



# 7<sup>th</sup> Discussions in Structural Molecular Biology

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## Conference Committee

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### Lectures - Thursday, March 12, Session I

L1

## STRUCTURE OF THE MOTOR SUBUNIT AND TRANSLOCATION MODEL FOR EcoR124I RESTRICTION-MODIFICATION COMPLEX

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### Introduction

Although type I restriction-modification systems of bacteria were the first to be discovered and characterized, their lack of specific cleavage sites relegated them to the sidelines of early DNA enzymology while the site-specific type II systems were developed into the essential reagents that today make molecular cloning routine. Nevertheless, the intriguing complexities of type I mechanisms led to an extensive body of sometimes puzzling results, in sharp contrast to the straightforward mechanisms of the type II restriction-modification systems (RMs). Type I RMs are multisubunit, multifunctional molecular machines that recognize specific, typically asymmetric, DNA target sequences of ~13 to 17 bp. Depending on the methylation status of adenine residues in the target, three enzyme subunits either act together as a typical methyltransferase or recruit a pair of endonuclease motor subunits that initiate translocation of DNA through the enzyme and eventually cleave non-specifically at apparently random sites. The protein complex remains bound at the target sequence while up to thousands of bp are pumped through the enzyme by tracking along the helical pitch at rates of up to hundreds of bp per second. Translocation is driven by helicase-like motor subunits that consume ~1 ATP per ~1 bp without separating the strands.

### Results and Discussion

The type I restriction-modification enzymes differ significantly from the type II enzymes commonly used as molecular biology reagents. On hemi-methylated DNAs type I enzymes act as conventional adenine methylases at their specific target sequences, but unmethylated targets induce them to pull thousands of basepairs through the enzyme before cleaving distant sites nonspecifically. Biochemical,

biophysical, and molecular biological studies of their translocation and cleavage mechanisms offer a wealth of detail that has lacked a structural framework. We report the first X-ray crystal structure of the subunit responsible for DNA translocation and cleavage by the type I enzyme EcoR124I, resolved at 2.6 Å [1]. Understanding how the cooperation of subunits, domains, substrates, and cofactors enables type I RMs to carry out their diverse and peculiar activities is likely to be enhanced by knowledge of their molecular structures. Here reported crystal structure of the HsdR motor subunit of plasmid-borne type I RM EcoR124I is used to develop a model for the complete translocation complex with bound DNA, using structures of related methylase and specificity subunits and constraints from experimental data on the pentameric enzyme complex on DNA. The model predicts the rearrangements and cooperation of subunits and domains required to initiate and stabilize the translocating complex as it tracks on DNA. The model accounts for many known features of type I RMs, and makes a number of experimentally testable predictions about their structural and functional organization and mechanism and provides a structural framework for duplex DNA translocation by RecA-like ATPase motors.

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L2

## STRUCTURE OF SPOIISA-SPOIISB TOXIN-ANTITOXIN COMPLEX FROM *BACILLUS SUBTILIS*

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SpoIISA proteic toxin from *Bacillus subtilis* was shown to affect cell membrane of either mother cell during sporulation process or, when artificially over-expressed, vegetatively growing cells. The toxin was predicted to be composed of two domains – a membrane spanning domain consisting of three helices and a negatively charged cytosolic domain (C-SpoIISA) [1]. Our previous results indicate that neither of these two domains alone is sufficient for toxic effect [2]. In the normal conditions, toxicity of SpoIISA is abolished by tight binding of positively charged SpoIISB antitoxin to the toxin's cytosolic domain [1].

Crystals of C-SpoIISA-SpoIISB complex were obtained in three different conditions, however unit cell content and parameters are strikingly similar for all of these. Although we first measured native datasets, the structure was solved using data collected to 1.3 Å from Se-Met protein crystal, which was used as well for phase problem solution by MAD. The solved molecular structure reveals

C-SpoIISA dimer binding of 2 molecules of SpoIISB and appears to shed some more light into mechanism of SpoIISA toxicity and its neutralization by SpoIISB binding. Residues, which were previously identified by genetics experiments to unleash SpoIISA toxicity [1] are observed to be directly involved in the contacts between C-SpoIISA and SpoIISB. On the other hand, dimerization of C-SpoIISA might be responsible for bringing membrane spanning parts of SpoIISA into each other proximity allowing it to exhibit its lethal properties.

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L3

## THE SMALL LACCASE FROM *STREPTOMYCES COELICOLOR*

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Laccases are enzymes capable of oxidation of phenolic compounds with concomitant reduction of molecular oxygen to water. The so called "large laccases" have been studied extensively and both functional and structural data are available [1]. Large laccases comprise three domains and their active sites are localized within one protein chain. Laccases utilize one copper ion of type I at the oxidation site and a trinuclear copper cluster in the interior of the enzyme facilitates electron transfer from substrate to oxygen. The gene encoding for the two-domain small laccase (SLAC) has been found in *Streptomyces coelicolor*, a known antibiotic producing bacterium. Phylogenetic and sequence comparison studies indicated the possible structure arrangement of small laccases as trimers [2].

Recombinant enzyme characterization lead to results supporting the idea of both dimeric and trimeric functional

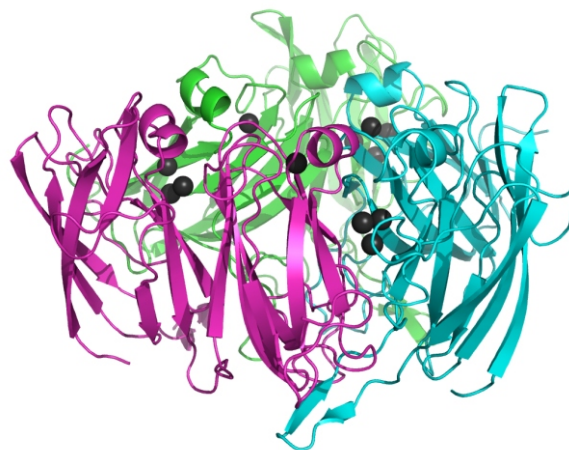
forms. SLAC expressed in *Aspergillus oryzae* and purified by a two-step ion exchange protocol was subjected to crystallization experiments and the best crystal form for structural studies grew with PEG 550 MME as precipitant [3].

The structure of SLAC was solved by multiple wavelength anomalous dispersion method (MAD) with diffraction data collected at beamline BM-14 of the European Synchrotron Radiation Source in Grenoble. SLAC forms trimers with the trinuclear clusters localized between individual chains and with specific trimerization features (Fig. 1). The first three-dimensional structure of this type of enzyme explains the characterization results, confirms the theoretical predictions and brings new questions regarding the catalytic action [4].

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**Figure 1.** The Small Laccase trimer, secondary structure elements shown, chains distinguished by colour/ degree of grey, copper ions as spheres. Graphics created with program Pymol.

L4

## FUNCTION ANALYSIS OF CYSTATHIONINE GAMMA-LYASE MUTANTS

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Cystathionine gamma-lyase (EC 4.4.1.1) abbreviated as CTH is a regulatory and metabolic enzyme with pyridoxal phosphate (PLP, vitamin B6) as a co-factor. It cleaves cystathionine (2-amino-4-(2-amino-2-carboxy-ethyl) thio-butanoic acid) into cysteine, ammonia and  $\alpha$ -ketobutyrate. It produces also hydrogen sulfide ( $H_2S$ ) putatively active in cell proliferation and neurotransmission. Deficiency in CTH function is associated with cystathioninuria often considered as a cause of severe psychomotorical or mental retardation, strabism or epilepsy. CTH deficiency is considered to be a common biochemical phenotype in the population of Central European ancestry (estimated frequency 1:3000).

Crystallographic analysis of human hCTH (4x403 residue complexes of apo-hCTH, hCTH-PLP and hCTH-PLP-PAG [1]) explains why its correct function is restricted to its tetrameric complexes only and why four catalytic sites of the tetramer are not equivalent. It also shows a way of substrate path through a deep catalytic cavity and large conformational changes (flaps opening) during PLP binding.

The analysis of structure effects of 8 genetically related mutations for which the physiological and clinical data are available on a sample of homozygote and heterozygote patients (Arg62His, Thr67Ile, Thr311Ile, Arg197Cys, Gln240Glu, Thr311Ile, Ser403Ile, deletion Gly57-

Gln196) showed clear correlation between the mutations and the observed plasma concentrations of metabolites [2]. A positive effect of the vitamin B6 administration for some mutations and zero responsiveness for other mutations is also evident from the molecular structure. However, frequent barriers or by-passes of processes in human body explain why the genotype-phenotype correlation, i.e. the expression of genotype into the actual patients health and mental state is not deterministic and has many exceptions.

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L5

## STRUCTURE CHANGES EVOKED BY BINDING OF LIGANDS INTO THE ACTIVE SITE OF GALACTOSIDASE FROM $\beta$ -ENZYME *Arthrobacter* C2-2

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The structures of three complexes of enzyme  $\beta$ -galactosidase from an Antarctic bacterium *Arthrobacter* sp. C2-2 are presented. Three of the obtained crystals with dimensions 100 – 400  $\mu$ m were soaked in different ligands (D-galactose, D-galactonolactone and IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside)) and used for X-ray diffraction data collection.

IPTG is an inhibitor for this enzyme, diffraction data were collected on an in-house source of X-ray radiation using rotating anode. The resolution limit is 3.3 Å. The data for complexes with D-galactonolactone and D-galactose were collected in ESRF in Grenoble using synchrotron radiation and the structures were determined at resolutions of 2.2 and 2.5 Å. D-galactonolactone is an inhibitor for this enzyme and the D-galactose is a product of the catalyzed reaction. The data were processed using HKL2000. All the crystals belong to monoclinic space group *P*21, the unit cells parameters are very similar but packing of the hexamers in the crystals differs.

The enzyme  $\beta$ -galactosidase (EC 3.2.1.23) belongs to the enzyme class called glycosylases which catalyze hydrolysis of the terminal  $\beta$ -D-galactosyl of  $\beta$ -D-galactosides and it is able to catalyze trans-glycosylation. It is attractive for research and industry because of its wide range of biotechnological applications (to reduce the energy costs, to treat lactose intolerance, to prevent crystallization in sweet products, to increase its sweetening power, to simplify fermentation during production of soured milk products, to modify the freezing point of ice creams, etc.).  $\beta$ -galactosidases are distributed in numerous microorganism, plants and animal tissues. Enzymes that exhibit  $\beta$ -galactosidase activity are divided into four distinct GHs, GH-1, GH-2, GH-35 and GH-42.  $\beta$ -galactosidase from *Escherichia coli*, which belongs to GH-2, is one of the most studied and commonly used  $\beta$ -galactosidases. The three-dimensional structure of  $\beta$ -galactosidase [1] from *Escherichia coli* has been determined.

This project is focused on  $\beta$ -galactosidase from an Antarctic bacterium *Arthrobacter* sp. C2-2. Psychrotrophic bacterium *Arthrobacter* sp. C2-2 was isolated in the Antarctic area as part of an environmental study. This psychrotrophic bacterium is able to exist at low temperature (which is typical for Arctic and Antarctic regions, and for mountains and deep oceans too).

The structure of native enzyme  $\beta$ -galactosidase [2] from *Arthrobacter* sp. C2-2 has been determined. There are

several structure features in  $\beta$ -galactosidase from *Arthrobacter* sp. C2-2. The biggest one is that this enzyme forms hexamer with molecular weight of 660 kDa. These hexamers were indicated in solution and in asymmetric unit of crystal in contrast to  $\beta$ -galactosidase from *E. coli* which forms homotetramers. Each monomer consists of five domains and contains 1023 residues and has an active site located in the TIM barrel domain - between the pair of catalytic residues Glu442 and Glu521. There are two distinct binding modes for the galactosyl group of ligands - shallow and deep. Each binding mode has specific hydrogen bonds between enzyme and ligands. In the cold adapted enzyme, the residue Trp552 is responsible for binding in the deep binding mode and the residue Cys999 for binding in the shallow binding mode.

IPTG is bound in the shallow binding mode. IPTG is located on the top of the active site and in contact with Cys999. IPTG is bound in the very similar position as IPTG in the structure of the mesophilic  $\beta$ -galactosidase.

The molecules of D-galactonolactone and D-galactose were found in the deep binding mode. In comparison with the shallow binding mode, the galactosyl moiety is rotated by about 90° and shifted deeper into the active site to rest on Trp552.

The molecule of D-galactose was found in a chair conformation in each monomer. Its oxygen atom 1 is in  $\beta$ -anomer configuration. The galactose 6-hydroxyl binds directly to the sodium ion. The binding is not accompanied by any conformational change of the enzyme as opposed to the complex with D-galactonolactone. There is only a small difference between the molecule of D-galactose and D-galactonolactone: D-galactonolactone has the double bond between carbon C1 and oxygen O1 atoms. This small difference causes a large structural change in the case of the complex with D-galactonolactone.

The molecule of D-galactonolactone was found in the active site of each monomer. The 6-hydroxyl binds directly to the sodium ion in all three structures. The binding is accompanied by an enzyme conformational change - Phe585 is rotated and the side chain of His335 moved up closer to the active site.

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## Lectures - Thursday, March 12, Session II

L6

### DIRECTED EVOLUTION OF HALOALKANE DEHALOGENASE TOWARDS HIGHER ACTIVITY IN BUFFER CONTAINING DIMETHYL SULFOXIDE

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Haloalkane dehalogenases (EC 3.8.1.5) are bacterial proteins which structurally belong to the superfamily of  $\alpha$ -hydroxylases. Haloalkane dehalogenases convert a broad range of halogenated hydrocarbons into corresponding alcohols by the hydrolytic cleavage of the carbon-halogen bond. Improvement of enzyme stability in organic co-solvents would make these enzymes more suitable for applications in biodegradation and decontamination of toxic compounds. In this study, the haloalkane dehalogenase DhaA from *Rhodococcus rhodochrous* NCIMB 13064 [1] was subjected to the random mutagenesis with the aim to obtain variants with improved activity in buffer containing organic co-solvent dimethyl sulfoxide. Dehalogenase gene was mutated at an average rate of 1.5 nucleotide substitutions per gene using error-prone polymerase chain reaction. Mutated genes were heterologously expressed in the autolysis strain *Escherichia coli* XJb. Modified pH indicator assay [2] enabled screening of seven thousand colonies of the mutant library for activity with 1,2-dibromoethane in the buffer containing 42% dimethyl sulfoxide. Four variants showing enhanced activity were detected: D73G +

E98V, L95V+A172V, F144I+C176G, and V245L. These positive variants were purified to homogeneity by affinity chromatography and characterized by spectroscopic analysis and steady-state kinetics. Circular dichroism spectroscopy revealed that all four enzymes possessed proper folding. During thermal denaturation, all mutant enzymes, except the variant F144I+C176G, exhibited improved thermostability in comparison with the wild-type enzyme. Activity assays with the substrates 1,2-dibromoethane and 1-iodohexane showed that the mutant L95V+A172V possessed increased activity in the presence of 40% dimethyl sulfoxide, compared to the wild-type enzyme. Structure-function relationships are currently being analyzed for this mutant.

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L7

## A STRUCTURAL INTERPRETATION OF THE SEQUENTIAL PREFERENCES

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It will be shown that probability of amino acids occurrence in proteins (P1) differs across organisms and species. Based on these propensities a difference between the real occurrence and a level of expectancy for sequential motifs of di- (P2), tri- (P3) and tetrapeptides (P4) will be demon-

strated. The connection between structural representations of the over or under-populated motifs will be discussed in terms of their phi/psi distribution in proteins from PDB. Potential utilization of the findings will be discussed including protein design and ligand engineering.

L8

## QM/MM STUDY OF $\beta$ -1,4-GALACTOSYLTRANSFERASE-1

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In the absence of  $\alpha$ -lactalbumin, the enzyme  $\beta$ -1,4-galactosyltransferase-1 (Gal-T1) catalyses transfer of galactose residue from UDP-Gal to the C-4 hydroxyl group of N-acetylglucosamine. The catalytic mechanism of Gal-T1 was investigated using hybrid quantum mechanical/molecular mechanical (QM/MM) method with QM part containing 253 atoms treated with density functional theory (DFT) at the BP/DZP level. The remaining parts of Gal-T1 complex, altogether 4527 atoms were modeled using the AMBER molecular force field. A theoretical model of Michaelis complex was built using the X-ray structures of Gal-T1 containing donor or acceptor substrate respectively. The QM(DFT)/MM model identified a  $S_N2$ -type

transition state with D318 as the catalytic base for the reaction in the enzyme active site. In the transition state (TS), donor sugar is almost fully cleaved from pyrophosphate, while nucleophilic oxygen O4 remains protonated, with low barrier hydrogen bond transfer to the catalytic base. The structure of TS is characterized by the  $O_A4-C1$  and  $C1-O1$  distances 2.703 Å and 2.092 Å respectively. The activation barrier for the proposed reaction was estimated to be ~14 kcal/mol. This modeling study provides detailed insight into the mechanism of the Gal transfer catalyzed by Gal-T1.

*Acknowledgement: This work has been supported by MRTN-CT-2006-035866 (REVCAT) and VEGA 2/0176/09*

L9

## STUDY OF WATER MOLECULES DYNAMICS AT THE TUNNEL OPENINGS OF HALOALKANE DEHALOGENASES

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Haloalkane dehalogenases (3.8.1.5.) are bacterial enzymes cleaving the carbon-halogen bond of halogenated aliphatic compounds by hydrolysis. Active site of haloalkane dehalogenases is buried inside the protein. The active site cavity is connected with the protein surface by tunnels which serve as the transport routes for substrates and products. The tunnel openings belong to the evolutionally most variable regions among haloalkane dehalogenases. Dynamics of solvent molecules may influence substrate

binding and catalytic activity and it is therefore of importance to study solvation dynamics in different proteins.

In this study we employed time resolved fluorescence spectroscopy and molecular dynamic simulations to investigate behavior of water molecules in the vicinity of the tunnel mouth of haloalkane dehalogenases DbjA and DhaA. Specific labeling of DbjA and DhaA is based on formation of a covalent bond between specific ligand and protein during enzymatic reaction. In the wild-type enzyme, this complex is further hydrolyzed by a water molecule

which is activated by the catalytic histidine. Histidine substitution impairs hydrolysis step leading to the formation of stable protein ligand complex [1]. In this study, we have developed a protocol for specific labeling of the tunnel opening and elimination of all unbound and non-specifically bound coumarin molecules.

Acrylamide quenching and time-resolved anisotropy experiments confirmed the selective labeling of enzyme by coumarin and complete removal of unbound molecules of coumarin. Steady-state and time-resolved emission spectra measurements showed significant differences in the polarity, accessibility and mobility of the dye and its microenvironment for both studied haloalkane dehalogenases. Coumarin bound in haloalkane dehalogenase DbjA is more flexible and more hydrated in comparison with coumarin bound in DhaA. Microenvironment dis-

plays higher polarity and lower viscosity than in DhaA. The obtained experimental data showed good agreement with the results obtained by molecular dynamics calculations. These results reflect geometry of the tunnel mouths evident from the crystal structures [2].

Solvent dynamics in the tunnel mouth will be further studied in other natural haloalkane dehalogenases and their variants. Comparison of solvent dynamics for various constructs will help us to better understand how this dynamics influences functional properties of the enzymes with buried active sites.

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## Lectures - Friday, March 13, Session III

L10

### ELECTRONEGATIVITY EQUALIZATION METHOD – FAST METHOD FOR CHARGE CALCULATION

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The progress which appeared in the latest decades in the computational field established computational chemistry methods to be of comparable quality to experimental methods. The number of cases in which computational chemistry methods can be successfully applied is still increasing and these methods are nowadays used with profit for modeling molecular systems and detailed studies of various structural and functional properties.

Partial atomic charge is a molecular property which is very often used in chemistry, particularly for clarification of differences in structure or reactivity between molecules. Unfortunately, partial charges are not obtainable from experiment, but they can be derived using the quantum chemistry methods. In the quantum chemistry, there are more approaches, how to solve this task, but none of them can be considered to be the best one and what is more, these methods are on one hand very precise, but also quite time-demanding. For some types of calculations it is not acceptable to wait for the results for such long time and for that reasons also some alternative approaches appeared. These approaches are based mostly on the semi-empirical principles and the Electronegativity Equalization Method is one of them.

The Electronegativity Equalization Method was developed as a semi-empirical method based on the Density Functional Theory [1] and it is a fast way how to obtain ap-

propriate partial charges for arbitrary molecule. The methodology is based on the Sanderson's Electronegativity Equalization Principle [2] which is applied to the Density Functional Theory. Due to its semi-empirical character, it is necessary to parameterize the Electronegativity Equalization Method before the first usage and the parameterization process influences the quality of resulting charges. We have already parameterized the Electronegativity Equalization Method on very large sets of organic, organohalogen and organometal molecules from the Cambridge database of crystallographic structures (CSD) and the National Cancer Institute 3D structure database (NCI DIS). Based on these training sets, very robust parameterization was performed and the number of so far parameterized elements was increased [3, 4]. The obtained parameters were carefully validated and resulting partial atomic charges were in a very good agreement with quantum mechanically calculated partial atomic charges.

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