 Jackson Hall, USA

EMAIL gf203@cam.ac.uk

α-synuclein (αS) is a protein involved in neurotransmitter release in presynaptic terminals, and whose aberrant aggregation is associated with Parkinson's disease. In dopaminergic neurons, αS exists in a tightly regulated equilibrium between water-soluble and membrane-associated forms. Using a combination of solid-state and solution-state NMR spectroscopy, we characterised the conformations of αS bound to lipid membranes mimicking the composition and physical properties of synaptic vesicles. The study evidences key regions of the protein possessing distinct structural and dynamical properties and having specific roles in determining the way the protein partitions between membrane-bound and unbound states. Taken together, our data define the nature of the interactions of αS with biological membranes and provide insights into their roles in the function of this protein as well as in the molecular processes leading to its aggregation.

Rethinking NMR Resources at PDBe

Aleksandras Gutmanas, Pieter MS Hendrickx, Swanand Gore, Glen van Ginkel, Sameer Velankar and Gerard J Kleywegt

Protein Data Bank in Europe, European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI), Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SD, UK

EMAIL gutmanas@ebi.ac.uk

Protein Data Bank in Europe (PDBe; pdbe.org) partners with the RCSB PDB, PDBj and BMRB to form the wwPDB consortium. The wwPDB members jointly manage all aspects of deposition, annotation, remediation and distribution of the PDB archive, and are engaged in a friendly competition in developing advanced value-added services based on the PDB data for use by the scientific community.

PDBe is currently engaged in a process of redesigning PDB entry pages, which will provide richly annotated information on function, structure and validation of biomacromolecules and their complexes. The output of the new wwPDB X-ray, NMR and 3D CryoEM validation pipelines will be available for all publicly released PDB entries and will be included in the planned PDBe Validation portal.

At a wwPDB workshop convened by PDBe, the developers of the most commonly used software packages for structure determination by NMR have agreed to specify and implement a new mmCIF/STAR-like format to capture experimental NMR restraints and peaks to enable automatic validation of these types of data. Once the new format is defined, the NMR validation pipeline will be updated and the output will be included in the validation reports and portal.

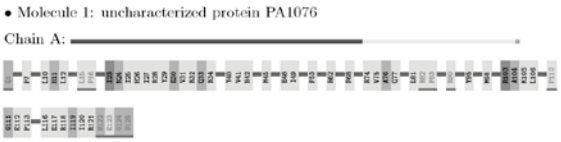
The wwPDB validation pipeline. Geometry, ensembles and shifts

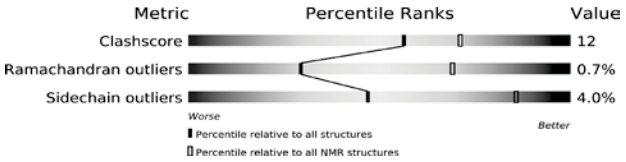
Pieter MS Hendrickx*, Aleksandras Gutmanas* and Gerard J Kleywegt

*Protein Data Bank in Europe (PDBe), EMBL-EBI, Wellcome Trust Genome Campus, Cambridge, UK

EMAIL pieter@ebi.ac.uk

The Protein Data Bank in Europe (PDBe; pdbe.org) is a founding member of the Worldwide PDB (wwPDB; wwpdb.org) consortium, which manages the Protein Data Bank (PDB), the single global archive of three-dimensional structures of biological macromolecules and their complexes. PDBe has been tasked by the wwPDB partners to develop software pipelines implementing the recommendations of the wwPDB and EMDataBank validation task forces (VTFs) for X-ray crystallography, Nuclear Magnetic Resonance and 3D Electron Microscopy.

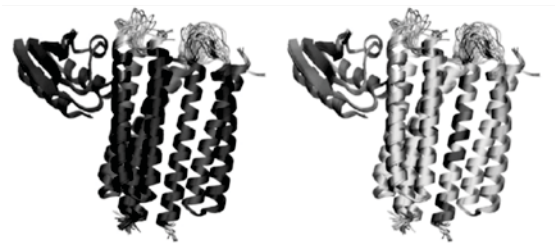




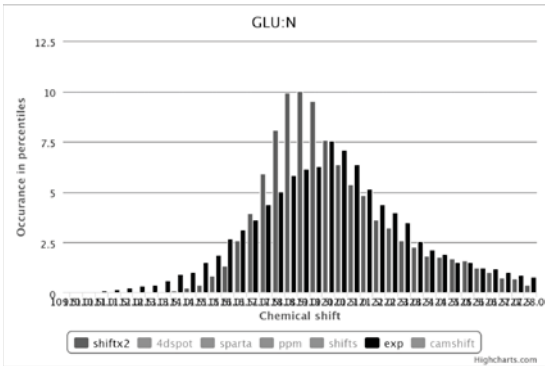
These pipelines are developed for use in structure deposition and annotation by depositors and wwPDB curators and are (being) integrated into the new wwPDB Deposition and Annotation tool that was launched in early 2014. The pipelines produce concise validation and annotation reports, which can be submitted along with manuscripts describing the structures and will become publicly available with the release of PDB entries. In addition, the validation pipelines are made available as stand-alone anonymous servers.

As recommended by the wwPDB NMR VTF, the NMR pipeline applies the same knowledge-based validation criteria for structure quality assessment as for crystal structures. However, global parameters are reported only for well-defined structural regions of proteins. The validation of chemical shifts is presently limited to completeness of assignments, referencing corrections and identification of statistical outliers.

Here we present the results of a bulk analysis of the NMR PDB archive, comparing different methods for determining well-defined structural regions of proteins (dihedral order, NmrCore, Cyrange and FindCore) and chemical shift outlier detection (ShiftX2, Ppm, 4dSpot, Camshift, Sparta+,…). This analysis will aid the NMR VTF to recommend suitable software suites to be used in the unified wwPDB validation pipeline.



Additionally, running this new validation pipeline on the whole PDB archive creates a vast amount of data. We present a thorough analysis of the structure quality assessment by Molprobity for the whole PDB archive, comparing NMR and X-ray structures with respect to year of deposition and software package used for structure refinement.



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Small molecule compounds from *Salvia sclareoides* for use as novel therapeutics in Amyloid related diseases

Amy Irvine[1], Peter Davis[1], Catarina Dias[2], Nadia Makaya[2], Matthew Cliff[4], Andrea Hounslow[1], Abigail Williams[1], Jennifer Louth[3], Beining Chen[3], Amelia Rauter[2],Jon Waltho[1,4], Rosemary Staniforth[1]

[1]Department of Molecular Biology and Biotechnology, University of Sheffield, UK.
[2]Center of Chemistry and Biochemistry, Carbohydrate Chemistry Group – Faculdade de Ciências da Universidade de Lisboa, Ed C8, Piso 5, Campo Grande, 1749-016 Lisboa, Portugal
[3]Krebs Institute, Department of Chemistry, University of Sheffield, UK
[4] Manchester Institute of Biotechnology, University of Manchester, UK

Salvia species have been used in European and Chinese folk medicine as an antiseptic, treatment for memory loss and insomnia. The Staniforth group has been studying *Salvia sclareoides*, a native Portuguese aromatic herb related to common sage, for its medicinal qualities in treating amyloid related diseases.

Salvia sclareoides butanol and water extracts have been shown to disassemble preformed fibres of our amyloid model protein, Cystatin B, using a thioflavin T fluorescence spectroscopy assay and electron microscopy.

HSQC NMR has been performed with Cystatin B soluble protein and cellular human prion protein. Experiments with extracts from *Salvia* showed disappearance of residues specific to the dimer form of Cystatin B and the formation of species undetectable by NMR such as amorphous aggregate. In addition, some extracts showed attenuation of specific prion C-terminal residue resonances, suggesting a binding event.

NMR and mass spectrometry are currently being utilised to identify the active compound(s) within the extracts. NMR STD, DOESY and NOESY experiments are all being used to look at interactions between the extracts and Aβ monomers and oligomers. Ultimately a mechanism of activity will also be defined. Our current data suggests therapeutic activity could be a stabilisation of intermediates along the amyloid formation pathway.

Cell toxicity assays have also been performed with extracts from *Salvia*. An SMB PrP^{sc} expressing cell line in conjunction with a dot-blot assay has been used to show that *Salvia sclareoides* water extract is effective in reducing levels of PrP^{sc} in cells. Even at high concentrations, the MTT assay showed that the extracts were not toxic to the cells and, indeed, human neuroblastoma (SH-SY5Y) cells. Cell assays are now being developed to show that the extracts prevent toxicity of Amyloid β oligomers.

The development of novel therapeutics to treat amyloid disease is of paramount importance and research so far suggests that *Salvia sclareoides* extracts are a promising source of novel therapeutic compounds.

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Ben Johnson Masters Student University of Sheffield

Do amyloidogenic proteins compete with each other during fibrilisation?

Department of Molecular Biology and Biotechnology,
University of Sheffield, UK.

Protein misfolding has been implicated in a number of different pathologies, including Alzheimer's disease (AD), Huntington's Disease (HD) and Parkinson's Disease (PD). AD is characterised by the formation of large, extracellular protein aggregates composed predominantly of Aβ peptides, which also form toxic oligomeric species. Although it is accepted that aggregation of these peptides is likely to be responsible for neurotoxicity associated with AD, current data suggests the involvement of multiple amyloid-forming proteins in the pathophysiology of AD. Transthyretin (TTR), is a homotetrameric thyroid hormone transporter, associated with certain systemic amyloidoses such as Familial Amyloid Polyneuropathy (FAP) and Familial Amyloid Cardiomyopathy (FAC). However, it has been observed to inhibit Aβ fibril formation. It has also been noticed that there is an inverse relation between the amyloidogenic potential of TTR and affinity to bind Aβ peptide. TTR mutants (S85A & Wild-Type) with different monomer and tetramer stabilities were made by site directed mutagenesis and purified using pMMHa system. Thioflavin T fluorescence, SEC-HPLC assays and TEM were performed to characterize high molecular weight oligomers of TTR. Thioflavin T assays were used to examine the ability of these different species to suppress Aβ fibrilisation *in vitro*. 2D HSQC experiments were then conducted to aid the characterisation of the interactions between TTR and Aβ and to establish which conformer of TTR favours binding.

Catalytic intermediate of β-phosphoglucomutase trapped through mutation of the general base

Luke A. Johnson[A], Yi Jin[A], Andrea M. Hounslow[A], Matthew W. Bowler[B], Matthew J. Cliff[C] & Jonathan P. Waltho[C]

[A]Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, United Kingdom

[B]Structural Biology Group, European Synchrotron Radiation Facility F-38043 Grenoble Cedex, France

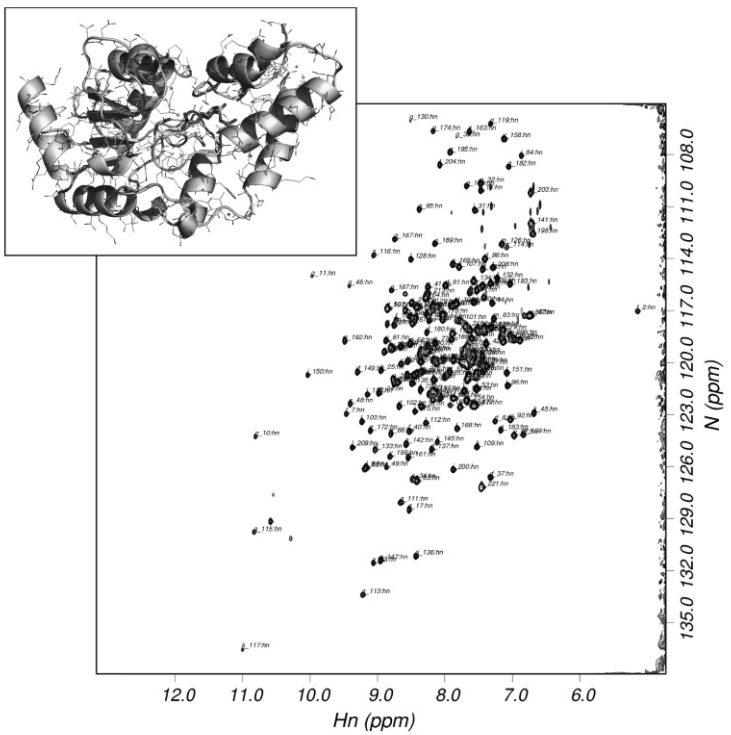
[C]Manchester Institute of Biotechnology, University of Manchester, Manchester M1 7DN, United Kingdom

E-mail: mba08lj@sheffield.ac.uk

Understanding how enzymes progress through their reaction coordinate is crucial to explaining the mechanisms by which they function. Beta-phosphoglucomutase (β-PGM) from *Lactococcus lactis* catalyses the interconversion of β-glucose-1-phosphate and β-glucose-6-phosphate through a β-glucose-1,6-bisphosphate intermediate. It is a well-studied phosphoryl transfer enzyme for which metal fluoride analogues have been indispensable in its investigation. These analogues form isoelectronic and isosteric mimics of both transition and ground states, providing stable complexes amenable for structural and spectroscopic investigation. However, it has been impossible to characterise β-glucose-1,6-bisphosphate intermediates with these techniques.

Through mutation of β-PGM general base Asp10 to Asn, we successfully formed a β-glucose-1,6-bisphosphate complex. The ^{1}H, ^{13}C and ^{15}N backbone resonances were assigned and an X-ray crystal structure was established. β-glucose-1,6-bisphosphate is found to orientate with the C1 phosphate in the active site. The structure represents a near attack conformer where Asp-8 is positioned for 'in-line' nucleophilic attack, and Asn10 δ2 amide hydrogen bonds to the bridging oxygen.

Kinetic investigation reveals that the D10N mutant is catalytically active. Utilising BEST-TROSY techniques it is apparent that upon exposure to substrates that a slow conformational change occurs resulting in trapping of the β-glucose-1,6-bisphosphate intermediate. The nature of this change remains to be addressed, but provides an intriguing observation which may provide further detail into the mechanism of β-PGM.



Dynamics of protein-ligand interactions – Impact on drug discovery

Outi Kamarainen[*ϟ], Geoff Holdgate[†], Andrew Scott[†], Steve Homans[#], Anastasia Zhuravleva[ϟ⍰], and Sarah Harris[*⍰]

[**]School of Physics and Astronomy, University of Leeds, E C Stoner Building, Leeds LS2 9JT

[ϟ]Faculty of Biological Science, University of Leeds, Garstang Building, Leeds LS2 9JT

[†]AstraZeneca, Discovery Sciences, Block 50, 50F49, Mereside, Macclesfield, Cheshire, SK10 4TG

[#]Faculty of Science, Agriculture and Engineering, Newcastle University, Newcastle upon Tyne, NE1 7RU

[⍰]Corresponding authors

EMAIL O.Kamarainen13@leeds.ac.uk

Introducing a new drug to market is an extremely lengthy and expensive process (typically 10-15 years and $1.2billion). Better understanding of how and why a drug molecule binds to a target and what changes in the structure and chemistry could improve the binding affinity may dramatically shorten the drug design process. In addition to structure-based approaches, in recent years the role of molecular motions in binding selectivity and efficiency has attracted increasing attention from drug design research. Molecular dynamics simulation of the drug and target protein is a potentially very powerful tool providing detailed atomic descriptions of movements and interactions of biological macromolecules and ligands. However, despite the great success for characterization of protein dynamics, MD simulations are still limited by two main challenges: the accuracy for MD characterizations of protein-ligand interactions is critically dependent on the force field and dynamic studies are limited by relatively short time scales (hundred ns – a few μs) of MD simulations. Indeed, whilst computer simulations have been used to obtain information on protein-drug binding (including estimations of individual contributions from different types of entropy), due to these limitations, it is ongoing debate how well the *in silico* methods represent experimental data. Consequently, extensive evaluation of different force fields using experimental data is an essential step for developing computational methods for estimating entropic (dynamic) contributions upon ligand/drug binding.

For this study, we use the well-characterized N-terminal domain of the chaperone protein Hsp90 as a model system to study the vibrational entropy of Hsp90 binding to different small-molecule inhibitors. We employ the NMR spectroscopy to validate the data from molecular dynamics simulations and to improve our *in silico* methods. The aim of this project is to develop a generic computational approach that can be used to predict dynamic features for different protein-ligand/drug systems and thus, to improve drug design and lead optimisations processes in pharmaceutical sector.

Visualization of Transient Protein-Protein Interactions that Promote or Inhibit Amyloid Assembly

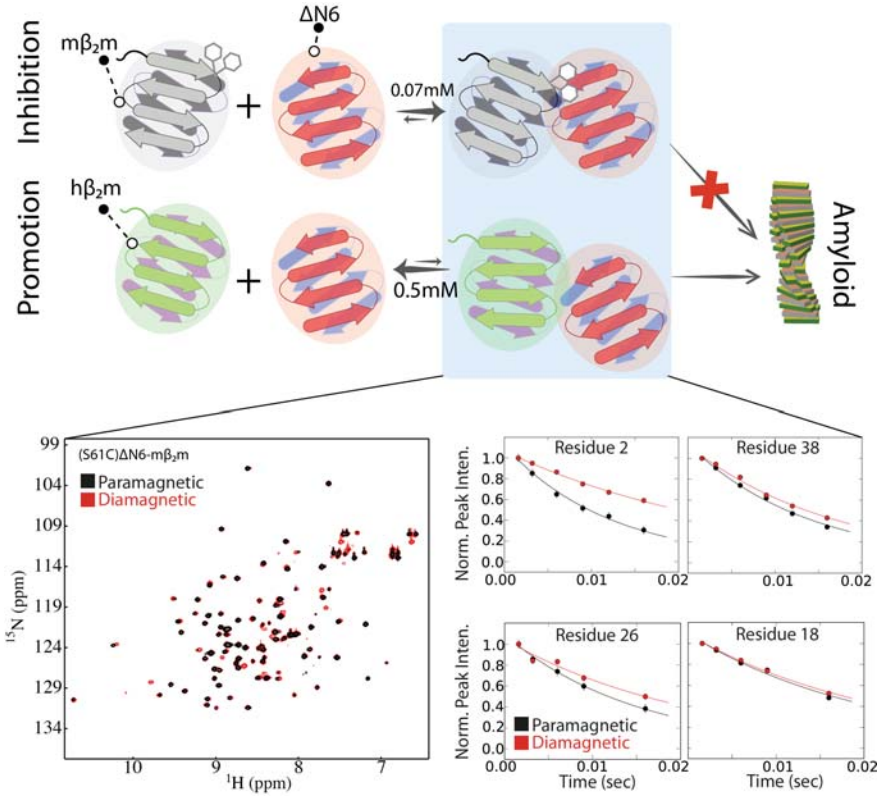
Theodoros K. Karamanos, Arnout P. Kalverda, Gary S. Thompson,

Sheena E. Radford

Astbury Centre for Structural Molecular Biology and School of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, UK

T.Karamanos@leeds.ac.uk

In the early stages of amyloid formation heterogeneous populations of oligomeric species are generated, the affinity, specificity and nature of which may promote, inhibit, or define the course of assembly. Despite the importance of the intermolecular interactions that initiate amyloid assembly, our understanding of these events remains poor. Here, using amyloidogenic and non-amyloidogenic variants of β_2-microglobulin (β_2m) we identify the interactions that inhibit or promote fibril formation in atomic detail. The results reveal that different outcomes of assembly result from biomolecular interactions involving similar surfaces. Specifically, inhibition occurs via rigid body docking of monomers in a head-to-head orientation to form off-pathway dimers. By contrast, the promotion of fibrillation involves relatively weak binding which results in conformational changes in the initially non-fibrillogenic partner. The results highlight the complexity of interactions early in amyloid assembly and reveal atomic level information about species barriers in amyloid formation.



"Different modes of binding of substituted hydroxyquinoline inhibitors in the active site of *Pseudomonas* γ-butyrobetaine hydroxylase (BBOX)- An SAR by NMR study"

"**Amjad Khan**, Ivanhoe K. H. Leung, Christopher J. Schofield, Timothy D. W. Claridge".

"Department of Chemistry, University of Oxford, Chemistry Research Laboratory, 12 Mansfield Road, Oxford, OX1 3TA".

"amjad.khan@chem.ox.ac.uk"

"γ-butyrobetaine hydroxylase (GBBH or BBOX) is a 2-oxoglutarate (2OG) and Fe (II) dependent oxygenase that catalyzes the biosynthesis of L-carnitine by hydroxylation of γ-butyrobetaine (GBB) in animals. Carnitine is essential for the transportation of long chain fatty acids from cytosol into mitochondria to produce metabolic energy (ATP) through TCA cycle. BBOX is a current inhibition target for the treatment of diseases, including cancer, ischemia, inflammation, anaemia and myocardial infarction. Most of the inhibitors of 2OG dependent oxygenase family are 2OG mimics competing for chelating Fe (II) in the enzyme active site.

Here we report a series of hydoxyquinoline derivatives as inhibitors of BBOX and their different modes of binding to the enzyme by NMR reporter ligand (GBB and 2OG) displacement assay. When the reporter ligand (s) is bound to the protein, significant broadening of its resonances is observed due to high transverse relaxation rates in the bound state. In the presence of a competitive binder, however, the reporter ligand (s) will be displaced, which will lead to sharpening and recovery of its signal (see figure below). To boost the sensitivity of line broadening, the native catalytic Fe (II) was substituted by paramagnetic Mn (II). These studies suggest that FG2216 inhibits BBOX by competing against only 2OG while FG2216 with a hydrophobic residue (substituent) in the side chain inhibits BBOX by competing against both GBB and 2OG. The assay is therefore potentially useful in determining the mode of inhibition of different classes of 2OG oxygenase inhibitors".

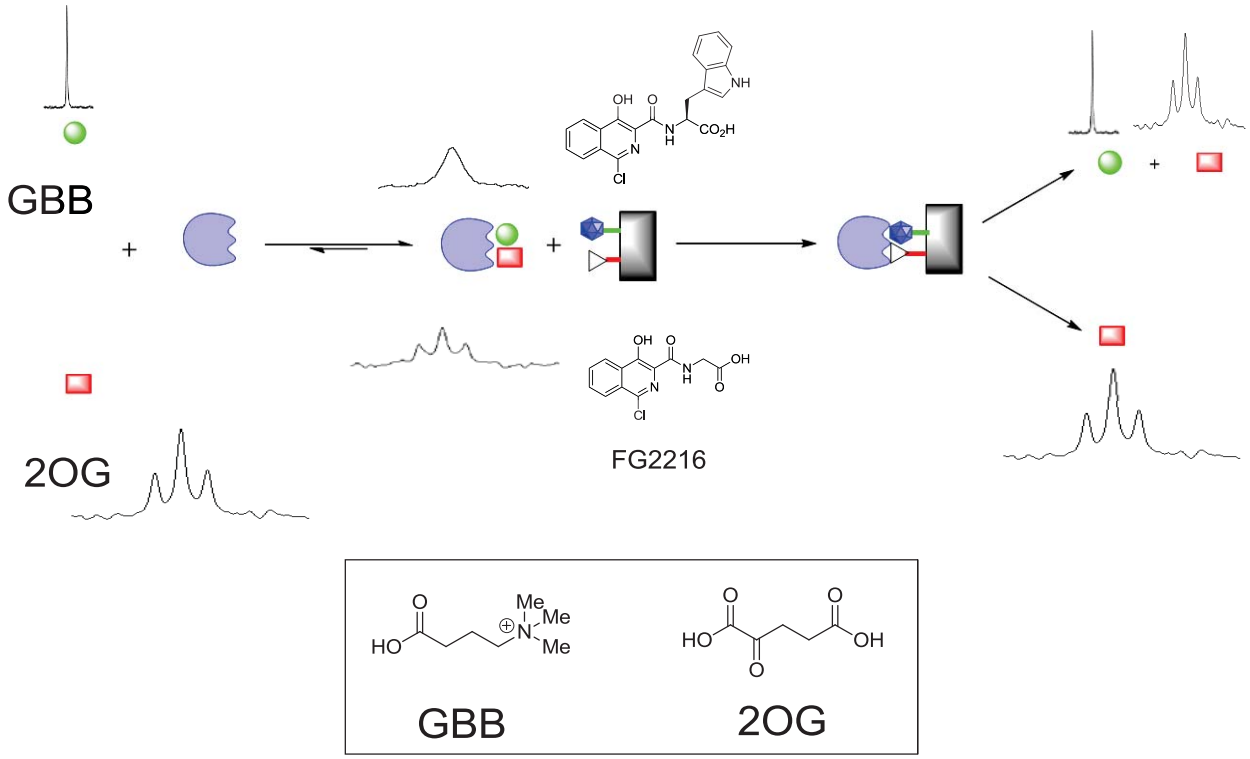


Figure - Principle of NMR reporter screening assay. When the reporter ligand is bound to the protein, the corresponding NMR signal is broadened and its intensity is lowered. In the presence of a competitive ligand, the reporter ligand is displaced from the protein binding site and its signal intensity is recovered.

Real-time pure shift ^{15}N HSQC with band-selective proton decoupling

Peter Kiraly*, Liladhar Paudel*, Ralph W. Adams*, Juan A. Aguilar*[‡], Mohammadali Foroozandeh*, Matthew J. Cliff[#], Péter Sándor[¥], Jonathan P. Waltho[#], Mathias Nilsson*[‖] and Gareth A. Morris*

*School of Chemistry, University of Manchester, Oxford Road, Manchester, M13 9PL (UK)

[‡]Department of Chemistry, Durham University, South Road, Durham, DH1 3LE (UK)

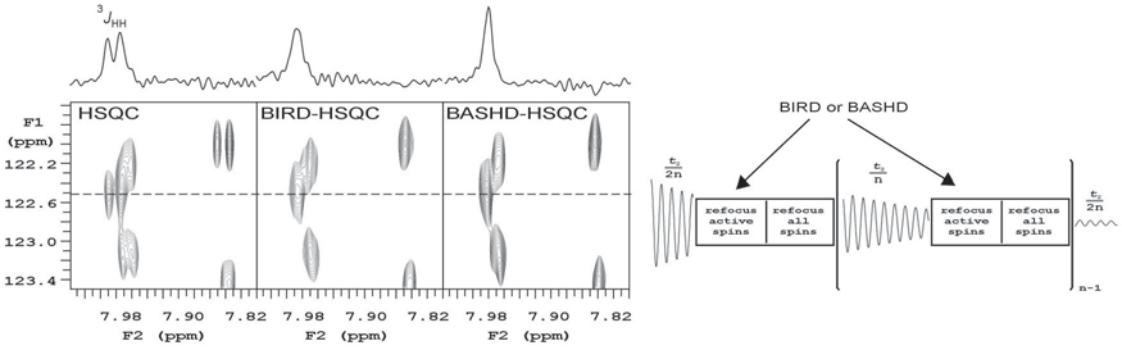
[#]Manchester Institute of Biotechnology, 131 Princess Street, Manchester, M1 7DN (UK)

[¥]Agilent Technologies R & D a. Marketing GmbH & Co. KG, Hewlett-Packard Strasse 8, 76337 Waldbronn (Germany)

[‖]Department of Food Science, University of Copenhagen Rolighedsvej 30, 1958 Frederiksberg C (Denmark)

EMAIL peter.kiraly.nmr@gmail.com

Band-selective proton decoupling has been used previously, to good effect, for improving the resolution of NMR experiments [1, 4-6]. Here we present a new real-time method that simultaneously improves both resolution and sensitivity in HSQC, illustrating it with data for uniformly ^{15}N-labelled proteins. The multiplet structure of amide proton resonances in the direct dimension is collapsed by collecting FIDs as chunks of data [2] interspersed with pairs of spin echoes, with chunk widths small compared to the inverse of the homonuclear coupling. The first echo uses a semi-selective pulse on the resonances to be observed (e.g. NH protons), while the second refocuses all spins. The net result is to refocus J modulation while leaving chemical shift evolution unchanged. The composite FID acquired in this way has in effect all homonuclear couplings to resonances outside the selected region suppressed. (This is in contrast to conventional band-selective methods, in which the selective irradiation is applied to the passive rather than to the active spins.) No special data processing is needed, and standard hardware may be used provided that it supports windowed acquisition. As all multiplet signals from one spin is collected in a single resonance the sensitivity is often increased even if there is a slight signal loss and line broadening from T_2 relaxation during the double echoes and from pulse imperfections. Similar pure shift HSQC spectra can be achieved using BIRD [3], but for ^{15}N HSQC the band-selective method is less sensitive to relaxation. Illustrative spectra of ubiquitin and beta-amyloid peptide 1-42 are shown.



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Determination of the individual roles of the linker residues in the interdomain motions of calmodulin using NMR chemical shifts

Predrag Kukic, Carlo Camilloni, Andrea Cavalli and Michele Vendruscolo*

*Chemistry Department, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW

Many protein molecules are formed by two or more domains whose structures and dynamics are closely related to their biological functions. It is thus important to develop methods to determine the structural properties of these multidomain proteins. Here, we characterize the interdomain motions in the calcium-bound state of calmodulin (Ca^{2+}-CaM) using NMR chemical shifts as replica-averaged structural restraints in molecular dynamics simulations. We find that the conformational fluctuations of the interdomain linker, which are largely responsible for the interdomain motions of CaM, can be well described by exploiting the information provided by chemical shifts. We thus identify ten residues in the interdomain linker region that change their conformation upon substrate binding. Five of these residues (Met76, Lys 77, Thr79, Asp80 and Ser81) are highly flexible and cover the range of conformations observed in the substrate-bound state, while the remaining five (Arg74, Lys75, Asp78, Glu82 and Glu83) are much more rigid and do not populate conformations typical of the substrate-bound form. The ensemble of conformations representing the Ca^{2+}-CaM state obtained in this study is in good agreement with residual dipolar coupling (RDC), paramagnetic resonance enhancement (PRE) and small-angle X-ray scattering (SAXS) measurements, which were not used as restraints in the calculations. These results provide initial evidence that chemical shifts can be used to characterize the conformational fluctuations of multidomain proteins.

A Fast and Robust Method to Re-weigh Populations of Protein Conformations

H.T. Alvin Leung*, Olivier Bignucolo*, Simon Bernèche*, Stephan Grzesiek*

*Biozentrum, University of Basel, Klingelbergstrasse 50/70, Basel, CH - 4056

EMAIL alvin.leung@unibas.ch

The determination of protein ensembles remains a challenging problem. Unfolded protein ensembles may be under-constrained due to the large ensemble size and few experimental data available. The use of restrained simulations and post simulation re-weighing are some of the common strategies to obtain a better agreement between the experimental and back-calculated NMR parameters. Here a fast and robust method based on convex optimisation and the principle of maximum entropy to re-weigh populations of conformations is presented. The method was applied to the 9-residue peptide MD simulation trajectory. It was shown that the helical content was slightly under-represented in the MD simulation. Also, only a small re-weighing was needed to give a better agreement with the experimental RDC values and the re-weighing energy was similar to the error of the force field.

NMR studies of an oxygen sensing enzyme

Ivanhoe K H Leung*, Adam P Hardy*, Francois-Xavier Cantrelle†, Isabelle Landrieu†, Christopher J Schofield* and Timothy D W Claridge*

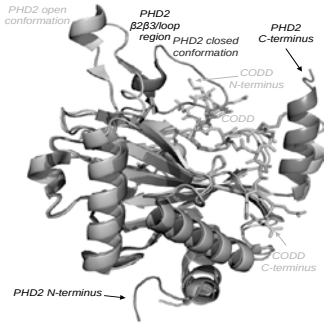
*Department of Chemistry, Chemistry Research Laboratory, 12 Mansfield Road, Oxford OX1 3TA, United Kingdom

† Unité de Glycobiologie Structurale et Fonctionnelle, CNRS UMR 8576, Université Lille 1, 59655 Villeneuve d'Ascq, France

EMAIL ivanhoe.leung@chem.ox.ac.uk

Prolyl hydroxylase domain containing protein 2 (PHD2) is a Fe(II) and 2-oxoglutarate (2OG) dependent oxygenase that is involved in a key step in human oxygen sensing as part of the response to limiting oxygen levels (hypoxia).[1] PHD2 initiates the oxygen dependent degradation of the α-subunit of hypoxia inducible factor (HIF-1α) by catalysing the hydroxylation of two conserved prolyl residues: proline-564, which is located in the C-terminal oxygen dependent degradation domain (CODD), and proline-402, which is located in the N-terminal oxygen dependent degradation domain (NODD). PHD2 has been studied extensively by protein X-ray crystallography both in its substrate-free and substrate (CODD)-bound forms.[2,3] The structures imply that PHD2 undergoes a large conformational change upon CODD binding. However, to date, there is no structural information about how NODD binds to PHD2. In addition, the molecular mechanisms of how PHD2 recognises and chooses its substrates and the roles and dynamics of the flexible regions in substrate recognition are still unknown.

I studied PHD2-substrate (CODD/NODD) interactions in solution by monitoring chemical shift perturbation. In addition, I have also studied PHD2 backbone amide relaxation. These experiments allow the identification of regions that are involved in CODD/NODD selectivity and further our understanding about the relation between the motion of PHD2 to its function and its substrate specificity.



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Characterisation of an endotoxin-sensing molecular switch

Karen McClymont, S. M. Kelly and B. Smith

Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow G12 8QQ UK

k.mcclymont.1@research.gla.ac.uk

Bacterial endotoxin is the lipopolysaccharide (LPS) found in the outer cell membrane of gram-negative bacteria. It is the first indicator of a gram-negative bacterial infection and is thought to be the primary cause of sepsis – a severe reaction to a systemic infection which can result in multiple organ failure and often death. The Limulus Amebocyte Lysate (LAL) test developed by Levin and Bang in 1968 is used for the detection of endotoxin. It is extremely sensitive to LPS, simple and reliable and is in high demand due to the lack of a more sensitive test.

Characterisation of the key proteins involved in binding endotoxin and a better understanding of the molecular details surrounding LPS is necessary to advance knowledge and develop new, highly sensitive biosensors to benefit pharmaceutical and medical device screening as the increase in demand for horseshoe crab blood has led to an unsustainable burden on the wild population of horseshoe crabs. My project will involve characterising factor C (Figure 1) to understand and exploit its recognition of LPS and to identify the conformational changes that activate the enzyme as a consequence of this. Factor C is a serine protease zymogen that works as a 'biosensor' in response to LPS. The protein binds LPS and becomes enzymatically active as a consequence. Such activation events must involve a conformational change in the enzyme in response to LPS binding.

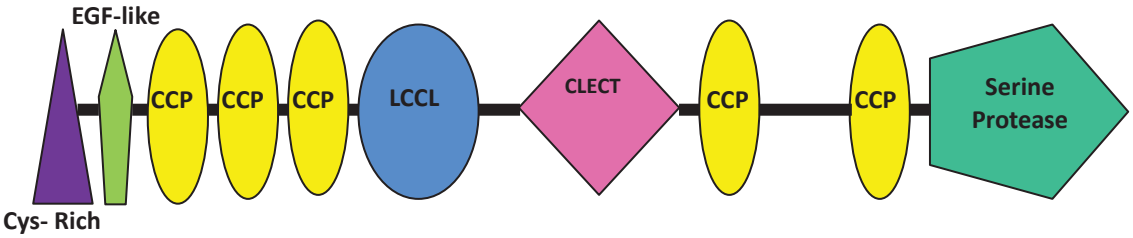

Figure 1: Diagrammatic representation of the domain structure of Factor C. In the mature protein, the N-terminal region of the heavy chain, made up of a Cys-rich domain, an EGF-like domain and three of five Complement Control Protein (CCP) domains is responsible for LPS recognition. The rest of the heavy chain includes another CCP, a C-type lectin domain and the LCCL domain. The light chain consisting of the C terminal CCP domain and the serine protease domain is cleaved to form the A and B chains.

The ultimate goal of this work will be to design a highly sensitive and specific novel endotoxin sensor to replicate the sensitivity of Factor C without the use of the enzymatic activity as a read out. This may help to decrease the chance of false negative or positive results that have prevented current synthetic factor C based LPS testing products from gaining regulatory approval along with their significantly lower differences in sensitivity (up to 300-fold) compared to LAL-based tests. In order to achieve this I aim both to purify full length Factor C to characterize how its conformation changes on binding LPS using low resolution techniques, and to express and purify smaller LPS-binding fragments to derive a detailed picture of how the protein recognizes LPS specifically. For both strands of the project I will have to develop methods that allow me to produce multi-milligram quantities of purified protein. Full length factor C will be purified in its native form from the amebocytes themselves as well as from recombinant sources, and biophysical techniques to determine its size and shape applied. LPS binding fragments will be purified exclusively from recombinant sources, and since the aim here is to determine three dimensional structures of the protein fragments in complex with LPS, expression systems that are the most convenient for the production of NMR and X-ray crystallography samples are being explored first.

Insights into infectious sheep prions by solid-state NMR spectroscopy

Henrik Müller*, Oleksandr Brener*, Olivier Andreoletti†, Timo Piechatzek*, Dieter Willbold*, Giuseppe Legname‡ and Henrike Heise*

* Institute of Complex Systems (ICS-6), Research Centre Jülich GmbH (FZJ), Jülich, Germany and Institute for Physical Biology, Heinrich-Heine-Universität Düsseldorf, Germany

† Interactions Hôtes Agents Pathogènes, Ecole Nationale Vétérinaire de Toulouse, France

‡ Department of Neuroscience, Scuola Internazionale Superiore di Studi Avanzati (SISSA), Trieste, Italy

henrik.muller@chem.ox.ac.uk

The infectious form of the mammalian prion protein (PrP^{Sc}) has been the cause of mad-cow disease as one of the major health crises in the United Kingdom in the last century. PrP^{Sc} is known to be formed by β-sheet rich amyloid fibrils. Its detailed structural architecture, however, is still elusive due to the failure of biophysical structure analysis on insoluble, non-crystalline, heterogeneous, and infectious protein fibrils. Since all model concepts available to date are based on low-resolution data, it is a current matter of intense debate which part of the PrP-sequence forms the β-sheet core, whether PrP^{Sc} contains β-helices[1] or β-sandwiches[2], and how the presence of infectious as well as non-infectious PrP-fibrils can be explained structurally. The rapid methodological developments of solid-state NMR in the last years established the possibility of applying NMR-spectroscopy also to solid biological macromolecules such as amyloid fibrils[3].

In our contribution, the first focus will be on explaining our protocols for the preparation of infectious samples of full-length ovine (ov) recombinant (rec) PrP^{Sc}(25-233) in NMR-sufficient amounts by seeded fibril growth from brain extract. Based on biophysical characterisation and bioassays in transgenic mice, we will demonstrate that our *in vitro*-generated ovrecPrP^{Sc} closely resembles prions in living organisms. Thereafter, we will report on our experimental progress in using high resolution solid-state NMR indicating that ovrecPrP^{Sc} is characterised by enough homogeneity to draw first structural conclusions. Firstly, our data indicate a semi-mobile N-terminus, some residues with secondary chemical shifts typical for α-helical secondary structure in the middle part between ~115 to ~155, and a distinct β-sheet core C-terminal of residue ~155. These findings enable distinction between current PrP^{Sc}-models. Secondly, by comparing fingerprint spectra we provide evidence that samples with different infectivity may not differ in the overall arrangement of secondary structure elements but rather in the flexibility of protein segments outside of the β-core region. Thirdly, structural order in ovrecPrP-fibrils can be improved by selectively amplifying a more homogeneous fibril structure from a heterogeneous initial state using a repeated seeding protocol.

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NMR investigations of the Y2 receptor: Providing selectively labelled receptor using cell-free expression

Paul Mueller*, Kristina Witte*, Frank Bernhard[#], Peter Schmidt*

*Institute for Medical Physics and Biophysics, Medical Department, University Leipzig, Härtelstrasse 16-18, D-04107 Leipzig, Germany

[#]Institute of Biophysical Chemistry, Centre for Biomolecular Magnetic Resonance, J.W. Goethe-University, D-60438 Frankfurt a.M., Germany

EMAIL paul.mueller@medizin.uni-leipzig.de

The Y receptors, a family of class A G protein-coupled receptors (GPCRs), are well known to be involved in numerous physiological processes (e.g. memory retention, food intake, energy homeostasis). In spite of their clinical relevance little is known about the structural basis of their highly divers functions. Here, we report the establishment of a cell-free expression system for human neuropeptide Y receptor type 2 (Y2R), which provides specifically labelled receptor in low milligram quantities for NMR experiments. So far, up to 600 µg/mL Y2R have been expressed, purified and reconstituted in DHPC/DMPC bicelles (q = 0.25). Expression yields were increased by an N-terminal Ser-tag (MKSSSSSG). Furthermore, it was shown by ligand binding assays as well as in G protein activation experiments that the reconstituted Y2R is functional. The Y2R will be used in NMR studies for further characterization in terms of its structure and even dynamics. Because the cell free expression allows for selective incorporation of isotope labelled amino acids the resulting NMR spectra are less complex in comparison to the fingerprints of uniformly labelled receptor samples. It is therefore possible to investigate specific receptor sites.

Generating a high affinity IGF2 receptor.

Ryan Nicholls[1], Christopher Williams[1], Susana Frago[2], Bassim Hassan[2] and Matthew Crump[1],

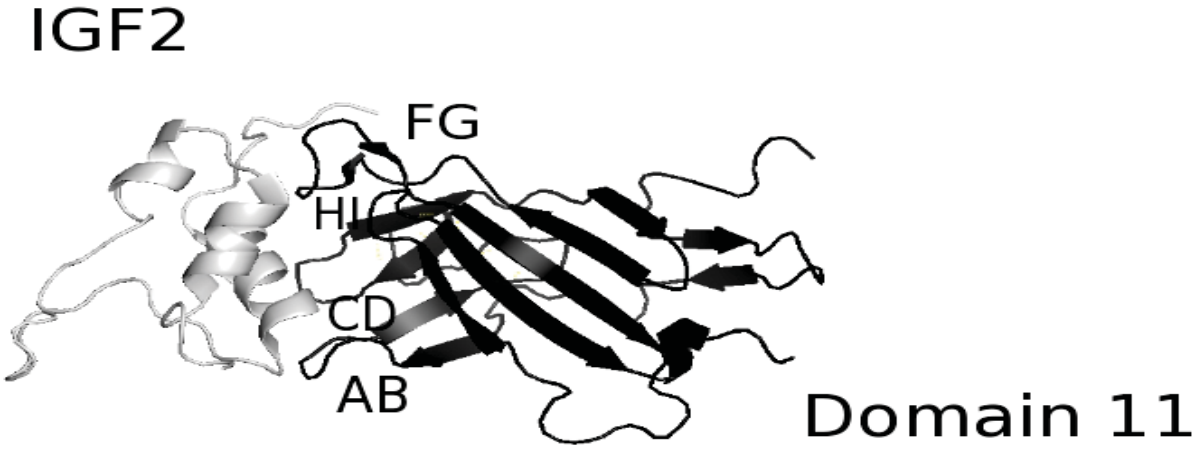
[1]Orgainc and Biological Section Chemistry, School of Chemistry, University of Bristol, Cantock's Close, Bristol. BS8 1TS

[2]Cancer Research UK Tumour Growth Control Group, Oxford Molecular Pathology Institute, Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE.

EMAIL: rn12576@bristol.ac.uk

The misregulation of the embryonic growth factor Insulin-like growth factor 2 (IGF2) has been linked to the growth and proliferation of many cancer cell types [1]. Here we seek a novel IGF2 targeting therapeutic through directed evolution of an existing IGF2 binder.

The Insulin-like growth factor 2 receptor (IGF2R), a 300kDa trans-membrane receptor composed of 15 homologous extracellular repeats, is involved in the transport and bio-availability of IGF2; as well as many other ligands, including mannose-6-phosphate tagged proteins. Domain 11 (D11) of IGF2R is responsible for IGF2 binding with assistance from domain 13 which increases D11 affinity for IGF2 [2]. D11 is composed of a β-barrel core with four loops (AB, CD, FG and HI) that form the IGF2 binding site. The assistive fibronectin insert of domain 13 interacts with the AB loop, changing its structure to generate higher affinity binding compared with lone D11. Previous work uncovered an AB loop mutant (E4) that had a 10 fold increase in IGF2 affinity, which was stable enough to generate a NMR structure of the IGF2-D11 complex [3].



Cartoon representation of domain 11 mutant E4 in complex with IGF2 (PDB:2L29), NMR structure, with associated binding loops labelled (AB,CD,FG and HI).

Herein we have continued to engineer a recombinant high binding affinity domain 11 for IGF2 by further mutating the binding loops of D11. This has been achieved by systematically mutating residues in the loops to generate increased electrostatic and hydrophobic interactions with the IGF2-domain 11 complex. This work has led to a >100 fold increase in affinity of D11 for IGF2 with the potential therapeutic applications in treating IGF2 related tumours. My current work is to fully understand the structural and dynamic basis for the increased IGF2 affinity.

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Dynamics and translational diffusion of Protein G at high concentrations by NMR spectroscopy

Stanislava Panova*, Alexander Golovanov*

* Faculty of Life Sciences, Manchester Institute Of Biotechnology, The University of Manchester, 131 Princess Street, Manchester, M1 7DN, UK

stanislava.panova@postgrad.manchester.ac.uk

Protein self-association is a process intrinsic for all proteins at high concentrations. It leads to increased viscosity, gelation and possible precipitation, which cause problems in protein manufacturing, stability and delivery. If protein drugs require high dosing, special approaches are needed. At high concentrations proteins experience conditions close to crystal state, therefore interactions in solution could potentially coincide with crystal lattice contacts. A range of diverse methods are used to study this process, but the complexity of the mechanism makes it hard to build a reliable model. Here, the self-association of streptococcal Protein G (PrtG) was studied using Nuclear Magnetic Resonance (NMR) spectroscopy in solution. The properties of protein-protein interactions at high concentration, up to ~ 200 mg/ml, were studied on residue-level resolution. Residue specific information on protein dynamics was obtained using ^{15}N relaxation measurements. The experiments were carried out at multiple concentrations. Variation in the rotational correlation time over these concentrations showed changes in the protein dynamics, which reflected oligomerisation processes occurring in solution. Pulsed-field gradient NMR spectroscopy was used to monitor translational diffusion coefficients in order to estimate the degree of protein self-association. Oligomer formation was also monitored by looking at variations in ^{1}H and ^{15}N amide chemical shifts. Future thorough analysis of the relaxation data and calculation of chemical exchange contributions will help to allocate specific sites involved in oligomer formation. Better understanding of protein self-association mechanisms under different conditions could assist in developing methods to reduce the level of reversible protein self-association in solution at high protein concentrations.

Quantifying the Interaction of α-Synuclein with the Ribosome Surface by NMR Spectroscopy.

Jack Paton Annika Weise, Lisa D. Cabrita, John Christodoulou and Christopher A. Waudby

Institute of Structural and Molecular Biology, UCL and Birkbeck College, Gower Street, London, WC1E 6BT

j.paton.12@ucl.ac.uk

Understanding protein folding as it occurs during translation is crucial for understanding the *in vivo* process, and the biological implications of the presence of the ribosomal particle on the emerging polypeptide. This area has been studied in great detail using numerous biophysical methods, such as force microscopy [1], fluorescence anisotropy decay measurements [2] and NMR spectroscopy [3, 4]. NMR in particular presents the advantage of providing residue specific information on co-translational events, and has been used to show the presence of interactions between the vectorially emerging polypeptide and the ribosomal surface.

These surface interactions are of great importance in modulating the folding pathway of the translating protein, however our understanding of their nature is limited in terms of important thermodynamic and kinetic information. Hence, to complement these investigations, we have carried out titrations between 70S bacterial ribosomes and the isolated, ^{15}N labelled form of a model disordered protein, α-synuclein. Using ^{1}H-^{15}N HSQC data, we report on line-broadening effects that can be attributed to an interaction between synuclein and the ribosome surface, and show, using ^{1}H spin-echo relaxation measurements, that this broadening is consistent with a simple 1:1 interaction model. This reveals a weak interaction (K_d > 1 mM) with fast kinetics close to the diffusion limited association rate. Thus, we have shown that this approach is able to characterise extremely weak ribosome-protein interactions at a residue specific level. We also note that the observed K_d is comparable to the effective concentration of tethered nascent chains during translation, and therefore even such apparently weak interactions have the potential to significantly perturb the co-translational folding process.

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Resolution and sensitivity enhancement by real-time pure shift HSQC

Liladhar Paudel*, Ralph W. Adams*, Péter Király*, Juan A. Aguilar*[‡], Mohammadali Foroozandeh*, Matthew J. Cliff[#], Péter Sándor[¥], Jonathan P. Waltho[#], Mathias Nilsson*[‖] and Gareth A. Morris*

*School of Chemistry, University of Manchester, Oxford Road, Manchester, M13 9PL (UK)

[‡]Department of Chemistry, Durham University, South Road, Durham, DH1 3LE (UK)

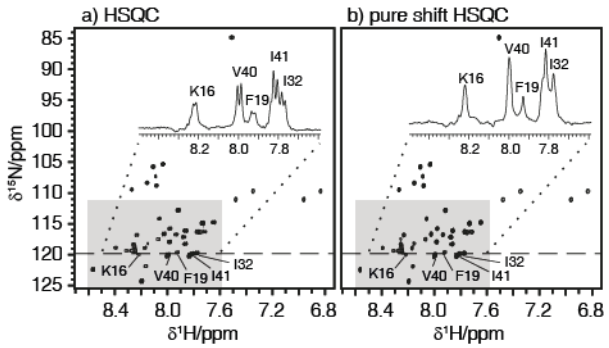
[#]Manchester Institute of Biotechnology, 131 Princess Street, Manchester, M1 7DN (UK)

[¥]Agilent Technologies R & D a. Marketing GmbH & Co. KG, Hewlett-Packard Strasse 8, 76337 Waldbronn (Germany)

[‖]Department of Food Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C (Denmark)

EMAIL liladhar.paudel@manchester.ac.uk

A pulse sequence for acquiring F_2-pure shift HSQC spectra in real time is described, in which ^{1}H-^{1}H broadband decoupling is achieved using BIRD pulse trains [1,2]. FIDs for each t_1 increment are acquired in real time by windowed acquisition of chunks of data between BIRD elements in explicit sampling mode. J_{HH} evolution from one chunk to the next is refocused by a hard 180°/BIRD sequence element (except in the case of geminal methylene protons), and the effect of J_{XH} is suppressed by broadband heteronuclear decoupling. Homodecoupling is effective as long as the time between refocusing elements is $<<1/J_{HH}$ [3]; more frequent refocusing gives cleaner spectra, but at the expense of some line broadening due to the signal losses imposed by imperfect pulses and T_2 relaxation. Here, we show that this homonuclear decoupling acquisition scheme in HSQC gives both resolution and sensitivity enhancement. This new method is well-suited to ^{1}H-X (X=^{13}C or ^{15}N) correlations at natural abundance, as well as to labeled systems where labels are sparse. As illustrated with the ^{1}H-^{15}N correlated spectra shown below of ^{15}N-labeled beta-amyloid peptide 1-42 (Aβ) [4], the method is potentially useful in reducing signal overlap in spectra of macromolecular systems such as proteins and nucleic acids.



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Structural insights into RNA recognition by the human microRNA biogenesis protein TRBP

Matthieu P. Benoit [(1)], Christine Ebel [(1)], Lionel Imbert [(1)], Andrés Palencia [(2)], Julien Pérard [(3)], Jérôme Boisbouvier [(1)] and **Michael J. Plevin [(1,4)]**

(1) Institut de Biologie Structurale, CNRS/UJF/CEA, Grenoble, France

(2) European Molecular Biology Laboratory, Grenoble, France

(3) Unit of Virus Host-Cell Interactions, CNRS/UJF, Grenoble, France

(4) Department of Biology, York Structural Biology Laboratory, University of York, UK

michael.plevin@york.ac.uk

Micro-RNAs (miRNA) are small non-coding RNAs that regulate gene expression through RNA interference (RNAi). Human miRNAs are generated via a series of enzymatic processing steps. The precursor miRNA (pre-miRNA) is recognized and cleaved by a complex containing the RNase III enzyme, Dicer, and several non-catalytic accessory proteins. HIV TAR element binding protein (TRBP) is a constituent of the Dicer complex which augments complex stability and potentially functions in substrate recognition and product transfer to the RNA-induced silencing complex (RISC). Here we have analyzed the interaction between the RNA-binding region of TRBP and the oncogenic human miRNA, miR-155, at different points in the miRNA biogenesis pathway. We show that the region of TRBP that binds miRNA precursors comprises two independent double-stranded RNA binding domains (dsRBDs) connected by a long flexible linker. No evidence of contact between the two dsRBDs was observed either in the apo- or RNA-bound state. Pre-miR-155 interacts non-co-operatively with the RNA-binding region of TRBP to form a complex with two protein molecules per RNA. Finally, we determined that the RNA-binding region interacts with pre-miR-155 and miR-155/miR-155* via the same binding surfaces and with similar affinity, suggesting that TRBP could function before and after processing of pre-miRNAs by Dicer.

Structural Studies of the Variable Heavy Domains of Heavy Chain Antibodies

C. E. Prosser[*,†], L. C. Waters[†], L. M. Griffin[*], F. W. Muskett[†], V. Veverka[†], P. W. Addis[†], T. S. Baker[*], A .D. G. Lawson[*], A. J. Henry[*], R. J. Taylor[*] and M. D. Carr[†]

* UCB, 208 Bath Road, Slough, SL1 3WE, United Kingdom.

† Department of Biochemistry, Henry Wellcome Building, University of Leicester, Lancaster Road, Leicester, LE1 9HN, United Kingdom

EMAIL cep23@le.ac.uk

Complementarity determining regions (CDRs) are responsible for the affinity and specificity of antibody-antigen recognition. Camelid heavy chain antibodies (HCAbs) are found alongside conventional antibodies in camels, llamas and some species of shark. Unique features of HCAbs, which make them an attractive tool for probing antibody-antigen interactions, include:

- Longer CDRs than conventional antibodies, thought to provide greater diversity in antigen binding
- All antigen binding functionality encoded into the CDRs of a single domain, the variable heavy domain of the HCAb (termed the VHH domain)

We have used NMR-based approaches to solve the solution structures of several representative anti-IL-6 VHHs with differing CDR lengths to gain an insight into behavior of the CDRs and how this may influence antigen recognition. Chemical shift-based experiments have been used to compare the effects of the VHH and conventional antibodies binding to equivalent functional sites on IL-6.

The results show that VHHs may be an attractive alternative to using Fab or scFv fragments to map binding sites and guide drug discovery.

Measurement of protein reduction potentials using ^{1}H-^{15}N HSQC NMR

Michelle Rowe, Samantha Taylor†, Harriet Crawley-Snowdon*, Jane Wagstaff*, Mark Shepherd, Richard Williamson and Mark Howard

School of Biosciences, University of Kent, Giles Lane, Canterbury, CT2 7NJ

† Current address: Department of surgery and cancer, Imperial College, South Kensington Campus, London SW7 2AZ

* Current address: MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge CB2 0QH

EMAIL m.rowe@kent.co.uk

In order to function properly a protein must adopt the correct fold and in many proteins the formation of native structure is aided by the presence of disulphide bonds. Proteins belonging to the protein disulphide isomerase (PDI) family of proteins assist in the formation and rearrangement of disulphide bonds in substrate molecules, thereby ensuring they adopt the correct structure.

The determination of the reduction potential of a protein correlates with its function *in vivo* and, as in the case of PDI, the redox potential can be delicately poised to allow rapid cycling between oxidised and reduced forms that facilitate formation and isomerisation of disulphide bonds. Conventional methods of redox analysis has involved monitoring tryptophan fluorescence, using HPLC, or radiolabelling to quantify relative amounts of oxidised and reduced forms of the protein. However these methods can only be applied to proteins with one active site. The measurement of the redox potential of PDI that contains two redox active sites has been successfully determined using a differential alkylation approach followed by mass spectrometry.[1]

We have used NMR spectroscopy as a tool for the simple measurement of reduction potentials of redox active proteins by following multiple ^{15}N HSQC protein resonances.[2] Analysis of a titration series using mixtures of oxidised and reduced glutathione enables the ratio of oxidised or reduced protein present to be monitored. The fraction reduced has then been used to produce Hill plots and the reduction potential determined using the Nernst equation. If resonance assignments are available it is possible to differentiate between multiple redox active sites present in the protein under investigation. In addition data redundancy is generated where the change in oxidation state affects residues in proximity to the disulphide bond. This method gives comparable results to those achieved using current methods as well as being simple, rapid and non-destructive.

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Transient structure and dynamics of HDAC2: How does an intrinsically disordered domain regulate protein function?

Andrea C. Sauerwein, John Kirkpatrick, and D. Flemming Hansen

Institute of Structural and Molecular Biology, University College London, Gower Street, London, WC1E 6BT

EMAIL a.sauerwein@ucl.ac.uk

Histone deacetylases (HDACs) are modification enzymes that catalyse the removal of acetyl groups from the lysine ε-amino group. Their action is balanced by histone acetyl transferases (HATs) which add acetyl groups to the lysine ε-amino group. This interplay between HDACs and HATs makes them key players in eukaryotic gene expression. Whilst HDACs regulate other proteins, they are also regulated themselves by means of subcellular localisation, association with other proteins into multisubunit complexes, and posttranslational modifications.

In human histone deacetylase 2 (HDAC2) many posttranslational modifications are located in the mainly intrinsically disordered C-terminal domain, which is also referred to as the regulatory domain. To understand how this intrinsically disordered domain (150 AA) can regulate protein function it is essential to acquire a detailed knowledge of its structure in the absence and presence of posttranslational modifications. Following a challenging chemical shift assignment transient structural elements and transient interactions were quantified using a combination of chemical shifts, residual dipolar couplings, relaxation experiments, and spin-labelling. Of particular interest is that, when a virus phosphorylation of the C-terminal HDAC2 tail is mimicked by site-directed mutagenesis, significant changes are observed in the transient long-range interactions of this very dynamic species.

Characterization of the allosteric landscape for the ER 70 kDa molecular chaperone BiP

Saeid Shahidi*, Anastasia Zhuravleva*

*Faculty of Biological Science, University of Leeds, Garstang Building, Leeds LS2 9JT

EMAIL: s.shahidi@leeds.ac.uk

Study and manipulation of protein homeostasis and the stress response raise the possibility of altering many pathological processes, including neurodegenerative diseases, diabetes and cancers. Our research deals with understanding the molecular mechanisms of stress responses in the endoplasmic reticulum (ER) and is focused on a central player in the ER homeostasis network, a 70 kDa heat-shock protein called BiP. Similar to other Hsp70s, BiP contains two domains, the N-terminal nucleotide-binding domain (NBD) and the substrate-binding domain (SBD), which is further subdivided into a β-sandwich subdomain (βSBD) and an α-helical domain (αLid). The majority of BiP chaperone functions relies on the ability of the SBD to bind and release protein substrates under the tight nucleotide-dependent control of the NBD. In turn, substrate binding to the SBD regulates the ATP hydrolysis rate of the NBD. Several factors including posttranslational modifications, BiP controllable aggregation, interactions with other components of ER protein control network and binding to different substrates have been shown to play a key regulatory role for BiP chaperone activity and significantly impact BiP functions *in vivo.*

Our project aims to understand the molecular details on how the key BiP function (i.e., substrate binding and release under control of ATPase hydrolysis) is regulated *in vitro* and *in vivo*. Recently, we examined similar ligand-driven structural changes and conformational flexibility in *E. coli* Hsp70, DnaK and elucidated a detailed mechanism by which an allosteric signal was transferred between its two domains: SBD and NBD (Cell 2012 and PNAS 2011). To tackle the BiP multicomponent and dynamic chaperone system, we are using a similar 'divide-and-conquer' approach that combines several biophysical tools (i.e., ITC, size-exclusion chromatography and mass spectrometry), molecular biology methods, structural techniques (mostly NMR, but also X-ray and cryo-EM), the evolutional sequence analysis and computational modelling. Our preliminary results suggest that despite the high sequence similarity between human BiP and DnaK, BiP has several unique features. Particularly, small sequence variations between these two proteins result in distinct effects on the BiP conformational landscape. Moreover, BiP aggregation plays an important 'buffering' role to control the BiP allosteric cycle. We believe that the outcomes of this project will provide a novel view of the human chaperone network and open a new avenue in the design of new therapies.

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Solvent and J-modulation Suppression in PFGSTE Diffusion Measurements

Davy Sinnaeve*

* NMR and Structure Analysis Unit, Department of Organic Chemistry, Ghent University, Krijgslaan 281 S4, B-9000 Gent, Belgium

EMAIL Davy.Sinnaeve@UGent.be

The measurement of molecular translational diffusion in solution by NMR pulsed field gradient experiments has become an established methodology with a broad range of applications, such as the study of intermolecular interactions or complex mixture analysis. One of the greatest sources of error during the extraction of diffusion coefficients is spectral overlap between peaks of different compounds. It has been shown before that homonuclear scalar coupling evolution during the pulse sequence is particularly notorious in this respect, as this increases resonance line-width due to dispersive line-shape contributions. [1] These J-modulation artefacts result in increased peak overlap, leading to either higher or lower erroneous diffusion coefficients, depending on whether the overlap occurs with the positive or negative tail of the dispersive peak, thus complicating subsequent 2D DOSY plot analysis.

When solvent suppression is required in diffusion experiments, the most successful schemes that have previously been applied are WATERGATE and excitation sculpting. [2,3] However, the long spin echoes introduced by these schemes to accommodate the soft rf-pulses lead to significant scalar coupling evolution. Recently, it has been brought to the attention that the use of rf-pulses with a phase orthogonal to the in-phase magnetization can cancel out the anti-phase magnetization, effectively suppressing J-modulation line-shape artefacts. [4,5] This method has already been applied in solvent suppression ("Perfect-Echo WATERGATE"). [6] Here, a new PFGSTE-based diffusion NMR experiment based on the Perfect-Echo WATERGATE is presented that suppresses both the solvent peak and homonuclear J-modulation artefacts. An excellent quality solvent suppression next to near-perfect in-phase peak line-shapes is achieved.

Some attention will be given on to how to avoid artefacts affecting the quality of the solvent suppression resulting from the interference between the diffusion encoding/decoding gradients and the coherence selection gradients.

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XCamshift: Initial Results from a Cython Implementation of the Camshift Chemical Shift Force-field

Gary S. Thompson Astbury Centre for Structural Molecular Biology,
University of Leeds Leeds LS2 9JT UK.

The use of chemical shifts alone for the calculation of structures has provided both a simpler way to calculate folds of proteins and also provides a tool for calculating structures of transient states, which are not accessible from traditional restraints such as nOes. Camshift is the only currently available implementation of a force-field which uses chemical shifts to efficiently restrain the calculation of structures during molecular dynamics simulations. XCamshift and the accompanying chemical shift force-field framework Locsmith provide a complete implementation of the Camshift force-field in the popular and frequently used xplor nih package . Xcamshift uses the cython dialect of python to provide a force-field written in the python scripting language that is familiar to the users of xplor-nih, but at the same time works at the same speed as native C code.

Results from the latest version of xcamshift, will be used to:

1. Investigate the speed of Xcamshift energy and force calculations by comparing them with other force-field components such as non bonded interactions so as to demonstrate the efficiency of using chemical shifts in structure calculation protocols.
2. Demonstrate the use of the Xcamshift in restraining nOe poor structure calculations
3. Provide an overview of an initial implementation of an ensemble-averaged version of the force-field. This implementation is suitable for the analysis and restraint of chemical shifts in regions of proteins which are dynamic in nature and whose measured shifts are an average from a number of states
4. Look at the effect of scaling terms within the force-field during molecular dynamics based structure calculations so as to avoid local minima

Structural and Mechanistic Insights into the FusB Family of Fusidic Acid Resistance Proteins

Jennifer H. Tomlinson, Gary Thompson, Arnout Kalverda, Anastasia Zhuravleva, Steve Homans and Alex J. O'Neill

School of Cell and Molecular Biology, Faculty of Biological Sciences, Garstang Building, University of Leeds, Leeds, LS2 9JT

EMAIL j.h.tomlinson@leeds.ac.uk

The antibiotic fusidic acid inhibits protein synthesis in the bacterial pathogen *Staphylococcus aureus* by blocking release of the translocase, elongation factor G (EF-G), from the ribosome. Resistance to fusidic acid is most commonly mediated by proteins of the FusB family, which bind to EF-G and protect it from the inhibitory effect of fusidic acid, although the mechanism by which FusB-type proteins interact with EF-G to mediate this resistance is not fully understood. In this study NMR has been used to characterise the interaction between a truncated form of EF-G and FusB. Chemical shift perturbation analysis has been used to locate the binding site for FusB in domain 4 of EF-G while loss of amide signals from domain 3 on binding suggest that FusB has a significant long-range effect on the dynamics of this domain. Amide RDC measurements show reorientation of domains 4 and 5 with respect to each other upon FusB binding while the structure of each domain remains unchanged. RDC measurements for both binding partners have allowed us to determine the likely relative orientation of the two proteins, permitting docking studies to be performed between the crystal structures. PRE measurements provide distance restraints across the protein-protein interface to refine the docked structure, building towards a model of the EF-G-FusB interaction. An understanding of this protein-protein interaction will provide insights into this clinically-important mechanism of antibiotic resistance.

Probing Ras: Sos: nucleotide interactions using Nuclear Magnetic Resonance

Uybach Vo[1], Kevin Embrey[2], Alexander Breeze[2], Alexander Golovanov[1]

1 U. Vo, A.P. Golovanov: *Faculty of Life Sciences and Manchester Institute of Biotechnology, The University of Manchester, 131 Princess Street, Manchester M1 7DN, UK*

2 K.J. Embrey, A.L. Breeze: *Discovery Sciences, AstraZeneca Research & Development, Mereside, Alderley Park, Cheshire, SK10 4TF, UK.*

Ras proteins are mutated in 30% of all human tumours contributing to several malignant phenotypes including abnormal cell growth. The activity of Ras is partly regulated by the binding of guanine nucleotide exchange factors, such as Sos. The mechanism of Ras activation via its interactions with Sos remains unclear making it a challenging system for effective drug targets. The aim of this work is to understand the molecular interactions of the Ras: Sos complex supported mainly by Nuclear Magnetic Resonance spectroscopy (NMR). Comprehensive signal assignments in the NMR spectra of Ras, which we have completed, allows observations of the changes to specific residues in the spectra upon Ras binding to its binding partner e,g, Sos. The sequence-specific signal assignments of K-Ras by NMR have provided details on important binding site regions of K-Ras that were missing in previous literature. This has allowed us to identify binding site hotspots in the NMR spectra of Ras upon interactions with Sos. To gain a further understanding into the binding events of the Ras: Sos complex, we carried out a series of NMR-titration experiments, whereby increasing concentrations of Ras were added to Sos and analysed by NMR. Analysis of these NMR spectra enables us to monitor signals at the Ras: Sos binding sites under near physiological conditions.

Mapping the Co-translational Folding Energy Landscape of the ddFln5 Immunoglobulin Domain

Christopher A. Waudby*, Maria-Evangelia Karyadi*, Sammy Chan*, Julian Schmidt-Engler*, Tomek Wlodarski*, Carlo Camilloni†, Michele Vendruscolo†, Lisa D. Cabrita* and John Christodoulou*

*Institute of Structural and Molecular Biology, UCL and Birkbeck College, Gower St, London, WC1E 6BT

†Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW

EMAIL c.waudby@ucl.ac.uk

Efficient protein synthesis and folding, avoiding the population of misfolded or aggregation-prone states, is critical to healthy cell function. During protein synthesis, nascent polypeptides are gradually extruded through the ribosome exit tunnel, and folding may therefore be initiated at the N-terminus of the protein and proceed in a vectorial manner before the C-terminus has fully emerged from the ribosome. This process cannot be described by a single free energy landscape or folding funnel, for the configurational space accessible to the nascent chain increases with the length of the polypeptide chain, and intermediate states may become favored and disfavored as the chain emerges. Therefore, the co-translational 'landscape' must instead be conceptualized as a nested series of landscapes spanning increasingly large conformational spaces. Developing a quantitative understanding of these surfaces, and understanding the perturbations that arise from the presence of the ribosome and associated factors, is currently a major experimental challenge that is currently being addressed by a variety of methods. Within our own group, we have pioneered NMR spectroscopy as a tool to study the length-dependent folding of stalled ribosome-nascent chain complexes [1], focussing particularly on the ddFln5 immunoglobulin domain from the *Dictyostelium discoideum* tandem repeat protein ABP-120 [2].

A complementary experimental approach to the study of stalled ribosome-nascent chain complexes is the use of N-terminal protein fragments to create a ribosome-free model of co-translational folding [3]. This has the particular advantage that the polypeptide fragments may be studied at high concentrations using high-resolution spectroscopic and biophysical methods to accurately characterize the onset of folding in structural and thermodynamical detail. Here we present such a series of N-terminal fragments of ddFln5, revealing a series of folding intermediates that we have characterized extensively using a combination of CD spectroscopy, real-time NMR, EXSY, diffusion and ^{15}N relaxation and relaxation dispersion measurements, and RDC and chemical shift based structure determination. These intermediates are related to the formation of long-range contacts within the protein, and to the isomerisation of a highly conserved native state *cis*-proline residue. These results provide an essential description of the 'ground state' co-translational landscape, against which perturbations in stalled ribosome-nascent chain complexes may be discerned and more fully understood.

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Characterisation of structural and dynamic properties of trigger factor dimerisation

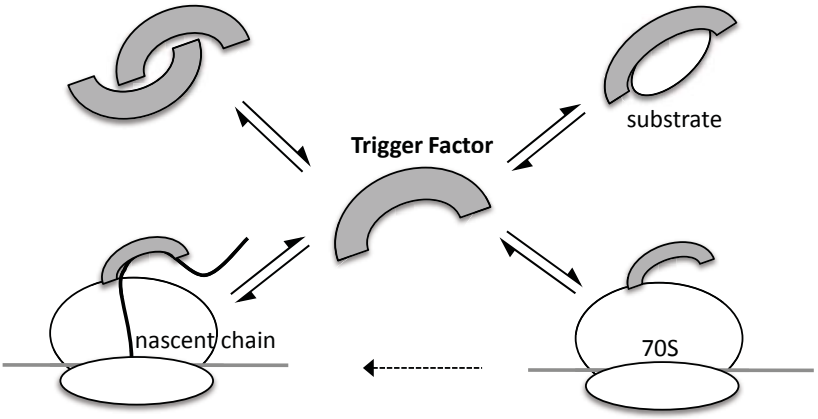
Anne S. Wentink, Christopher A. Waudby, Lisa D. Cabrita and John Christodoulou

Institute of Structural and Molecular Biology, UCL and Birkbeck College, Gower Street, London, WC1E 6BT

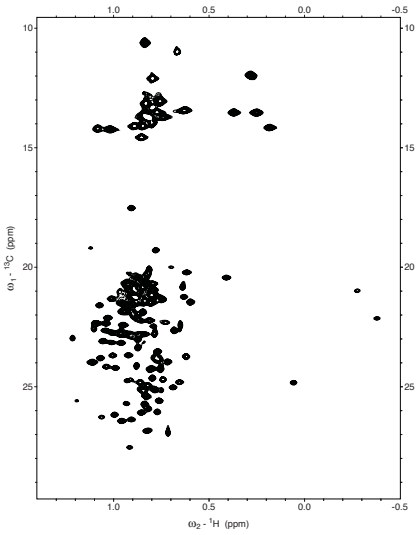
a.wentink@ucl.ac.uk

Trigger factor (TF) is a 48kDa ribosome-associated molecular chaperone involved in the early folding events of nascent polypeptide chains at the ribosomal exit tunnel. Within the bacterial cell, TF exists as a homodimer in a dynamic equilibrium between various substrate-bound states. TF interacts with the ribosome with micromolar affinity, preferring however active, translating ribosomes but has also been found to interact with isolated hydrophobic protein substrates. Although most interactions with substrates occur through monomeric TF, the TF dimerisation reaction may play an important role in regulating interaction with substrates by competing with weaker contacts and to act as a storage form of excess TF in the absence of high affinity substrates. The ongoing competition between substrate interaction and dimerisation might form the basis of a TF release mechanism from folding competent nascent chain client proteins at the ribosome surface.

The structure of the TF dimer has previously been characterised by X-ray crystallography, but differences across the obtained structures suggest the dimer interface is highly dynamic. No structures of the TF monomer have thus far been determined. Here, we therefore use NMR to investigate the dimerisation of the TF chaperone molecule using a range of NMR techniques to probe the solution structure of the TF dimer, and to characterise the thermodynamics and kinetics of the dimerisation process. This study provides a residue specific understanding of the TF dimerisation interface, which helps to elucidate the multi-step mechanism of the dimerisation reaction. We also report a first look at the NMR properties of the TF monomer in solution, which has thus far been elusive to other structural techniques.



Trigger Factor five state equilibrium



TF ^{1}H-^{13}C HMQC spectrum

Ions and side-chains: probing functional hotspots in histone deacetylase 8

Nicolas D. Werbeck*, John Kirkpatrick*, Havva Yalinca* and D. Flemming Hansen*

*Institute of Structural and Molecular Biology, University College London, Gower Street, London WC1E 6BT

EMAIL: n.werbeck@ucl.ac.uk

Histone deacetylases (HDACs) play a key role in a variety of cellular events including transcription, protein degradation and apoptosis. By deacetylating lysine side chains of their target proteins – histones and non-histone proteins - they constitute the counterparts of histone acetyltransferases (HATs). In this project we study human HDAC8 (42 kDa) as a model system to investigate the mechanism, activity and regulation of histone deacetylases from a structural and dynamical perspective using nuclear magnetic resonance (NMR) experiments. We make use of established NMR-experiments and extend the experimental repertoire to probe side-chains and ions in functionally relevant parts of HDAC8.

Cystatin C and Alzheimer's Disease

Abigail Williams, Adham Elshawaihde, Emma Keeley, Andrea Hounslow and Rosemary Staniforth
Department of Molecular Biology and Biotechnology, University of Sheffield, UK

The production of insoluble protein aggregates (amyloid) is characteristic of many neurodegenerative diseases. Alzheimer's disease (AD) is the most common cause of dementia, and is defined by amyloid plaques of amyloid β (Aβ) protein. Both fibrils and on-pathway oligomeric species are thought to be toxic. Aggregation of Aβ is modulated in the presence of other amyloidogenic proteins, including human cystatin C (hCC), a cysteine-protease inhibitor which can cause a hereditary form of cerebral amyloid angiopathy (CAA). Co-deposition of the two proteins is seen in parenchymal and vascular amyloid deposits in the brains of AD patients, and co-localisation has been shown at the cell surface in cell assays. [1, 3] Aβ fibril and oligomer formation is inhibited through interaction with hCC, as well as directly protecting neuronal cells from Aβ-induced toxicity.[3] hCC has been shown to interact directly with $A\beta_{40}$ and $A\beta_{42}$.[1, 4]

NMR HSQC titration was utilised as an approach for protein-ligand binding analysis. The Staniforth group have examined the nature of the interaction between hCC and Aβ by titrating monomeric hCC with different molecular forms of Aβ. The absence of chemical shift changes suggests there is no direct interaction between these species, implying these are not responsible for the observed effects *in vivo*. However, careful analysis of HSQC cross peak intensities revealed small localised changes, potentially indicating weak binding of the cystatin C monomer to a minority species in two of the Aβ samples: $A\beta_{42}$ monomer and Aβ-GM1 ganglioside. Cross peak amide intensity mapping localises the Aβ binding site within hCC to the helix motif and residues in close contact with it. This motif is key to the correct folding of cystatin monomers and is displaced from the fold in order for cystatin C to form dimers and fibrillise. It is possible that this part of the molecule becomes available for tight binding in a higher oligomeric form of human cystatin C.

It is proposed that the interaction between hCC and Aβ is not a simple monomer-dependent process as previously thought, and that the determination of the relevant conformations of the interacting Aβ and hCC is a key step to uncovering the molecular mechanism of hCC's activity in AD and other Aβ amyloid diseases. Protocols have been established for the production of stable, high molecular weight oligomeric species of hCC, as classified by TEM. NMR HSQC experiments and thioflavin T fluorescence assays have been utilised to examine the nature of the interaction between different species of hCC and Aβ. Comparison with other Aβ-modulating systems, such as transthyretin, neuroserpin and gelsolin, will allow the general mechanism for *in vivo* protection to be determined and could potentially lead to identification of a therapeutic peptide.

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Assignment impossible? *De novo* assignment of large protein complexes for mechanistic studies

Michael S. Barber, Iva Pritisanac, Andrew J. Baldwin

Department of Physical and Theoretical Chemistry, University of Oxford, South Parks Road, Oxford, OX1 3QZ.

Email: Michael.Barber@chem.ox.ac.uk; Iva.Pritisanac@chem.ox.ac.uk

The small heat shock proteins (sHSPs) play a key role in protein homeostasis by maintaining proteins in their native fold. Furthermore the sHSPs are connected to several human diseases such as cataracts and Alzheimer's and are known to form large scale oligomers. sHSP16.5, produced by the volcanic archea *Methanococcus jannaschii*, forms an octahedral assembly with 24 subunits and a combined molecular mass of ~400 kDa. Due to its large size, selective MILV methyl labelling, methyl-TROSY and 4D NOESY based techniques were employed to study oligomers by NMR. A putative methyl group assignment was generated by an in-house data-driven *de novo* assignment algorithm. This was validated via the 'divide and conquer' strategy. Namely, 75% of a 25 kDa truncated dimer was assigned by backbone polarisation transfer and CCC-TOCSY experiments. The assignment algorithm solely uses experimental chemical shifts and NOE contacts to create NOE connectivity graphs and match these to protein X-ray structures, yielding putative methyl assignments. A feature of the algorithm is the exhaustive sampling which reveals ambiguity among putative assignments. A combination of these assignment strategies are presented here for sHSP16.5 along with data describing milli-second dynamics which may suggest a possible mechanism for activity.

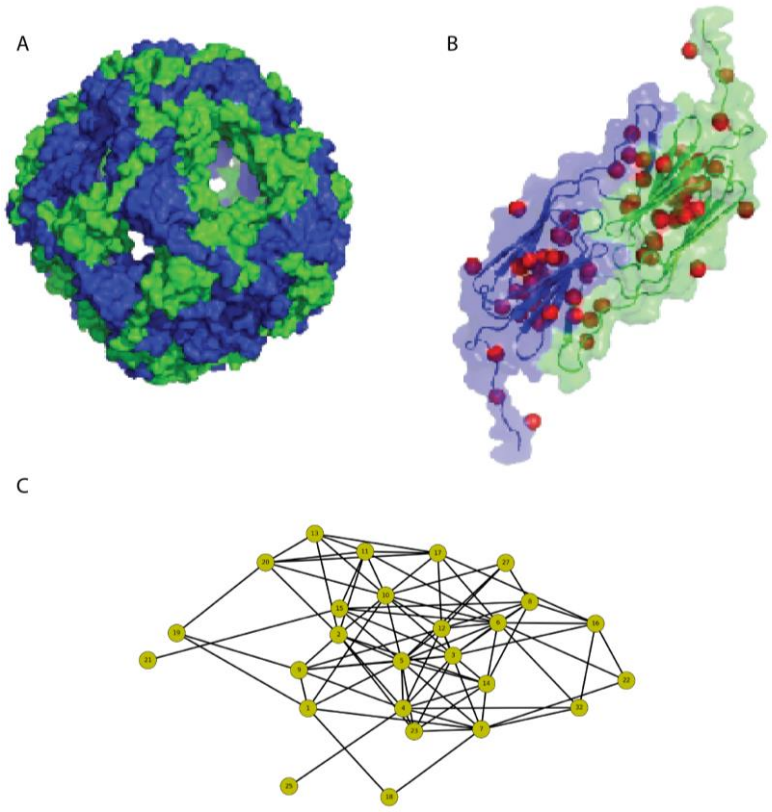

Figure 1: The structural models of HSP16.5 A) 24-mer displaying polyhedral geometry B) Truncated dimer with monomers shown in blue and green. Red spheres mark methyl groups of labelled MILV residues; C) Data connectivity graph based on NOE contacts in 4D NOESY experiment on an MILV methyl labelled HSP16.5 oligomer.

Fragment Screening by Microflow STD NMR

Matthew Goodwin[a], Hannah Maple[b], Christopher Williams[a], Ian Whitcombe[c], Richard Taylor[c] and Matthew Crump[a]

[a]Organic and Biological Section, School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS

[b]School of Social and Community Medicine, University of Bristol, Oakfield House, Oakfield Grove, Bristol, BS8 2BN

[c]UCB Celltech, 208 Bath Road, Slough, Berkshire, SL1 3WE

EMAIL mg12331@bristol.ac.uk

Fragment screening by tube based NMR requires high sample consumption.[1] One way of overcoming this is to use lower volume NMR tubes such as capillary tubes, however, these can have lower signal to noise (S/N) due to the narrow tube widths resulting in poor filling factors. Shigemi tubes offer an expensive alternative, although it would be unmanageable for screening thousands of compounds.

By using a microflow NMR system on a cryoprobe equipped 600 MHz NMR, 50 µL injections are routinely carried out, overcoming many of these problems. The flow cell consists of a glass cell of 3 mm width that can be lowered into the NMR. A liquid handler can inject samples from 96 or 384 well plates, and the sample flows into the cell as a plug of liquid inside of an immiscible fluorinated solvent. This fluorinated solvent has a magnetic susceptibility within 1% of that of D_2O, and so acts as a liquid Shigemi tube. This allows ^{1}H-^{15}N HSQC spectra to be acquired on **87 µg** of protein and STD spectra on **2.6 µg** in automation.

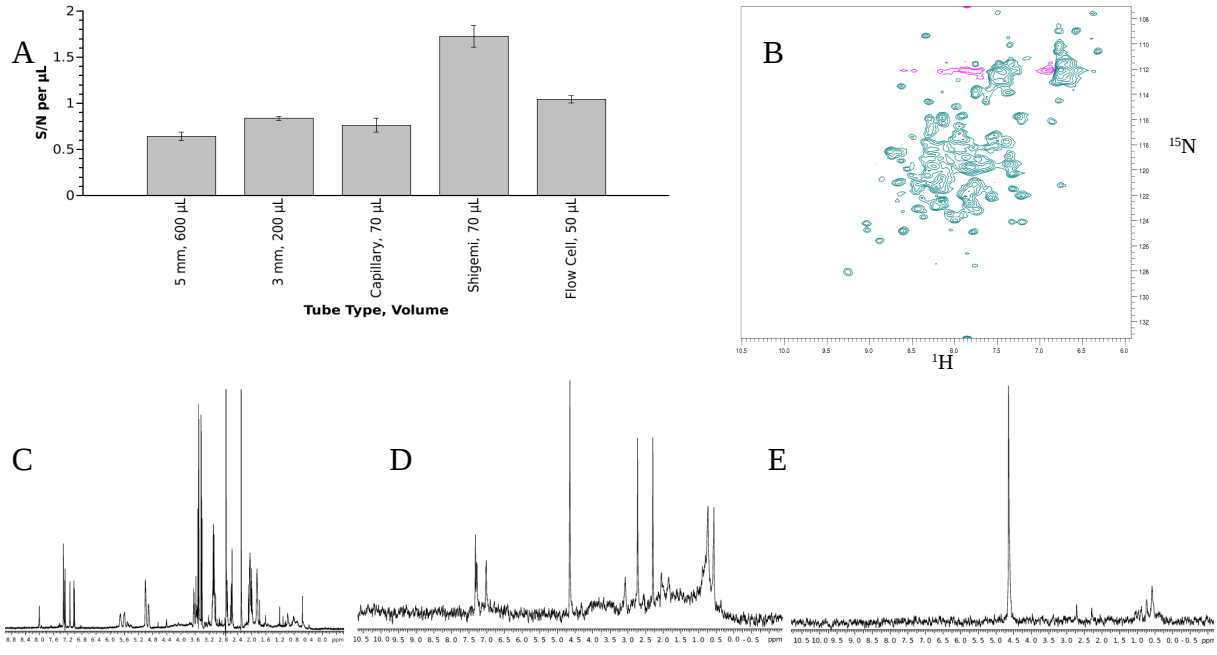

Figure 1. A. Comparison of S/N per µL of different tube types. The highest value is obtained by the shigemi tube, however the flowcell compares favourably. B. ^{1}H-^{15}N SOFAST HMQC spectrum obtained on 87 µg (100 µM, 50 µL) of protein in 50 mins. C. 5 min ^{1}H 1D spectrum of 1mM known binder ligand with 2.6 µg (12 µM, 50 µL) of protein. D. 40 min STD spectrum of same sample. E. Control STD with no protein present.

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Structural Evidence for a Critical Protein-Protein Interaction in the Bacterial Tol Pal Complex

ABSTRACT

Peter Holmes, Karthik Rajasekar, Colin Kleanthous

Department of Biochemistry, University of Oxford

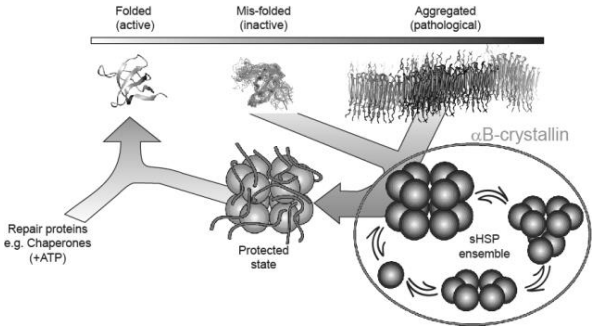
For life of all known lineages, regulation of protein activity via protein-protein interactions (PPIs) is critical for mediating both signal transduction and normal cell function. During periods of environmental stress, Gram-negative bacteria attack neighbouring strains by hijacking membrane-bound and soluble proteins of the target cell to deliver a potent cytotoxic domain. These domains, known as bacteriocins, first parasitize specific cell surface receptors, then via a series of PPIs that have yet to be fully elucidated and which possibly involve the proton motive force, translocate across target cell membranes to destroy the cell from within. Bacteriocin import is consequently a valuable model system for understanding the role of protein-protein interactions in cell import and inter-bacterial warfare. Recent research studies in the Kleanthous laboratory suggest that the multi-protein periplasmic Tol-Pal complex is critical for bacteriocin translocation. It is unclear how bacteriocins subvert Tol-Pal in order to catalyse their transfer into the cell. It is also unclear what the full physiological role of Tol-Pal is in Gram-negative bacteria. Current understanding indicates Tol-Pal may be involved in stabilizing the bacterial outer membrane during cell division. Using solution NMR spectroscopy, we demonstrate biophysical evidence that the intrinsically-disordered N-terminal region of the TolB protein in *Pseudomonas aeruginosa* introduces significant conformational change in the C-terminal domain of TolA through formation of a stable complex. Atomic details of this critical protein-protein interaction will facilitate a greater understanding of the functional role of the Tol-Pal complex within the cell, and its relation to bacteriocin import and the proton motive force.

Allosteric communication in αB-crystallin: insights from NMR, mass spectrometry and computational modelling

Olga Tkachenko, Benedict Girling, Elodie Limer, Georg K. A. Hochberg, Weston Struwe, Justin L. P. Benesch, Andrew J. Baldwin

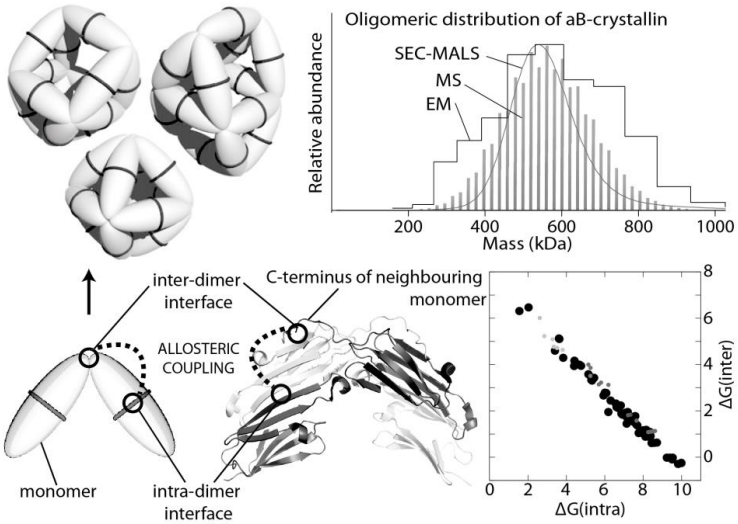
Chemical Research Laboratory, University of Oxford, Mansfield Road, Oxford, OX1 3TA

EMAIL olga.tkachenko@sjc.ox.ac.uk



αB-crystallin is a member of the small heat shock protein family, a class of chaperones represented in all kingdoms of life. In humans, αB-crystallin is a major component of the eye lens but is also expressed in many other tissues including the brain and muscle. It is found associated with protein deposits in patients with diseases such as Alzheimer's, Alexander's and Parkinson's, and αB-crystallin mutations are associated with cardiomyopathies, cataracts and neuropathies[1].

αB-crystallin consists of a 90-residue beta-sheet core domain with flexible N- and C-terminal tails, about 70 and 25 residues long respectively. The isolated core domain can form dimers and has comparable activity in preventing protein aggregation to the full-length molecule[2]. Full-length αB-crystallin further assembles into polydisperse subunit-exchanging oligomers containing between approximately 10 and 40 monomers[3]. This complex distribution can be explained using a simple model with just two interactions independent of oligomer size, namely the intra- and inter-dimer contacts shown schematically in the figure on the right[4,5]. Although these interfaces are over 13 Å apart, there is energetic compensation between them under a wide range of perturbations[4,6]. This implies an allosteric communication pathway across the molecule, the precise details of which are the object of our study. We are using NMR and mass spectrometry (MS) to look at the effect of adding peptides mimicking the C-terminal region of the protein (both wild-type and mutant) to the isolated core domain. Simulation of the titration NMR data to extract and compare the chemical exchange parameters reveals residue-level detail of cross-talk between the interfaces. Ion mobility MS allows us to resolve different peptide-bound states in the monomer-dimer equilibrium, and to study the effect of the mutations on full-length oligomer distributions. As part of a collaborative effort, we are using all available experimental data to build atomic-resolution models of full-length oligomers using Rosetta. Thus a multidisciplinary approach provides complementary ways to interrogate the mechanism of molecular dynamics of αB-crystallin, which is an important prerequisite to understanding its chaperoning action.



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NMR Discussion Group Spring Meeting. Cambridge 10th and 11th April 2014

Wednesday 9th April 2014

14:00	Check in at Downing College and St Catharine's College from 14:00 (Porters' Lodge)		

Thursday 10th April 2014

08:00	09:00	Breakfast for those staying at Downing or St Catharines	
10:00	13:15	Registration at Chemistry from 10:00 (bag drop available at Chemistry)	
14:00	Check in at Downing College and St Catharine's College from 14:00 (Porters' Lodge)		

Scientific session 1: Bristol Meyers Squibb Lecture Theatre, Department of Chemistry

13:25	13:30	**Welcome**	
13:30	13:35	**Jennifer Potts**	*Iain Campbell remembered*
13:35	14:15	**Gerhard Wagner**	*NMR studies of integral membrane proteins*
14:20	14:50	**Bernd Reif**	*Amyloid Aggregates and Large Soluble Protein Complexes*
14:55	15:25	**Claudio Dalvit**	*Recent developments in ^{19}F NMR spectroscopy for fragment screening and drug design*
15:30	16:00	Tea (Wolfson practical laboratory)	
16:00	16:20	**Roberto Buratto**	*Beyond the millimolar range: measuring ultra-weak ligand-protein affinities using NMR of long-lived states*
16:20	16:40	**Wing Ying Chow**	*Applying a solid-state NMR approach to probe atomic changes in collagen matrices in health and disease*
16:40	17:10	**Jennifer Potts**	*Molecular interactions in staphylococcal infections*
17:15	17:45	**Thomas Prisner**	*Advances in high field DNP and EPR*
17:50	19:30	Wine reception and posters (Wolfson practical laboratory)	
19:30	20:30	Cafeteria Dinner at Downing College (if booked)	

Friday 11th April 2014

08:00	09:00	Breakfast for those staying at Downing or St Catharines	
09:30		Checkout: r**ooms MUST be vacated by 09:30** (bag drop available at Chemistry)	

Scientific session 2: Bristol Meyers Squibb Lecture Lecture Theatre, Department of Chemistry

09:00	09:30	**Sjors Scheres**	*Recent advances in high-resolution cryo-EM structure determination*
09:35	10:05	**Torsten Herrmann**	*Turning failure into success: New perspectives for unsupervised NMR studies of proteins*
10:10	10:30	**Justin Lecher**	*Analysis of the ion channel gating mechanism in solution by NMR spectroscopy*
10:30	11:00	Coffee (Wolfson practical laboratory)	
11:00	11:30	**Elisabetta Chiarparin**	*Fragment based drug discovery: challenges and opportunities*
11:35	11:55	**Hiroki Takahashi**	*Dynamic nuclear polarisation enhanced solid-state NMR spectroscopy and magnetic resonance force microscopy for structural biology*
11:55	12:15	**Peter Schmidt**	*The Structure of Neuropeptide Y bound to its G protein-coupled Y2 receptor*
12:15	13:45	Lunch at Downing College - this is a served meal so please move swiftly over to Downing	
13:45	14:15	**Mario Schubert**	*Insights into protein–carbohydrate recognition by NMR spectroscopy*
14:20	14:40	**Christopher Waudby**	*Mapping the co-translational folding energy landscape of the ddFln5 immunoglobulin domain*
14:40	15:05	**Andrew Baldwin**	*NMR spectroscopy, mass spectrometry and electron microscopy elucidate the structure and dynamics of αB-crystallin oligomers*
15:10	15:50	**Ad Bax**	*Probing motions and structural rearrangements by RDCs*
15:55		Tea & Close (Wolfson practical laboratory)	