Session 23: Entanglement in Biology – from Proteins Folding to Drug Design

Lectures

L23.1

Targeting allosteric site in drug discovery – a case of tryptophan synthase

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The discovery of antibiotics with novel targets is greatly needed, but has been challenging. While there are numerous functions required for cell survival and growth, only a small fraction of these processes – primarily those involved in macromolecular synthesis – are inhibited by current antibiotics. The increasing occurrence of pathogens resistant to the existing therapeutics requires redirecting drug discovery effort. Largely unexplored opportunities exist in central metabolism, where many enzymes are only conditionally essential and therefore their successful exploitation requires understanding of metabolic fluxes and pathway regulations under certain environmental conditions. Tryptophan biosynthesis is one of the most well-characterized processes in bacteria with enzymes from *Salmonella typhimurium* and *Escherchia coli* serving as model systems. These enzymes are highly regulated at both the transcriptional and allosteric levels. The survival of many bacteria, including *Mycobacterium tuberculosis (Mtb)*, depends on the ability to synthesize the L-Trp whenever it is not available from the environment. The L-Trp biosynthetic pathway was validated as conditionally essential for *Mtb* survival *in vivo*. In *Mtb,* the multistep L-Trp synthesis involves several enzymes: TrpEG, TrpD, TrpC and TrpAB. The whole-cell screening against diversity-oriented synthetic library identified a novel bactericidal azetidine derivative, BRD4592 that specifically inhibits tryptophan synthase. TrpAB is a heterotetrameric, PLP-dependent enzyme catalyzing two last reactions in L-Trp synthesis, namely the conversion of indole-3-glycerophosphate to indole and glyceraldehyde 3-phosphate (TrpA) followed by indole and L-Ser condensation to yield L-Trp (TrpB). During the catalytic cycle, indole is transferred from the TrpA to TrpB active site through 25 Å channel to react with the L-Ser-PLP adduct. The enzymatic activities and substrate channeling between the two active sites are coordinated by complex allosteric communication between the subunits to optimize indole utilization. This regulation involves cycling between various conformational states, with both subunits adopting either open (inactive) or closed (active) state. Detailed kinetic, biophysical and structural evidence reveals that BRD4592 is an allosteric, mixed-type inhibitor, which binds specifically to a previously uncharacterized site within the intersubunit channel, at the subunit-subunit interface. The molecule interacts with both states of TrpB, open and closed, but it promotes closed, active state of the TrpB subunit and increases affinity for L-Ser substrate. BRD4592 acts on three levels: it limits flexibility of the interface disrupting mutual activation of the subunits, prevents indole translocation and increases product inhibition providing means to successfully inhibit both, TrpA and TrpB reactions. These results indicate that allosteric binders might represent an effective tool to deactivate enzymes involved in central metabolism, which often are allosterically regulated and might be predisposed for this type of inhibition.

L23.2

tRNA methylation: a determinant of mitochondrial pathogenesis

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Methylation of tRNA is the most common form of post-transcriptional modification. Although the addition of just one methyl group to a nucleobase or a backbone group can help tRNA folding in cell-free assays, the biological context of the methylation is unknown. Here we show that the m^1G9 methylation is a determinant of the pathogenicity of the MELAS disease, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes. This methylation is catalyzed by a protein knot-fold that is active only in the complex with two other proteins. The most frequent mutation of MELAS is the 3243A>G mutation in human mt-$tRNA^{Leu/UUR}$. Using both cell-free and cell-based assays, we show that the single m^1G9 methylation to mt-$tRNA^{Leu/UUR}$ separates the mutant from its wild-type counterpart. While the wild-type tRNA is assisted to the native structure, the mutant is led to an aberrant structure that is rapidly degraded. This work provides the framework for understanding how tRNA methylation by a knotted protein fold determines the cell-fate of a pathogenic mitochondrial tRNA.

Oral presentations

O23.1

Visualizing supramolecular machines in action by methyl TROSY-NMR – substrate processing by AAA+ unfoldases

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In the cell, proteins are continuously synthesized and degraded. Degradation as well as unraveling of aggregated proteins depends on the activity of a family of ATP-driven ring-shaped molecular machines that catalyze unfolding of protein substrates by threading them through central ring channels. Knotted proteins represent a challenge for biological unfoldases due to their unprecedented stability. Even though protein remodeling by AAA+ enzymes is central for maintaining proteostasis in a living cell, a detailed structural description of how this is accomplished at the level of the substrate molecules that are acted upon is lacking. We have developed a methodology that combines chemical cross-linking and methyl TROSY-NMR spectroscopy to study, at atomic resolution, the stepwise unfolding and subsequent refolding of the two domain substrate calmodulin by the VAT AAA+ unfoldase from *Thermoplasma acidophilium*. The use of transverse relaxation-optimizes-based NMR experiments in a conjunction with methyl-specific isotopical labelling in the otherwise perdeuterated background allowed us to study complexes that are larger than 400 kDa. The approach presented generates an atomic resolution picture of substrate unfolding and subsequent refolding by unfoldases that can be quite different from results obtained via in vitro denaturation experiments. Moreover, it paves an avenue for structural studies of knotted protein targets as they pass through the central pore of biological unfoldases.

O23.2

Characterization of substrate binding and function of a knotted protein

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Methyltransferases (MTs), as enzymes with crucial biological functions, are widely represented in various organisms – from bacteria to humans. Distinct topologies are found within this class of proteins, including ones containing a knot. All of the knotted MTs have the simplest trefoil knot, which creates a crevice for the methyl donor to bind. The structure of the knotted region is strictly evolutionary conserved, implying the importance of that motif in the proteins.

I will show the differences in the mode of binding of the substrate between knotted and unknotted methyltransferases and its conformations and dynamics. Moreover, some of the knotted methyltransferases were shown to function asymmetrically, with one active site available for catalysis at a time [1-3]. I will focus on the knotted protein TrmD, that methylates tRNA, and show that the knotted region is implicated in the asymmetrical behavior and the function of this protein.

References:

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3. Zhang H, Wan H, Gao ZQ, Wei Y, Wang WJ, Liu GF, Dong YH (2012) Insights into the catalytic mechanism of 16S rRNA methyltransferase RsmE (m3U1498) from crystal and solution structures. *Journal of Molecular Biology* **423**: 576-589.

Posters

P23.1

In vitro protein folding – development of the method of receiving the therapeutically relevant recombinant bovine IFN-α and anti-staphylococcal endolysin $LysK_{CA}$

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Technology of cloning genes let obtain various recombinant proteins (RP) in large amount and short terms both for research and industrial application, in particular for the production of the therapeutic proteins.

However, deposition of the most RP in prokaryotic host-cells occurs in inactive state in the form of insoluble inclusion bodies (IBs). Activation of the target protein requires the development of individual approach to its isolation, purification and refolding.

The complex and stepped method of receiving the recombinant two-domain endolysin $LysK_{CA}$ and single-domain bovine interferon-α from IBs has been developed. The first stage is determination of the optimal isolation and ion-exchange chromatography purification conditions. The second stage includes the screening of refolding by dilution conditions (pH and temperature value, red-ox potential and anti-aggregative additives of the refolding buffer). The last step is the selection of the renaturation method – by dilution or on the resin matrix (MAR).

Scaling up the purification and refolding after screening its conditions gives the possibility to get the target protein with biological activity and in the homogenous state. It was found that renaturation by dilution can be applied to all proteins whereas MAR does only for multi-domain proteins.

Given technique was also efficient in obtaining other therapeutically relevant RP such as multi-domain ephrin-A5, LysK and single-domain ovine IFN-α and CHAP-domain of endolysin LyK.

P23.2

Identification of amino acid residues indispensable for metalloid antiport function of Acr3

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Acr3 is a plasma membrane As(III)/H^+ and Sb(III)/H^+ antiporter that confers resistance to both metalloids in yeast cells. This protein is involved in metalloid detoxification pathway not only in budding yeast but also prokaryotes, fungi and lower plants. Acr3 has a 10-transmembrane span topology but its spatial structure has not been experimentally determined yet. However, Acr3 3D hypothetical model has been generated with homology modelling method and is based on an $ASBT_{Yf}$ crystal structure. It allows to group neighbouring amino acid residues that confer similar functions but belong to different transmembrane regions. In order to understand how metalloids are translocated, it is necessary to identify residues crucial to substrate binding/coordination and transport specificity. Random mutagenesis and phenotype screening allow to identify amino acid residues that are both important to a protein function and difficult to choose and analyse with site-directed mutagenesis techniques. Using these methods we identified amino acid residues potentially indispensable for transport activity or specificity of Acr3. These findings may allow a better insight into the molecular mechanism of metalloid transport mediated by Acr3.

P23.3

The arsenite transporter Acr3 undergoes Rsp5-dependent UbK63-linked oligo-ubiquitination

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Acr3 is a plasma membrane transporter, a member of the bile/arsenite/riboflavin transporter (BART) superfamily, that confers high-level resistance to arsenicals in the yeast *Saccharomyces cerevisiae*. Arsenic toxicity causes a global health problem affecting millions of people. Chronic arsenic toxicity results in multisystem diseases. We have previously shown that Acr3 acts as a low affinity As(III)/H^+ and Sb(III)/H^+ antiporter. Here, we examined Acr3 degradation and showed that the Acr3 transporter undergoes endocytosis and vacuolar degradation through multivesicular bodies (MVB) pathway. Our results demonstrate that Acr3 is poli-ubiquitinated with UbK63 chain and that this modification is crucial for its efficient sorting to MVB and subsequent degradation in the vacuole. Moreover, Acr3 ubiquitination and internalization is mediated by the Rsp5 ubiquitin ligase. Based on the ten-transmembrane transporter model of Acr3, we selected eleven cytoplasmically oriented lysine residues potentially subjected to a post-translational modification by ubiquitination and replaced each residue with arginine. Although single K to R substitution did not increase Acr3 stability, multiple mutants accumulated in the plasma membrane, endosomes and the vacuolar membrane. K to R mutation did not affect Acr3 activity or its ability to confer As(III) resistance in yeast cells.

P23.4

Recovery of Herpesvirus entry mediator (HVEM) from inclusion bodies of *Escherichia coli*

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Herpesvirus entry mediator (HVEM) is a human cell surface receptor of the TNF-receptor superfamily. Protein is expressed on certain tumor cell types (for example, melanoma) as well as on tumor-associated endothelial cells. The system of HVEM-interacting molecules is complex. The binding of HVEM to CD160 and BTLA on T cells delivers a co-inhibitory signal to T-cell activation (Watanabe *et al.,* 2003; Sedy *et al.,* 2005; Cai *et al.,* 2008), while the binding of HVEM to LTα and LIGHT stimulates host immune responses (Ware *et al.,* 1995; Granger & Ware, 2001). The discovery BTLA and CD160 (Ig superfamily member) binding to TNFRSF member herpes virus entry mediator (HVEM) (Cai *et al.*, 2008; Sedy *et al.,* 2005) is a rare example of a direct interaction between the two superfamilies
HVEM was expressed in *Escherichia coli.* Unfortunately, protein was accumulated in inclusion bodies. Reactivation of proteins was a complex and time-consuming process. Experimental optimization of the process conditions for HVEM was required. We have tested the most often used methods for protein renaturation: dilution, dialysis and affinity chromatography. Final purification was performed using size exclusion chromatography.

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P23.5

Possible chromophore formation in the Trp-Cage miniprotein variants – investigation of new potential fluorescent miniprotein

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The GFP protein is a versatile biological marker for monitoring physiological processes, visualizing protein localization and detecting transgenic expression *in vivo*. GFP among all its incontestable advantages, has one significant disadvantage: it is relatively large to affect the function of fused protein of the interest. The fluorescent center in GFP is a tripeptide consisting of SYG residues, which are located in the central alpha-helix. In this study, we took an attempt to synthesize a chromophore analogous to that existing in GFP on the basis of synthetic Trp-Cage (TC) miniprotein. TC is a small 20-residue long polypeptide adopting a stable folded structure with a compact hydrophobic core packed around Trp6 residue.
The idea of our study was to modify the original sequence of TC to construct the tripeptide fluorophore in its hydrophobic core. We chemically synthesized two TC variants: TC(L7G) and TC(N1D, A2L, I4A, L7G) and carried out the incubation in different conditions (pH, temperature, polar and non-polar solvents). The MS analysis of the incubated samples reveals the appearance of the new chemical entity which we investigated by means of NMR spectroscopy.

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