Session V: The state-of-the-art integrated structural biology – a hawk-eyed view of biomolecular structure

Lectures

L5.1

Membrane proteins structure and dynamics – they both matter

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Keywords: membrane proteins; dynamic structure

To understand the molecules we not only need to see how they look like, i.e. to determine their high-resolution static structures, we also need to know and understand how they move, we need to study their motions. In the course of our recent studies we have established an NMR spectroscopy-based approach to determine the dynamic structures of mammalian integral membrane proteins at high accuracy and resolution. On the examples of α-helical mitochondrial translocator protein (TSPO) and β-barrel human voltage-dependent anion channel (hVDAC1) we have shown the importance of the interplay of the protein structure and dynamics and their relevance in the functional context.

In summary we clearly demonstrate that the traditional static description of the membrane protein structures is not sufficient to understand their functions and describe the mechanistic aspects of their work.

L5.2

Structural analysis of the spliceosomal complexes by cryo-EM

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Keywords: spliceosome; structural analysis; electron cryo microscopy

The spliceosome is a dynamic RNA-protein complex, which assembles on pre-mRNA substrates by the sequential binding of five canonical subunits - small nuclear ribonucleoprotein particles (U1, U2, U4/U6 and U5 snRNPs) - and numerous non-snRNP factors. Hierarchical assembly of the complex is initiated by the recognition of the 5'-splice site and branch site by U1 and U2 snRNPs. Subsequent recruitment of U4/U6.U5 tri-snRNP results in a fully assembled but catalytically inactive complex B. A series of complex structural and compositional rearrangements leads to the formation of a group II intron-like RNA catalytic core required for two trans-esterification steps of splicing catalysis. The enormous complexity and highly dynamic nature of spliceosomes have limited high-resolution structural analysis by x-ray crystallography to individual proteins and smaller snRNPs sub-complexes. Recent developments in the field of electron cryo microscopy (cryo-EM), including introduction of direct electron detectors and statistical image processing, provide solutions to some of these problems, as illustrated in our 3D reconstruction of 1.5 MDa spliceosomal U4/U6.U5 tri-snRNP at 3.7 Å resolution. We will present our recent progress in structural analysis of the spliceosomal complexes by means of cryo-EM.

References

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L5.3

Prokaryotic cell biology by electron cryotomography

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Keywords: electron cryotomography; prokaryotic cell biology

The prospect of understanding simple cells in complete molecular detail is truly exciting. Electron cryotomography (ECT) is a powerful technique that enables 3D visualisation of intact cells at macromolecular resolution, essentially bridging the so-called 'resolution gap' between light microscopy and data derived from high-resolution imaging techniques (e.g., X-ray crystallography, single particle electron cryomicroscopy).

Recent technological advances in ECT hardware development, sample preparation, and automated data collection have proven particularly useful in studying prokaryotic organisms. Until recently bacterial and archaeal cells were perceived as bags of mishmashed enzymes. However, displaying their architecture at nanometre scale has yielded a much more intriguing picture of spatiotemporally organised entities where distinct processes such as plasmid segregation or cell division are tightly controlled.

Here, I briefly introduce the principles of ECT and discuss its advantages and limitations. I then report how ECT has been utilised to elucidate the architecture of the cytokinetic ring formed by the tubulin homologue FtsZ (FtsZ-ring) in bacterial cell division. Addressing this issue is particularly crucial since the membrane constriction mechanism has remained elusive. In the presented study, a detailed 3D arrangement of individual FtsZ protofilaments has been obtained *in vivo* in *Escherichia coli* cells and *in vitro* inside constricting liposomes of sizes corresponding to those of a bacterial cell. The observed FtsZ-ring architecture favours a mechanism of cell membrane constriction that is accompanied by filament sliding and allows us to speculate on where the energy for constriction comes from.

L5.4

How folded is unfolded and how unfolded is folded?

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Keywords: protein folding/unfolding; NMR spectroscopy

Protein folding and unfolding are crucial for a range of biological phenomena and human diseases. These complex processes do not follow a simple two-state model. Defining the structural properties of the involved transient species is therefore of an interest. Moreover to retain their biological functions proteins can be either structured or intrinsically disordered (IDPs). This is the reason why an access to the information to all these states in which proteins work is crucial for our understanding of the physiological and pathological functions of these macromolecules in living organisms. The NMR spectroscopy assisted with other biophysical techniques gives an access to the both structural and dynamical information on different stable and transient states that proteins can adopt under different conditions. This presentation is a demonstration of a great diversity of different states adopted by proteins that are reachable by modern biophysical techniques, with the emphasis of NMR spectroscopy.

Posters

P5.1

The localization of the polymerization site in fibrin αC region

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Keywords: fibrinogen; monoclonal antibody; fibrin polymerization; fibrin αC region

Introduction: αC regions are the carboxy terminal two-thirds of the Aα chains of fibrin(ogen) molecule. Numerous data suggests that αC regions take place in fibrin polymerization but no sites are localized in αC region yet. The aim of this study was to localize the site of fibrin polymerization in αC region.

Methods: Monoclonal antibody (monAb) I-5A to the Aα509-610 region of fibrin(ogen) was isolated from hybridoma culture medium by affinity chromatography on fibrin-sepharose. Fab-fragment of monAb I-5A was prepared on papain-sepharose in PBS. Protease 2, which cleaves from human fibrinogen Aα505-610 fragments, was obtained from *Bacillus thuringiensis* culture medium. Fibrin polymerization in the presence or absence of monAb I-5A or Fab-fragment and polymerization of fibrin desAα505-610 were investigated by turbidity analysis and electron microscopy, performed in a H-600 electron microscope (Hitachi, Japan).

Results: The results of turbidity analysis show that monAb I-5A and its Fab-fragment at equivalent concentration increase lag-period 1,1- and 1,3-fold respectively and decrease maximum rate of fibrin polymerization 7,25- and 7,5-fold respectively. Electron microscopy data indicate that monAb I-5A and its Fab-fragment inhibit both stage of fibrin polymerization – protofibrils formation and their lateral association. It was shown the impairment of fibrin desAα505-610 polymerization: lag-period was increase 3 times and final clot turbidity – 2 times.

Conclusions: Our data let us to suggest that Aα505-610 contain the site of fibrin polymerization.

P5.2

Molecular effects of mutations in cytochrome b related to mitochondrial diseases: large-scale motion of iron-sulfur protein and superoxide generation

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Keywords: cytochrome bc1; mitochondrial complex III; mitochondrial diseases; reactive oxygen species; domain movement

Cytochrome bc1(mitochondrial complex III) is a key enzyme of the respiratory chain. It's catalytic core consists of cytochrome b, cytochrome c1, and iron-sulfur protein (ISP) which performs a movement during the enzymatic cycle. Several mutations related to mitochondrial diseases are located at the cytochrome b-ISP interface. Using the purple bacteria model we recently showed that one of such mutations, G167P, shifts the equilibrium position of the ISP away from the Qo catalytic site and is associated with increased reactive oxygen species (ROS) levels. Here, we compare the molecular effects of the G167P mutation with another mutation from that group, G332D (G290D in human, found in patients with exercise intolerance). Spectral analyses of cofactors and kinetic measurements suggest that although it has a similar effect on the ISP shift, its consequences on enzymatic activity and ROS production are less severe. We suggest that even a small difference in the shift may result in a significant difference in ROS generation.

P5.3

The pathway of *D*-Tyr in *Thermus thermophilus*: aminoacylation and deacylation steps

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Keywords: *D*-amino acids; aminoacylation; deacylation

Amino acids are chiral molecules that exist in *D* and *L* forms. Normally, only *L*-amino acids are incorporated into proteins, and biosynthetic pathways involve different enzymes to prevent misincorporation. The first step includes the aminoacylation of cognate amino acids by aminoacyl-tRNA-synthetases (aaRS). These enzymes are specific to *L*-amino acids, but they can also charge tRNAs with *D*-forms. *D*-aminoacyl-tRNA-deacylase (DTD), an additional editing enzyme, exists to make corrections in the second step of protein biosynthesis. DTD was first described in 1967 (Calendar & Berg, 1967), and since then, the mechanism of hydrolysis by DTD has been investigated (Soutourina et al., 1999; Zheng et al., 2009).

The aim of our research was to study the contribution of the aminoacylation step in distinguishing between *L*-Tyr and *D*-Tyr by TyrRS and the editing mechanism against *D*-Tyr-tRNATyr by DTD in *T. thermophilus*. We first performed a tRNATyr labeling assay using [□ -32P]-ATP to identify Km/Vmax values for *D*-Tyr and *L*-Tyr in the aminoacylation reaction. We confirmed that this step showed no discrimination between *D*- and *L*-isomers by TyrRS from *T. thermophilus*. We then developed a purification procedure for DTD to yield a high quality enzyme (Rybak et al., 2015) and tested its activity in [□ -32P]-AMP formation and deacylation assays. To investigate the mechanism of *D*-Tyr-tRNATyr hydrolysis, we performed molecular modelling, molecular dynamics simulations and site-directed mutagenesis studies of the proposed DTD active site and tRNATyr variants with a substitution of the2'- and 3'-OH group.

P5.4

Purification, characterization and study of fibrinolytic properties of the novel *Bacillus thuringiensis* var. *israelensis* protease

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Keywords: proteases; *Bacillus thuringiensis*; fibrinogen; purification

The finding and characterization of new enzymes targeted to the fibrinogen molecule are of interest for modern biotechnology and medicine. The purpose of this work was isolation and purification of the fibrino(geno)lytic protease of *Bacillus thuringiensis* var. *israelensis* IMV B-7465, and study its physico-chemical properties and mechanisms of action on the fibrinogen. Purification was performed with DEAE 650(M), HW-55 and Sepharose 6B. Protease's action on fibrinogen was estimated by SDS-PAGE with further immunoprobing using the mouse monoclonal 1-6B (anti-Aα509-610) antibody. The final specific activity of the purified protease increased up to 33-fold, with a 26.7% recovery compared with the initial activity. The enzyme showed optimum activity at around pH 10.0, 50 °C, with preferential specificity of the enzyme to the chromogenic substrates with Leu, Ala or Lys in S1 position. The enzyme demonstrated highest specificity to the Glp-Ala-Ala-Leu-pNA and *D*-Val-Leu-Lys-pNa substrates (Km were 570 μM and 211 μM respectively). Inhibitory assay showed that the protease is a metalloprotease. The enzyme preferentially cleaved the Aα-chain of fibrinogen with the formation of peptide (10 kDa) that corresponded to the C-terminal part of Aα-chain. Proteolytic action on fibrinogen is at the AαAla494-Ser495 peptide bond. The lag-period of thrombin-induced polymerization of the protease-affected fibrinogen was four times longer compared to the uncleaved fibrin.

Thus, the novel fibrinogen-specific metalloprotease of *B. thuringiensis* var. *israelensis* IMV B-7465 is distinctly directed to inhibit the polymerization ability of fibrinogen. The enzyme could be used in the study of fibrinogen functioning and in the design of anti-thrombotic agents.

P5.5

Tailor-made lipopeptide biosurfactant biosynthesis

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Keywords: *Bacillus subtilis*; surfactin; surfactin analogue; HPLC-UV

Lipopeptides are one of the most promising groups of microbial biosurfactants because of their properties and potentially large diversity [1]. Surfactin is one of the strongest lipopeptides produced by *Bacillus subtilis*, which lowers the surface tension of water from 72 to 27 mN/m at a concentration of 0.005%. The aim of the study is to design and produce tailor-made lipopeptides using a broth and solid-state fermentation (SSF) process. *B. subtilis* strain KB1 [2] produced a mixture of five surfactin analogues with the alkyl chain ranging from C12 to C16. SSF of rapeseed cake showed that the amount of oxygen influences the amount of each surfactin structural analogue. High oxygen levels resulted in mainly C15 (more than 50%) while lowering the oxygen in the C12 analogue. To determine the oxygen effect on surfactin analogue production, we used 11-ml tubes filled with different volumes (20%, 50% and 80%) of carbon from various sources (glucose, fructose, sucrose & mannose) and adjusted the pH. The highest yield of surfactin was obtained for pH 7.5 using 4% glucose and a 20% filling volume.

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P5.6

The role of Ca^{2+}-dependent structure changes and gamma chains crosslinking in plasminogen activation on fibrin fragment DD

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Keywords: plasminogen activation; fibrin fragment DD

Ca^{2+}-binding sites of fibrinogen/fibrin D-domains and crosslinkage of γγ-chains of D-domains by FXIII are known to provide fibrin stability under polymerization process but their role in fibrinolysis is still unclear. The aim of present study was to investigate the role of Ca^{2+}- and γγ-covalent binding- dependant structure changes in fibrinolytic process initiation on fibrin fragment DD.

The study was performed using fibrinogen fragment D, fibrin fragment DD and cross-linked fibrinogen fragment DD, which were obtained from plasmin hydrolysate of human fibrinogen/fibrin. Chelation treatment of fragments was used to modulate Ca^{2+}-content in fragments.

Study of plasminogen activation by tissue-type plasminogen activator (tPA) on fibrinogen fragment D and fibrin fragment DD have shown the intensification of plasmin formation in case of the fragments pretreatment by EDTA and EGTA. Potentiation of tPA-mediated plasminogen activation by chelating agents treated fragments D and DD decreases in presence of Ca^{2+}. Apparently, the calcium ions removal leads to plasminogen activation sites exposure. Fibrinogen cross-linked fragment DD had similar to EDTA-treated DD effect on plasmin formation by tPA. Thus γγ-crosslinkage can also be responsible for plasminogen activation site unclosing.

Our results reveal the requirement of γγ-crosslinking and Ca^{2+}-dependent structure changes in D-domains for tPA-mediated plasminogen activation sites exposure and initiation of fibrinolysis.