Saturday, March 21, Session VIII

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MIRACLE OF MULTI-DOMAIN PROTEINS: A CASE STUDY OF ROCK KINASE

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Globular polymer molecule is an archetypal image of proteins presented in all school books. But many proteins consist of several such globular subunits called domains. In fact, majority of proteins have multi-domain architecture so that the archetypal protein image should be revisited. Moreover, proteins often lack conventional globular fold in some of their parts. If they are unstructured, polypeptide chain forms random coil; most typical fold of structured non-globular domain is coiled-coil.

ROCK kinases belong to the proteins possessing coiled-coil fold in a large extent - own kinase domain comprises only about 30% of its 1354 residues while coiled-coil motif 45%. More then a decade of effort has been dedicated to a very simple question: how the structure of whole ROCK kinase looks like. As it is (currently?) impossible to determine the structure of such large protein at once we are reliant on the combination of various techniques. How such combination helped us to construct the model of full-length ROCK kinase (Fig. 1) and can in general help to answer that very basic question crucial for myriad of proteins will be presented during the talk.

This, however, is still just one part of the story. Second part, perhaps even more essential, is the relationship between the overall architecture of multu-domain protein and its function. How far have we come in this aspect concerning ROCK kinase will be also discussed. Putting it in the context of whole family of protein will finally demonstrate that the understanding of the interplay of diverse domains within the proteins is generally a next, level-up challenge in biology.

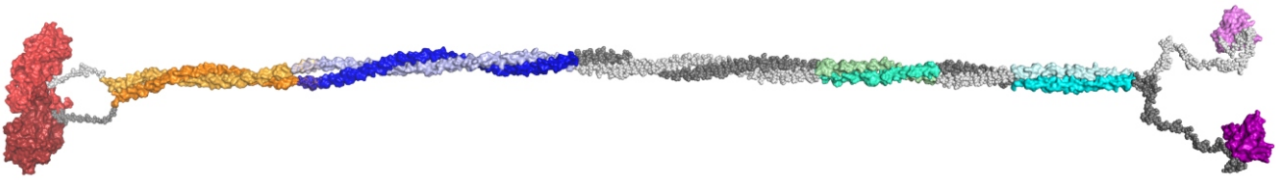

Figure 1. Overall model of full-length ROCK kinase.

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UNDERSTANDING STRUCTURE-FUNCTION RELATIONSHIPS OF HALOALKANE DEHALOGENASES BY ANCESTRAL SEQUENCE RECONSTRUCTION

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Ancestral sequence reconstruction represents a powerful approach for empirical testing of structure-function relationship of diverse proteins. This paleomolecular technique allows resurrection of ancient enzymes based on sequences predicted by a phylogenetic analysis. Starting from an alignment of modern sequences, the phylogenetic tree is inferred and statistical methods are used to predict the most likely ancestral sequences at the internal nodes of the tree. Genes that encode the inferred ancestral sequences can then be synthesized, expressed in cultured cells and experimentally characterized [1-2]. In this study, the sequences of representative members of haloalkane dehalogenase subfamily II were selected as targets for prediction of recent common ancestor of haloalkane dehalogenase DbjA [3-5] and DbeA [6], ancDbjA-DbeA-node1, and additional ancestors corresponding to the deeper nodes of the branch leading towards the present-day enzymes, ancDbjA-DbeA-node2, ancDbjA-DbeA-node3, ancDbjA-DbeA-node4 and ancDbjA-DbeA-node5. The genes encoding predicted sequences were synthesized; the ancient proteins were overexpressed in *Escherichia coli BL21 (DE3)*, purified to homogeneity by metallo-affinity chromatography and biochemically characterized. All resurrected enzymes were correctly folded and revealed enhanced thermodynamic stability up to 20 °C compared to the modern enzymes. Moreover, the ancestral enzymes exhibited different oligomeric states compared to descendant haloalkane dehalogenases. Steady-state kinetics revealed high catalytic efficiency of constructed enzymes towards 1,2-dibromoethane. The substrate specificity of the ances-

tors was determined spectrophotometrically towards a set of thirty different halogenated substrates and compared with substrate specificity profiles of the corresponding descendant enzymes. Multivariate statistical analysis of collected data uncovered significant differences in substrate specificity profiles of ancestral and modern enzymes. On-going crystallisation and structural analysis of selected ancestral enzymes will provide insight to unique properties of ancestral enzymes.

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PROBING METAL-ION-ASSISTED INTERACTION IN SELF-PROCESSING MODULE OF FrpC PROTEIN

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In the present work we describe a simple experimental model protein tailored to study the formation of metal ion-assisted interaction during ligand-induced protein folding. The model system is based on a calcium-binding protein, SPM, a self-processing module, derived from the internal segment of the bacterial FrpC protein, which mediates a Ca^{2+}-dependent autocatalytic cleavage of the highly specific Asp-Pro peptide bond and covalent linkage of the carboxy-terminal group of the splinter segment to -amino group of a lysine residue of an adjacent protein. SPM is a polypeptide that is intrinsically disordered in the absence of calcium and folds upon binding of Ca^{2+} ions into a compact and stable structure. The cleavage of the Asp-Pro bond is accompanied with the Ca^{2+}-dependent folding of SPM that is unambiguously characterized by the formation of Ca^{2+}-assisted interaction between a pair of unique tryptophan residues. Therefore, Ca^{2+}-dependent folding linked to enzymatic activity would prove SPM to be an excellent model to investigate the formation of Ca^{2+}- interaction during the ligand-induced transition from an unfolded to the folded conformation.

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STRUCTURAL BASIS FOR TRANSCRIPTION TERMINATION COUPLED TO RNA PROCESSING/DEGRADATION

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In *Saccharomyces cerevisiae*, production of both, protein-coding (mRNAs) and non-coding (ncRNAs) RNA, is performed by an enzyme termed RNA polymerase II (RNAPII). RNAPII consists of the core where the process of transcription occurs, and a flexible C-terminal domain (CTD) that is connected with the RNAPII core by a short linker. Termination of transcription is highly dependent on the size of RNA molecule to be terminated, and it is divided into two pathways, Nrd1p-dependent and Rtt103p- dependent for ncRNAs and mRNAs, respectively. For each pathway, the interaction between transcription termination machinery and CTD of RNAPII is crucial for the proper termination of transcription. The interaction with CTD is achieved by the CTD-interacting domain (CID) of either Nrd1p or Rtt103p.

We demonstrate that transcription termination of ncRNAs is physically coupled to RNA degradation/processing performed by the nuclear exosome. The coupling is achieved by the CID of Nrd1p that recognizes a mimic of CTD (Nrd1p-interactin motif, NIM) within the TRAMP (Trf4p-Air2p-Mtr4p) complex, a cofactor of the nuclear exosome. We report the structure of Nrd1p CID bound to the Trf4p NIM. Binding assays show that Nrd1p binds Trf4p NIM with a K_D of ~ 1 M, whereas the binding affinity for CTD has a K_D of ~ 100 M. Mutational analyses of the binding surface of both Nrd1p CID and Trf4p NIM confirm our structural findings. What is more, competition experiments show that Nrd1p CID binds NIM and CTD in a mutually exclusive manner.

Since the interaction between CID, of either Nrd1p or Rtt103p, and CTD of RNAPII is crucial for the proper transcription termination, we have hypothesized that transcription termination of mRNAs may also be coupled to RNA processing/degradation performed by the nuclear exosome/TRAMP. Pull-down experiments show that Rtt103p CID also recognizes the NIM, and another region of Trf4p specific only to Rtt103p, the RIM (Rtt103-interacting motif). Specifically, we report the structure of Rtt103p CID bound to Trf4p NIM, and initial structural studies of the complex between Rtt103p CID and Trf4p RIM. We show that Rtt103p CID utilizes the same pocket to bind both CTD and the NIM. Overall, our results unveil a novel role of CID, of Nrd1p and Rtt103p, in not only transcription termination but also in RNA processing/degradation by the nuclear exosome.

Saturday, March 21, Session IX

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FUNCTIONAL ANALYSIS OF A NOVEL HALOALKANE DEHALOGENASE DsvA ISOLATED FROM THERMOPHILIC BACTERIUM *SACCHAROMONOSPORA VIRIDIS* DSM 43017

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Microorganisms able to grow in extreme conditions, including high concentration of salts, alkaline pH, low or high temperature and organic solvent medium have been an important source of robust enzymes for various practical applications [1]. Haloalkane dehalogenases (HLDs; EC 3.8.1.5) are predominantly bacterial enzymes that catalyze hydrolytic cleavage of a carbon-halogen bond in a broad range of halogenated aliphatic compounds, producing a corresponding alcohol, a halide anion and a proton [2]. These enzymes can be use in bioremediation, decontamination of warfare agents, synthesis of optically pure compounds, biosensing and cell imaging [3].

A novel member of HLD family, DsvA from *Saccharomonospora viridis* DSM 43017 isolated from peatbogs of Ireland, has been subjected to detailed biochemical characterization in this study. The enzyme was successfully expressed in *Escherichia coli* BL21(DE3) cells and purified to homogeneity by metalloaffinity chromatography. Proper folding and thermostability was assessed by circular dichroism spectroscopy. Although DsvA exhibited comparable melting temperature (Tm = 58 °C) with other HLDs isolated from mesophilic organisms, its kinetic stability determined at 45 and 60 °C was significantly higher than the kinetic stability of other HLDs. Interestingly, DsvA possesses only one, instead of two, halide-stabilizing residues in its active site previously observed in majority of characterized HLDs. Despite unusual composition of catalytic residues, DsvA exhibited clear dehalogenase activity. The highest activity of the enzyme was determined towards 1-bromoheptane, 1-iodohexane and1-bromohexane, whereas no activity was detected in the reaction with bulky and cyclic chlorinated substrates. The temperature and pH optima of DsvA were measured with 1-iodohexane. Maximal activity was detected between 45 and 50 °C and at pH 8.9. Steady state kinetic analyses were performed with 1-iodohexane, 1,2-dibromoethane, 1-iodohexane and 1,3-dibromopropane. The complex kinetic mechanism of the enzyme with 1-iodohexane was determined and revealed substrate inhibition. The kinetics of DsvA with 1,3-dibromopropane followed a simple Michelis-Menten dependence, while the kinetics of the enzyme with 1,2-dibromoethane followed a mechanism involving positive cooperative substrate binding. Crystallization of DsvA for structural analysis is currently in progress.

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