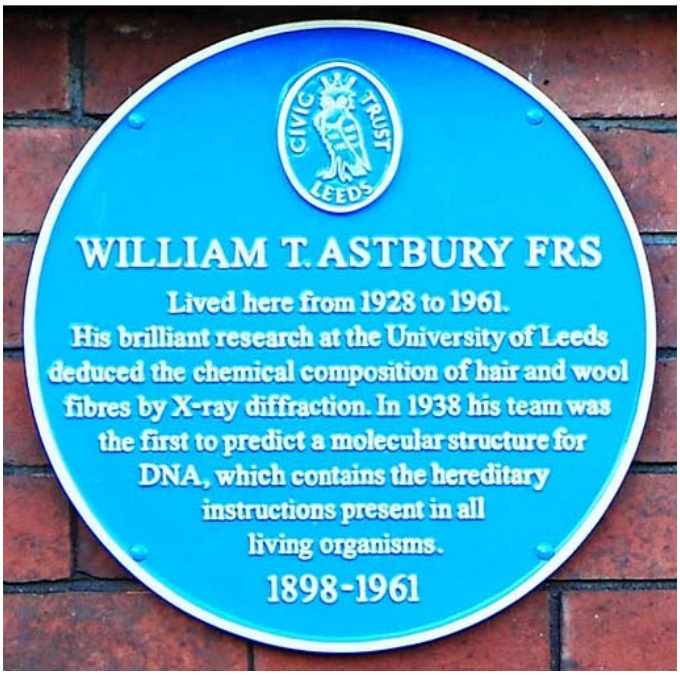
Astbury Centre for Structural Molecular Biology

UNIVERSITY OF LEEDS



ASTBURY CENTRE

Annual Report 2010

Front cover illustration: Leeds Civic Trust blue plaque on Bill Astbury's house in Headingley that was unveiled in November 2010.

Acknowledgement

The Astbury Centre for Structural Molecular Biology thanks its many sponsors for support of the work and its members for writing these reports. The report is edited by David Brockwell.

This report is also available electronically via http://www.astbury.leeds.ac.uk

Mission Statement

The Astbury Centre for Structural Molecular Biology will promote interdisciplinary research of the highest standard on the structure and function of biological molecules, biomolecular assemblies and complexes using physico-chemical, molecular biological and computational approaches.

Introduction

I am delighted to write the Introduction to the Annual Report on the activities of the Astbury Centre in for Structural Molecular Biology in 2010. The Centre brings together scientists from diverse backgrounds from across the University of Leeds with the goal of understanding the basis of life in atomic resolution. This annual report provides a snapshot of the Astbury Centre's research portfolio in 2010. Many of the reports demonstrate the power of interdisciplinary approaches to address major problems in modern biology.

2010 was a buoyant and successful year for the Centre with successes on many fronts. A major highlight of 2010 was the research away day that was attended by around 130 members of the Centre, of whom over 50 made oral or poster presentations on their research. This gave us a chance to meet academically and socially and to experience a day of brilliant science that was enjoyed by all. The Astbury Centre also continues to host a vibrant and highly international seminar programme. In total, there were 23 seminars in 2010, half of which were delivered by international visitors.

During 2010, many members of the Centre were recognised for the quality and impact of their research. I'm delighted to report that Sheena Radford has been elected as a fellow of the Academy of Medical Sciences. Many postgraduate and postdoctoral members of the centre were externally recognised for the excellence of their research. Martin Fisher won a prize at an RSC conference; Martin Fascione received an award to present at the International Carbohydrate Symposium in Tokyo; Tom Branson won the first prize for his poster at the "Summer Course Glycosciences" in Wageningen; Susie Turrell was awarded the 2010 British Society for Cell Biology Science Writing Prize; Vincent Postis, together with collaborators from Oxford, won the best poster prize at the Membrane Transport Proteins Gordon Research Conference at Biddeford, USA; and Dale Shepherd won the Barber prize for the best student lecture at the annual British Mass Spectrometry Society conference. We welcomed four new academic staff to the Centre in 2010: Stephen Muench, Richard Foster, Robin Bon and Kevin Critchley. We were also delighted to welcome 41 new PhD students to the Centre, and 34 of our students were successful in defending their PhD theses. Congratulations to all!

Members of the Astbury Centre published 133 papers in leading journals in 2010 including ten papers in the *Nature* series, *Science* and *PNAS*. The Biology Faculty of 1000 highlighted research from the Centre including papers on interactions of Kaposi's sarcoma-associated herpesvirus ORF57 protein (Whitehouse); the transition state for the folding of a membrane protein (Radford, Brockwell, Baldwin); responses to RNA virus infection (Macdonald, M Harris); and the molecular mechanism of the transporter protein Mhp1 (Henderson).

Astbury Centre members continue to be very successful in raising external grant income, including many of our newly appointed staff who have succeeded in getting their first major grants funded. New larger grants involving our members included two Wellcome Trust programme grants (Radford, Homans, Hewitt; Baldwin), and a second BBSRC-funded Longer Larger (LoLa) grant (involving Westhead). In addition, Lorna Dougan, with David Brockwell as a collaborator, secured the Centre's second prestigious European Research Council (ERC) Starter grant. In addition, our drive to develop strong and sustained links with industry is bearing fruit, with many new collaborative links with companies being established. At the end of 2010, Astbury Centre members held a £37M share of grants totaling £57M.

Postdoctoral researchers and postgraduate students continue to make major contributions to the activities of the Centre. Lucy Woods and Adam Daniels have done a superb job leading the Astbury Society which has organised many enjoyable social and scientific events this year including the Sports Day and barbecue associated with the Astbury annual lecture. The

Astbury lecture was given by Janet Thornton, FRS, who delivered a stunning lecture describing how bioinformatics may shed insights into the function of biological macromolecules. Nicole Timms and Adam Daniels organised, with the West Yorkshire branch of the British Science Association, an event at the Thackray medical museum entitled "Astbury's influence on medical science through to the 21st century and beyond": the event featured four speakers from the Centre, and was attended by three generations of the Astbury family.

The Centre was involved in a wide range of events to enhance the public's understanding of science during 2010, in addition to the event noted above that was organised by the Astbury Society. Bill Astbury's distinguished career was featured in an exhibition which formed part of the Royal Society's celebration of its 350th anniversary. The exhibition was held at the Thackray medical museum, and highlighted Bill Astbury's work in founding the field of structural molecular biology: the exhibition showed how current day research within the Astbury Centre has evolved from Astbury's diffraction images of DNA and protein fibrils. In November, I was honoured to be invited by the Leeds Civic Trust to unveil a blue plaque on Bill Astbury's house in Headingley, emphasising the importance of Astbury in Leeds' history: the unveiling was attended by the Astbury family, Astbury Centre members past and present, and members of the Leeds Civic Trust. In conjunction with the plaque unveiling, Bruce Turnbull appeared on the BBC Radio Leeds breakfast show, where he discussed with great aplomb how Astbury's X-ray studies on wool, hair and poached eggs underpin our understanding of many phenomena from hair perming to Alzheimer's disease!

2011 promises to be an exciting year for the Centre. We are planning our third residential research retreat which will be held at a venue on the edge of the Peak District national park in the autumn. In addition, the Astbury Society is organising a Young Life Scientists' Symposium as part of the centenary celebrations of the Biochemical Society: the event will be entitled "Protein Evolution and Engineering: From Research to the Real World', and promises to showcase how protein evolution may be used to tackle a range of important challenges facing global society.

This annual report (as well as those from previous years) is also available as a PDF document that can be downloaded from our website.

Finally, I would like to thank all of our members for their enthusiasm for Structural Molecular Biology and their help and efforts to make the Centre the success it is today. Many thanks also to our editor, David Brockwell, for leading the preparation of this Report.

Adam Nelson

Director, Astbury Centre for Structural Molecular Biology

Leeds, March 2011

Contents

Contributions indexed by Astbury Centre Principal Investigator

Biomolecular mass spectrometry

James Ault, Tom Knapman, Victoria Morton, Helen Beeston, Henry Fisher, Lynsey Jones, Aneika Leney, Bethny Morrissey, George Preston, Caroline Pritchard, Dale Shepherd, Lucy Woods, Peter Stockley, Sheena Radford, Nicola Stonehouse, Andrew Wilson, Peter Henderson, Sarah Harris and Alison Ashcroft

Introduction

The main focus of our research is the development and application of mass spectrometric techniques to investigate the tertiary and quaternary structures of biomolecules. We use non-covalent electrospray ionisation mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) to determine the mass, conformational properties, stoichiometry, stability, and binding characteristics of proteins and protein complexes. We are also pioneers of ion mobility spectrometry-mass spectrometry (IMS-MS), which offers a unique opportunity to separate co-populated biomolecular entities on the basis of their physical shape and to measure their mass and cross-sectional area (Ω) in a single, rapid (≤ 2 mins) experiment.

Results

The MS2 phage capsid consists of a *T*=3 icosahedral protein lattice with 180 copies of the coat protein (CP) in the form of 90 non-covalent dimers (CP_2) (Figure 1(a)). The reaction profile of capsid assembly *in vitro*, triggered by adding stem-loop RNA (TR) to CP_2, can be monitored in real-time by ESI-IMS-MS and we have identified two non-covalently bound, transient capsid intermediates, namely the hexamer and decamer (Figure 1(b)).

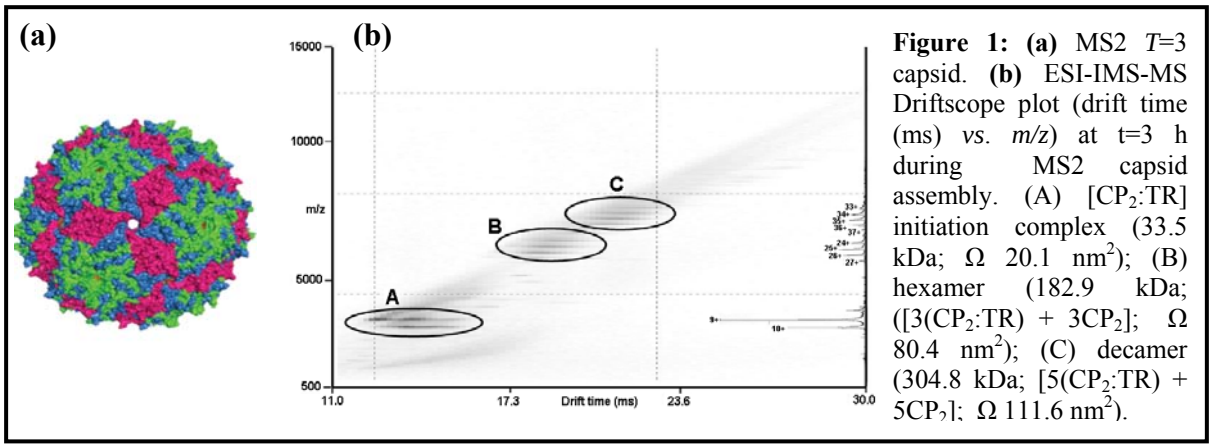


Figure 1: (a) MS2 *T*=3 capsid. **(b)** ESI-IMS-MS Driftscope plot (drift time (ms) *vs. m/z*) at t=3 h during MS2 capsid assembly. (A) [CP_2:TR] initiation complex (33.5 kDa; Ω 20.1 nm^2); (B) hexamer (182.9 kDa; ([3(CP_2:TR) + 3CP_2]; Ω 80.4 nm^2); (C) decamer (304.8 kDa; [5(CP_2:TR) + 5CP_2]; Ω 111.6 nm^2).

To provide insights into the quaternary architecture of these intermediates, we used a combination of IMS-MS coupled to collision induced dissociation (CID) MS/MS (Figure 2). The hexamer and decamer were found to exhibit different dissociation behaviour. Expulsion of a monomer from the hexamer resulted in a large decrease in the observed Ω of the residual complex, consistent with a proposed ring-like structure in which removal of a monomer reveals a cavity which could lead to structural collapse. The residual decamer showed little change in Ω after monomer removal by CID, consistent with the proposed structure which should not be disrupted by removal of a monomer (Figure 2).

Publications

Ashcroft, A. (2010) Mass spectrometry and the amyloid problem – how far can we go in the gas phase? *J. Am. Soc. Mass Spectrom.* **21**:1087-1096.

Basnak, G., Morton, V., Rolfsson, O., Stonehouse, N., Ashcroft, A. & Stockley, P. (2010) Viral genomic ssRNA directs the pathway towards a *T*=3 capsid. *J. Mol. Biol.* **395**:924-936.

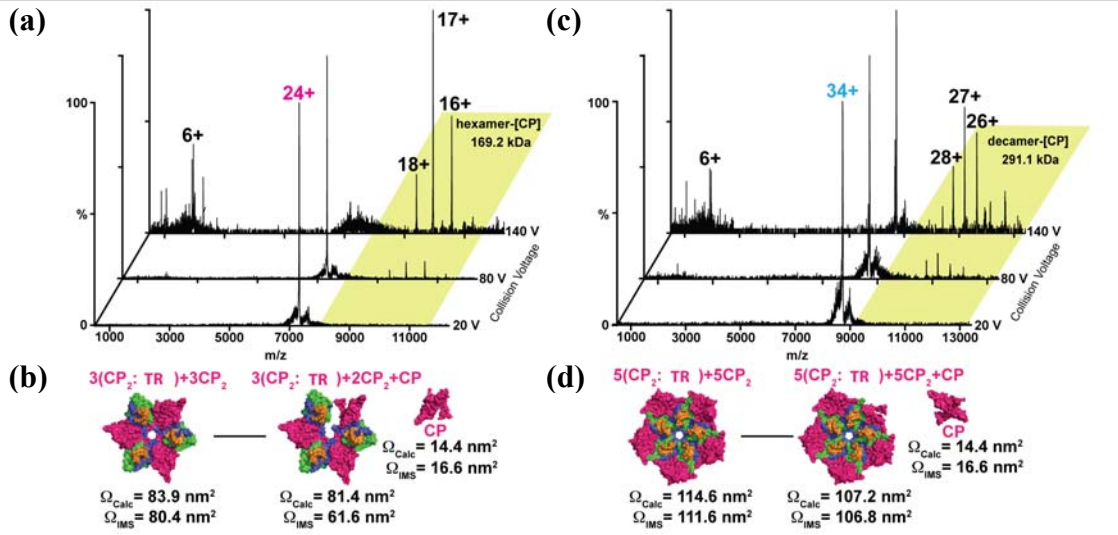


Figure 3: (a) and **(c)**: ESI-MS-CID-IMS-MS spectra from the precursor 24+ and 34+ charge state ions of the (a) hexamer and (c) decamer capsid assembly intermediates, respectively, showing the loss of a monomer sub-unit; **(b)** and **(d)**: potential structures of the (b) hexamer and (d) decamer assembly intermediates after dissociation with loss of a monomer modelled using an in-house developed algorithm.

Jones, L., Baldwin, S., Henderson, P. & Ashcroft, A. (2010) Defining topological features of membrane proteins by ESI-MS. *Rapid Commun. Mass Spectrom.* **24**:276-284.

Knapman, T., Berryman, J., Campuzano, I., Harris, S. & Ashcroft A. (2010) Considerations in experimental and theoretical collision cross-section measurements of small molecules using travelling wave IMS-MS. *Int. J. Mass Spectrom*. **298**:17–23.

Knapman, T., Morton, V., Stockley, P., Stonehouse, N. & Ashcroft, A. (2010) Determining the topology of macromolecular protein complexes. *Rapid Commun. Mass Spectrom.* **24**:3033–3042.

Morton, V., Dykeman, E., Stonehouse, N., Ashcroft, A., Twarock, R. & Stockley, P. (2010) The impact of viral RNA on assembly pathway selection. *J. Mol. Biol***. 401**:298-308.

Morton, V., Burkitt, W., O'Connor, G., Stonehouse, N., Stockley, P. & Ashcroft, A. (2010) RNA induced conformational changes in a viral coat protein studied by HDX-MS, *Phys. Chem. Chem. Phys.* **12**:13468- 13475.

Muldoon, J., Ashcroft, A. & Wilson, A. (2010) Selective protein-surface sensing using ruthenium (II) tris-(bipyridine) complexes. *Chem. European J.* **16**:100-103.

Smith, D., Radford, S. & Ashcroft, A. (2010) Elongated oligomers in β_2-microglobulin amyloid assembly revealed by IMS-MS. *Proc. Nat. Acad. Sci. USA*, **107**:6794-6798.

Funding

This work was funded by the BBSRC, the EPSRC, the Wellcome Trust, Waters UK Ltd., LGC, AZ, and GSK. We thank the BMSS for student travel grants.

Collaborators

Dr G. O'Connor (LGC, UK), Prof. G. Waksman (Birkbeck College, London), Prof. C. Cambillau (University of Marseilles, France), Dr j. Hoyes (Waters UK Ltd.), Dr M. Anderson (AZ), Dr U. Gerhardt (GSK).

Phase behaviour and transitions of peptides and proteins

Raffaela Cabriolu and Stefan Auer

Introduction

My research is focused on the application of theoretical computational tools developed in soft condensed matter physics to investigate the phase behaviour and transitions of complex systems of biomolecules. From a purely statistical mechanical point of view an ensemble of many peptides and proteins represents a new and important system which should lead to bridge our understanding of colloidal systems, polymers, and proteins. The research highlights in the year 2010 were the calculation of a phase diagram of natively folded α-helical and unfolded β-sheet forming peptides and the application of nucleation theory to amyloid fibril formation.

Phase diagram of α-helical and β-sheet forming peptides.

The intrinsic property of proteins to form structural motifs such as α helices and β sheets leads to a complex phase behavior in which proteins can assemble into various types of aggregates including crystals, liquidlike phases of unfolded or natively folded proteins, and amyloid fibrils. In this work we use a coarse-grained protein model that enables us to perform Monte Carlo simulations for determining the phase diagram of natively folded α-helical and unfolded β-sheet forming peptides. The simulations reveal the existence of various metastable peptide phases. The liquidlike phases are metastable with respect to the fibrillar phases, and there is a hierarchy of metastability.

The application of nucleation theory to amyloid fibril formation.

The assembly of proteins into amyloid fibrils is a widespread and much-studied phenomenon, because it has wide implications ranging from biotechnology to human disease. Yet, the nucleation of such nanofibrils is poorly understood. In our work we try to illustrate that amyloid formation might follow a common fibril nucleation mechanism which could be treated in the framework of existing general theories of nucleation of new phases. Progress to support this view was made in our recent work, where we used concepts from the theory of overall crystallization to describe the kinetics of overall protein aggregation, and applied classical and atomistic nucleation theories to describe the nucleation of amyloid fibrils**.**

Publications

Auer, S. & Kashchiev, D. (2010) Phase diagram of α-helical and β-sheet forming peptides. *Phys. Rev. Lett.* **104**:168105.

Auer, S. & Kashchiev, D. (2010) Insight into the correlation between lag time and aggregation in the kinetics of protein aggregation. *Proteins* **78**:2412.

Cabriolu, R., Kashchiev, D. & Auer, S. (2010) Atomistic nucleation theory of amyloid fibrils formation. *J. Chem. Phys.* **133**:225101.

Kashchiev, D. & Auer, S. (2010) Nucleation of amyloid fibrils. *J. Chem. Phys.* **132**:215101.

Funding

This work was supported by the EPSRC-GB Grant No. EP/G026165/1.

Collaborators

Dimo Kashchiev (Sofia).

Molecular mechanisms of nutrient and drug uptake by cells

Vincent Postis, Xiaobing Xia, Jean Ingram, Jocelyn Baldwin, Michael McPherson and Stephen Baldwin

Introduction

Members of the Proton-dependent Oligopeptide Transporter (POT) family are widely distributed in living organisms and transport short peptides, amino acids or nitrate across cell membranes, driven by the inwardly directed proton (H^+) electrochemical gradient. They represent a subfamily of the very large Major Facilitator Superfamily (MFS) of transporters. The human family members PepT1 and PepT2 are responsible for the uptake of di- and tri-peptides in the small intestine and kidney, respectively. In addition to being a major route for the absorption of dietary nitrogen, they are also involved in the uptake of many orally administered drugs, including the β-lactam antibiotics. Further exploitation of these proteins as routes for drug uptake requires an understanding of the molecular basis by which they recognise and translocate substrates. Towards this end, work in our laboratory has focused recently on the use of biophysical and other approaches to probe the structure/function relationships of such transporters. This research forms part of a larger programme aimed at understanding the mechanisms involved in transmembrane transport of solutes, and exploitation of transporters both as drug targets and as routes for drug uptake.

$PepT_{So}$, a prokaryote homologue of human PepT1 and PepT2

Because large scale production of human membrane proteins is typically very difficult, our studies have focused on bacterial homologues of the human peptide transporters, as part of the BBSRC-funded UK Membrane Protein Structure Initiative (MPSi). Bioinformatic searches revealed that a transporter from *Shewanella oneidensis*, which we have designated $PepT_{So}$, shows a much greater sequence similarity (~30%) to the human proteins than most other bacterial peptide transporters. Functional studies on the protein, expressed in *Escherichia coli*, revealed that it was also closely related functionally to PepT1, not only in its substrate selectivity and apparent affinity for dipeptides and tripeptides, but also in its proton-dependence. We were able to express and purify the protein in large amounts and it proved readily crystallizable. The structure of the transporter was determined by X-ray crystallography in a collaboration with Simon Newstead and other members of So Iwata's Membrane Protein Crystallography Group at Imperial College and the Membrane Protein Laboratory at the Diamond Light Source, Oxfordshire. The protein was revealed to have a typical MFS transporter structure, with N- and C-terminal six-transmembrane (TM) helix bundles, related by a pseudo two-fold symmetry axis running perpendicular to the membrane

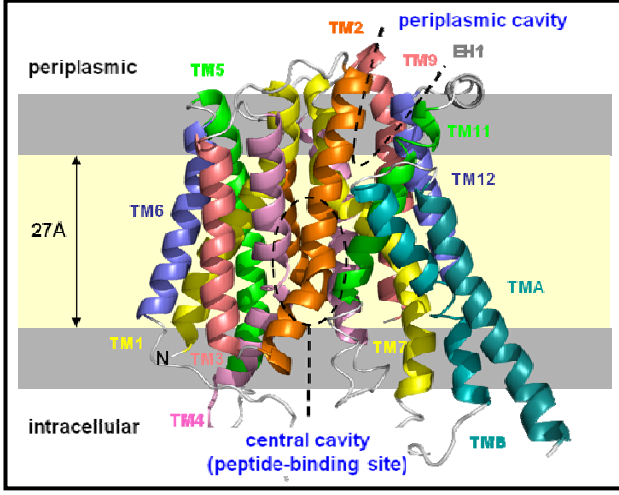


Figure 1: cartoon representation of $PepT_{So}$ embedded in a lipid bilayer. Pairs of related transmembrane (TM) helices in the N- and C-terminal halves of the protein, likely to originate from a gene duplication event, are coloured the same, except for the two poorly-conserved TMs (TMA and TMB) near the centre of the sequence, which are both shown in cyan.

plane (Figure 1). Two additional TM helices (TMA and TMB in Figure 1), inserted into the cytoplasmic loop connecting the N- and C-terminal bundles, are poorly conserved in the POT family and unlikely to play a key functional role.

The N- and C-terminal TM helix bundles of $PepT_{So}$ surround a central hydrophilic cavity, likely to represent the substrate binding site. This is lined by conserved residues implicated in peptide recognition in functional studies on homologous mammalian transporters. In the current crystal structure, this cavity is occluded both from the periplasmic and the cytoplasmic sides of the membrane. Conformational changes during the translocation cycle are predicted to alternately allow access to the site from the two sides of the membrane, enabling movement of the peptide across the lipid bilayer (Figure 2). Such conformational changes are likely to be coupled to the proton gradient via protonation of a highly conserved residue, His_{61}, which is located at the base of a second hydrophilic cavity extending into the protein from the periplasmic side of the membrane (Figure 1). Mutation of this residue to cysteine prevents peptide transport.

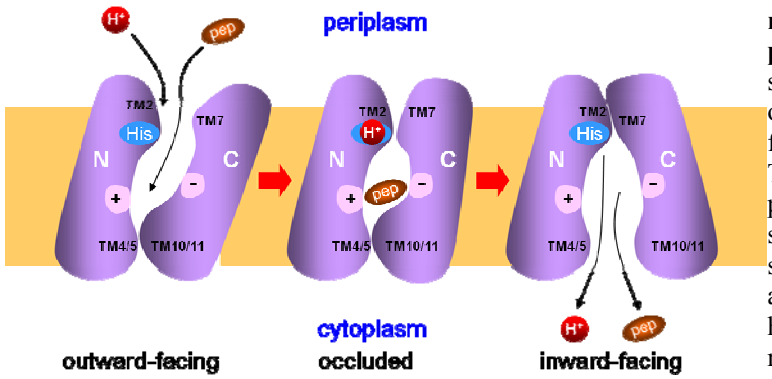


Figure 2: predicted model for the mechanism of proton-driven peptide transport. The current crystal structure represents the occluded state of the transporter, in which gates formed by association of the indicated TM helices prevent access to the peptide (pep) binding site from either side of the membrane. The binding site includes multiple conserved acidic and basic residues, while a conserved histidine residue likely plays a key role in the coupling of transport to the transmembrane proton gradient.

Current Work

A programme of site-directed mutagenesis coupled with functional assays of transport is currently underway, with the objective of elucidating the roles of conserved residues in substrate binding and translocation, and in the coupling of peptide transport to the proton gradient across the membrane.

Publications

Newstead, S., Drew, D., Cameron, A., Postis, V., Xia, X., Fowler, P., Ingram, J., Carpenter, E., Sansom, M., McPherson, M., Baldwin, S. & Iwata, S. (2011) Crystal structure of a prokaryotic homologue of the mammalian oligopeptide-proton symporters, PepT1 and PepT2. *EMBO J.* **30**:417-426.

Funding

This work was funded by the BBSRC.

Collaborators

Simon Newstead, David Drew, Alexander D. Cameron, Elisabeth P. Carpenter and So Iwata (Division of Molecular Biosciences, Imperial College, London, UK), and Philip W. Fowler and Mark S.P. Sansom (Department of Biochemistry, University of Oxford, Oxford, UK). We also thank Peter J.F. Henderson and all the other members and advisors of the MPSi for advice and support.

Diverse aspects of negative stranded RNA virology

Cheryl Walter, Diane Munday, Rebecca Surtees, Stephen Carter, Weining Wu, Julian Hiscox and John Barr

Introduction

The current research carried out in our laboratory focuses on a group of viruses that possess negative stranded RNA genomes. These include human respiratory syncytial virus (HRSV) that is responsible for extensive global outbreaks of childhood respiratory disease, and Crimean Congo hemorrhagic fever virus, which is responsible for a devastating infection with life-threatening consequences. A summary of two very different projects investigating the molecular and cellular biology of these viruses is described below.

Investigating the structure and function of the viral RNP

While these viruses are extremely diverse in their disease causing ability, they possess one common structural characteristic that is at the heart of their respective life cycles; a ribonucleocapsid (RNP). This assembly is an association of the RNA genome with a virus-encoded nucleocapsid (N) protein, and its formation is essential for several fundamental aspects of the virus replication cycle including gene expression and virus assembly.

One aspect of our research is to try to understand how the structure of the RNP dictates and relates to its function. Towards this aim, we have developed cell-based assays that allow the assembly of viral RNPs from their RNA and protein components. By site-directed mutagenesis of specific N protein residues we are currently forming a detailed picture of how the RNP binds RNA, homo-typically multimerizes, and thus forms a competent template for both RNA replication and mRNA transcription. New grants awarded in 2010 will allow us to obtain detailed structural information of the RNP, which will provide a more detailed molecular framework to direct further investigation of the structure-function relationship.

Investigating the virus/host interaction.

A common theme of all viruses is that they are involved in extensive interactions with the host, and these interactions can either hinder or aid in the viral replication cycle. Negative stranded RNA viruses are no exception to this, and to investigate this process further we have used quantitative proteomics to examine how the host-cell proteome is altered following HRSV infection of human epithelial cells. This work revealed HRSV infection induced extensive changes in the expression and distribution of cellular proteins associated with membrane bound fractions within the cytoplasm, and in particular identified profound mitochondrial dysfunction. This work further supports an emerging concept that sub-cellular organelles are platforms for the innate immune response.

Publications

Munday, D., Emmott, E., Surtees, R., Lardeau, C., Wu, W., Duprex, W., Dove, B., Barr, J. & Hiscox, J. (2010) Quantitative proteomic analysis of A549 cells infected with human respiratory syncytial virus. *Mol. Cell. Proteomics*. **9**:2438-2459.

Munday, D., Emmott, E., Surtees, R., Lardeau, C., Wu, W., Duprex, W., Dove, B. Hiscox, J. & Barr, J. (2010) Quantitative proteomic analysis of A549 cells infected with human respiratory syncytial virus. *Proteomics* **10**:4320-4334.

Walter, C. & Barr, J. (2010) Bunyamwera virus can repair both insertions and deletions during RNA replication. *RNA* **16**:1138-1145.

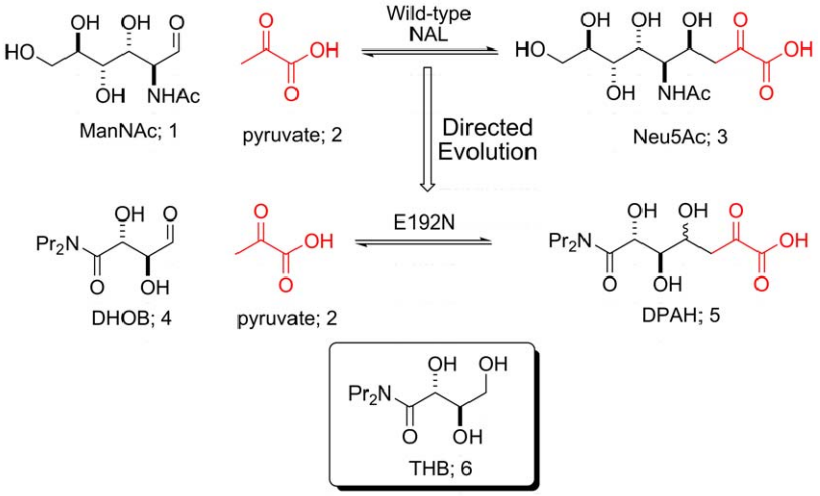
Funding
JB is the grateful recipient of two project grants from The Wellcome Trust. The work described above was also funded by the Medical Research Council, the BBSRC and a CASE studentship awarded in collaboration with the Health Protection Agency.

Novel enzymes based on the *N*-acetyl neuraminic acid scaffold

Ivan Campeotto, Chi Trinh, Tom Harman, Nicole Timms, Adam Daniels, Arwen Pearson, Adam Nelson and Alan Berry

Introduction

N-acetylneuraminic acid lyase (NAL) catalyses the reversible aldol condensation of *N*-acetyl mannosamine (ManNAc; **1**) with pyruvate (**2**) to yield the sialic acid, *N*-acetyl neuraminic acid (Neu5Ac; **3**). As such this enzyme plays an important role in controlling the cellular levels of sialic acids as they are being incorporated into growing complex sugars. In our continuing studies of altering aldolases for a variety of uses in constructing complex biomolecules, we have used rational protein engineering and directed evolution to alter a number of properties of these enzymes.



In previous studies on NAL, we have successfully evolved novel enzymes with altered substrate specificity and stereochemistry. The substrate specificity of the *Escherichia coli* NAL was switched from the natural condensation, **1** + **2** → **3**, to an aldol condensation which generated *N*-alkylcarboxamide analogues of Neu5Ac (**5**). This was achieved by the single mutation of Glu-192 to Asn. In order to analyze the structural changes involved and to more fully understand the basis of this switch in specificity, we isolated all 20 variants of the enzyme at position 192 and determined the activities with a range of substrates. We also determined five high-resolution crystal structures.

Results

In order to determine the relationship between the amino acid at position 192 and the activity with various compounds, we isolated all 20 possible variants of NAL at position 192 and assessed their activity with either the natural substrate **3** or the new analogue **5**. These results (Figure 1) show that the activity towards Neu5Ac is highest for the wild-type enzyme (E192), and only the E192Q variant shows significant activity towards this substrate whereas the activity profile of the E192X library with DPAH, **5** differs dramatically from that with Neu5Ac **3**, as many more of the variants (for example E192F, E192H, E192M, E192N, E192Q, E192P and E192V) show significant activity with the new substrate. These results suggest that specific hydrogen bonding interactions are important for the wild-type enzyme and its natural substrate, but that a variety of mechanisms are in play to allow the variants to bind and utilize DPAH as a substrate.

In order to gain further insights into the mechanism of substrate specificity in the E192 variants we embarked on an X-ray crystallo-graphic analysis of the 'best' enzyme for DPAH

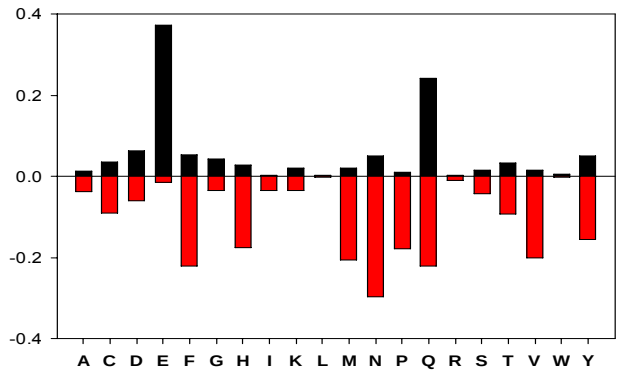


Figure 1: the activity profile of the E192X variants with Neu5Ac (black) and with DPAH (red).

cleavage, namely E192N, and structural modeling of the possible binding modes of interaction of the DPAH with other E192 variants.

Five high resolution crystal structures of NAL were solved; the structures of the wild-type *E. coli* NAL in the presence and absence of pyruvate, the E192N variant in the presence and absence of pyruvate and the E192N variant in the presence of pyruvate and a competitive inhibitor (2*R*, 3*R*)-2, 3, 4-trihydroxy-*N*, *N*-dipropylbutanamide (THB; **4**). This latter structure (Fig. 2) revealed that the inhibitor mimics the substrate and can bind in two orientations in the enzyme active site. It also reveals details of the mechanism of the specificity switch. The natural E192 would clash with the substrate explaining why the natural enzyme only poorly accepts DPAH, whereas the extensive hydrophobic surface generated in the active variants can all accept the new substrate.

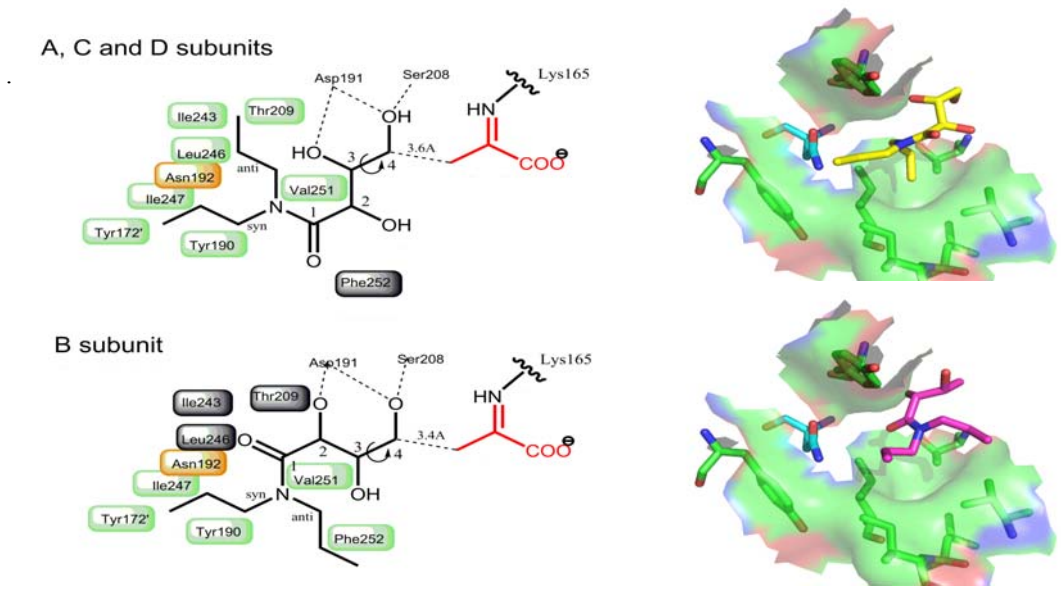


Figure 2: **right**; structures of *E.coli* NAL in complex with pyruvate and the competitive inhibitor DHOB (**6**) (yellow and purple) in subunits A, C and D (upper) and subunit B (lower). Active site residues forming the hydrophobic surface of the active site are shown in green and the introduced Asn-192 is shown in cyan. **Left**; schematic representations of the conformations of the substrates inferred from these structures showing the two major conformations of binding. Asn-192 lies at the bifurcation of the dipropylamide

This work demonstrates the subtleties of enzyme-substrate interactions and the importance of determining structures of enzymes produced by directed evolution, where the specificity determinants may change from one substrate to another.

Publications

Campeotto, I., Bolt, A., Harman, T., Dennis, C., Trinh, C., Phillips, S., Nelson, A., Pearson, A. & Berry, A. (2010) Structural insights into substrate specificity in variants of *N*-acetylneuraminic acid lyase produced by directed evolution. *J. Mol. Biol.* **404**:56-69.

Horsfall, L., Nelson, A. & Berry, A. (2010) Identification and characterization of important residues in the catalytic mechanism of CMP-Neu5Ac synthetase from *Neisseria meningitidis*. *FEBS J.*, **277**:2779-2790.

Tunio, S., Oldfield, N., Berry, A., Ala'Aldeen, D., Wooldridge, K. & Turner, D. (2010) The moonlighting protein fructose-1, 6-bisphosphate aldolase (fba) of *Neisseria meningitidis*: Surface localization and a role in host cell adhesion. *Mol. Micro.*, **76**:605-610.

Funding
This work was supported by The Wellcome Trust, EPSRC and by a BBSRC doctoral training grant.

Exploring proteins and their complexes using force

Neal Crampton, Khalid Al-Zahrani, Simon Connell and David Brockwell

Introduction

The first reports of the unfolding of single protein molecules by application of force were published fifteen years ago. Concurrent with and partly driven by this achievement, it became evident that many cellular processes are triggered or catalysed by force. For example, mechanical forces are utilised in many diverse cellular activities including communication, translocation and even the catalytic activation of proteins. As mechanical deformation fundamentally alters the underlying energy landscape of the non-covalent interactions that stabilise both proteins and their complexes, application of force can often result in startling effects if only biophysical parameters derived from non-force methods (e.g. affinities, stabilities and off-rates) are considered. To understand mechano-biology it is thus vital to understand the fundamental effects of force on biomolecules, especially the relationship between structure and mechanical stability for both structural and catalytic proteins and for protein-protein interactions. To achieve this aim we use the atomic force microscope (AFM) to unfold proteins or break apart protein complexes.

Expanding the experimental tool box

1. Dynamic force spectroscopy (DFS) is a powerful method able to probe the underlying features of the potential energy landscape of non-covalent interactions. This is achieved by applying a mechanical force in a defined direction to the particular interaction under study. Application of a force tilts the potential energy landscape exponentially increasing the unbinding or unfolding rate. When this force is applied at differing loading rates a dynamic force spectrum is obtained, from which the position (x_u) and height (k_u) of significant transition barriers along the unfolding (or rupture) pathway can be identified. To characterize fully the unfolding landscape, however, it is necessary both to explore the entire force spectrum and to characterize each species populated during unfolding. Both of these are difficult to achieve using conventional AFM techniques that use a deflecting (compliant) cantilever to measure the force applied onto the biomolecule. To address these problems we have developed a new technique called constant deflection AFM (CD-AFM) in which cantilever is locked to a pre-defined set-point using a secondary high powered feedback-controlled laser.

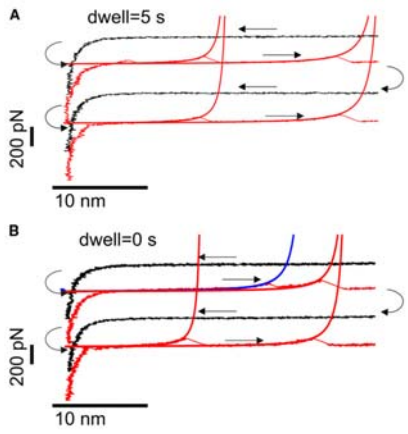


Figure 1: repeat unfolding/refolding of protein L by CV-AFM reveals the refolding intermediate. After pickup of a full-length (protein L)$_5$, the construct is repeatedly relaxed and extended for a distance that allows only two domains to unfold. Extend traces (showing unfolding events) are shown in red and relaxed traces in black for a surface dwell time of 5 or 0 s (**A** and **B**, respectively). Sequential relax-extend-relax-extend force curves showing the unfolding of two completely folded domains (red WLCs). The separation between the two events is as expected for completely unfolding protein L.

This has allowed identification of two novel features of the (un)folding of protein L: (i) a high –energy barrier to unfolding that is rate-limiting at high unfolding rates and (ii) the presence of a refolding intermediate populated at low applied force (Figure 1). These data reveal that even topologically simple proteins may fold over unexpectedly rough energy landscapes.

2. The unfolding force of a protein domain is affected by the length and type of the polyprotein construct in which it resides. To obviate these effects it is necessary to extract the parameters that coarsely define the underlying energy landscape (x_u and k_u) by Monte Carlo methods. However, for heteropolymeric constructs (comprising two domain types), the probability of unfolding one domain type at a particular position in an unfolding sequence will depend on k_u, x_u and the compliance. Development of a statistical model to analyse the unfolding patterns of heteropolymeric proteins has thus allowed x_u and k_u of one protein to be deduced if the other is known.

3. In addition to their static, elastic behaviour it also important to understand the time-dependent visco-elastic properties of biomolecules. In a collaboration with the Kawakami group we have examined the visco-elasticity of the coiled-coil of single myosin rods under application of increasing force loads. By measuring the response of a magnetically driven oscillating AFM cantilever under different force loads it is possible to extract values for the friction and elastic constants as a function of myosin extension. This demonstrated that at forces below 30 pN, both the elastic and friction constants increase rapidly compared to an unstructured polypeptide, indicating that the two-stranded coiled-coil structure is both statically and dynamically rigid. The coiled coil of myosin could be useful in muscle function as a molecular shock absorber, whereby sudden loads are dissipated in the rod structure, preventing uncoiling of the coiled coil and thereby offering more robust muscle function.

Publications

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Funding

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Collaborators

Prof. Godfrey Beddard, School of Chemistry, University of Leeds
Prof. Colin Kleanthous, Department of Biology, University of York
Dr Emanuele Paci, Institute of Molecular and Cellular Biology, University of Leeds.
Dr Masaru Kawakami, School of Materials Science, Japan Advanced Institute of Science and Technology, Japan.

Structural studies of the motor proteins dynein and myosin

Bara Malkova, Yusuke Kato, Hiroshi Imai and Stan Burgess

Introduction

Dynein is a family of minus-end directed microtubule motors that function in a wide diversity of cellular processes in eukaryotes including the trafficking of numerous cargoes (e.g. vesicles, mRNA, mitochondria), the positioning of the nucleus, Golgi apparatus and the mitotic spindle. Dyneins also drive the propagated-bending waves of cilia and flagella. Dynein is one of three different families of molecular motors, the others being kinesin and the actin- based motor myosin, and by far the least well understood. Dynein is large (~ 520 kDa), with a motor domain ~ten times larger than that of the other microtubule-based motor kinesin and has an evolutionary origin within the AAA+ superfamily of mechanoenzymes, unlike kinesin and myosin.

My lab is interested in discovering the mechanism of action of the motor domain of dynein and how dimeric cytoplasmic dynein steps along a microtubule processively. We have shown previously by electron microscopy (EM) that each dynein heavy chain has a stalk-head-tail structure. The head is ring-like and contains six AAA+ domains. ATP hydrolysis primarily in AAA1 drives the conformational changes associated with the power stroke and those governing its binding to and release from, the microtubule track via a small domain at the end of the ~12nm long anti-parallel coiled coil of the stalk.

Results

Studies in my lab are focused on understanding the structure and mechanisms of the molecular motor dynein alongside continuing studies of myosin motors (Oke et al. 2010) in collaboration with Prof.s Peter Knight and John Trinick within the Astbury Centre.

My lab is pursuing the 3D structures of flagellar dynein (in collaboration with Prof. Kazuhiro Oiwa's group, KARC, Kobe, Japan) and of recombinant cytoplasmic dynein (in collaboration with Prof. Sutoh University of Tokyo and Dr. Kon, University of Osaka), funded by BBSRC. In collaboration with Dr Andrerw Carter (LMB-MRC) we are examining by EM recombinant dynein's from fungi. We use negatively stained and frozen-hydrated (cryo-EM) techniques and single particle image processing techniques.

A project funded by the Human Frontiers Science Program (HFSP) is investigating the biochemical and biophysical properties of dimeric cytoplasmic dynein bound to microtubules by cryo-EM. The collaborators in my team are Dr. Takahide Kon and Prof. Hideo Higuchi (University of Tokyo) and Dr. Andrej Vilfan (Ljubljana, Slovenia).

Publications

Oke, O., Burgess, S., Forgacs, E., Knight, P., Sakamoto, T., Sellers, J., White, H. & Trinick J. (2010) Influence of lever structure on myosin 5a walking. *Proc. Natl. Acad. Sci. USA* **107**:2509-2514.

Funding

This work is funded by BBSRC, HFSP and The Wellcome Trust.

Collaborators

Prof. Kazuhiro Oiwa and Dr Hitoshi Sakakibara, KARC, Kobe, Japan
Prof. Kazuo Sutoh University of Tokyo, Japan
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Dr Matt Walker, MLW Consulting (Cornwall, UK)
Dr Anthony Roberts, Harvard Medical School, Boston, USA

pH-responsive biodegradable polymers for intracellular drug delivery

Rongjun Chen

Introduction

One of the main aims of drug delivery research is the efficient intracellular delivery of therapeutics, particularly macrodrugs such as proteins and nucleic acids. After being internalised by endocytosis, drugs face several obstacles such as lysosomal degradation before they can reach their target organelles or cell nuclei. One strategy to prevent lysosomal degradation is through endosomal membrane disruption using pH-responsive polymers. These polymers can undergo pH mediated coil-globule changes in conformation and this property enhances their membrane-disruptive behaviour.

Another important aspect of drug delivery is the transport of drugs through extracellular barriers before they reach the cell surface. Ideally, the drug should reach all cells in a tumour after leaving the vasculature. However, high interstitial pressure within tumours, diffusion limitations and the extracellular matrix present significant obstacles to effective drug delivery. Therefore relatively high drug concentrations were frequently used to overcome these problems. This inevitably led to toxic side effects in patients. Drug delivery systems serve to reduce the systemic toxicity by enhancing the delivery of therapeutics to specific diseased sites at a lower dose.

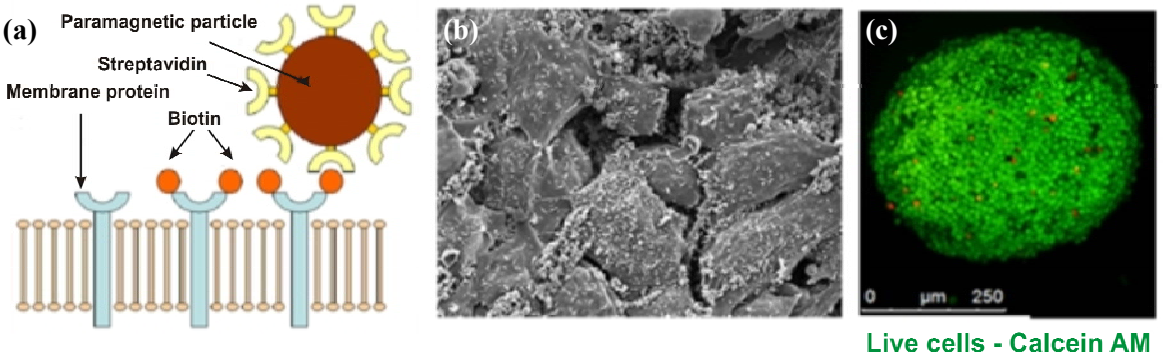

Figure 1: **(a)** Schematic of magnetic labeling of cell surface. **(b)** SEM image of 3D TE671 multicellular structures assembled by magnetic manipulation. **(c)** Live/dead staining of 6-day-old magnetic HeLa multicellular spheroids using fluorescein diacetate and propidium iodide. Viable cells appear as green, while non-viable cells appear as red.

Results

We have recently developed a class of biodegradable, pH-responsive, endosomolytic polymers to mimic factors that enable efficient viral transfection. The parent polymer is a polyamide, poly(L-lysine isophthalamide), and hydrophobic amino acids were grafted onto its pendant carboxylic acid groups to manipulate its amphiphilicity and structure. Recent studies indicated that L-phenylalanine grafted polymers, such as PP-75 (M_n = 24.9 kDa, degree of grafting = 63.2%), have vastly superior membrane-disruptive activity at endosomal pHs and could be used for intracellular drug delivery.

Drug delivery systems are often studied using two dimensional (2D) cell monolayers which cannot reproduce the complex three dimensional (3D) environment in tissues or organs. To better model the actual in vivo conditions, we have recently developed magnetic multicellular spheroids (Figure 1(c)) from magnetically labeled cells (Figure 1(a), (b)). The 3-D culture system was used as an avascular tumour model to investigate how drug delivery systems

transport through extracellular barriers and reach individual cells. These spheroids can be easily separated using a magnetic separator within a few seconds without the need for centrifugation. This allows for facile separation of spheroids after incubation with drugs or drug delivery systems for further examination using other analytical techniques.

The distribution of the fluorescent polymer, PP-75-FITC, in the magnetic HeLa multicellular spheroids was investigated. As shown in Figure 2, the 3D delivery of PP-75-FITC is dependent on both polymer concentration and incubation duration. More polymers can be delivered into the spheroids when there is a higher polymer concentration or longer incubation time, as shown by the stronger fluorescence.

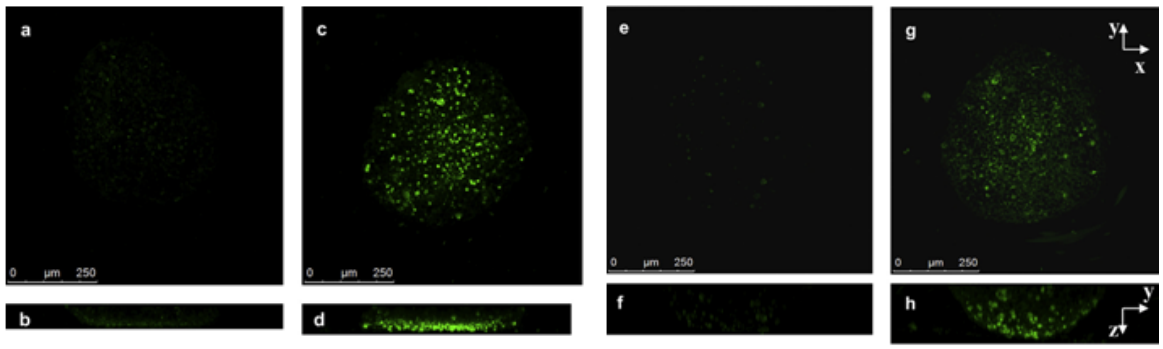

Figure 2: confocal microscopy images of HeLa spheroids treated with PP-75-FITC at **(a, b)** 0.05 mg mL^{-1} for 1 h, **(c, d)** 4 mg mL^{-1} for 1 h, **(e, f)** 1 mg mL^{-1} for 2 min and **(g, h)** 1 mg mL^{-1} for 2 h. Samples were imaged after 3.5 h further incubation. (a, c, e, g) show the image in the X-Y axis, while (b, d, f, h) show the image in the Y-Z axis.

In our study above, PP-75 was able to penetrate efficiently into the 3D multicellular spheroids, reach almost all the cells (~93%) in the spheroids and achieve a high cell entry. When the polymers were internalised, they were able to disrupt endosomal membranes for effective intracellular release of endocytosed payloads. The transport of PP-75 in the spheroids might be less obstructed due to its small size (37 nm in diameter at pH 7.4), which makes it easier to diffuse through the extracellular space. As PP-75 migrated through the spheroids, it experienced decreasing pH and could undergo further size reduction (17 nm in diameter at pH 6.0). This could have actually aided in the penetration of PP-75 towards the core of the multicellular spheroids and resulted in a higher proportion of cells which internalised the polymer. In addition, grafting with the hydrophobic amino acid L-phenylalanine can facilitate effective cell surface binding, which is necessary for uptake of the polymers.

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Collaborators
Prof Nigel Slater, University of Cambridge.

Molecular self-assembly in a model amphiphile system

Lorna Dougan

Introduction

Biological processes are intimately linked to the unique properties of water and the versatility with which it interacts with a wide variety of biomolecules. These interactions are thought to be of major significance in the structure, dynamics and activity of proteins, the formation of biological membranes and the transport of ions and co-solutes. Of particular interest is water's behaviour with respect to non-polar molecules or non-polar groups of biomolecules, which have a tendency to adhere to each-other to minimize their exposure to the solvent. The process by which molecules containing both non-polar and polar regions adopt a defined arrangement is termed molecular self-assembly. Given the complexity of typical biological self-assembly systems it is useful to consider a model system which retains the key elements of interest for the study of self-assembly. One such model system are the alcohols which contain both a polar group (OH) and non-polar methyl groups (CH_3) and offer the opportunity to study the properties of a small amphiphile in an aqueous environment.

Beyond the 'iceberg' model

The thermodynamic signatures associated with the hydration of alcohols are striking. When water is mixed with an amphiphilic molecule, the entropy of mixing is non-ideal. In fact the excess entropy is frequently negative, as occurs in the instance of methanol in water. Historically, the most familiar and favoured explanation for this general behaviour has been based on the notion of enhanced water ordering in the vicinity of hydrophobic groups. According to this model, the structural enhancement of solvent explains the negative deviation from ideal entropy of mixing and thereby leads to an entropic driving force for hydrophobic association of non-polar groups. Despite the attractiveness of this description, its validity has now been questioned by a number of diffraction measurements which report no discernible structural enhancement of water near non-polar groups. In a recent paper we showed that the excess entropy could be quantitatively explained in terms of a simple model which explores the observed partial molecular-scale demixing of alcohol and water in solution. It does this without resort to the idea that water is more structured around the hydrophobic headgroups, the so-called "iceberg" model. This work suggested that it is the amphiphilic nature of a molecule that determines the self-assembly process in aqueous solution. In the present study we continue this theme, invoking recent data on the effect of temperature and pressure on the mixing of alcohol-water.

This approach now presents an attractive framework for examining the behaviour of model amphiphile systems under cooling and compression. Given the important contributions of both enthalpic and entropic contributions to the self-assembly of biological systems, use of a simple model system allows detailed examination of the interplay between molecular self-assembly and thermodynamics. In this study we extend our analysis to methanol-water mixtures far from ambient conditions to separate the effects of pressure and temperature on the excess entropy. This allows us to draw conclusions about the clustering behaviour in this important model system. Furthermore, by studying a model amphiphile system we have the possibility of making predictions about the behaviour of biological amphiphile systems of greater complexity.

Our results suggest that under increased pressure and reduced temperature, the average cluster size of both water and methanol clusters, while the system becomes better mixed. This apparent contradiction can be explained by a change in topology of the clusters, with water

clusters becoming more sheet-like, rather than globular, and hence interacting more with the surrounding methanol clusters. This work suggests that structural properties of a system, driven by molecular self-assembly, determine the thermodynamics. Ongoing work will examine the molecular self-assembly of cryoprotectant aqueous solutions which exhibit interesting thermodynamic properties.

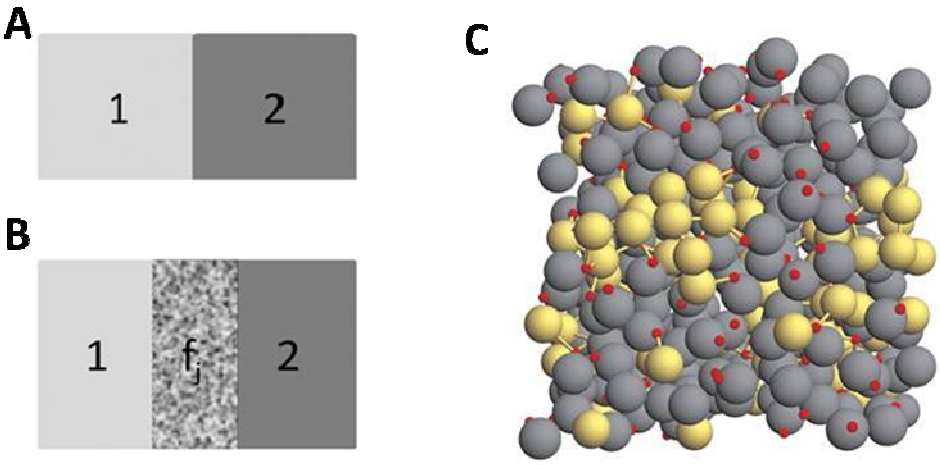

Figure 1: self-assembly of water and methanol in the liquid state. The schematics of two models are shown in **(A)** and **(B)**. In (A) the 'fully demixed' model there is a sharp boundary between the water and alcohol clusters, depicted in the schematic as 1 and 2. There is no mixing at the atomic level. In (B) the 'interface' model water and alcohol again exist at clusters but there is now an interfacial region containing both water and alcohol. The molecules assigned to the interface are treated as randomly mixed and so make negligible contribution to the excess entropy of the system. The entropy of the system is determined not only by the size of the clusters but also by their topology and the topology of the interfacial region. **(C)** Neutron diffraction data coupled with computational modelling allows for examination of the clustering of water and alcohol. Applying the 'interface' model we can accurately calculate the excess entropy of the system, which is in good agreement with experimental data. This approach shows that structural insight into a system can provide an insight into important thermodynamic properties of a system.

Publications

Dougan, L., Crain, J., Finney J. & Soper A. (2010) Molecular self-assembly in a model amphiphile system, *Phys. Chem. Chem. Phys.* **12**:10221 -10229.

Funding

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Collaborators

Prof. Alan Soper, Rutherford Appleton Laboratories, United Kingdom
Prof. John Finney, University College London, United Kingdom

The structure of the Pumilio homology domain of murine Pum2

Huw Jenkins and Thomas Edwards

Introduction

Human fertility is directly affected by the regulation of gene expression during gamete formation. Post-transcriptional regulation of gene-expression through control of translation by RNA-binding proteins has recently been implicated as a critical factor in this process. Two RNA-binding proteins that have been shown to interact in order to control translation during spermatogenesis are the deleted in azoospermia (DAZ) family member, DAZ-like (Dazl) and a homologue of the *Drosophila* Pumilio Puf protein – Pum2. Members of the Puf family of RNA-binding proteins bind to the 3' UTR of selected transcripts causing repression of translation. The RNA is bound to a C-terminal Puf (Pum and FBF) domain. This domain is also the interaction site for Dazl.

Structure of murine Pum2

In order to start to examine the detail of the interaction between Dazl and Pum2 on target RNAs we have solved the 1.6 Å resolution crystal structure of the murine Pum2 Puf domain (Figure 1). The structure was solved by molecular replacement using the human Pum1 Puf domain structure as a search model. We demonstrated that this domain is capable of binding with nanomolar affinity to RNA sequences from the *hunchback* Nanos response element (NRE) and a previously identified Pum2 binding element (PBE). We are also screening RNA sequences that were bound with high affinity by Pum2 in SELEX experiments with the aim of solving the co-crystal structure of Pum2 bound to a high affinity target RNA sequence. We now also have high resolution diffraction data from Dazl:RNA complexes. Future work will focus on solving the structure of the Dazl:Pum2:RNA complex.

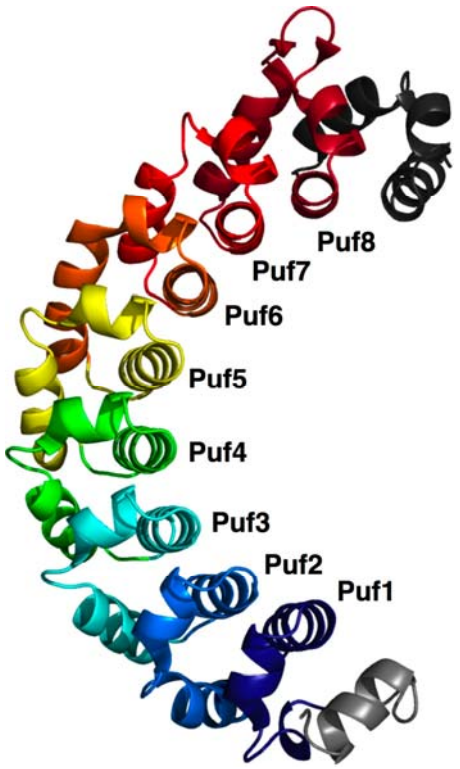

Figure 1: mouse Pum2 Puf domain. Cartoon of the cystal structure of the murine Pum2 Puf. The domain contains 8 tandem Puf repeats coloured from blue (Puf 1) to red (Puf8).

Publications

Gupta Y., Lee T., Edwards T., Escalante C., Kadyrova L., Wharton R. & Aggarwal A. (2009) Co-occupancy of two Pumilio molecules on a single hunchback NRE. *RNA* **15**:1029-35.

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Collaborators

Aneel Aggarwal (Mount Sinai School of Medicine, New York); Robin Wharton (Ohio State University); Howard Cooke and Nicola Gray (MRC Human Genetics Unit, Edinburgh).

Development of small- molecule tools to probe or inhibit biomolecules and their complexes

Martin McPhillie, Lee Shearer, Ian Chopra, John Findlay, Peter Johnson, Alex O'Neill and Colin Fishwick

Design, synthesis and biological evaluation of novel inhibitors of bacterial RNA polymerase.

Bacterial RNA polymerase (RNAP) catalyses the synthesis of RNA from a DNA-template. Since this enzyme is essential for the growth and survival of bacteria, inhibition of bacterial RNAP is an established strategy for antibacterial therapy; for example the rifamycin antibiotics (known RNAP inhibitors) are used in the frontline treatment of tuberculosis. RNAP is considered an attractive antibacterial drug target since it is well conserved amongst bacterial pathogens, but has distinct structural differences from eukaryotic RNAP. This qualifies bacterial RNAP as a target for broad-spectrum antibacterial therapy and permits the design of selective inhibitors. Despite this the rifamycins are currently the only class of bacterial RNAP inhibitor approved for clinical use, highlighting the need for new RNAP inhibitors and the overall challenge of RNAP as a drug target.

Combating the rise in bacterial resistance to antibiotics is an important strategy in drug discovery. Novel antibacterial agents with new modes of action offer the best possibility to overcome existing resistance mechanisms. New opportunities to explore bacterial RNAP as a target have arisen following the recent discovery of the Myxopyronin B **1** (MyxB) binding site (Figure 1). MyxB is an antibiotic produced from the myxobacterium *Myxococcus fulvus* Mxf50, which inhibits bacterial RNAP and the growth of Gram-positive and Gram-negative bacteria. MyxB binds to the switch region of RNAP, where its mode of action has been proposed to involve either inhibition of the opening and closing of the beta prime subunit, or stabilisation of the refolding of the β'-subunit switch-2 region, leading to an inactive conformation that cannot bind to template DNA. As bacteria have become resistant to the rifamycins, the MyxB binding site presents itself as an excellent opportunity for the design of new RNAP inhibitors as the two antibiotic binding sites are distant from each other.

Using the co-crystal structure of *T. thermophilus* RNAP-myxopyronin B complex (PDB ID 3EQL) the MyxB binding site was explored using SPROUT. Given the large volume of this cavity we focussed on the region around the enecarbamate side chain, as it was narrow and offered a bound inhibitor the potential for making hydrogen-bonding interactions with residues Trp1039 and Glu1041 (Figure 1).

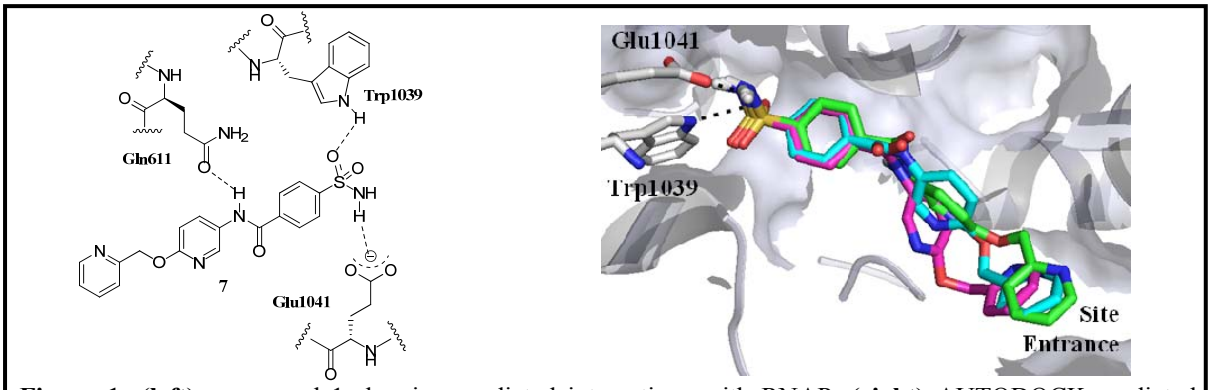

Figure 1: (left) compound 1 showing predicted interactions with RNAP. **(right)** AUTODOCK predicted docking poses of compound 1 (blue) within RNAP (grey): lowest energy pose (pink) and highest populated pose (green).

Using SPROUT, a substituted pyridyl-benzamide scaffold was predicted to fill this volume and make interactions with residues Trp1039, Glu1041 and Gln611 (Figure 1). A library of compounds (e.g. **1,** Figure 1**)** have been synthesised and tested in an *in vitro E. coli* RNAP assay using a Kool NC-45^{TM} RNA polymerase template (Epicentre Biotechnologies). A number of these molecules displayed encouraging *in vitro* potency (9 – 46 mM), and appear to be selective for the bacterial as opposed to the human enzyme.

Development of small- molecule tools to probe the protein-protein interactions of RBP
Lipocalin receptors are a new family of membrane proteins. The best characterized functionally is that for retinol binding protein (RBP) which mediates the uptake of retinol. Recent work at Leeds has started to yield important information on the precise nature of the interaction of RBP with its receptor (RBPr). Indeed, there is now considerable evidence to suggest that the interactions of lipocalins such as RBPr with other proteins (eg, in addition to RBP, RBPr interacts with transthyretin (TTR)), play a crucial role in diseases such as type II diabetes (Fig 2). Although the crystal structure of RBP is known, there is much still to be established for RBPr and its interactions with other proteins. This project will utilise small focussed libraries of small molecules to probe the details of these protein-protein interactions

We have used *in silico* methods to design and synthesise a range of small-molecule libraries targeting the various interactions (Figure 2). Using a range of approaches including gel-shift assays, fluorescence quenching, and SPR, a number of molecules have been identified which modulate these various interactions and we are now building up a picture of the interplay of the various protein-protein interactions involving RPB as well as the role of these within the cellular environment.

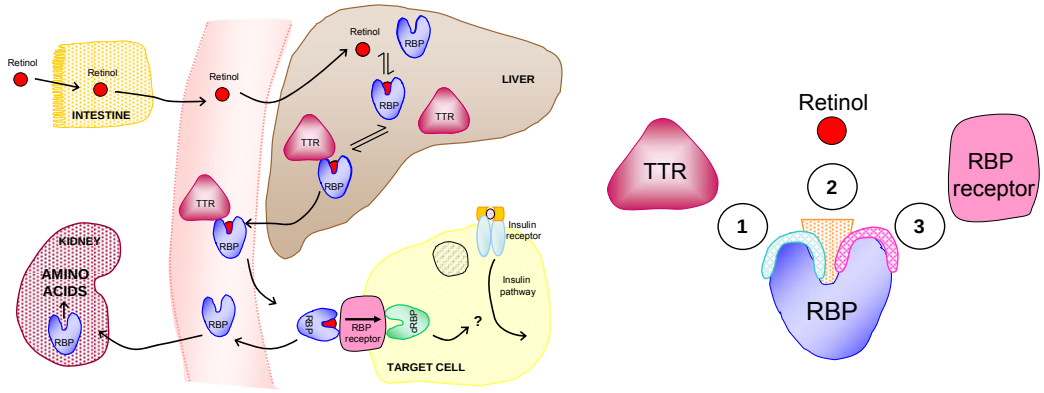

Figure 2: (left) The "life history" of retinol binding protein (RBP) from its retinol dependent secretion from the liver to its excretion via the kidney following the release of retinol to the cell via a specific membrane receptor; **(right)** Target sites on RBP for small molecule intervention-1. Primary TTR binding site, 2. Retinol Binding cleft and 3. Membrane Receptor interaction region.

Publications

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Funding

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Viroporins and enveloped virus assembly as antiviral targets

Toshana Foster, Matthew Bentham, Lynsey Corless, Ranjitha Tatineni, Mark Verow, Jamel Mankouri, Elizabeth Atkins, Barnabas King, Carsten Zothner, David Rowlands, Mark Harris and Stephen Griffin

Introduction

Antiviral drugs represent the only means to control/eliminate many virus infections. New molecular and *in silico* methods now permit the rational development of bespoke inhibitors of viral processes, creating a burgeoning field poised to shape future therapies.

The formation of new virus particles within the cell requires the hijacking of cellular secretory and vesicle trafficking pathways. In this regard, many viruses encode small hydrophobic ion channel proteins, termed "viroporins" that perform vital functions during virus egress. Drugs blocking viroporin function have been used clinically to treat influenza, providing a precedent to develop novel inhibitors targeting other clinically important viruses.

HCV infects up to 170 million people causing severe liver disease including liver cancer. Current interferon-based therapy for HCV is inadequate and a new era of combination therapy with specifically targeted antivirals is fast approaching. We first discovered that the HCV p7 protein functions as a viroporin and that it could be blocked by small molecule inhibitors, preventing the production of infectious HCV particles. We have undertaken a multi-disciplinary approach to understand how HCV particles are assembled, how p7 facilitates this process and to develop antivirals targeting p7 as a component of new combination therapies. We are also targeting other viroporins for therapy as well as creating translational clinical links for the treatment of HCV infection and liver cancer.

Development of p7 inhibitors as HCV therapeutics

We have utilised molecular modelling and *in silico* drug docking studies as a guide to the mode of action for p7 inhibitors. Combined with knowledge of strain-dependent drug sensitivity, we were able to predict inhibitor binding sites and engineer specific resistance mutations into HCV genomes, some of which had been documented in clinical trial patients. Such mutants prove the specific antiviral effects of prototype p7 inhibitors, many of which are already licensed for clinical use (see below). Working with Dr Richard Foster (School of

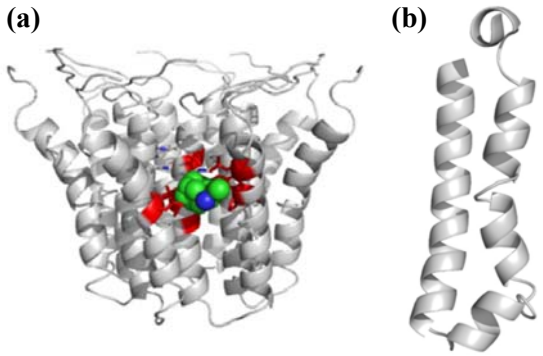

Figure 1: (a) rimantadine docked to a heptameric p7 channel model. **(b)** solution structure of monomeric p7. Foster, Verow *et al.* submitted

Chemistry), we are pursuing a rational approach to the design of novel p7 inhibitors, incorporating vHTS and molecular design led programmes, as well as establishing an *in vitro* HTS for p7 function.

High resolution structural studies of the p7 ion channel complex

Although molecular modelling represents a powerful approach for rational drug design, atomic resolution structures are desirable for high fidelity approaches. With Prof Steven Homans, we have recently solved the solution structure of monomeric p7. This will prove

invaluable to both ongoing molecular design and drug binding dynamic studies and the structure of the complete p7 channel complex is making good progress.

Assembly and trafficking of HCV particles

In close collaboration with Prof Steve Weinman (University of Kansas, USA), we recently showed the first conclusive proof that p7-mediated proton flux was directly required to protect acid-sensitive intracellular HCV particles. This activity is blocked by p7 inhibitors.

We are currently investigating the switch between intra- and extracellular HCV particles.

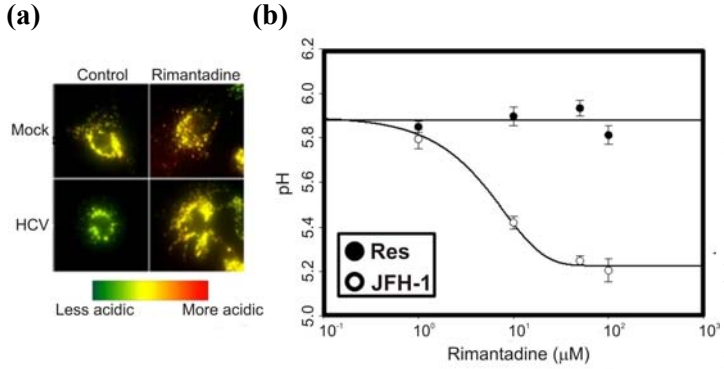

Figure 2: live cell measurement of p7 proton channel activity. HCV infected cells were labelled with lysosensor blue/yellow DND-160 +/- rimantadine and vesicle pH determined by ratiometric fluorescence. (a) fluorescence image of labelled cells. (b) quantitation of pH for wild-type JFH-1 HCV and JFH-1 with rimantadine resistance mutation (Res) in the presence of rimantadine.

We are investigating how HCV virions leave the cell once they are assembled. Dogma states that HCV egress is closely associated with the VLDL secretory pathway, yet we have found that disrupting late acting ESCRT component and Rab GTPases involved in endosomal trafficking prevents secretion of infectious virus without affecting VLDL or global cellular secretion.

Targeting other viroporins for therapy

We can apply many of our *in vitro* and drug discovery methodologies to viroporins from other clinically important viruses where a pressing need for novel antiviral therapies exists. At LIMM we have begun programmes targeting the viroporins of W.H.O. emerging viruses, which are becoming an increasing threat with accelerating globalisation and climate change. With Richard Foster, Dr Paul Targett-Adams (Pfizer, UK) and Prof Wendy Barclay (Imperial College, London), we are developing novel inhibitors of adamantane-resistant influenza A virus M2 channels. In addition, we are working closely with Dr Andrew Macdonald (FBS) characterising a novel viroporin encoded by high risk human papillomavirus, the major cause of cervical cancer. We also collaborate with Dr John Barr (FBS) on the SH viroporin of Respiratory Syncitial Virus.

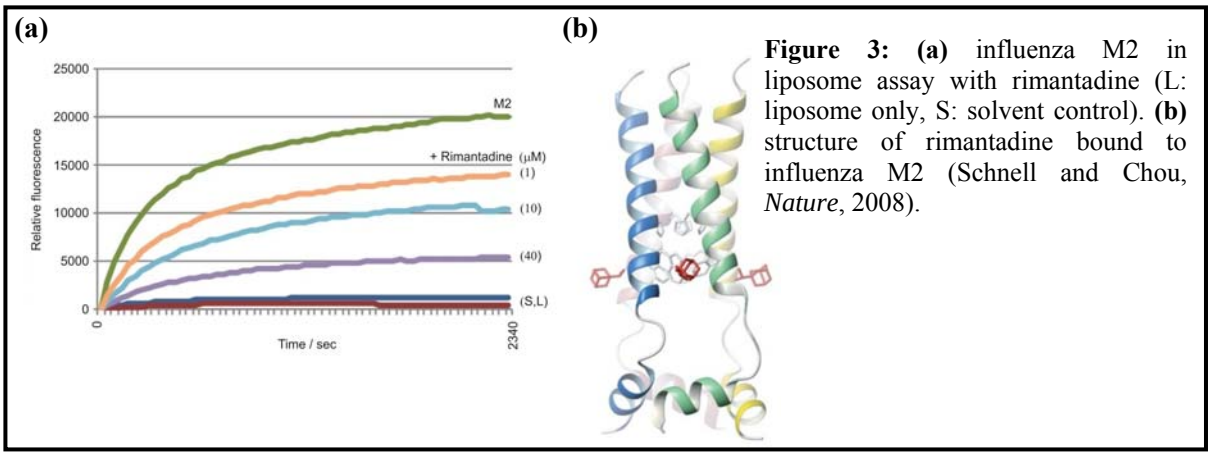

Figure 3: (a) influenza M2 in liposome assay with rimantadine (L: liposome only, S: solvent control). **(b)** structure of rimantadine bound to influenza M2 (Schnell and Chou, *Nature*, 2008).

Translational clinical trials for HCV and hepatocellular carcinoma
We are working with the considerable clinical resource at LIMM and within St James University Hospital (SJUH) to undertake clinical trials, both to validate p7 inhibitors in patients and for future studies of hepatocellular carcinoma. Currently, we are undertaking a biological endpoint trial with Dr Mark Aldersley, Dr Lynsey Corless and Dr Charlie Milson (SJUH Liver Unit), and Prof Alan Melcher and Prof Chris Twelves (SOCR), validating the antiviral effect of rimantadine during HCV treatment.

Publications
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Mankouri, J., Tedbury, P., Gretton, S., Hughes, M., Griffin, S., Dallas, M., Green, K., Hardie, D. Peers, C. & Harris, M. (2010) Enhanced hepatitis C virus genome replication and lipid accumulation mediated by inhibition of AMP-activated protein kinase. *Proc. Natl. Acad. Sci. USA* **107**:11549-54.

Wozniak, A., Griffin, S., Rowlands, D., Harris, M., Yi, M., Lemon, S. & Weinman, S. (2010) Intracellular proton conductance of the Hepatitis C Virus p7 Protein and its contribution to infectious virus production. *PLoS Pathog.* **6**:e1001087.

Funding
This work is funded by Yorkshire Cancer Research, CRUK, the Biomedical Health Research Centre (University of Leeds) and the MRC.

Collaborators
Weinman (University of Kansas, USA), Russell (Memorial University, Newfoundland), Gretch (University of Washington, USA),Rose (NIBSC), Barclay (Imperial), Tuthill (IAH Pirbright), McCormick (Southampton), Targett-Adams (Pfizer).

University of Leeds: Macdonald, Foster, Homans, Rowlands, Fishwick, Barr.

Studies on hepatitis C virus replication and pathogenesis

Jamel Mankouri, Yutaka Amako, Mair Hughes, Toshana Foster, Bjorn-Patrick Mohl, Barnabas King, Zsofia Igloi, Doug Ross, Elizabeth Atkins, Carsten Zothner, Steve Griffin and Mark Harris

Introduction

Hepatitis C virus (HCV) infects 170 million individuals and is a major cause of chronic liver disease, including fibrosis, cirrhosis and hepatocellular carcinoma. The virus has a single stranded positive sense RNA genome of 9.5kb that contains a long open reading frame encoding a single polyprotein of 3000 amino acids which is cleaved into 10 individual polypeptides by a combination of host cell and virus specific proteases. The molecular mechanisms of virus replication and pathogenesis remain to be elucidated, however, recently a clone of the virus that is able to replicate in cell culture has been identified and we are using this model extensively to understand how the virus replicates and causes pathology.

Research overview

A major focus of work is NS5A, a three-domain pleiotropic protein with multiple roles in the virus lifecycle. We showed that NS5A interacts with the proto-oncogene β-catenin (Milward *et al*.) and stimulates its transcriptional activity potentially contributing to development of hepatocellular carcinoma, and are currently analysing the interactions between NS5A and cellular SH3 domain containing proteins.

We are also investigating the role of NS5A in virus replication. We are using a mass spectrometric approach to identify sites of phosphorylation (Nordle Gilliver *et al.*) within the protein. We have analysed the ability of NS5A to bind to RNA and have shown that all three domains contribute to this activity (Foster, Belyaeva *et al.*). We are also generating a series of NS5A mutants, particularly in domains II and III to characterise the role of these interactions in viral replication.

We are also very interested in NS2 and are conducting both structural (Foster, Tedbury *et al*.) and functional studies on this protein. Ongoing studies are analysing the membrane topology of NS2 and its role in virus assembly and release. The latter is part of a broader project to define the biochemical and cell biological processes underpinning the assembly and release of infectious HCV particles.

Further studies have shown that HCV infection inhibits the key cellular metabolic regulator of AMPK (Mankouri *et al*.). AMPK agonists including well characterised drugs such as metformin override this effect and inhibit virus replication, raising the potential for development of new therapeutic approaches.

Publications

Foster, T., Belyaeva, T., Stonehouse, N., Pearson, A. & Harris, M. (2010) All three domains of the hepatitis C virus non-structural NS5A protein contribute to RNA binding. *J. Virol.* **84**:9267-9277.

Foster, T., Tedbury, P., Pearson, A. & Harris, M. (2010) A comparative analysis of the fluorescence properties of the wild-type and active site mutants of the hepatitis C virus autoprotease NS2-3. *Biochim. Biophys. Acta* **1804**:212-222.

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Mankouri, J., Tedbury, P., Gretton, S., Hughes, M., Griffin, S., Dallas, M., Green, K., Hardie, D., Peers, C. & Harris, M. Enhanced hepatitis C virus genome replication and lipid accumulation mediated by inhibition of AMP-activated protein kinase. (2010) *Proc. Natl. Acad. Sci. USA*, **107**:11549.

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Funding
This work is funded by the Wellcome Trust, Yorkshire Cancer Research and BBSRC. Doug Ross holds an Astra Zeneca CASE studentship, Bjorn Patrick Mohl holds a Glaxo-Smith Kline CASE studentship and Elizabeth Atkins holds a Pfizer CASE studentship.

Collaborators
Chris Peers, Faculty of Medicine and Health, University of Leeds.
Grahame Hardie, University of Dundee
William Irving, University of Nottingham
Kalle Saksela, University of Helsinki, Finland
John McLauchlan, MRC Virology Unit, Glasgow

Structure-activity relationship of the bacterial galactose-H+ symport protein: homologue of the human GLUT transporters

Preethi Sukumar, Kim Bettaney and Peter Henderson

Introduction

Membrane transport proteins are encoded by 5-15% of the genome in all organisms from microbes to man and play a crucial role in the metabolism of living cells by mediating the influx of nutrients and the efflux of toxins and other waste products. Over 600 families of membrane proteins have been identified by extensive sequence analysis and out of these the major facilitator superfamily (MFS) of transport proteins is one of the largest. However, with very few exceptions, little three-dimensional information has been obtained. MFS proteins are generally deduced to have 12-membrane spanning α-helices comprised predominantly of hydrophobic residues, with helices 6 and 7 connected by a cytoplasmic domain containing 60-70 hydrophilic amino acids. The galactose-H^+ symport protein of *Escherichia coli* is one such transport protein and is importantly, a structural and functional homologue of the family of mammalian passive facilitated glucose transporters (GLUT). It is also homologous to the L-arabinose-H^+ symporter and the D-xylose-H^+ symporter of *E.coli* with 34 and 64% identity respectively. The sugar specificities of the *E.coli* transporters vary in the sense that GalP primarily transports hexoses and AraE and XylE transport pentoses. Nevertheless, the sugar specificities of GalP, GLUT1 (found in the human erythrocyte) and GLUT4 (rat adipocytes) are similar, suggesting that GalP is a bacterial equivalent of GLUT1. This proposition is further strengthened by the observation that the antibiotics cytochalasin B and forskolin, which are proven to be potent inhibitors of glucose transport in GLUT1, GLUT2, GLUT3 and GLUT4 also inhibit sugar transport by GalP. GalP is therefore an ideal model for studies of the structure/function relationship of GLUT1 and sugar transporters in general.

Although atomic resolution crystal structures of soluble proteins have been reported in an increasing number, such progress has not been made in terms of transporters and other membrane proteins, which have proven extremely difficult to crystallize. GalP has also resisted rigorous crystallisation attempts for many years, which could partly be due to its inherent conformational flexibility that makes it difficult to obtain stable crystals. The aim of the work here was to identify specific residues of GalP that influence substrate/inhibitor recognition and increase the overall stability of the protein, favourable for crystallisation. Up to 23 different mutants were constructed in order to analyse the role of individual residues in the structure and function of the protein. We describe the characteristics of one such mutant that has glycine in the place of native aspartate (Asp312Gly) at the interface of TM8 and TM9, so changing the residue in GalP to that found at the corresponding position in the inositol transporter, IolF, from *Bacillus subtilis*.

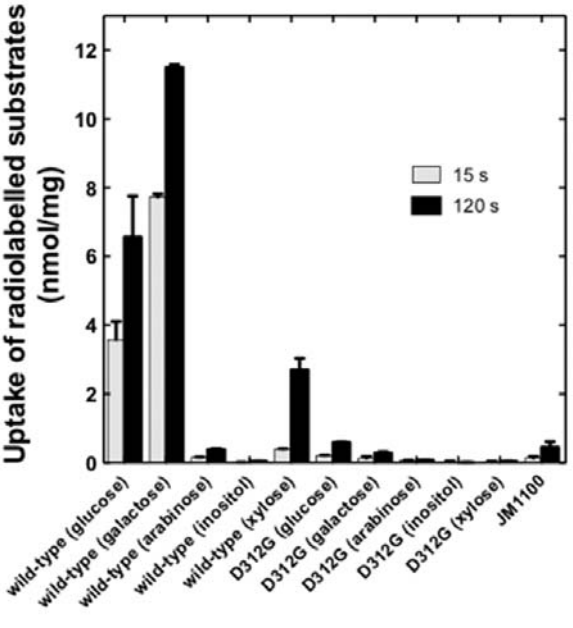

Figure 1: sugar transport assay.

Results

The ability to transport sugars was abolished in the GalP protein with an Asp312Gly mutation (Figure 1). However, fluorescence measurements showed that the protein was still able to bind galactose or glucose substrate. The specificity for other sugars was lost (Figure 1). Furthermore, in the mutant the binding of the inhibitors, cytochalasin B and forskolin, was actually enhanced (Figure 2). Reconstitution of this protein into liposomes also revealed the loss of counterflow activity (Figure 2).

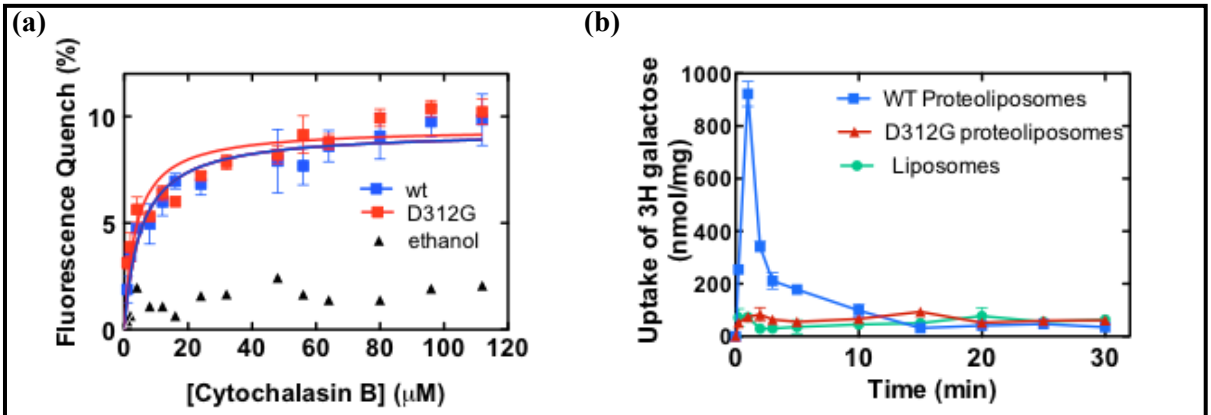

Figure 2: (a) fluorescence binding assay for wild-type and D312G GalP. **(b)** activity of wild-type and D312G GalP reconstituted into liposomes.

The detergent-solubilised and purified mutant protein exhibited much greater thermal stability than the wild-type, especially in maltoside detergents (not shown), and is being taken forward into crystallisation trials.

The results show that the Asp312Gly mutation specifically blocks the structural changes necessary for substrate translocation with little or no effect on ligand binding, creating a stabilised protein more suitable for crystallisation trials than the wild-type.

Publications

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Funding

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Collaborators

Stephen Baldwin (Leeds), Jocelyn Baldwin (Leeds), Heather Findlay (Bristol), Paula Booth (Bristol)

Conformational changes during β_2-microglobulin amyloid assembly

Timo Eichner, Arnout Kalverda, Gary Thompson, Sheena Radford and Steve Homans

Introduction

Numerous studies of amyloid assembly using different protein systems under a variety of conditions have indicated that partially unfolded states are responsible for initiating aggregation *in vitro* and *in vivo*; however, little is known about the structure of key amyloid intermediates in atomic detail. Here we use ΔN6, a truncation variant of the naturally amyloidogenic proteiņ β_2-microglobulin (β_2m), to determine, for the first time, the structure of a non-native amyloidogenic intermediate at high resolution in solution using nuclear magnetic resonance (NMR)

Real-time NMR refolding studies confirm the structural analogy of ΔN6 and I_T

In order to validate whether the non-native slow folding intermediate I_T shares a common structure with ΔN6, wild-type β_2m was denatured in 8 M urea and then refolded by ~10-fold dilution. The re-equilibration back to the native state *via* the trapped amyloidogenic intermediate I_T was monitored using SOFAST- ^{1}H-^{15}N HMQC spectra acquired ~2 min after initiation of refolding by dilution. Figure 1(a) shows the superposition of the ^{1}H-^{15}N spectra of ΔN6 and the kinetic intermediate I_T. After ~2 min of refolding the spectrum is predominantly (>75%) I_T and reveals 68 peaks which are indistinguishable (^{1}H/^{15}N within ± 0.05/0.5 ppm) from those obtained for the truncation variant ΔN6.

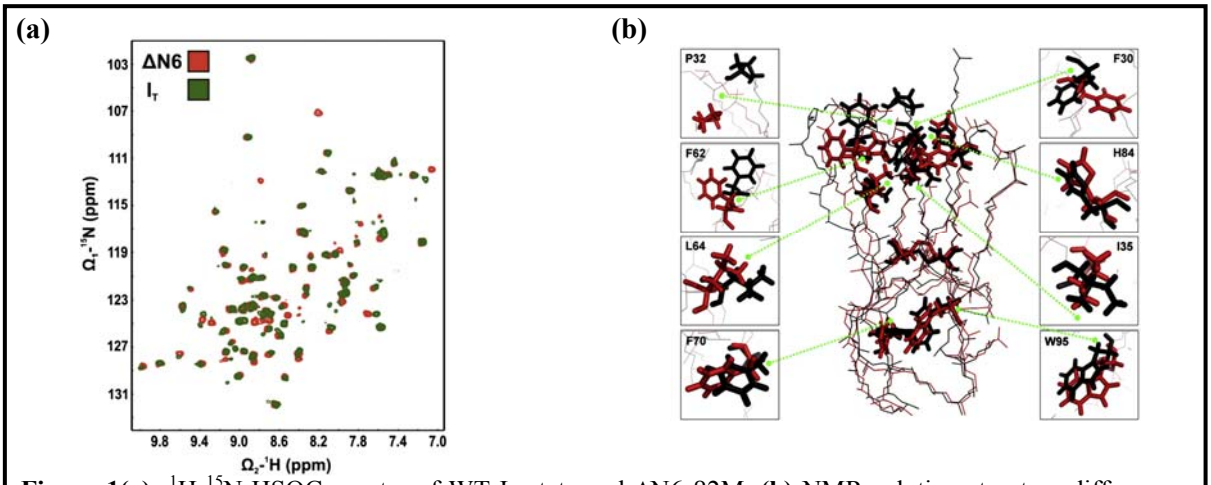

Figure 1(a): ^{1}H-^{15}N-HSQC spectra of WT I_T state and ΔN6 β2M. **(b)** NMR solution structure differences between WT (red) and ΔN6 β2M. (black).

The high-resolution solution structure of I_T reveals a native-like Ig fold

After having validated that ΔN6 mimics structurally the amyloidogenic intermediate I_T a full chemical shift assignment and structure calculation of the wild-type protein and ΔN6 was carried out at pH 7.5, and 25°C. The resulting structural ensembles (Figure 1(b)) revealed that ΔN6 has a native-like Ig β-sandwich fold, with a major repacking of the hydrophobic core to accommodate the non-native peptidyl-prolyl trans-isomer at Pro 32

Publications

Eichner, T. & Radford, S. (2009) A generic mechanism of β_2-microglobulin amyloid assembly at neutral pH involving a specific proline switch. *J. Mol. Biol.* **105:**8926-31.

Funding

This work was funded by the University of Leeds.

Proteolysis and protein:protein interactions in neurodegenerative diseases

Lizzie Glennon, Heledd Griffiths, Kate Kellet, Harry King, Vicki Lewis, Jo Rushworth, Nicole Watt, Isobel Whitehouse and Nigel Hooper

Introduction

Alzheimer's disease (AD) is the commonest neurodegenerative disease of old age. Currently, there are no drugs available to halt or slow the progression of this devastating disease which is placing a huge burden on patients and carers. AD is characterised by the deposition in the brain of senile plaques that are composed of the amyloid-β peptide (Aβ). Through mechanisms that are poorly understood, Aβ oligomers, fibrils and/or aggregates are toxic to nerve cells. Aβ is derived from the larger transmembrane amyloid precursor protein (APP) through proteolytic cleavage by the β- and gamma-secretases (Figure 1a). The β-secretase (BACE1) cleaves within the APP sequence at the N-terminus of the Aβ peptide, with the gamma-secretase complex cleaving the resulting membrane-bound stub at the C-terminus of the Aβ sequence. Inhibition of both the β- and gamma-secretases are being considered as potential therapeutic approaches to combat AD.

The prion protein is probably best known for its role in the transmissible spongiform encephalopathies or prion diseases, such as Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy in cattle. In these diseases the normal cellular form of the prion protein (PrPC) undergoes a conformational conversion to the infectious form, PrPSc. We have shown that PrPC inhibits the β-secretase cleavage of APP, lowering the amount of Aβ produced and, therefore, potentially protecting against AD. In both cell models and mice, reduction of PrPC levels resulted in an increase in Aβ production. BACE1 co-immunoprecipitated with PrPC from cells and brain samples, suggesting a direct interaction between the two proteins. More recently, PrPC was identified as a high affinity receptor for Aβ oligomers (Figure 1c).

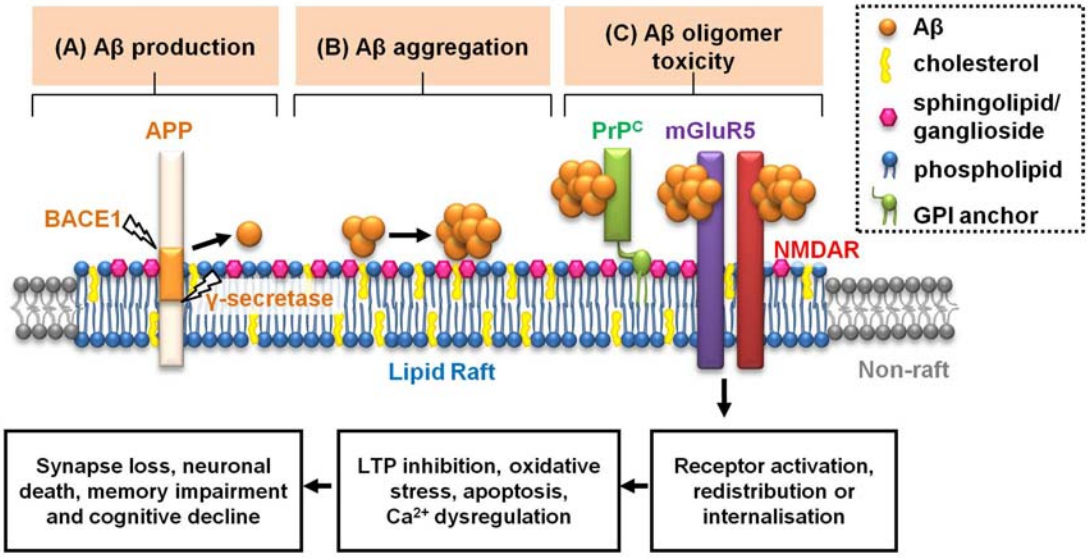

Figure 1: production, aggregation, neuronal binding and toxicity of Aβ oligomers.

Protein:protein interactions in Alzheimer's disease

Several other proteins, in addition to PrPC, have been reported to regulate the proteolytic processing of APP. For example, some proteins bind to APP and/or alter its subcellular

trafficking to modulate its proteolytic processing, including ApoER2. Thy-1, contactin and neurofascin, along with PrPC, were recently identified to interact with APP in vivo, i.e. be components of the brain interactome of APP, while other proteins have been identified through genome-wide association studies. From these studies, it is evident that APP processing and Aβ generation can be modulated by a diverse number of interacting proteins in various cellular compartments. This modulation could involve direct binding to BACE1 or APP itself, thereby influencing enzyme activities or the susceptibility of APP to cleavage. Alternatively, the mode of action may be indirect, involving the segregation of the secretases and APP into either the same or different membrane domains or cellular compartments. The molecular and cellular mechanisms underlying the modulation of APP processing in this way clearly need to be understood in order to provide a complete knowledge of AD pathogenesis. The components of the APP and BACE1 interactomes could potentially be exploited therapeutically to modulate Aβ production.

Prion protein as a receptor for Aβ oligomers

Soluble oligomers of Aβ cause neurotoxicity, synaptic dysfunction and memory impairments which underlie AD. A plethora of Aβ assemblies have been isolated from natural sources and prepared synthetically, which vary in size and morphology, although which Aβ assemblies bind to PrPC and whether these correspond to pathologically relevant Aβ oligomers that are elevated in AD brains remains unclear. Using conformation-specific antibodies, we report that fibrillar Aβ oligomers recognised by the OC antibody, that have been shown to correlate with the onset and severity of AD, bind preferentially to human neuroblastoma cells expressing PrPC. The green tea polyphenol (-)-epigallocatechin gallate (EGCG) and the red wine extract resveratrol both re-modelled the fibrillar conformation of Aβ oligomers and reduced significantly their binding to PrPC-expressing cells. Further, EGCG and resveratrol neutralised the toxicity of fibrillar Aβ oligomers towards neuroblastoma cells expressing PrPC. These data indicate that a fibrillar conformation is required for the binding of Aβ oligomers to PrPC and suggest that remodelling Aβ oligomers may prevent the neurotoxicity arising from their binding to PrPC.

Prion protein is a novel zinc transporter

Zinc is released into the synaptic cleft upon exocytotic stimuli, although the mechanism for its reuptake into neurons is unresolved. Using zinc specific fluorescent dyes we report that PrPC enhances the uptake of zinc into neuronal cells. This PrPC-mediated zinc influx was dependent on the octapeptide repeats in PrPC but did not require the endocytosis of the protein. The PrPC-mediated zinc uptake was blocked by selective antagonists of AMPA receptors and PrPC interacted with both GluA1 and GluA2 subunits. Zinc-sensitive tyrosine phosphatase activity was decreased in cells expressing PrPC and increased in the brains of PrPC null mice, providing evidence of a physiological consequence of the process. Furthermore, this PrPC-mediated zinc uptake was ablated in cells expressing a range of familial prion disease-associated mutants of PrPC and in prion-infected cells, suggesting that this loss of zinc uptake may contribute to the neurodegeneration observed in prion diseases.

Publications

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Funding
This work was funded by the MRC, Wellcome Trust, Alzheimer's Research UK and BBSRC.

Collaborators
Tony Turner, Colin Fishwick, Chris Peers.

Electrodes to study membrane proteins

Duncan McMillan, Qingshan (Rachel) Mu, Nikolaos Daskalakis, Lukasz Krzeminksi, James Kendall, Steve Evans, Richard Bushby, Peter Henderson and Lars Jeuken

Introduction

Membrane proteins, which are estimated to account for a third of the human genome, perform a myriad of functions in biology. They perform key roles in signal processing and bioenergetics. Our lab develops new techniques to study membranes and membrane proteins and we focus on ion channels and redox enzymes. Ion channels have key roles in signal transduction, while redox enzymes catalyse redox reactions in many vital processes, including photosynthesis and metabolism. We aim to link membranes and membrane proteins to electrode surfaces, which allows us to probe electron consumption/production (redox enzymes) or transmembrane charge transport (ion channels) using electrochemical methods.

Cholesterol tethers to 'wire' membranes

have prepared electrode surfaces which enables the characterisation of redox-active membrane enzymes and ion-channels in a native-like environment. For this, we have used two approaches. In the first approach, tethered bilayer lipid membranes (tBLMs) are prepared, in which lipid bilayers are attached to the electrode surface via special chemical anchors that are bound to the surface on one side and insert into a bilayer leaflet at the other (Figure 1). Cholesterol derivatives have been synthesised, which, via a hydrophilic linker, are connected to a thiol group that form self-assembled monolayers (SAMs) on gold electrodes. These cholesterol-lipids have been mixed with small thiols to provide space for transmembrane proteins. In the second approach, lipid vesicles are attached intact on solid surfaces. By appropriate modification of the gold-electrode surface, the vesicles do not loose their integrity when adsorbed, as indicated by their ability to retain encapsulated fluorescent dyes.

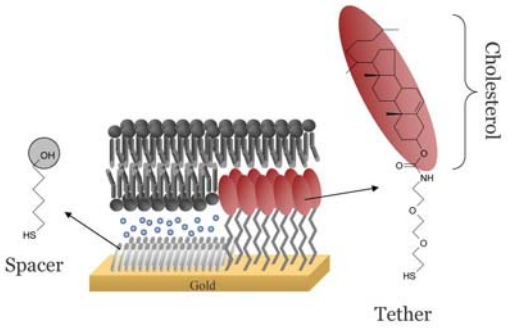

Figure 1: chemical structures of 6-mercaptohexanol (spacer) and the cholesterol tether molecule used to form tBLMs.

Ion channels

Studies were performed to provide a proof-of-principle that our tBLM system can be used to characterise ligand gated ion-channels. For this purpose, tBLMs were formed on either (a) pure tether lipid or on (b) mixed self-assembled monolayers (SAMs) of tether and spacer molecules (see Figure 1). While the tether lipid is required to form a tBLM with high resistivity, the spacer molecule dilutes the cholesterol content in the lower leaflet of the bilayer forming "ionic reservoirs" required for the sub-membrane hydration. By using simple ion-channels (gramicidin) and ionophores (valinomycin) we have shown that these ionic reservoirs are required for ion transport through the membranes. This is most likely due to the thermodynamic requirements of ions to be solvated once transported through the membrane. Unexpectedly, electrochemical impedance spectroscopy (EIS) shows an increase of capacitance upon addition of gramicidin, while valinomycin addition decreases the membrane resistance in the presence of K^+ ions. We hypothesise that this is due to previously reported phase separation of tether-lipid and spacer molecule on the surface.

Proton-pumping enzymes

Surface adsorbed vesicles were used to study a proton-pumping enzyme, cytochrome bo_3, which is a terminal oxidase and proton-pumping enzyme from *Escherichia coli*, Cytochrome bo_3 was reconstituted in lipid vesicles, which were subsequently 'loaded' with a pH-responsive fluorescent dye. Cytochrome bo_3 oxidises lipophylic ubiquinol to ubiquinone and reduces molecular oxygen to water. Ubiquinone included in the vesicles can be electrochemical reduced to ubiquinol and this property was exploited to drive the formation of a proton gradient in the adsorbed lipid vesicles as schematically indicated in Figure 2. In nature, transmembrane proton gradients are formed using energy supplied by light or chemical reactions, but in our biomimetic or hybrid organic–inorganic systems we have for the first time shown that surface-applied electrochemical potentials can also be used as an energy source.

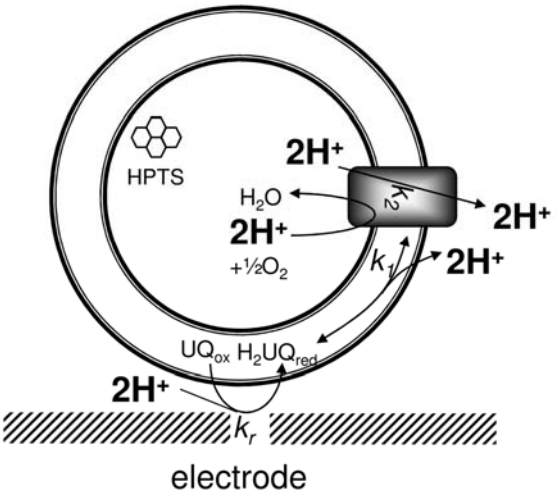

Figure 2: illustration of the experimental setup used and the four protons removed from the intravesicular compartment per 2 electrons required to reduce ubiquinone to ubiquinol. This illustration assumes a single orientation of the enzyme in the

Future directions

We aim to continue to the development of the proton-pumping system with the ultimate aim to use this system to study the enzymes on the single molecule level. Other quinone enzymes, the NapC and CymA are also being studied with the particular focus on properties that made UQ a special substrate when compared to aqueous solutes. The proteins CymA and NapC both have a single α-helix that binds them to the The work with ion channels will be continued by testing more complex ligand-gated ion-channels. Finally, EU funding has been obtained to study if the tBLM platform can be used to study the interaction between nanoparticles and lipid membranes, with the aim to characterise potential toxicological effects of nanoparticles.

Publications

Kendall, J., Johnson, B., Symonds, P., Imperato, G., Bushby, R., Gwyer, J., van Berkel, C., Evans, S. & Jeuken, L. (2010) Effect of the structure of cholesterol-based tethered bilayer lipid membranes on ionophore activity, *Chem. Phys. Chem.*, **11**:2191-2198**.**

Weiss, S., Bushby, R., Evans, S. & Jeuken, L. (2010) A study of cytochrome bo3 in a tethered bilayer lipid membrane. *Biochim. Biophys. Acta* **1797**:1917-1923.

Funding

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Structural and functional studies of copper amine oxidases

Thembi Gaule, Mark Smith, Lucy Chappell, Arwen Pearson, Peter Knowles and Mike McPherson

Introduction

We use structure-based protein engineering and structural analysis to dissect the mechanisms of oxygen entry routes and catalytic mechanisms of a range of oxidases. Most activity in 2010 was focussed on the *E. coli* copper amine oxidase.

Results

We have been studying the role of two peripheral calcium ions (Figure 1) on the formation of the post-translationally modified TPQ cofactor, and on the ability of oxygen to access the active site. Structural and biochemical studies have been used to explore the removal of the calcium ions (Smith *et al*., 2010) We have also generated a number of mutational variants of residues that line a hydrophobic channel identified as the putative oxygen entry pathway in a number of amine oxidases, including ECAO. The mutation of a residue within one of the calcium binding sites essential abolishes the formation of TPQ while a second site mutation within the channel restores TPQ biogenesis. We are using the suicide inhibitor 2-hydrazinopyridine and structural studies to explore the structural flexibility of the various mutational forms of ECAO. We have also been engaged in generating heterodimer forms of copper amine oxidases in which one subunit carries a mutation. These studies are designed to address the proposal that these enzymes display half-of-site reactivity.

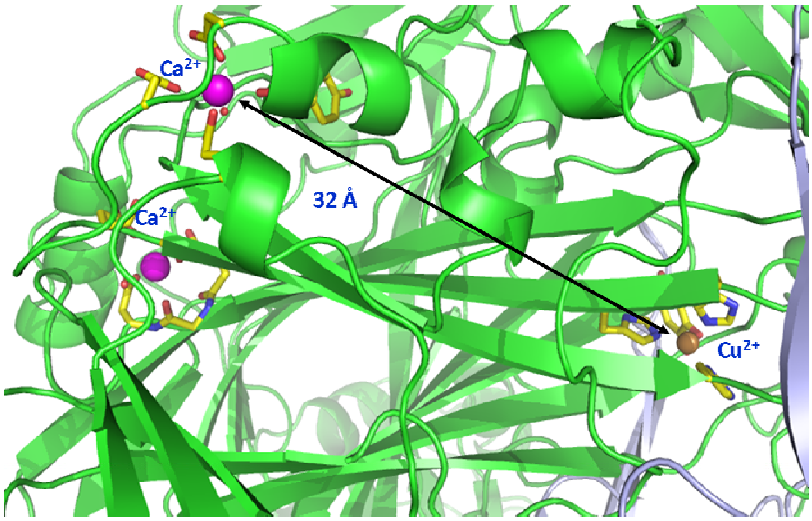


Figure 1: location of two peripheral calcium ions (pink) relative to the active site copper in *E. coli* copper amine oxidase.

Publications

Smith, M., Pirrat, P., Pearson, A., Kurtis, C., Gaule, T., Knowles, P., Phillips, S. and McPherson, M. (2010) Exploring the roles of the metal ions in *Escherichia coli* copper amine oxidase. *Biochemistry* **49**:1268-1280.

Funding

This work was supported by funding from BBSRC and the Wellcome Trust.

Development of methods for the synthesis of skeletally-diverse alkaloid-like small molecules

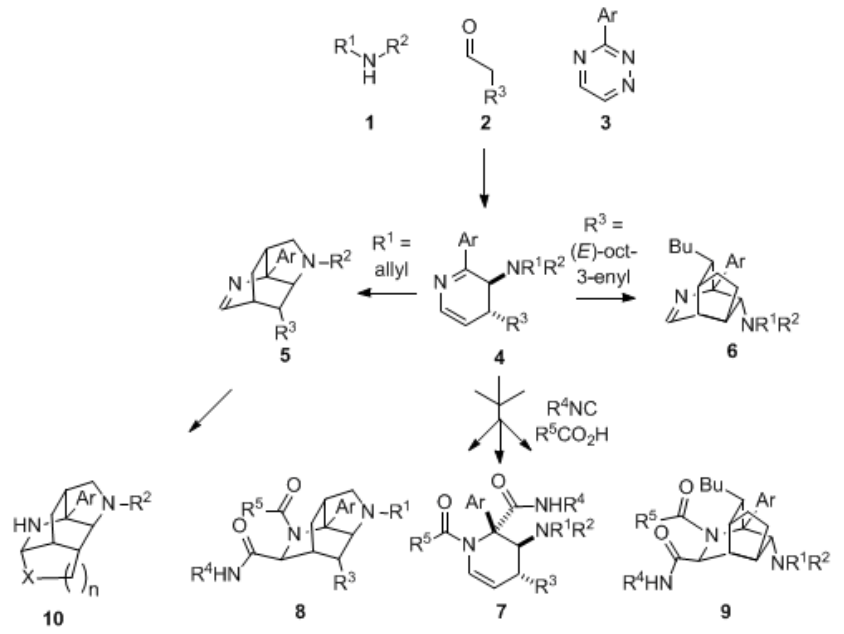
Sushil Mauyra, Sarah Murrison, Christian Einzinger, Stuart Warriner and Adam Nelson

Introduction

Historically, chemists have explored chemical space in an uneven and unsystematic manner. An analysis of the scaffolds of the 25 million known cyclic small molecules (in 2008) revealed that one sixth of the compounds are based on just 30 (out of the 2.5 million) known molecular scaffolds! To address this historic uneven exploration, we have developed synthetic methods that allow the scaffolds of small molecules to be varied combinatorially and systematically. Here, we report two approaches to the synthesis of skeletally-diverse small molecules that are broadly inspired by the alkaloid class of natural products.

An approach based on two three-component reactions

We have exploited two consecutive three-component reactions in the synthesis of skeletally-diverse alkaloid-like small molecules. The outcome of the reaction between amines **1**, carbonyl compounds **2** and triazines **3** depended on the substituents (Scheme 1). Following formation of a 2-aza-diene **4**, further cyclisation – to give either **5** or **6** – was observed with appropriate unsaturated substituents; further decoration with a Joullié-Ugi reaction was also possible (→ **7**, **8** or **9**). In addition, with an appropriately appended nucleophilic group, direct cyclisation to products of general structure **10** was also possible. We used this overall approach to prepare 43 alkaloid-like molecules based on 28 distinct frameworks. Crucially, each of the molecules was, in general prepared from 5 components; the substituents in these components determined the final scaffolds of each small molecule.

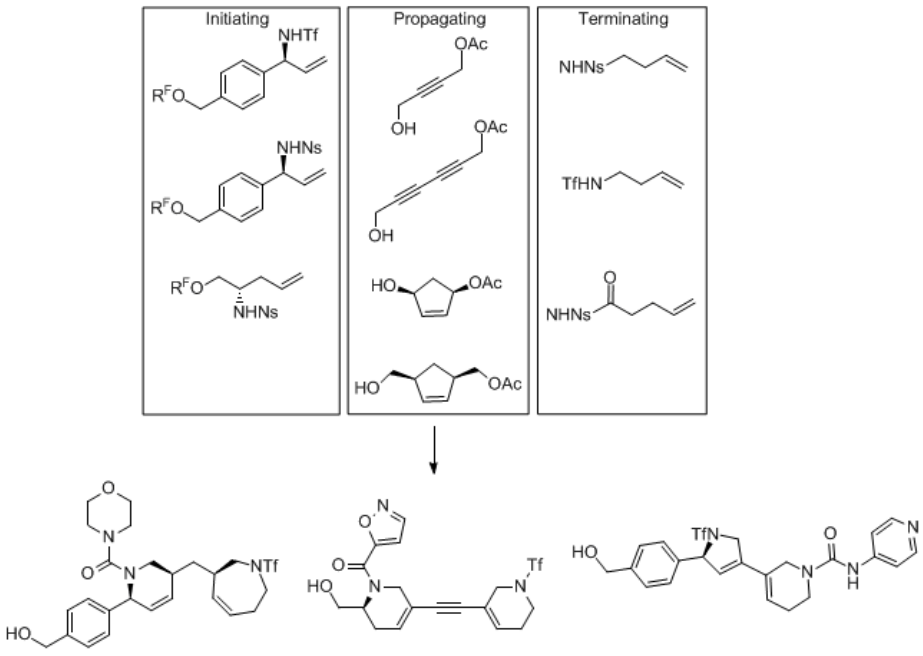


Scheme 1: synthetic approaches to skeletally-diverse alkaloid-like compounds based on two three-component reactions.

A building block-based approach exploiting metathesis cascade chemistry

We have also used a building block-based approach in the preparation of skeletally-diverse alkaloid-like compounds. Initially, combinations of 'initiating', 'propagating' and 'terminating' building blocks were converted into a wide range of metathesis substrates

(Scheme 2). After subsequent metathesis cascade reaction, and decoration, skeletally-diverse alkaloid-like compounds were obtained. Some examples of the final compounds prepared are shown in Scheme 2. Crucially, by varying the building blocks exploited, and the reactions used to link them together, it was possible to vary combinatorially the scaffolds of the small molecule products prepared.



Scheme 2: a building block-based approach to skeletally-diverse alkaloid-like compounds.

Summary

We have developed a range of approaches for the synthesis of skeletally-diverse small molecules. We are now exploiting these synthetic methods in the discovery and optimisation of ligands for a range of protein targets. The results of these research programmes will be described in due course.

Publications

O'Leary-Steele, C., Pedersen, P., James, T., Lanyon-Hogg, T., Leach, S., Hayes, J. & Nelson, A. (2010) Synthesis of small molecules with high scaffold diversity: exploitation of metathesis cascades in combination with inter- and intramolecular Diels-Alder reactions. *Chem. Eur. J.* **16**:9563-9671.

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Funding

We thank EPSRC, the EU and AstraZeneca for support.

Collaborators

Dr Ben McKeever-Abbas, AstraZeneca and Professor Steve Marsden, University of Leeds.

Biophysical theory of lipid bilayers and proteins

Richard Bingham, Ed Causton, Chinmay Das, Simon Connell, Stephen Smye, David Brockwell and Peter Olmsted

Introduction

Lipid bilayer membranes are an essential component of all biological systems, forming a functional barrier for cells and organelles from the surrounding environment. The advent of many new experimental techniques has led to an explosion in work on the basic physical mechanisms behind membranes. Over the past year our group has studied a number of these properties. Proteins undergo stress and mechanical forces in a number of situations, including degradation, ligand binding, cell division and motility, and muscle and molecular motor motion. Moreover, the use of proteins for nanotechnology purposes can also center around their force response, and processing of proteins (e.g. antibodies) often exposes them to mechanical forces. We are interested in the fundamental physics of proteins under forces.

Lipid bilayers in electric fields

Electric field-induced formation of pores in bilayers is widely exploited as an experimental protocol (*e.g.* for drug delivery), although a complete theory of the process does not yet exist. The lipid molecules that form membranes contain both permanent and induced dipoles, and an electric field can induce the formation of pores when the transverse field is sufficiently strong (electroporation). We have developed a phenomenological free energy to model the response of a bilayer to a transverse static electric field. The model contains a continuum description of the membrane dipoles and a coupling between the headgroup dipoles and the membrane tilt. The membrane is found to become unstable through buckling, rather than peristaltic, modes. The instability occurs on a length scale (nm) that similar to that of pore formation and at a critical transmembrane voltage of order 0.3V, similar the magnitude reported in experiments. The instability is found to depend strongly on the strength of the coupling between the dipolar headgroups and the membrane tilt as well as the degree of dipolar ordering in the membrane. Our most recent work in this area involved recasting the previous theory for membrane deformation to explicitly account for thickness changes in the two leaflets of a lipid bilayer. This is important for understanding membrane dynamics in processes such as invagination, endocytosis, immune response, and trafficking (in addition to membrane poration).

Stratum corneum bilayers

Stratum corneum (SC), the outermost layer of skin, consists of keratin-filled rigid non-viable corneocyte cells surrounded by multilayers of lipids; it is responsible for the barrier properties of skin. In the previous year we studied how the composition (ceramide NS-24:0, free fatty acid 24:0 and cholesterol) controls the SC membrane structural properties, such as high density, low permeability, and the nature of the gel phase of these bilayers. One important result is that the bilayer phase is probably most likely to form (rather than a disordered inverse micellar phase) in the presence of external fields or templating effects, such as the keratin protein that takes up a large fraction of the SC. Our simulations suggest that the composition ratio in native SC lipid layers is responsible for both the good barrier properties and the stability of the lipid structure against mechanical stresses.

We have developed a method to use AFM to extract the local stretching modulus of lipid bilayers. This is the first technique that can be used to measure the mechanical properties of different regions of the same membrane, and we have demonstrated the technique on a three component mixture (DOPC, cholesterol, sphingomyelin) that exhibits liquid-liquid phase separation analogous to lipid rafts. We have shown that mechanical force can induce a

transition from the liquid-ordered to liquid-disordered phases, and we have interpreted this in terms of the breaking of hydrogen bonds that stabilize the cholesterol-rich liquid-ordered phase.

We have recently won funding to continue this work, in collaboration with Unilever, and the Universities of Bradford and Hull.

Multidimensional aspects of mechanical unfolding of proteins

Proteins form the molecular machinery of biological systems. A thorough understanding of their response to external forces is essential both in order to understand their operations within cellular environments and to contrive new uses for their use in medicine and nanotechnology. In the laboratory, we may try to replicate the mechanical forces experienced *in vivo* using Atomic Force Microscopy (AFM) force spectroscopy experiments. The challenge here is to interpret the distribution of rupture events in terms of a simple model that captures the salient features of the protein's behavior. The usual model is of thermally activated escape from a simple potential well along a single reaction coordinate direction, as modified by the external force. We have examined the flaws inherent with one dimensional models and studied a simple symmetric multidimensional well, which represents the many different directions in configuration space in which an unforced protein can unfold. Such a model can then be projected onto an effective one dimensional model with renormalized parameters (e.g. the transition state displacement). Such a model can produce "catch-bond" behaviour, simply due to the dramatic dimensional reduction of phase space by the applied force. We also derive and verify a new method for constructing the 'natively folded protein free energy' based on the Jarzynski Relation, which relates the work from non-equilibrium experiments to equilibrium free energies.

Publications

Bingham, R., Olmsted, P. & Smye, S. (2010) Undulation instability in a bilayer lipid membrane due to electric field interaction with lipid dipoles, *Phys. Rev. E* **81:**051909.

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Funding

This work was funded by the EPSRC, Yorkshire Forward, the EU (FP7 Network of Excellence SOFTCOMP), and Unilever.

Collaborators

The stratum corneum work was carried out in close collaboration with Massimo Noro, and Alex Lips at Unilever Research Port Sunlight; and with Jamshad Anwar at Bradford University.

Free-energy landscapes of proteins from simulation and experiment

Richard Malham, Kostas Papachristos, Zu Thur Yew, Sergei Krivov and Emanuele Paci

Introduction

The properties of proteins, and their biological function, are directly related to their free-energy landscape which in turn is uniquely defined by the aminoacid sequence. In our group, and in close collaboration with Dr Sergei Krivov, we use multiscale modelling to determine free-energy landscapes and from them predict experimentally measurable macroscopic properties of proteins.

An example of the utility of atomistic and theoretical modelling to make full use of advanced experimental techniques is the response of proteins to mechanical probes.

Results

Experiments have been mostly analyzed with one-dimensional models of the free-energy landscape. We have recently found that, as the two ends of a protein (filamin in this case) are pulled apart at a speed tending to zero, the measured mechanical strength plateaus at ~30 pN instead of going toward zero, deviating from the almost universally assumed *Bell model*. The deviation can only be explained by a switch between parallel pathways. Insightful analysis of mechanical unfolding kinetics needs to account for the multidimensionality of the free-energy landscapes of proteins, which are crucial for understanding the behaviour of proteins under the small forces experienced in vivo.

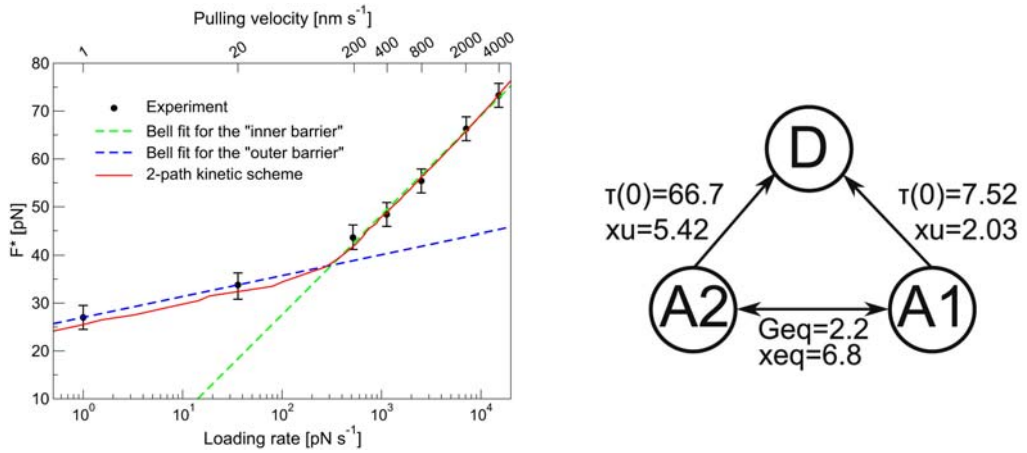

Figure 1: the modal unfolding force, F*, as a function of the loading rate, RF*, calculated at each F*. The pulling velocities at which the F* were determined are indicated (top of the graph). (Dashed lines) Piecewise fits of the data at 200–4000 nm/s (blue dashed line) and at 1–20 nm/s (green dashed line) to $k_BTln(RF^*)/x_{in/out}+C_{in/out}$ (the Bell model). The force-dependencies are xF^*_{in}=4.6 Å and xF^*_{out}=21.6 Å for the high- and low-velocity regimes, respectively (red continuous line). Fit to the three-state model with parallel pathways shown on the right.

Characterizing the conformational properties and dynamics of biopolymers and their relation to biological activity and function is an ongoing challenge. Single molecule techniques have provided a rich experimental window on these properties, yet they have often relied on simple one-dimensional projections of a multidimensional free energy landscape for a practical interpretation of the results. We recently studied three mostly disordered 10- or12-residue peptides with different structural propensity in the presence or absence of a force applied to their ends. Each peptide produces fluctuation power spectra with a characteristic dynamic fingerprint consistent with persistent structural motifs of helices, hairpins, and random coils. The spectra for helix formation shows two well-defined relaxation modes, corresponding to local relaxation and cooperative coil to uncoil interconversion. In contrast, both the hairpin

and random coil are polymer-like, showing a broad and continuous range of relaxation modes giving characteristic power laws of -5/4 and -3/2, respectively; the 5/4 power law for hairpins is robust and has not been previously observed. Our results demonstrate the yet unexploited potential of single molecule fluctuation spectroscopy to probe more fine scaled properties of proteins and biological macromolecules and how low dimensional projections may cause the loss of relevant information.

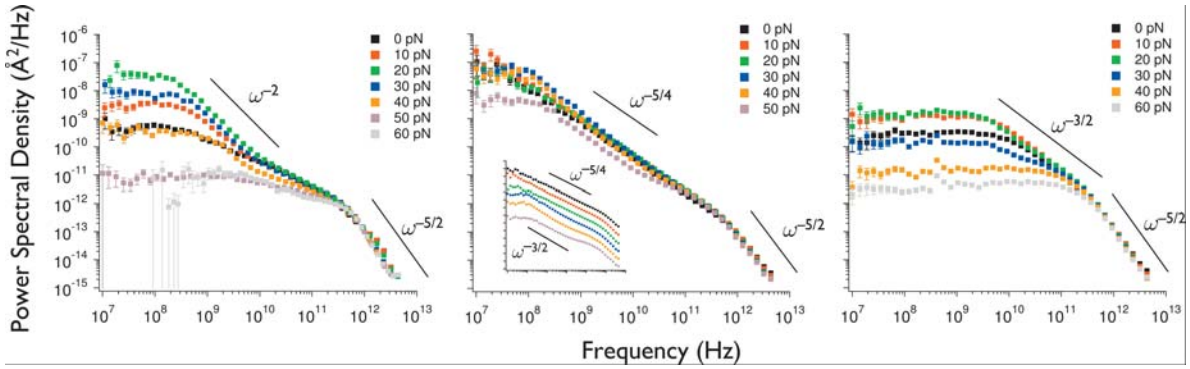

Figure 1: power spectral density of fluctuations from atomistic simulations on a log-log scale, for forces between 0 and 60 pN, for three different peptides. The PSD provide a fingerprint of the different dynamics of the peptides, partly related to their structural propensities. Such PSDs can in principle be measured experimentally (see Taniguchi 2010). In collaboration with Masaru Kawakami we are developing a method to charcterise disordered proteins through their noise spectra.

Besides the mechanical properties of proteins, we are also working on the interpretation of terahertz spectra of protein crystals, modelling of mesoscopic models of protein assemblies from cryoEM data and development of novel methods and force-fields.

Publications

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Funding

This work was funded by EPSRC and Wellcome Trust.

Collaborators

We thank Bhavin Khatri, Masaru Kawakami, Jane Clarke, Matthias Rief, Michael Schlierf.

Dynamic structural science: developing the tools to probe biological mechanism in atomic detail

Saskia Bakker, Andrew Burnett, Lucy Chappell, Thembaninkosi Gaule, James Gowdy, Elena. Kovaleva, Katarzyna Tych, Briony Yorke and Arwen Pearson

Introduction

The fundamental aim of both physical and life scientists engaged in structural studies is to understand how structure leads to function. To achieve this it is vital to be able to watch chemical reactions and biological processes as they occur, and an explicit time resolved structural description at atomic resolution is therefore essential.

X-ray crystallography is a well established technique that enables the direct visualisation of molecular structure at atomic resolution. However, particularly for the study of macromolecular samples, there remain methodological deficiencies that limit the achievable resolution and hence the information that can be derived from the data. Research in our group is aimed at developing complementary experimental approaches that can be combined with the X-ray diffraction experiment in order to overcome some of these limitations. The methods we develop are being applied to a number of biological systems, including copper and iron containing metalloenzymes, the amino acid decarboxylases and a number of viruses.

Results

1. Single crystal spectroscopy

UV/Visible, Fluorescence and Raman spectroscopy are sensitive techniques that provide information about electronic structure of bound chromophores (UV/visible) or local protein and ligand structure (Raman). We have developed an on-axis single crystal UV/visible and Raman multi-mode single crystal spectrometer that can be mounted on a synchrotron beamline that is able to measure spectroscopic data that can be directly correlated to diffraction data. This enables us to monitor any X-ray radiation induced changes during diffraction data collection and provides spectroscopic information to support the assignment of the chemical state of ligands and cofactors, important in structural studies of enzyme mechanism. An in-house single crystal UV/Visible spectrometer is also available which can be used to monitor functional changes in the crystalline state, i.e. enzyme turnover, and can thus identify the optimal time-points to cryo-trap meta-stable reaction intermediates for structure determination.

Whilst UV/Visible, Fluorescence and Raman spectroscopy provide detailed information about local structure, they report only indirectly on global macromolecular dynamics. We have recently developed instrumentation to measure Terahertz time domain spectra from protein single crystals. Terahertz time domain spectroscopy is sensitive to ps-ns motions (i.e. side chain motions), as well as long range order making it an ideal technique to study protein dynamics.

2. Time resolved structural studies

X-ray crystallography is traditionally viewed as a static technique, enabling the determination of an atomic resolution structure averaged over both the many molecules within the crystal and the time required to acquire a diffraction dataset. However, time-resolved studies are possible and, using pump-probe Laue crystallography, sub nanosecond time-resolutions have been achieved for a small number of protein systems. This approach currently requires an absolutely reversible reaction and this has limited its application to the majority of biological systems. Taking advantage of recent developments in automation and software, in collaboration with Diamond Light Source, we are developing a new multi-microcrystal

methodology that will allow time-resolved studies of any biological macromolecule that can a) be crystallised and b) for which the reaction can be rapidly and uniformly triggered (i.e. by light, temperature or pH jump).

Publications

Campeotto, I., Bolt, A., Harman, T., Dennis, C., Trinh, C., Phillips, S., Nelson, A., Pearson, A. & Berry, A. (2010) Structural insights into substrate specificity in variants of N-acetylneuraminic acid lyase produced by directed evolution. *J. Mol. Biol.* **404**:56-69.

Cedervall, P., Dey, M., Pearson, A., Ragsdale, S. & Wilmot, C. (2010) Structural insight into methyl-coenzyme M reductase chemistry using coenzyme B analogues. *Biochemistry* **49**: 7683-7693.

Foster, T., Belyaeva, T., Stonehouse, N., Pearson, A. & Harris, M. (2010) All three domains of the hepatitis C virus nonstructural NS5A protein contribute to RNA binding. *J. Virol.* **84**: 9267-9277.

Foster, T., Tedbury, P., Pearson, A & Harris, M (2010) A comparative analysis of the fluorescence properties of the wild-type and active site mutants of the hepatitis C virus autoprotease NS2-3. *Biochim. Biophys. Acta* **1804**:212-222.

Smith, M., Pirrat, P., Pearson, A., Kurtis, C., Trinh, C., Gaule, T., Knowles, P., Phillips, S. & McPherson, M. (2010) Exploring the roles of the metal ions in *Escherichia coli* copper amine oxidase. *Biochemistry* **49**:1268-1280.

Funding

This work is funded by the EPSRC, BBSRC, Wellcome Trust, The Leverhulme Trust, RCUK, The Royal Society and the EU.

Collaborators

Leeds: Michael Webb (Chemistry), John Cunningham (Electronic and Electrical Engineering), Alan Berry (Biological Sciences), Adam Nelson (Chemistry), Mike McPherson (Biological Sciences), Mark Harris (Biological Sciences), Peter Stockley (Biological Sciences).

External: Robin Owen (Diamond Light Source, UK), James Holton (Laurence Berkeley National Laboratory, CA, USA), Eddie Snell (Hauptmann Woodward Medical Research Institute, NY, USA), Peter Moody (Leicester, UK).

Siamycin I directly inhibits the pheromone-stimulated autophosphorylation activity of the bacterial FsrC quorum sensor and phosphorylation-dependent enzyme activities

Pikyee Ma, Kenzo Nishiguchi, Hayley Yuille, Lianne Davis, Jiro Nakayama and Mary Phillips-Jones

Introduction

Enterococcus faecalis is a significant agent of nosocomial infection which continues to be of concern in the clinic, particularly with regard to levels of reportable VRE (vancomycin-resistant enterococci). One of the most important virulence factors that contributes to the adherence properties of these enterococci is gelatinase (GelE) which is under the control of the quorum sensing Fsr signal transduction pathway. Quorum sensing has been identified as a promising target for the design of novel antibacterial agents.

During the past year, we have developed further our characterisation of intact FsrC, the membrane sensor kinase component of the FsrC pathway, including its molecular interactions with the activating pheromone signalling ligand, GBAP, and with siamycin I an inhibitor of FsrC activity that we identified previously (Ma *et al*., 2008; Astbury Report 2009). Here we report on our recent studies of the mode of action of siamycin I, particularly with regard to its mechanism of growth inhibition of enterococci (MIC ~ 5 $\mu g.ml^{-1}$).

Results

Following the identification of FsrC activity as a direct target of inhibition by siamycin I, we sought to identify further sites of inhibitory action, particularly those that might account for its lethality towards enterococci. Therefore initially an investigation of the effects of siamycin I on the activities of other membrane sensor kinases in *E. faecalis* was explored, including the VicK (EF1194) sensor kinase which is a component of the VicKR two-component system that is essential for viability in *E. faecalis*. In addition to intact FsrC (EF1820) and VicK (EF1194), purified preparations of further intact membrane sensors were also tested (Fig. 1). Autophosphorylation assays of each protein in the presence and absence of 100 μM (20-fold excess) siamycin I were performed. All of these membrane sensors, including VicK, exhibited reduced levels of phosphorylated protein (and therefore activity) in the presence of siamycin I, and a wide variation in levels of inhibition are observed (Figure 1). These proteins all possess a histidine phosphorylation site and share the ability to dimerise, and to bind and hydrolyse ATP. A wider range of (related) protein types was therefore included (Table 1). The activity of each purified protein was assayed in the presence and absence of 100 μM siamycin I.

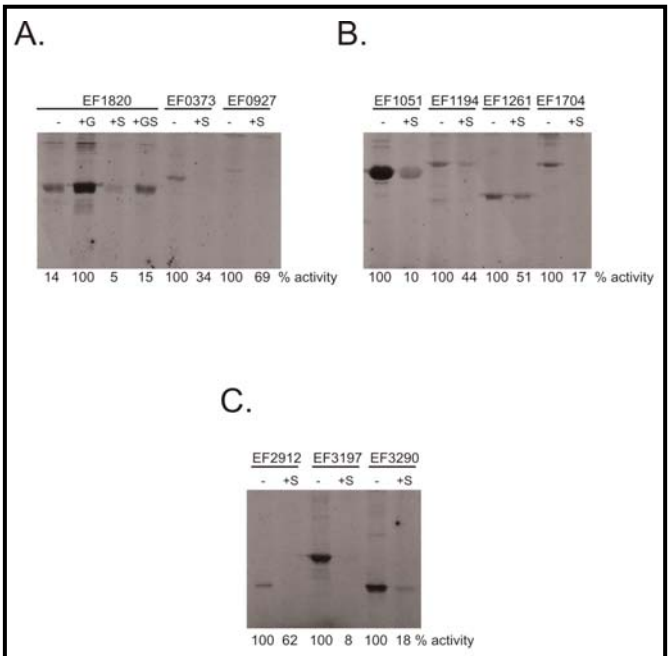

Figure 1: inhibition by siamycin I of a collection of purified intact membrane sensor kinases from *E. faecalis* V583.

Siamycin I significantly inhibited PrrB autophosphorylation activity, confirming that the inhibitor is likely to inhibit the activities of membrane sensor kinases from a range of different bacteria, including Gram-negative species.

Enzyme	Control	+ siamycin I	% inhibition
PrrB (RegB) (intact membrane sensor kinase from *Rhodobacter sphaeroides*). (Densitometry units)	6528.0 ± 1372.8	2212.7 ± 377.2	66
Na^+-dependent ATPase (from porcine cerebral cortex) ($A_{660} \times 10^{-3}$)	235.7 ± 7.1	148.2 ± 6.1	38
Protein kinase A (soluble catalytic subunit from bovine heart) (Densitometry units)	34249 ± 1124	29641 ± 1953	13
β-galactosidase (from *Escherichia coli*) (ΔA_{420}. min^{-1}. mg $protein^{-1}$ ($\times 10^{-3}$)).	84.7 ± 13.9	92.0 ± 9.9	0

Table 1: enzyme activities in the presence and absence of siamycin I

The effect of siamycin I on the activities of ATPase and the soluble catalytic subunit of a protein kinase purified from bovine heart (which involve ATP binding/hydrolysis reactions in common with membrane sensor kinases) were also significantly inhibited. However, the activity of unrelated purified β-galactosidase of *Escherichia coli* exhibited no significant change in the presence of the inhibitor (Table 1). β-galactosidase activity relies on the formation of tetramers; presumably then, the mechanism by which siamycin I inhibits is not through disruption of the oligomerisation events.

Taken together, these results confirm that siamycin I exhibits specific and direct inhibition of the membrane sensor kinase FsrC, confirming at least one target in the Fsr pathway previously implicated in the siamycin I-mediated inhibition of gelatinase and GBAP production. However, it is clear from the present study that siamycin I is likely to inhibit the activities of a range of different enzymes, including membrane sensor kinases (including essential VicK), protein kinases and ATPases involved in ATP-dependent reactions in a wide range of organisms, exerting inhibitory effects on downstream cell processes and thereby providing an explanation for the mechanisms by which bacterial growth is inhibited by siamycin I.

Publications

Ma, P., Nishiguchi, K., Yuille, H., Davis, L., Nakayama, J. and Phillips-Jones, M. Siamycin I directly inhibits the pheromone-stimulated autophosphorylation activity of the bacterial FsrC quorum sensor and phosphorylation-dependent enzyme activities (*submitted*).

Funding

Much of this work has been funded by the BBSRC.

Collaborators

Professor Jiro Nakayama and Dr Kenzo Nishiguchi (Kyushu University). PM is grateful to Peter Henderson for supervisory guidance.

Exploring protein folding energy landscapes

Alice Bartlett, Claire Friel, Gerard Huysmans, Lindsay McMorran, Gareth Morgan, Clare Pashley, Sara Pugh, Stephen Baldwin, David Brockwell and Sheena Radford

Introduction

Understanding the role of a protein's primary amino acid sequence in controlling the search for the native conformation is of fundamental importance. Progress towards this goal requires the structural and energetic characterisation of all species encountered on the folding energy landscape. Work in our laboratory focuses on using a range of biophysical approaches to examine the folding landscapes of the small four-helical colicin immunity protein, Im7, and the β-barrel outer membrane protein PagP.

The transition state for folding of an outer membrane protein

In contrast to our detailed understanding of the energy landscape for water-soluble protein folding our knowledge of how integral membrane proteins fold to a stable native structure remains limited. This study used mutational analysis to describe the transition state involved in the reversible folding of the outer membrane β-barrel protein PagP from a disordered state in 10M urea to the native structure embedded in a lipid bilayer. The equilibrium stability and unfolding kinetics for 19 variants spanning all regions of the PagP structure were analysed. The results reveal a highly polarized transition state structure in which the C-terminal β-strands are highly structured whereas the N-terminal β-strands and the α-helix remain poorly organized (Figure 1). The data are consistent with a tilted insertion of PagP into the membrane.

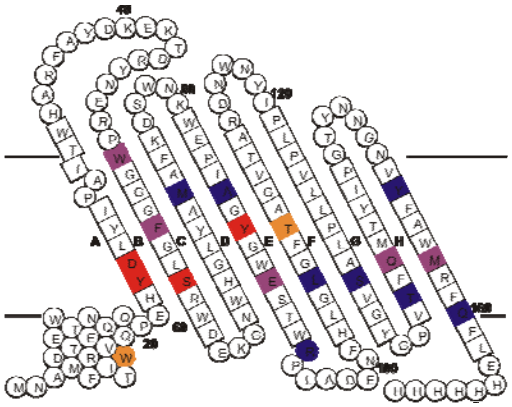

Figure 1: Φ_F-values obtained for PagP folding mapped onto a topology map of the protein structure. $\Phi_F > 0.5$ are in blue, $0.3 \leq \Phi_F \leq 0.5$ are in red and $\Phi_F < 0$ are in orange.

Perturbing the folding energy landscape of the bacterial immunity protein Im7 by site-specific N-linked glycosylation

Im7 folding has been interrogated using numerous biophysical methods and we now have a detailed understanding of how this small helical protein attains its native structure. Exploiting our knowledge of Im7 folding we have used expressed protein ligation to create site-specifically glycosylated variants of the protein. Glycans were introduced at seven sites within the Im7 sequence (Figure 2) and the kinetic and thermodynamic consequences of N-linked glycosylation analyzed. In several instances the introduction of a glycan was found to influence folding by modulating the local conformational preferences of the protein sequence. This effect was most evident when the glycosylated residue was located in the centre of a helix or in compact turn motifs between segments of ordered secondary structure. This study demonstrated that N-linked glycosylation affects protein folding rates and stability in a tunable manner that is predictable based on knowledge of the native protein structure.

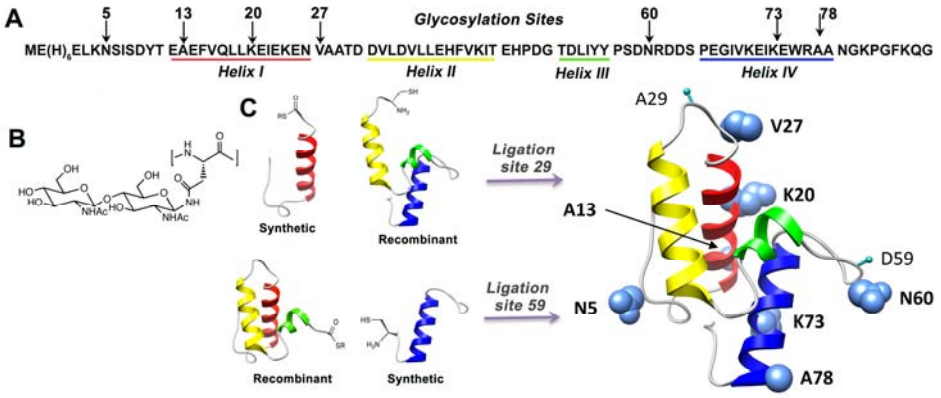

Figure 2: expressed protein ligation strategies for production of glycosylated Im7. A. Im7 sequence highlighting the 7 solvent exposed glycosylation sites. B. Structure of chitobiose-Asn; C. Cartoon of Im7 (PBD: 1AYI) illustrating positions 29 and 59 (cyan markers), which were mutated to Cys enabling the ligation strategies to introduce glyco-Asn. The native Im7 residues at each glycosylation site are shown in space-fill.

Current work

The transition states and intermediate populated during Im7 folding have been characterised in atomistic detail. By contrast our knowledge of the unfolded ensemble has remained sparse. Current work in our laboratory aims to address this. An Im7 variant containing destabilizing mutations has been designed so that the unfolded state is predominantly populated at equilibrium. Biophysical analyses to characterize this unfolded Im7 variant in are on-going.

Building on the studies of PagP folding our laboratory is now examining the folding mechanisms of two homologous β-barrel outer membrane proteins from *E.coli*: OmpT and OmpP. This project will shed light on whether there is a generic mechanism for folding of outer membrane proteins.

Publications

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Funding

This work is funded by the BBSRC and The Wellcome Trust. We thank Keith Ainley for technical support.

Collaborators

Barbara Imperiali (MIT, USA), Christopher Gell (Max Planck Institute of Molecular Cell Biology & Genetics, Germany), Alastair Smith (Avacta Group Plc, York).

Revealing the structure of β_2-microglobulin in amyloid fibrils by site-directed spin labelling and chemical labelling

Timo Eichner, Andrew Hellewell, Toral Jakhira, Theo Karamanos, Carol Ladner, Aneika Leney, Eva Petrik, Geoffrey Platt, Maya Pandya, Morwenna Porter, Claire Sarell, Alessandro Sicorello, David Smith, Ricardo Tomé, Nathalie Valette, Lucy Woods, Wei-Feng Xue and Sheena Radford

Introduction

Dialysis-related amyloidosis is a debilitating disorder in which β_2-microglobulin (β_2m) deposits in amyloid fibrils within cartilagenous-rich joints, resulting in carpel tunnel syndrome and pathological bone destruction. β_2m is a 99 residue protein which adopts a β-sandwich fold, where the two β-sheets are formed entirely from anti-parallel β-strands linked by an inter-sheet disulfide bond. Here we have probed the environment and accessibility of side-chains within amyloid fibrils formed *in vitro* from β_2m with a long-straight morphology by site-directed spin labelling and accessibility to modification with N-ethyl maleimide using 19 site-specific cysteine variants.

Site-directed spin labelling

In order to investigate the organization of β_2m in long-straight fibrils, 19 single cysteine mutants of the protein were quantitatively spin labelled with 1-oxyl-2,25,5-tetramethyl-D-pyrroline-3-methyl-methanethiosulfonate (MTSL) (Figure 1(a)) for analysis by electron paramagnetic resonance (EPR). The sites for mutation were selected because they are accessible according to the X-ray crystal structure of native β_2m and because they provide at least one probe within each native β-strand and many of the adjoining loop regions.

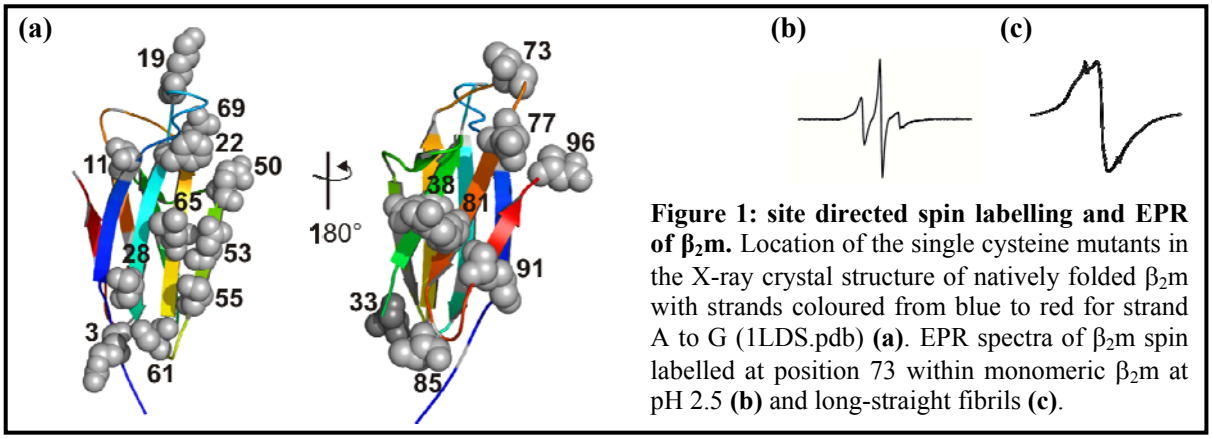

Figure 1: site directed spin labelling and EPR of β_2m. Location of the single cysteine mutants in the X-ray crystal structure of natively folded β_2m with strands coloured from blue to red for strand A to G (1LDS.pdb) **(a)**. EPR spectra of β_2m spin labelled at position 73 within monomeric β_2m at pH 2.5 **(b)** and long-straight fibrils **(c)**.

EPR of monomeric and long straight fibrils of β_2-microglobulin

To gain information about the environment and mobility of the side-chains in different β_2m conformations, EPR spectra of the spin labelled β_2m samples in solution in the monomeric state, as well as when assembled into fibrils were obtained. As illustrated for the derivative MTSL-labelled at position 73, the EPR spectrum of monomer at pH 2.5 results in sharp and narrowly spaced spectral lines indicating high mobility (Figure 1(b)). Upon formation of long-straight fibrils (Figure 1(c)), a very different EPR spectrum is obtained. This spectrum is dominated by a single spectral line that is indicative of spin exchange narrowing. Such spin exchange is caused by the close proximity of the multiple spin labels, previously estimated to be within 7 Å of each other, and causes the loss of well defined three line hyperfine structure.

EPR spectra of fibrils formed from the 19 different single cysteine variants, were acquired. Little or no detectable spin exchange is apparent in the EPR spectra for spin labels introduced in the N-terminal or C-terminal regions of the polypeptide chain indicating the

absence of a parallel, in-register stacking at these residues. In contrast exchange narrowed EPR spectra were observed for most sites in the core region, suggesting the core region has extensive segments in which the same residues come into close contact with each other, indicative of a parallel, in-register structure.

A continuous array of parallel, in-register β-strands involving most of the polypeptide sequence is inconsistent with the cryo-electron microscopy (cryo-EM) structure which reveals an architecture based on subunit repeats. Combined with information about side-chain mobility and accessibility we propose that each subunit within amyloid fibrils of β_2m consists of *ca.* six β_2m monomers organised in stacks with a parallel, in-register array (shown in Figure 2). The results suggest an organisation more complex than the accordion-like β-sandwich structure commonly proposed for amyloid fibrils. Further work is now ongoing to refine these models and to couple them with fibrils formed at neutral pH.

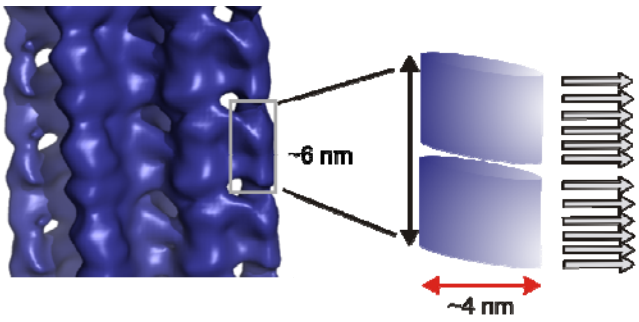

Figure 2: long-straight fibrils of β_2m are arranged as sets of β-strands stacked in parallel, in-register repeats. The subunit repeat shown by cryo-EM of β_2m fibrils indicates that there must be a break in the parallel in-register arrangement of the constituent β-strands within the cross-β array. Here two sets of six β-strands are proposed to fit into the *ca.* 6 nm repeat. Such an arrangement is fully consistent with residues in these strands giving rise to single line EPR spectra.

Publications

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Funding

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Collaborators

Our external collaborators include Ralph Langen, University of Southern California, Robert Griffin, Massachusetts Institute of Technology, Sandra Macedo-Ribeiro, Universidade do Porto, Helen Saibil, Birkbeck College, David Middleton, University of Liverpool, Michele Vendruscolo, University of Cambridge. We also collaborate closely with Alison Ashcroft, Sarah Harris, Eric Hewitt, Steve Homans, Peter Stockley and Stuart Warriner.

The binding of bacteriophage MS2 to its target receptor

Katerina Toropova, Peter Stockley and Neil Ranson

Introduction

Bacteriophage MS2 is a small icosahedral virus that infects *E. coli*. It has a single-stranded, positive-sense RNA genome of ~3.6 kbp that encodes just four gene proteins: maturation, lysis, coat and replicase. MS2 spontaneously assembles a *T*=3 icosahedral capsid around its' genomic RNA *in vivo*. This capsid contains 1 copy of the RNA, 180 copies of the coat protein, and a single copy of the maturation protein. All structural studies on MS2 to date (both EM and X-ray) have used icosahedral symmetry averaging, and all lack any information on the location or structure of the maturation protein. However, it is known that the maturation protein mediates the interaction between the virus and its natural receptor, the bacterial F-pilus.

Results

We have used cryo-EM and single-particle image processing to determine the location of the maturation protein within the MS2 capsid. Multiple copies of the virus (~285 Å in diameter) bind to the sides of the F-pilus (Figure 1a). We used a technique called post-imaging fiducial marking to identify the site of interaction. A white 'dot' was added to each bound virus at the point of interaction (Figure 1b). 3-D reconstruction & icosahedral symmetry averaging gives a structure with density above each 5-fold vertex, which implies the virus:pilus interaction is occurring at one of the virus' twelve 5-fold vertices (Figure 1c). As a result, the virus:receptor complex must have C5 rotational symmetry, which can be used to determine the 3-D structure of the complex.

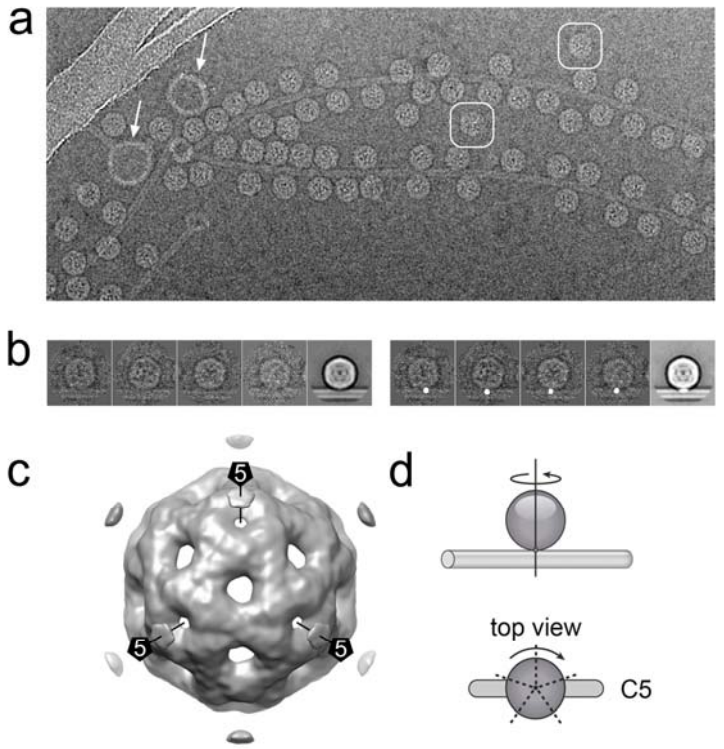

Figure 1: MS2 binding to the bacterial F-pilus. **(a)** Raw cryo-EM image of MS2 bound to the F-pilus. **(b)** post-imaging fiducial marking. **(c)** Icosahedral reconstruction of 'marked' images gives density above the 5-fold axes. This suggests that the virus binds at a 5-fold vertex **(d)** the deduced symmetry of the virus:receptor complex.

Such a 3-D structure, created using C5 rotational symmetry is shown in Figure 2 at ~20 Å resolution. The structure reveals for the first time the non-uniform distribution of the packaged genomic RNA within a native virion. Much of the density for RNA and/or maturation protein is found beneath the pilus-bound vertex, with relatively little at the vertex opposite the interaction with the pilus. Indeed a column of density is seen on the 5-fold axis itself, and appears to be poised for release from the virion during the later stages of the infection process. We have used the distribution of density determined here as a constraint in

mathematical modelling studies that are beginning to help explain the startling efficiency of virus assembly in vitro.

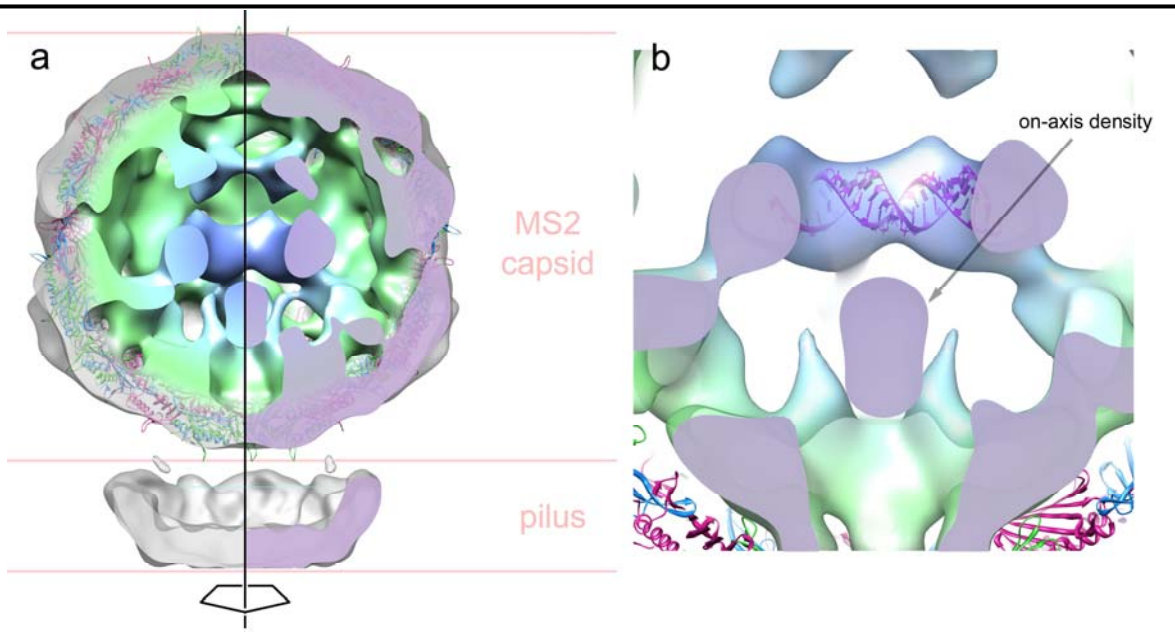

Figure 2: 3-D structure of MS2 bound to its target receptor, the bacterial F-pilus. (a) Rear-half of the receptor-bound virion. On the left-hand-side, density corresponding to the capsid proteins are coloured grey, whilst density corresponding to RNA and/or maturation protein is coloured with a radial scheme (from blue at low radius to green at high). On the right-hand-side, the cut surface is coloured purple for clarity. **(b)** Close-up view of a central section (~55 Å in thickness) through the receptor-bound virion. The on-axis density is labelled, and an A-type RNA duplex is shown docked into the EM density for size comparison. All density corresponding to capsid proteins (grey in (a)) has been masked away showing the fitted atomic coordinates.

Publications

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Funding

We thank the Leeds University Interdisciplinary Institute in Bionanotechnology for a studentship to K.T. This work was funded by The Leverhulme Trust.

We thank Dr. Gabby Basnak for technical support, Dr. Anthony Roberts (Harvard, USA) for help with preparation of figures, and Professor Reidun Twarock (York) for collaboration & discussion.

Biomimetic production of precise nanomagnetic particles using magnetic bacteria and their biomineralisation proteins

Johanna Galloway, Masayoshi Tanaka, Jonathan Bramble, Andrea Rawling, Stephen Baldwin, Stephen Evans and Sarah Staniland

Introduction

Scientific and economic interest in nanotechnology has grown in recent years. Within this the quest to produce tiny and highly tailored magnetic particles, or nanomagnets is crucial. Nanomagnets have a range of practical uses such as: the development of 3D information storage systems providing high density data storage; medical applications such as site specific targeted therapies and image enhancers for diagnostic medicine.

However, as nanotechnology grows, so too does the need to develop precisely engineered nanomagnets. Different applications demand different shapes and sizes of particles and different magnetic properties. Producing nanomagnets with highly controlled; composition, size and shapes, in large enough amounts to be of use to these industries, has therefore become a key goal of researchers.

Magnetotactic bacteria are the simplest organisms that perform biomineralisation. They take up iron ions from solution and produce nanoparticles of magnetite (Fe_3O_4) within lipid vesicles (which are termed magnetosomes) with precise control, resulting in a strain specific uniform size and morphology.

The aim of this group's research is to investigate, understand and then manipulate the biomineralisation process within these bacteria to enable the production of high-yields of customised nanomagnets for nanotechnological application using the genetic precision of nature.

The research

The magnetic composition of magnetosomes as been sucessfully altered in vivo by doping the magnetosomes with cobalt resulting in magnetosomes with an increased magnetic coecivity compared to control magnetosomes. This was acheived with the addition of cobalt ions into the bacterial growth media which were taken up and incorperated into the magnetite mineral in approximately 1% quanities. More recently we have also achieved in vivo magnetosome doping with Mn (1-2%) and Cu (10%) as well the induced the production of independant nanoaprticles of Te and Se within the cell. The doping % could not be increased in vivo due to the restriction imposed by the organism. We thus sought an in vitro route to offer more flexibility and higher-yields. Here, in collaboration with the lab of Prof. Matsunaga we build on their original method and developed a biomimetic route to more precise nanomagnets synthesied at room temperature using a protein mediated precipitation of particles. The protein used was Mms6 (magnetosome membrane specific, 6 KDa) which was found to be unique to the magnetosome mebrane and tightly bound to the crystal. when this protein was expressed and purified and used in vitro it was found to control particle size and shape. This protein was located along with 3 others (Mms 5, 7 & 13) and all of them have charactierstic similar to other biomineralisation templating proteins such as and LG repeat sequence.

Research is now being conducted in two parallel and complementary directions. Firstly, the physical investigation of how these proteins interact with the forming mineral and control the magnetite's formation and morphology is being investigated (BBSRC funded). Several new proteins are being identified and expressed while their interaction with magnetite is being assessed using a range of spectroscopy, electron/force microscopy and neutron scattering techniques. Once key motifs, peptide and binding sites can be identifed we could begin to design tailored addatives for high-yield industrial nanomagnet production.

Secondly, we are developing a range of methods using the expressed Mms proteins in vitro for more advanced synthesis. We are enhancing this with the addition of membranes and

vesicles to the systems (EPSRC funded). This is being furthered by experimenting with different proteins that affect the fucntionality of membranes. For example we are investigating novel metal ion transport proteins and vesicle deformation proteins which can be incorperation into vesicles along with Mms proteins to develop a range of novel, flexible biomimetic systems. For example we are patterning surfaces with Mms6 to attempt to form customised nanomagnetic arrays. This is just one of many biomimetic systems we are developing to create several novel mineral/membrane assemblies, some tethered/free and attached to surfaces. Additionally a recent TEM study has revealed how mangetic bacteria divide and what happens to the magnetosome chain within them duing this process.

Publications

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Collaborators

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The FMDV replication complex

Sophie Forrest, Kris Holmes, David Rowlands and Nicola Stonehouse

Foot-and-mouth disease virus (FMDV) causes a highly contagious and sometimes fatal disease of cloven hoofed animals. The disease has a global impact on animal husbandry – both in terms of reducing productivity and preventing trade. The cost of the U.K. outbreak in 2001 was estimated at costing over £9 billion.

FMDV is usually considered to be a rapidly replicating virus. In tissue culture FMDV can complete the entire replication process, from entry to exit, in under three hours. Less well know is that fact that FMDV can persist in its natural host, a state where viral replication is greatly reduced, and it is these persistently infected animals that prove to be a major stumbling block in controlling FMD.

Replication of the genome is primarily performed by 3Dpol – a virally encoded RNA-dependent-RNA polymerase. This provides templates for subsequent rounds of translation as well as genomes for packaging into viral particles. We have developed a 'minimal' functional polymerase assay, consisting of; template RNA, a primer, 3Dpol and UTP. Using this assay we have screened a large number of RNA aptamers to 3Dpol, some of which were inhibitory. We have also demonstrated that, in complex with RNA, 3Dpol is able to form higher order structures. These structures appeared as fibrils or filaments of varied lengths by transmission electron microscopy. All components of a polymerase assay were necessary for their formation. However, in the presence of the 3Dpol inhibitory aptamers no fibrils were detected.

Studies are now continuing with the use of a GFP-replicon system, which allows the full viral replication cycle to be undertaken in BHK cells. Our long-term aim is to gain a greater understanding of how FMDV replicates its genome during a rapid, lytic, infection and what causes the switch to a slowed/stopped replication state, to induce a persistent infection.

Funding
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Collaborators
Martin Ryan, St Andrews
Jurgen Haas, Edinburgh
Esteban Domingo, Universidad Autonoma de Madrid
Nuria Verdaguer, IBMB-CSIC, Barcelona
Graham Belsham, Danish Institute for Food and Veterinary Research, Denmark.

Targeting the functions of the Human Papilloma Virus 16 oncoproteins with RNA aptamers

Clare Nicol, Tamara Belyaeva, Eric Blair and Nicola Stonehouse

Human papillomaviruses (HPVs) are DNA tumour viruses that infect epithelial cells. More than 100 types have been identified and those which infect genital epithelia are classified as low or high-risk, dependent on the risk of development of cancer. High-risk viruses cause a range of anogenital and oropharyngeal tumours, most commonly cervical cancer and have also been associated with squamous cell carcinoma of the head and neck and with Bowen's disease. The major factors responsible for the initiation of carcinogenesis are the viral oncoproteins E6 and E7. E6 has been shown to promote degradation of the tumour suppressor p53, whereas E7 has been demonstrated to bind and destabilise the cell cycle control protein pRb. In addition to these well characterised roles, E6 and E7 have been shown to interact with at least 50 other cellular proteins. For example, E6 interacts with cell-cell adhesion and proliferation proteins containing a PDZ motif (e.g. hScrib, MUPP1) and E7 is reported to interact with chromatin-modifying enzymes and transcriptional co-factors (e.g. p300, CBP and pCAF).

We have developed RNA aptamers as molecular tools to investigate some of these protein-protein interactions. Our previous work has identified E7 aptamers which alter the cell cycle distribution and induce apoptosis in an E7-expressing cell line that originates from a human cervical carcinoma (SiHa cells). The aptamers have been shown to reduce the interaction between E7 and the retinoblastoma protein, pRb *in vitro* and to target E7 degradation in SiHa cells.

Our recent studies have resulted in the selection of aptamers to HPV16 E6. Several of these resulted in apoptosis when transfected into SiHa cells. We have shown that two of the E6 aptamers (termed F2 and F4) bound to the C-terminal region of E6, known to interact with PDZ proteins. As the affinity of the E6-PDZ interaction is 1-2 μM and as the aptamers bind E6 with higher affinity, they were used to compete with the PDZ proteins using MAGI1 PDZ1 tagged expression constructs. Aptamer F2 was able to interfere with the E6-PDZ interaction. The E6-p53 interaction was unaffected by these aptamers, demonstrating the specificity of this molecule.

We now propose to investigate the therapeutic potential of the aptamers in systems that better represent the complexities of human HPV-associated carcinomas and establish the efficacy of the aptamers with other HPV types.

Publications

Nicol C., Belaeva T., Forrest S., Bunka D., Blair G. & Stonehouse N. Effects of single nucleotide changes on the binding and activity of RNA aptamers to human papillomavirus 16 E7 oncoprotein. (2011) *Biochem. Biophys. Res. Commun.* **405**:417-21.

Funding

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Collaborators

Andrew MacDonald, University of Leeds;Lawrence Banks, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy;Gilles Travé, Strasbourg, France; Ramon Garcia-Escudero, Madrid

Defining the specificity of the Rep:PcrA interaction

Jessica Johnston, Hannah Cooper and Gerard Lynch and Christopher Thomas

Introduction

The *pcrA* gene is ubiquitous in Gram-positive bacteria and encodes an essential but poorly-processive helicase. Although the role of PcrA in Gram-positive bacteria such as the human pathogen *Staphylococcus aureus* remains unclear, it has been shown to be important for the rolling circle replication of the pT181 family of staphylococcal plasmids.

To date, our work has focussed on helicases obtained from two organisms: *Staphylococcus aureus* (*Sau*PcrA) and (*Geo*)*Bacillus stearothermophilus* (*Bst*PcrA). We have observed that the activity of both helicases is stimulated in the presence of the plasmid initiator protein RepD, encoded by the staphylococcal plasmid pC221. Furthermore, we have also observed that the *Bst*PcrA is recruited to the replication origin of pC221 by RepD [1]. However, no details of the Rep:PcrA interaction are currently known.

Recent Findings

In order to create a homologous system for the study of *Bacillus* Rep:PcrA interactions we have cloned and purified the Rep protein from the *Bacillus stearothermophilus* plasmid pSTK1. This protein, RepSTK, demonstrates sequence-specific type-I topoisomerase activity against plasmids carrying the cognate replication origin but with a temperature optimum of 65°C, considerably higher than that for the comparable activity of RepD.

RepSTK also demonstrates specificity for the cognate PcrA helicase. As indicated above, in oligonucleotide displacement assays PcrA alone is a poor helicase. Although RepD can stimulate both *Sau*PcrA and *Bst*PcrA activities, RepSTK failed to demonstrate stimulation of *Sau*PcrA (Table 1).

Table 1. Stimulation of helicase activity.

PcrA	RepD	RepSTK
Sau	✓	×
Bst	✓	✓
SBS	×	×
BSB	✓	✓

The two helicases share 59% sequence identity; the known structure of *Bst*PcrA indicates four domains (1A, 1B, 2A, 2B; see Fig. 1) plus an unstructured C-terminal tail. We have now begun the dissection of PcrA to identify the region(s) conferring specificity on the Rep:PcrA interaction. Deletion of the C-terminal domain has no effect on stimulation. We have also exchanged the 2B domain between helicases (differences being shown in red in Fig. 1). Although the *Bst*2B domain in the context of *Sau*PcrA (SBS) produced an inactive helicase, the complementary *Sau*2B in *Bst*PcrA (BSB) gave a protein which was stimulated by both Rep proteins. This argues against a specific interface between Rep and the 2B domain; current efforts are now focussed on 1A, 1B and 2A to find the site of interaction necessary for the stimulation of helicase activity.

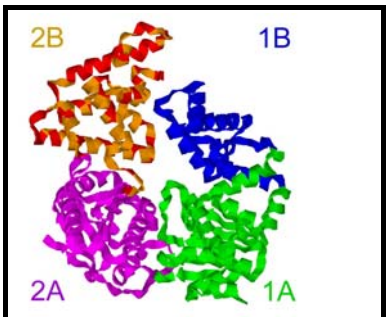

Figure 1: domains of PcrA.

Publications

Machon, C., Lynch, G., Thomson, N., Scott, D., Thomas, C. & Soultanas, P. (2010) RepD-mediated recruitment of PcrA helicase at the *Staphylococcus aureus* pC221 plasmid replication origin, *oriD*. *Nucleic Acids Res.* **38:**1874-1888.

Funding

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Single molecule microscopy with the AFM

Sergio Santos, Daniel Billingsley, Jennifer Kirkham, William Bonass, Neil Thomson

Introduction

Single molecule microscopy is an extensive field of research in bio nanotechnology. Studying fundamental biological processes, interactions and phenomena at the single molecule level allows details to be elucidated that can be lost or obscured in ensemble measurements due to averaging over many, many molecules. The atomic force microscope (AFM) is a well established tool for single molecule studies. The AFM is a versatile high resolution microscope that can image in native-like environments such as ambient and liquid. It is a surface profiling microscopy that measures force between a sharp probe and sample, using a cantilever force sensor, to map out the local topography and mechanical or chemical properties. It has the advantage of not requiring surface coatings or stains to obtain contrast, enabling molecules to be imaged in native-like environments.

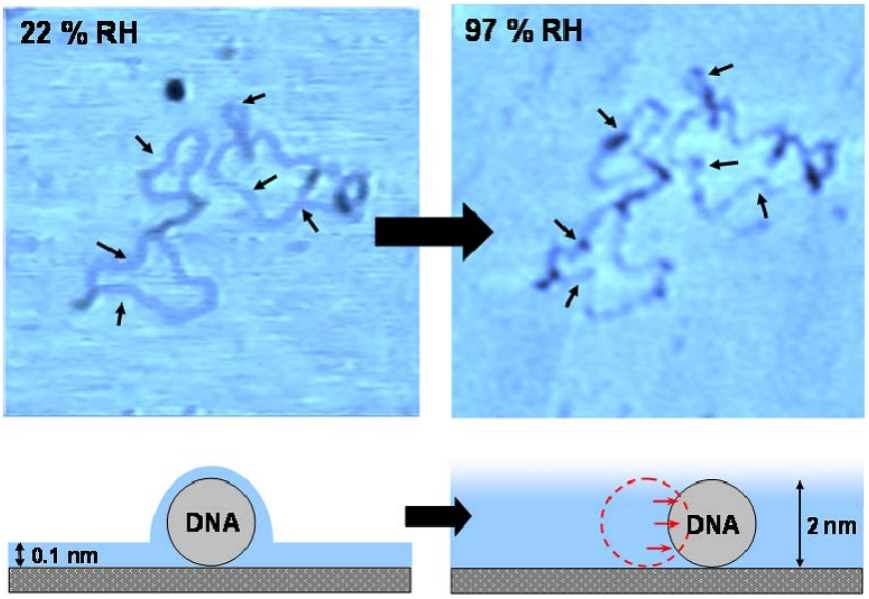

Figure 1: two supercoiled plasmid DNA molecules physisorbed onto mica surfaces imaged at 22% RH and 97% RH. The small black arrows mark regions where the local conformation of the molecules has altered due to the increase in the water layer depth, from sub-nanometre to around 2nm.

The role of hydration in ambient dynamic AFM

The AFM can be operated in the dynamic mode where the cantilever is vibrated while interacting with the surface. This mode is particularly important when imaging soft matter since frictional forces are almost eliminated. Furthermore, dynamic modes work well in ultra-high vacuum, ambient conditions and under liquid. Liquid imaging has some disadvantages to both sample preparation and instrument operation compared to ambient imaging. Molecular motion in liquid reduces stability and resolution. In ambient conditions a water layer typically forms on hydrophilic surfaces. The water layer is of the order of several angstroms to 2nm in thickness, depending on relative humidity. This layer has been commonly associated with instabilities, high adhesion and reduced resolution. We have found however, that the role of capillary interactions between probe and sample are strongly dependent on the cantilever characteristics. That is, while relatively un-sharp probes with compliant cantilevers tend to produce larger amounts of noise, stiffer cantilevers in combination with ultra sharp tips can greatly increase resolution.

Humidity-controlled AFM

The AFM can be operated under different humidity environments by enclosing the scan head in an environmental chamber. As the humidity is increased, the thickness of water layers on hydrophilic surfaces, such as mica crystals, increase to about 2nm at 90% relative humidity (RH). Recently, we have shown that this can be used to interrogate supercoiling of individual closed circular DNA molecules (Figure 1). As the thickness of the water layer approaches the diameter of the DNA, discrete and irreversible conformational changes in the molecules ensue. This is characterised by localised changes in DNA backbone, such as kinking, condensation and the rotation of loops. The interpretation is that strain energy initially taken up as DNA twist converts into local changes in the writhe of the molecule as the strain energy is released when the DNA molecules reside in a sufficient volume of water.

Apparent and true height in AFM

Since the field of Nanotechnology is defined through the dimensions of the nanoscale objects, it is clear that instruments capable of measuring true height and width with high precision are paramount for technological advances. While one of the main uses of the AFM is to measure the height of biomolecules, nanoparticles, the rugosity of the surface features, etc. The instrument, however, is notorious for always producing values which are lower the true ones. We have modeled the tip-sample interaction to show that this is due the area of interaction between the tip and the sample being finite. For example, even for the sharpest tip (i.e. an atom) the region of the sample interacting with it at relatively long distances is of the order of the size of atoms. This implies that there are certain limitations as to, for example, measure the true height of subatomic features even with a one atom tip. In terms of single molecular imaging, the finite geometry of the tip implies that there is a certain averaging between the height of the sample and the height of the supporting surface. Now that we have established the principle of height reconstruction is understood, recovering the true height is possible with reliable models of cantilever dynamics.

Publications

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Santos S., Barcons V., Font J. & Thomson N. (2010) Bi-stability of amplitude modulation AFM in air: deterministic and stochastic outcomes for imaging biomolecular systems. *Nanotechnology* **21**:225710.

Funding

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Nucleotide-dependent shape changes in the reverse direction molecular motor, myosin VI

Chun Feng Song, Kasim Sader and John Trinick

Introduction

Nearly 40 non-muscle myosins now been identified and most cells in the body express several of these at any one time for transport and tension generation roles. Myosin VI is a particularly interesting and puzzling myosin, because it walks along actin filaments in the opposite direction to all other myosins so far studied. Reverse directionality results from a 50 residue unique sequence, Insert 2, that redirects the lever arm through 120°, but this mechanism is not fully understood. Myosin VI is also unusual in having a much larger powerstroke, 120 nm, than expected from its lever arm which has only two calmodulin light chains; by comparison, muscle myosin II steps only 5 nm with a two light chain lever.

Results

We have studied the shape and flexibility of myosin VI molecules by electron microscopy of negatively stained specimens. Recovery of information from the micrographs was greatly improved using single particle analysis to produce image averages.

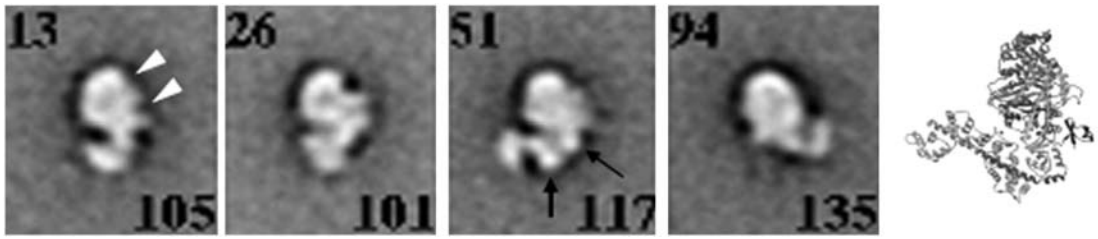

Figure 1: image averages of truncated myosin VI (S-1) without nucleotide. The characteristic 'face-profile' of the motor domain is seen in each panel (facing right except in panel 4). On one side, the motor shows two indentations that are the cleft between the upper and lower 50 kDa sub-domains and the gap between the lower 50 kDa and SH3 subdomains (arrowheads); on the other side, the motor curves smoothly. Insert 2 is visible (thin arrow), extending from which is the lever arm with two calmodulins, with their diagonally positioned lobes resolved in some cases (thick arrow). The numbers at the bottom right in each panel are the number of images in the average and at the top left are the class identification numbers. **Right:** the crystal structure of a similar apo myosin VI molecule showing a very similar shape (see particularly stain panel 3).

Our data also demonstrate a large shape change in myosin VI in response to ATP, ADP or ADP-gS (now shown) in which the angular throw of the lever is ~140° from the apo state, which may in part account for the large steps seen in myosin VI. Images of the full length apo molecules show the tail of the molecule folded back across the motor domain which may indicate a regulatory mechanism.

Publications

Song, C., Sader, K., White, H., Kendrick-Jones, J. & Trinick, J. (2010) Nucleotide-dependent shape changes in the reverse direction molecular motor, myosin VI. *Biophys. J.* **99**:3336-3344.

Funding

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Collaborators

Howard White (Eastern Virginia Medical School) and John Kendrick-Jones (MRC Laboratory of Molecular Biology, Cambridge).

Structural investigation of a fibronectin Type III -immunoglubulin domain tandem from A-band titin

Andras Czajlik, Gary Thompson, Arnout Kalverda, Ghulam Khan,
Steve Homans and John Trinick

Introduction

Single molecules of the giant protein titin extend across half of the muscle sarcomere, from the Z-line to the M-line, and have roles in muscle assembly and elasticity In the A-band titin is integral with thick filaments and in this region the sequence of titin contains fibronectin type III and immunoglobulin-like domains. These are arranged in regular patterns of eleven domain repeats called the large super-repeats. The large super-repeat occurs eleven times and this entire region thus forms nearly half of the titin molecule. We are studying the atomic structure, properties and the inter-domain arrangement of overlapping double and triple domain fragments of the large super-repeat (titin A59-A69 unit) by NMR spectroscopy.

Results

While A67-A68 has a well resolved ^{1}H-^{15}N HSQC spectrum side-chain experiments had poor spectral quality, even with a perdeuterated sample, preventing assignment. As the use of the standard 3D NOESY spectra was impractical, the A67-A68 domain tandem was first investigated by a mixture of chemical shift based structure determination and previously determined NMR structures. The fold of A68 was determined using CS23D based on ^{1}H, ^{15}N and ^{13}C chemical shifts. For A67 we used the NMR structure of A78 [C-terminal domain of the 3LPW], as this is the domain present at the same position as A67 in the next super-repeat. In order to investigate the validity of the model structures the measured and the back-calculated HN Residual Dipolar Couplings values (RDCs) [using a C12E6 - hexanol liquid crystal system] from models were compared for the N- as well as the C-terminal domains. Good correlation ($R > 0.9$) between the back calculated and measured RDC values was observed for resonances with good signal to noise drawn from secondary structure elements and this clearly shows that these structural models are valid as does the good agreement with amide ^{1}H nOe data as well.

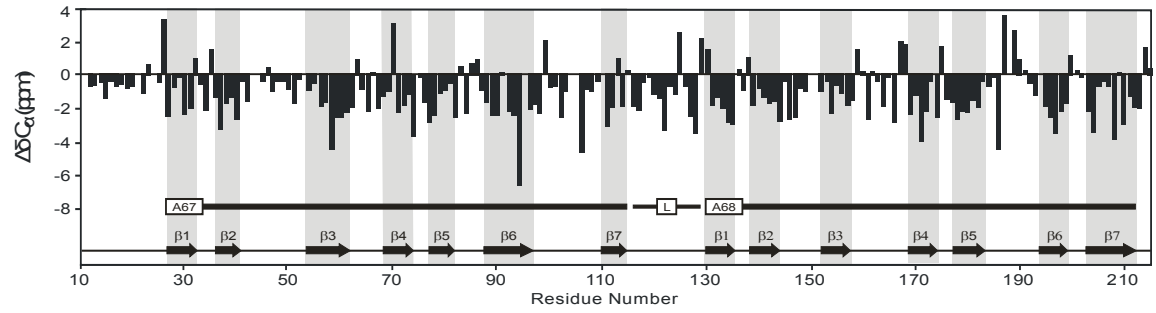

Figure 1: Cα chemical shifts and secondary structure assignments for A67-A68

RDC data only describe domain orientations up to an inversion Cartesian axes which does not change the handedness of alignment tensor frame; giving four possible structural solutions. Structures were calculated using rigid body minimization in using an RDC constraint term. This approach resulted in two physically possible structures. In order to find an unique structure measurements are in progress using a second liquid crystal system to give a second independent tensor frame.

Funding

This work is funded by the British Heart Foundation.

Pigment arrangement in chlorosomes from green filamentous bacterium *Chloroflexus aurantiacus*

Roman Tuma

Introduction

Chlorosomes are large light harvesting complexes found in sulphur (*Chlorobiaceae*) and filamentous (*Chloroflexaceae*) green photosynthetic bacteria. The high light harvesting efficiency of chlorosomes allows these bacteria to survive under extremely low-light conditions and hence to occupy unique ecological niches in the depths of lakes and oceans.

Experimental

We examined structure of chlorosomes from the green filamentous bacterium *Chloroflexus aurantiacus* (Cfx) by a combination of electron cryo-tomography and X-ray scattering (Figure 1).

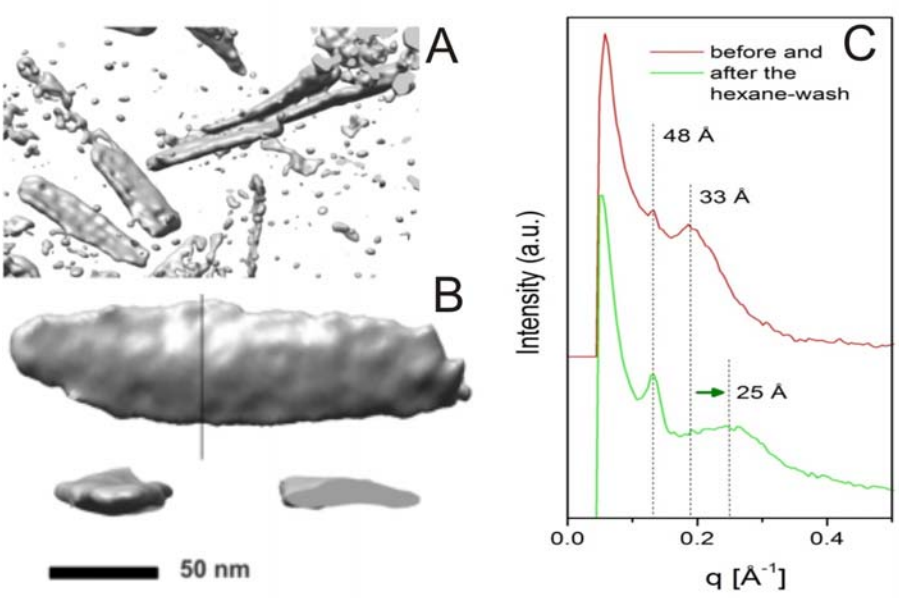

Figure 1: (A) 3-D tomographic reconstruction of volume containing several chlorosomes. **(B)** Overall shape and cross-section of a typical chlorosome from panel A. **(C)** X-ray scattering from chlorosomes prior (red) and after (green) hexane treatment. Note the different position of the lamellar diffraction peak (3.3 versus 2.5 nm) while the diffraction from photosynthetic membranes at 4.8 nm does not shift.

Results

A typical chlo rosome is a flat ellipsoidal body (200 x 50 x 20 nm, Figure 1B) containing about 10^5 bacteriochlorophyll *c* molecules (bchl *c*, the major chlorosomal pigment) and additional pigments such as carotenoids. In the interior bacteriochlorophyll molecules self-assembled into curved lamellae as demonstrated by the dominant diffraction peak with Bragg spacing 3.3 nm (Figure 1C).

Cfx chlorosomes contain a high proportion of carotenoids (30% of mass) and exhibit extremely fast energy transfer rates between carotenoids and bchl *c* (~50 fs). How might be such a large quantity of carotenoids accommodated within the lamellar system? It is known that non-polar solvents, such as hexane, remove carotenoids from chlorosomes without affecting the overall structure. Comparison of X-ray diffraction prior and after a hexane wash revealed a substantial decrease in the lamellar spacing (Figure 1C). This result suggests that carotenoids partition into the hydrophobic space between the aliphatic chains of esterifying

alcohols within the lamellae. Consequently, lamellar spacing increases with the length of the alcohol chains and the amount of carotenoids (Figure 2). This arrangement provides structural basis for the strong excitonic coupling between pigments within the chlorosome.

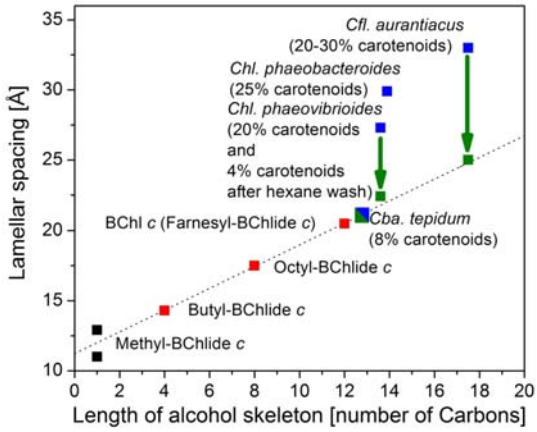

Figure 2: lamellar spacings of self-assembled, chemically modified pigments (red) bearing different lengths of esterifying alcohols compared with those of chlorosomes from various bacterial species prior (blue) and after hexane wash (green).

Publications

Psencik, J., Torkkeli, M., Zupcanova, A., Vacha, F., Serimaa, R. & Tuma, R. (2010) The lamellar spacing in self-assembling bacteriochlorophyll aggregates is proportional to the length of the esterifying alcohol. *Photosynth. Res.* **104**:211-219.

Funding

This work was funded by University of Leeds, Academy of Finland and the Grant Agency of Czech Republic.

Collaborators

This is an ongoing collaboration between several groups: Profs. Sarah Butcher and Ritva Serimaa at the University of Helsinki, Finland, Dr. Jakub Psencik at Charles University, Prague, Czech Republic. The work on Cfx chlorosome was done in collaboration with Prof. Robert Blankenship at Washington University, St. Louis, USA.

Studies on bacterial toxins

Thomas Branson, Simon Connell, Martin Fascione, Edward Hayes, Peter Johnson, Arnout Kalverda, Andrew Macdonald, Pintu Mandal, Emanuele Paci, Arwen Pearson, Neil Ranson, James Ross, Daniel Williamson, Michael Webb and Bruce Turnbull

Introduction

Diarrhoeal diseases such as cholera are still life threatening diseases in many parts of the world. Many diarrhoeal diseases are caused by protein toxins that have an AB_5 hetero-oligomeric structure. The proteins comprise a single toxic A-subunit and a pentameric B-subunit that interacts with specific cell surface glycolipids. Inhibitors of such protein-carbohydrate interactions could provide prophylactic treatments for these debilitating diseases, while mimicking the biological action of the toxins could provide a mechanism for delivering macromolecules into cells.

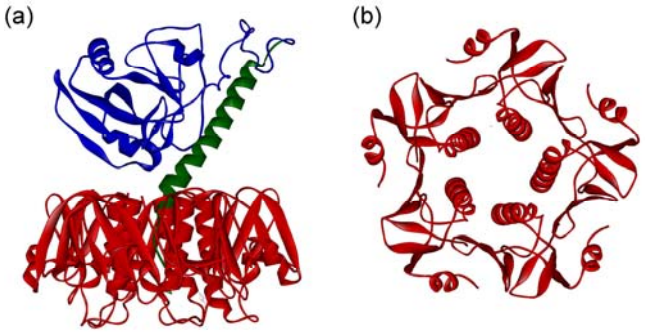

Figure 1: (a) Cholera toxin showing the toxic A1-subunit (blue), A2-linker peptide (green) and B-pentamer (red); **(b)** plan view of the B-pentamer.

We are investigating the binding properties of blood group oligosaccharides for a secondary binding site on cholera toxin and *E. coli* heat-labile toxin. It is believed that these interactions can influence how susceptible patients are to travellers' diarrhoea and certain strains of cholera. We are using a combination of virtual screening, molecular modelling, NMR spectroscopy, X-ray crystallography and synthetic chemistry for fragment-based design of small molecule inhibitors of cholera toxin. We are also investigating the use of protein engineering and site-specific chemical modification to direct the assembly of proteins into discrete three-dimensional structures to provide a general bottom-up strategy for synthetic biology.

Collaborators

David Andrews and Andrew Leach, AstraZeneca, UK.

Funding

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An ADP-dependent kinase dependent upon *N*-phosphorylation of histidine

Tom McAllister, Jeffrey Hollins, Zhenlian Ling, Nathalie Valette, Michael Nix, Arwen Pearson and Michael Webb

Introduction

Pyruvate, orthophosphate dikinase (PPDK) regulatory protein (PDRP) is responsible for the light/dark regulation of PPDK in C_3 and C_4 plants. This enzyme supplies phosphoenolpyruvate for temporary fixation of CO_2 in the mesophyll before shuttling to the bundle sheath cells for fixation by Rubisco. In the dark *in planta*, PDRP inactivates PPDK via an ADP-dependent phosphorylation of a threonine residue adjacent to the catalytic phosphohistidine residue (Figure 1); in the light, PDRP reactivates PPDK via a pyrophosphate-generating phosphotransferase activity. At present the mechanistic basis of neither the forwards nor the reverse reaction has been determined.

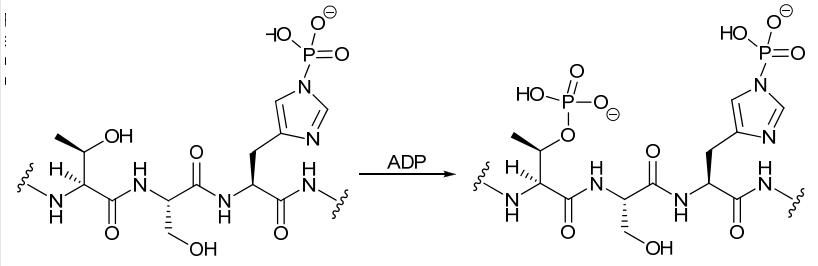

Figure 1: PDRP (PPDK regulatory protein) catalyses the ADP- and phosphohistidine dependent phosphorylation of PPDK.

Results

We have expressed and purified both the protein from maize and its sequence homologue from *E. coli*. The structure of neither protein is known and there are no close sequence homologues upon which to base a structural model. We are currently using isothermal titration calorimetry to understand the role of conserved residues in ADP-binding for both proteins and have identified several key residues involved in interactions with the phosphate residues of ADP. We have initiated crystallisation trials for both proteins and are attempting to determine the structure of this enzyme.

In parallel work, we are seeking to develop peptidic substrates for the enzyme: the enzyme requires the presence of a phosphohistidine residue for catalytic activity, however due to the hydrolytic instability of this modification it is not feasible to incorporate this into peptides directly. We therefore have synthesized the triazole analogue of phosphohistidine (Figure 2) and have optimised strategies for the incorporation of this mimic of a post-translational modification into peptides. We are now seeking to expand this work to investigate other post-translational modifications and to identify immunochemical reagents for their identification.

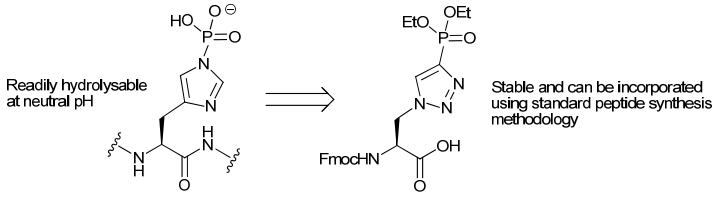

Figure 2: triazole analogue of phosphohistidine

Publications

McAllister, T. , Nix, M. & Webb, M. (2011) Fmoc-chemistry of a phosphohistidine analogue. *Chem. Commun.* **47**:1297-1299.

Funding

We would like to thank BBSRC and EPSRC for funding.

Collaborators

Dr Paul Ko Ferrigno (LIMM)
Dr Julian Hibberd (Plant Sciences, University of Cambridge)
Dr Chris Chastain (Minnesota State Moorhead)
Dr Ryan Mehl (Franklin & Marshall, USA)

Identification of the ribonucleoprotein complex required for efficient viral RNA processing in oncogeneic herpesviruses

Brian Jackson, Adam Taylor, Marko Norenberg and Adrian Whitehouse

Introduction

Post-transcriptional events which regulate mRNA biogenesis are central to the regulation of gene expression. As a consequence, cells have evolved a 'gene-expression production line' that encompasses the routing of a nascent transcript through multimeric mRNA–protein complexes that mediate its splicing, polyadenylation, nuclear export and translation. Of these events it has become clear that splicing is particularly important for mRNA nuclear export, as recruitment of multiprotein complexes required for mRNA export are bound to mRNA in a splicing dependent manner. Two multiple protein complexes, namely, hTREX and the EJC bind at separate locations on spliced mRNA. hTREX, which comprises Aly, UAP56 and the multiprotein ThoI complex, is recruited exclusively to the 5' end of the first exon, providing 5'-polarity and therefore directionality observed in mRNA export.

However, in contrast to the majority of mammalian genes, analysis of herpesvirus genomes has highlighted that most lytically expressed viral genes lack introns. Herpesviruses replicate in the nucleus of the host mammalian cell, and therefore require their intronless mRNAs to be exported out of the nucleus to allow viral mRNA translation in the cytoplasm. This therefore leads to an intriguing question concerning the mechanism by which the viral intronless mRNAs are exported out of the nucleus in the absence of splicing. To circumvent this problem, and to facilitate viral mRNA export, γ-2 herpes viruses encode the ORF 57 protein. ORF 57 interacts with Aly, binds viral RNA, shuttles between the nucleus and the cytoplasm and promotes the nuclear export of viral mRNA.

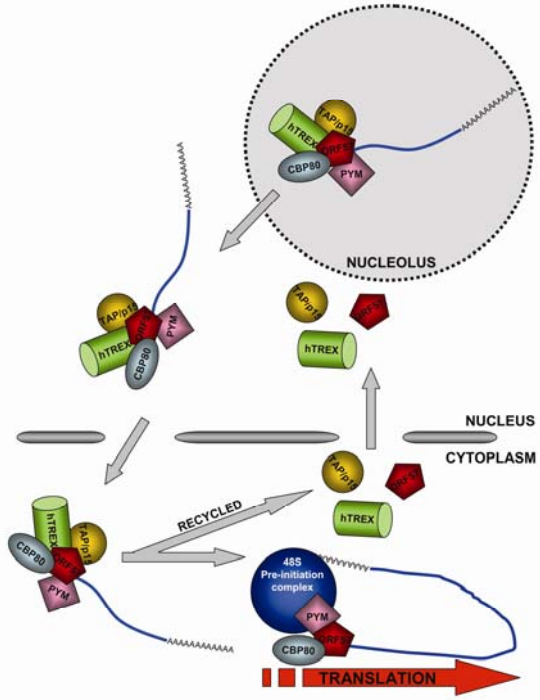

Figure 1: KSHV ORF57 mimics splicing to recruit the necessary nuclear export factors onto viral intronless transcripts and also mimics an EJC to enhance their translation.

Results

We are currently investigating how an intronless viral mRNP is assembled in KSHV and what role ORF57 plays in that process. We have shown that ORF57 interacts with hTREX and is essential for the recruitment of hTREX onto intronless viral mRNA transcripts. Importantly, ORF57 does not recruit the EJC to intronless viral transcripts. Moreover, we are currently determining how ORF 57 recognises the viral mRNA and allows recruitment of hTREX. This is the first system that has distinguished between hTREX and EJC *in vivo* and demonstrates that recruitment of hTREX alone to mRNA transcripts is sufficient for their nuclear export. Therefore, we believe this viral system is an exciting model to further study mRNA export mechanisms. We propose a model for herpesvirus mRNA export, whereby ORF57 mimics splicing in order to recruit the mRNA export machinery to intronless viral mRNAs.

Moreover, the inability of ORF57 to recruit the EJC may have implications for the efficiency of translation of the viral intronless mRNAs, as recent data suggests that the EJC promotes efficient translation of spliced mRNAs by several mechanisms. One such mechanism involves the cellular protein, PYM, which links the EJC to the 48S pre-initiation complex. Therefore, we have investigated whether KSHV ORF57 has any role in enhancing translation as well as nuclear export. We have shown that ORF57 sediments predominantly with the 40S ribosomal subunit and enhances translation of viral intronless transcripts. Moreover, we have demonstrated that ORF57 interacts with PYM and components of the 48S pre-initiation complex and functions to recruit PYM onto a viral intronless mRNA. Significantly, siRNA-mediated depletion of PYM ablates the interaction between ORF57 and components of the 48S pre-initiation complex and dramatically decreases the translation of KSHV intronless mRNAs. Therefore, we propose a model whereby ORF57 also mimics an EJC enabling efficient translation of intronless KSHV transcripts.

We are now determining the structure of the interaction interface at atomic-resolution between ORF57 homologues and the hTREX proteins, such as Aly. In collaboration with Dr Alexander Golovanov (University of Manchester) and Professor Stuart Wilson (University of Sheffield). This will provide a detailed comparison of the binding interfaces between ORF57 homologues and Aly using solution-state NMR. The regions of HSV ICP27 and HVS ORF57 involved in binding by Aly have been mapped as residues 104-112 and 103-120, respectively. We have identified the pattern of residues critical for Aly recognition, common to both ICP27 and ORF57. The importance of the key amino acid residues within these binding sites was confirmed by site-directed mutagenesis. The functional significance of the ORF57-REF/Aly interaction was also probed using an *ex vivo* cytoplasmic viral mRNA accumulation assay and this revealed that mutants that reduce the protein-protein interaction dramatically decrease the ability of ORF57 to mediate the nuclear export of intronless viral mRNA. Together these data precisely map amino acid residues responsible for the direct interactions between viral adaptors and cellular REF/Aly and provide the first molecular details of how herpes viruses access the cellular mRNA export pathway. Future work will utilise these identified binding interfaces as possible new drug targets, to be used in the future for anti-viral drug design efforts, for the prevention or treatment of KSHV-related malignancies.

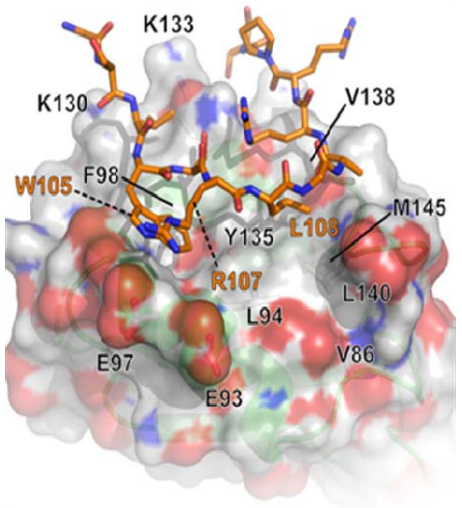

Figure 2: ORF57 homologue (HSV ICP27) recognizes and specifically binds to the surface of the RRM domain of cellular export factor REF/Aly

Publications

Boyne, J., Jackson, B., Taylor, A., Macnab, S. & Whitehouse, A. (2010). Kaposi's sarcoma-associated herpesvirus ORF57 interacts with PYM to enhance viral intronless mRNA translation. *EMBO Journal,* **29:**1851-1864.

Boyne, J., Jackson, B. & Whitehouse, A. (2010). Kaposi's sarcoma-associated herpesvirus ORF57: Master regulator of mRNA biogenesis. *Cell Cycle,* **9:**2702-2703.

Hiscox, J., Whitehouse, A. & Matthews, D. (2010). Nucleolar proteomics and viral infection. *Proteomics,* **10:**4077-4086.

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Proteomimetics as potential anticancer agents

Maria Filby, Kerya Long, Natasha Murphy, Panchami Prabhakaran, Alison Ashcroft, Thomas Edwards, Stuart Warriner and Andrew Wilson

Introduction

Protein-Protein interactions (PPIs) are crucial to virtually all biological processeses and as a result, provide an appealing target for small molecule intervention in a range of abberant conditions. *'Proteomimetics'* are synthetic molecules that can mimic the spatial projection of key binding residues from a secondary structural motif and have proven useful in the generation of inhibitors of several PPIs. *'Surface mimetics'* present recognition domains from a core scaffold in a multivalent manner to achieve high affinity protein surface recognition. This report will summarise our group's efforts using both approaches.

α-helix mimetic inhibitors

Our group have previously described two scaffolds based upon an aromatic oligoamide template having different side chains (Fig 1a and b) and demonstrated that these act as α-helix mimetic inhibitors of the oncogenic p53-hDM2 interaction. These *proteomimetics* were initially generated using solution-phase strategies. For the *N*-alkylated scaffold a manual solid phase method was also reported with a limited range of side chains. In the past year, we have started to explore the development of a solid-phase synthesis method for the *O*-alkylated scaffold and are now looking to adapt the approach for both scaffolds so as to permit automated synthesis using a CEM™ automated microwave assisted peptide synthesizer. This will allow us to assemble libraries for screening against a diverse array of helix mediated PPIs. We have successfully completed the synthesis of new Fmoc-protected monomers for assembly of both scaffolds (Figures 1a and b).

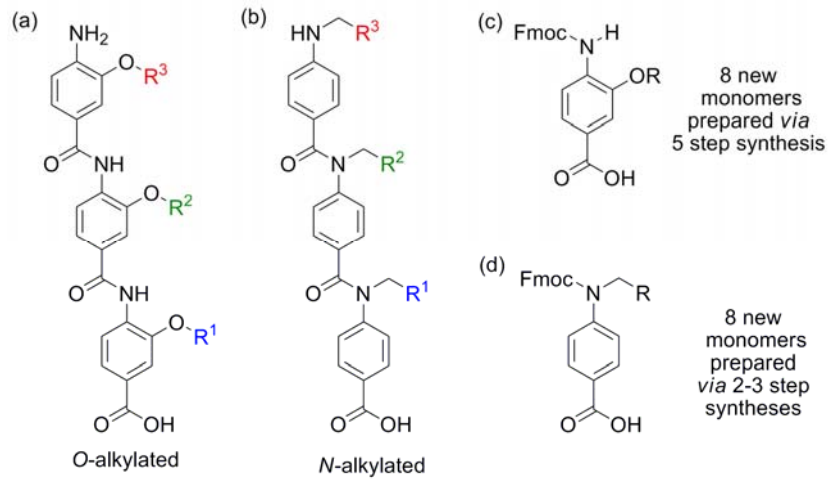

Figure 1: (a) structure of *O*-alkylated helix mimetic scaffolds **(b)** structure of *N*-alkylated helix mimetic scaffolds **(c)** structure of Fmoc-protected *O*-alkylated monomer **(d)** structure of Fmoc-protected *N*-alkylated monomer.

Surface mimetics

Following our success with ruthenium complexes bearing symmetrical disubstituted bipyridine ligands as protein surface mimetics, we endeavoured to exploit the variation in the 3D projection of functional groups that may be achieved using unsymmetrical monosubstitued bipyridines. We demonstrated that the geometrical arrangement of binding entities around a ruthenium(II) core leads to differences in protein-binding affinity towards cytochrome *c*. The

successful separation of four diastereomers of $Ru(bipy)_3$ (**1**) complex allowed us to test each of the optically pure isomers against a selection of proteins. The difference in binding affinity towards cytochrome *c*, obtained from fluorescence titrations between *fac*(**1**) and *mer*(**1**) geometrical isomers are different by one-order of magnitude (equivalent to 4 $kJmol^{-1}$ in free energy). On the other hand, little difference in affinity towards the target protein was observed between the stereoisomers Δ/Λ. Binding to non-metalloproteins of comparable pI such as α-chymotrypsin or lysozyme was observed to be weak and non specific. In addition, none of the compounds exhibited any quenching upon titration with 60% acetylated cytochrome *c*. Acetylation of surface exposed lysine residues prevents key electrostatic interactions involving these residues from taking place. Current and future studies are focused towards demonstrating these large macromolecules have biological relevance through cell based experiments.

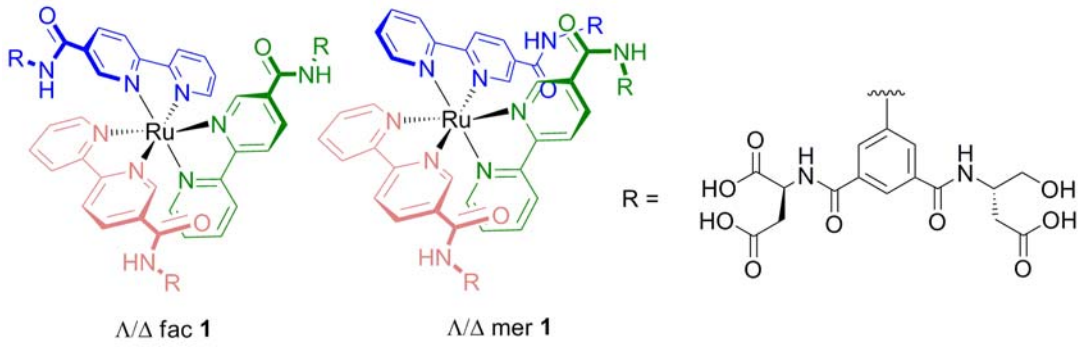

Figure 2: structure of $Ru(bipy)_3$ **(1)**

Publications

Campbell, F., Plante, J. P., Edwards, T. A., Warriner, S. L. & Wilson, A. J. (2010) *N*-Alkylated oligoamide α-helical proteomimetics. *Org. Biomol. Chem.*, **8**:2344-2351.

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Funding

We gratefully acknowledge EPSRC, the University of Leeds, The Wellcome Trust, Yorkshire Cancer Research and The European Research Council for financial support of this research.

Collaborators

We gratefully acknowledge Nicholas Fletcher and Serin Dabb (University of Belfast) for synthesis of bipyridine precursors.

Development of smart nanoparticle-aptamer sensing technology

Haiyan Zhang, Simon White, Michael Wilson, Lei Song, Peter Stockley and Dejian Zhou

Introduction

Aptamers are short single-stranded (ss) DNA/RNA molecules generated against specific targets with affinities often comparable to antibodies. They have several advantageous properties over antibodies as ligand binding agents, including a wider target choice; higher ligand specificity with comparable affinity (μM to pM); production by totally *in vitro* chemical synthesis (eliminating batch-to-batch variation); are more robust against thermal/chemical denaturation. As such aptamers are extremely usful in a variety of sensing and biomedical applications.

Metallic nanoparticles and quantum dots (QDs) have unique size-dependent properties that can be exploited for sensing applications. In this regards, gold nanoparticle (GNP) is very attractive because of its high stability, low toxicity, and versatile surface chemistry. Its strong plasmon absorption ($\varepsilon > 10^9$ $M^{-1}cm^{-1}$ for 20 nm GNP) is also size and aggregation-dependent: isolated GNPs are red but become purple/blue upon aggregation. The resulting colour change can be directly visualised by the naked eye, and is thus well-suited for simple, colorimetric sensors. With QDs, the unique, size-dependent, strong and stable fluorescence are excellently suited for fluorimetric sensors. The broad absorption and narrow, symmetric emission are well-suited for Förster resonance energy transfer (FRET) based sensors because this allows for greatly reduced background and higher sensitivity. Combining aptamers with GNPs/QDs, we are developing smart nanoparticle (SNP)-aptamer sensors that maybe exploited for rapid, sensitive detection of a wide range of targets: e.g. harmful food residues, disease biomarkers, environmental pollutants, and hazardous biological materials.

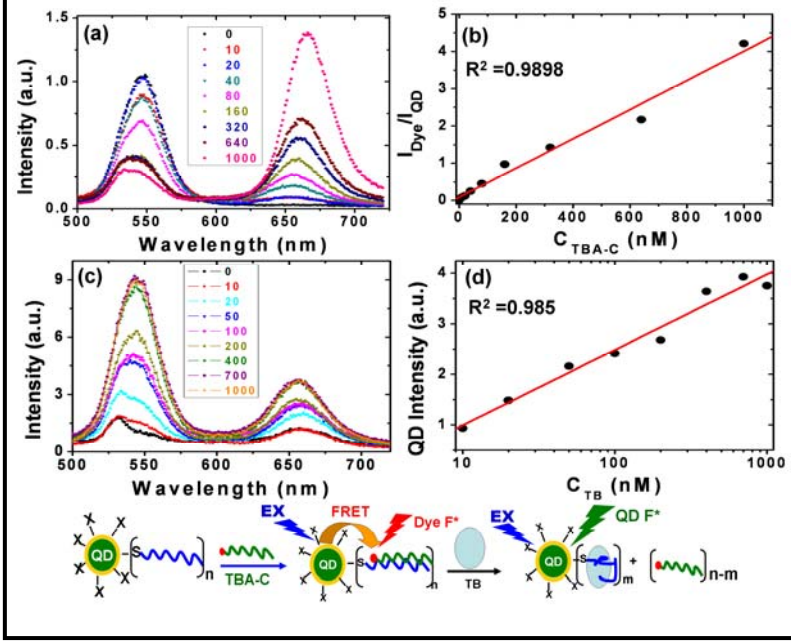

Figure 1: use of self-assembled DHLA-QD-TBA for DNA (top) and protein (bottom) detection. (**a**) Fluorescence spectra of self-assembled QD-$(TBA)_5$ (C_{QD} = 20 nM) after hybridisation to different amount of TBA-C (Atto-647N labelled). (**b**) A plot of Atto-647N to QD fluorescence ratio, I_{Dye}/I_{QD} *v.s.* TBA-C concentration. (**c**) Fluorescence spectra of QD-$(TBA/TBA\text{-}C)_5$ (C_{QD} = 10 nM) after a treatment with different amount of thrombin (TB). (**d**) A plot of the QD-fluorescence *versus* the TB concentration.

The QD-aptamer sensor is FRET based whose efficiency is strongly dependent on the donor-acceptor distance. For high sensitivity, the QD-aptamer sensor should be as compact as possible. We found that the self-assembly between a dihydrolipoic acid (DHLA) capped QD and thiolated anti-thrombin aptamer (TBA) provide a simple, straightforward means to make compact QD-aptamer conjugate (Figure 1). The resulting QD-TBA conjugate can hybridise specifically to its complementary DNA target (TBA-C, labelled with Atto-647N) to produce strong FRET (E > 80%) and the I_{Dye}/I_{QD} ratio increases linearly with the $C_{TBA\text{-}C}$ (Figure 1B),

suggesting it is suitable for TBA-C quantification. Introduction of thrombin (TB) to the system leads to the formation of TBA/TB complex and simultaneous detachment of the TBA-C and the recovery of the QD fluorescence. The QD fluorescence increases linearly with $\log(C_{TB})$, suggesting it is suitable for label-free nM TB quantification.

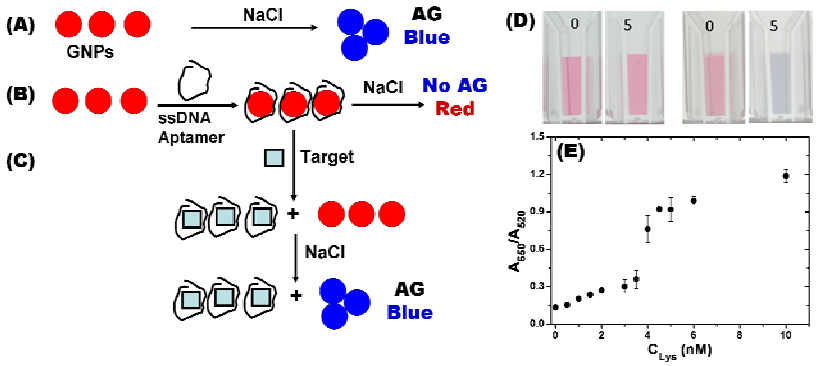

Figure 2: principle of the GNP-aptamer colorimetric sensor for protein detection. (**A**) GNPs readily aggregate upon addition of salt, leading to a red-to-blue colour change; (**B**) addition of ssDNAs (aptamers) to the GNPs produces stable GNP-aptamer physical conjugates which can protect the GNPs from salt-induced aggregation; (**C**) addition of targets leads to the formation of more stable aptamer-target complexes that depletes the GNP surface ssDNA aptamers, as a result, the GNPs ready aggregate when salt is introduced. (**D**) Photos of the GNP-anti-lysozyme aptamer sensors before (left) and after (right) 5 min of 100 mM NaCl addition, the lysozyme concentrations (0/5 nM) were shown on each cuvette. (**E**) A plot of the A_{650}/A_{520} ratio as a function of lysozyme concentration after 2 min of NaCl addition.

We have developed a simple label-free colorimetric sensor for lysozyme using unmodified GNP and aptamer probes by exploiting the different binding affinities of unstructured ssDNA aptamer and structured aptamer-target complex towards citrate stabilised GNPs (see Figure 2A-C for sensing mechanism). The resulting GNP-aptamer (anti-lysozyme) sensor can detect 5 nM lysozyme in minutes by direct visual inspection (Figure 2D). A plot of the A_{650}/A_{520} (a GNP aggregation state indicator) *v.s.* C_{Lys} reveals an interesting three-stage response: a slow steady increase between 0-3.5 nM, followed by a jump at 4 nM, and a more gradual increase as C_{Lys} increases further. The jump of A_{650}/A_{520} ratio indicates the onset of significant GNP aggregation, suggesting this system can detect 4 nM lysozyme by direct visual inspection.
In summary, we have developed two types of SNP-aptamer sensors: a QD based fluorimetric and a GNP-based colorimetric sensor, both of which can be used for rapid, label-free detection of low nM protein targets. Importantly, both sensing systems are general, and can be extended straightforwardly to other targets of interest by simply changing the aptamers used. Currently, we are focused on optimising the surface/conjugation chemistries to improve the sensor robustness, sensitivity and extending their target range. We aim to develop a general SNP-aptamer sensing technology that may have a range of applications: biosensing, disease diagnosis, and environmental and food safety monitoring etc.

Publications

Zhang, H., Stockley, P. & Zhou, D. (2011) Development of smart nanoparticles-aptamer sensing technology. *Faraday Discuss*. **149**:319-332.

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ASTBURY SEMINARS 2010

4th February 2010
Prof David Stuart, Division of Structural Biology, University of Oxford
"Chipping away at virus structure: prospects with new technology and some recent insight into the ancient origins of complex eukaryotic viruses"

18th March 2010
Dr Gilles Trave, CNRS, France
"Structure-function analysis of HPV E6 oncoprotein"

22nd April 2010
Prof Patricia Bassereau, Physical Chemistry Laboratory, Institut Curie, Paris
"Membrane nanotubes for studying intracellular trafficking"

27th May 2010
Prof Martin Blackledge, Institut de Biologie Structurale, Grenoble
"NMR studies of conformational fluctuations in folded and unfolded proteins in solution: towards a description of functionally important motions in biology"

17th June 2010
Annual Astbury Lecture
Prof Janet Thornton, EMBL European Bioinformatics Institute
"The reactions of life: enzymes are fun"

28th June 2010
Dr Allen M. Orville, Brookhaven National Laboratory
"Correlated single-crystal spectroscopy and X-ray crystallography at beamline X26-C of the NSLS"

1st July 2010
Dr Sjors Scheres, MRC Laboratory for Molecular Biology
"Multiple protein structures in one shot: maximum likelihood image refinement in 3D electron microscopy"

13th August 2010
Dr Doryen Bubeck, Wellcome Trust Centre for Human Genetics, University of Oxford
"Structural and functional characterization of RNaseH2 defines roles in replication, repair and autoimmunity"

9th September 2010
Prof James Bardwell, Department of Molecular, Cellular and Developmental Biology, University of Michigan
"Optimization of protein folding in vivo uncovers a new chaperone"

14th October 2010
Dr Jane Clarke, Department of Chemistry, University of Cambridge
"Exploring the protein folding landscape - unexpected diversions"

27th October 2010
Prof Daniel Otzen, Interdisciplinary Nanoscience Centre, Aarhus University
"Anti-aggregation strategies: a cure for Parkinson's disease?"

4th November 2010
Prof Christian Cambillau, University of Marseilles, France
"Structures of two lactococcal phage baseplates and their mechanism of activation deciphered using hybrid methods"

11th November 2010
Prof Rob Beynon, Proteomics Group, Institute of Integrative Biology, University of Liverpool
"Putting the 'Q' in proteomics: strategies for quantification"

9th December 2010
Dr Kay Grünewald, Division of Structural Biology, University of Oxford
"Integrative imaging of virus host interactions"

PUBLICATIONS BY ASTBURY CENTRE MEMBERS 2010

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