Saturday Morning Session VI - September 26

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STRUCTURAL STUDIES OF CYANOPHYCIN SYNTHETASE FROM *Anabaena variabilis*

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Many cyanobacteria are able to synthesize cyanophycin (multi-L-arginyl-poly-L-aspartate) as a storage compound for nitrogen, carbon and energy. Cyanophycin itself is considered as of biotechnological interest because the purified polymer can be chemically converted into polymers with reduced arginine contents. These derivates can be further utilized, like polyaspartic acid, as biodegradable substitutes for synthetic polyacrylate. Polyaspartic acid is also a key substance in various technical processes and biomedical applications.

It is established knowledge that the biosynthesis of cyanophycin is catalyzed by a dedicated enzyme, cyanophycin synthetase (CphA). This enzyme can be also used for *in vitro* production of cyanophycin. Thus, structural and functional studies of CphA are of great interest for applied sciences and industry. Despite numerous biochemical data on CphA, until now no structural information is available on this protein.

In this work we expressed and purified CphA from *Anabaena variabilis*. We crystallized some variants of the protein for X-ray structural analysis and applied various biochemical methods in order to get structural information of this enzyme and to understand the mechanism of its action in more detail.

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CRYSTAL STRUCTURE OF TETX MONOOXYGENASE REVEALED BY PHASING WITH 3-WL MAD

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The FAD-dependent monooxygenase TetX modifies several tetracycline antibiotics under regioselective hydroxylation of the substrate to 11a-hydroxy-tetracyclines that degrade non-enzymatically to not characterized products. The enzyme was found in anaerobic *Bacteroides fragilis* and confers resistance against tetracyclines on aerobically grown *Escherichia coli*[1]. The active enzyme requires molecular oxygen, NADPH and

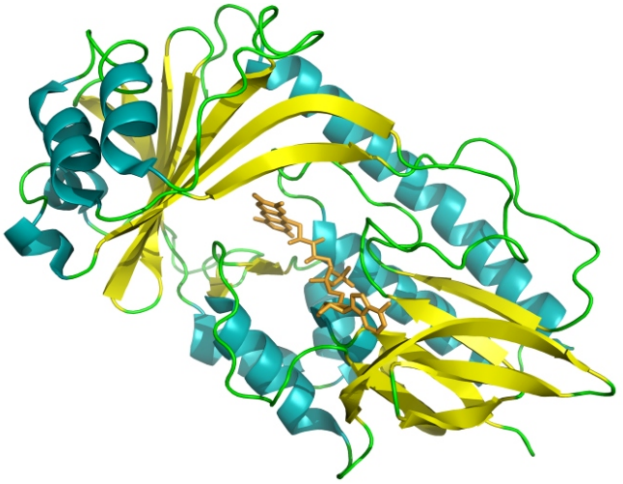

Figure 1. Ribbon structure of TetX monooxygenase with bound FAD in orange.

Mg^{2+} and has a broad substrate range amongst the tetracyclines up to the recently approved 3rd generation antibiotic tigecycline[2]. Recombinant TetX protein was expressed in *E.coli* BL21 (DE3) and *E.coli* B834 pRARE2 in minimal media M9 with selenomethionine. TetX protein was purified to homogeneity using affinity and size exclusion chromatography. With crystallization using hanging drop vapour-diffusion method, we obtained crystals in space group P1 and diffraction data were collected to a resolution of 2.5 Å in-house.

A 3-WL MAD data set of a crystal in space group $P2_1$ with incorporated selenomethionine was collected at the EMBL c/o DESY.

The 3D-crystal structure was solved using the Auto-Rickshaw protocol. After phase improvement and density modification with *SHARP* initial model building was performed manually in COOT. Model refinement was carried out with *REFMAC5* including TLS and non-crystallographic symmetry restraints. This is the first reported resistance mechanism against tigecycline which circumvents all known resistance mechanisms.

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2. Moore *et al.* (2005). *Biochemistry* **44**, 11829-1835.

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STRUCTURAL STUDIES OF THE COFACTORLESS OXIDASE PQQC

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In many cases cofactor biosynthesis based on enzyme catalyzed oxidation reactions describes complex and sophisticated mechanism and reactions. One of those intriguing biosynthises pathways is the biosynthesis of pyrroloquinolin quinone (PQQ). The pyrroloquinoline-quinone synthase C (PqqC) is a cofactorles oxidoreductases. It catalizes the very unusual reaction, which incudes ring closure and the eight electron oxidation of AHQQ to PQQ. Here we describe the reaction pathway which involves ring cyclization and eight electron oxidation of the substrate. Using PqqC active site mutants which trap a reaction intermediate we are able to give further insights into this pathway. PqqCH154S and PqqCY175F in complex with PQQ together with PqqCR179S in complex with an intermediate of the reaction show that cyclization to a first intermediate is independent from the conformational change of the protein and happens prior to any oxidation reaction. Furthermore we showed, that the recruitment of the molecular oxygen, which happens after the cyclization reaction is finished, is crucial for the conformational change. Not till then the oxidation can be facilitated.

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CRYSTAL STRUCTURE OF A METAGENOMIC DERIVED LACTONASE INVOLVED IN BIOFILM INHIBITION IN *Pseudomonas aeruginosa*

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Quorum sensing (QS), a gene regulation system in prokaryotes contingent on cell density, is driven by signaling molecules which enable bacteria to alter the expression of genes. Among affected genes are those coding for bio film formation, virulence or the production of extracellular proteins, making QS an interesting target for treatment with 2 possible routes of interference with the QS gene regulation system. Firstly quorum sensing inhibition by interference with the QS gene regulation system, and secondly quorum quenching by degrading the signaling molecules. Metagenomics offers an approach to find novel and naturally occurring quorum quenching strategies, other than variations of known mechanisms. N-(3-oxooctanyl)-L-homoserine lactone is one of the signaling molecules frequently utilized by bacteria. Cleavage of the lactone ring interferes with the signaling properties making it therefore an interesting target.

Proteins with the ability to inhibit bacterial growth where identified from a metagenomic library from environmental soil samples. Sample B09 (239 residues) inhibits biofilm formation and was shown to inactivate N-(3-oxooctanyl)-L-homoserine lactone, which is involved in quorum sensing. B09 has been expressed and purified by our collaborator at the University of Hamburg. Initial crystallization hits were improved (0.1M citric acid pH 4.0, 1.6 M ammonium sulfate) and complete data set

was collected to 2.65 Å resolution. A BLAST search against the protein data bank yielded several hits of moderate to low sequence identity. Molecular replacement was successful using the structure of an oxidoreductase (pdb 2ehd, Identity 38%) as a search model. The data were indexed in space group I422 with unit cell parameters a = b = 242.57 Å and c = 152.83 Å and 4 molecules in the asymmetric unit (75% solvent content) forming 2 dimers. The protein exhibits a typical Rossmann fold with an additional alpha-helix and beta-strand at the C-terminus. A long linker between -6 and -6 is highly flexible and shows no electron density possibly functioning as a lid on the active site upon substrate binding. The additional C-terminal beta-strand participates at the dimer interface by extending the 7-stranded beta sheet of one protomer to a 14-stranded antiparallel central beta-sheet within a dimer.

NADP+ was added to the protein solution prior to crystallization as it is needed for activity. Strong electron density could be observed in a difference fourier map at the topological switch point of B09. It was interpreted as ADP phosphorylated at the 2' hydroxyl. It appears that the nicotinamide-ribose in NADP+ is either freely rotating or that B09 binds a hydrolyzed version of NADP+. Surprisingly, crystals grown from native B09 also show difference density at the NADP+ binding site which strongly resembles 2' phosphorylated ADP. This indicates that B09 has scavenged a naturally occurring metabolite during expression in *E. coli*.

Our collaborators have identified several potential substrates which we intend to use in cocrystallization experiments to further characterize the enzyme's function and mechanism.

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CRYSTALLIZATION AND PRELIMINARY DIFFRACTION DATA ANALYSIS OF -ACTININ 2

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-Actinin belongs to the spectrin superfamily of proteins involved in cytoskeletal network, bundling or cross-linking actin filaments. Human -actinin isoforms are composed of an N-terminal actin-binding domain (ABD, formed by two calponin like domains), a central rod domain formed by four spectrin-like repeats (SR1, SR2, SR3 and SR4) and a C-terminal calmodulin-like domain (CaM, formed by four EF-hands). Its functional unit is an antiparallel homodimer leading to a molecular architecture that allows the protein to cross-link actin filaments. Four different -actinin isoforms are present in human cells: isoforms 1 and 4 are calcium sensitive and found in non-muscle cells, while isoforms 2 and 3 are calcium insensitive and found in muscle cells [1].

The muscle isoforms are regulated by the phosphoinositide PiP2 that upon binding to -actinin triggers conformational changes that enhance -actinin binding capacity to actin filaments and other muscle proteins. It was proposed that while the PiP2 head interacts with the N-terminal (ABD), the PiP2 hydrophobic tail disturbs the interaction between juxtaposed N-terminal and C-terminal regions in the áactinin antiparallel homodimer [2]. Nevertheless the molecular details on interaction between PiP2 and áactinin and concomitant conformational changes remain elusive.

Although a body of structural information is available on individual domains of -actinin [3-7], no high resolution structural information so far exists on the molecular architecture of the entire molecule. Therefore, a number of efforts based on biochemical, cellular and structural studies have been carried out in order to elucidate -actinin regulation at molecular level with the aim to better understand myofibrillogenesis and enhance insights into a number of diseases.

We underwent crystallographic study of -actinin muscle isoform 2 and show that diffracting crystals of -actinin muscle isoform 2 were obtained only after surface entropy reduction (SER) mutation in addition to protein reductive methylation. These approaches enhanced the propensity of -actinin full length isoform 2 to crystallize, yielding crystals diffracting to a resolution better than 3.5 Å at ESRF micro focus beamline (ID23-2) at 100 K. 15 datasets collected from different parts of several crystals were merged to yield a complete dataset at 3.4 Å resolution. Phase problem was solved using molecular replacement and known structures of -actinin domains (ABD, rod domain, EF hands) as search models. Structure refinement is currently on-going.

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