	Experiment title: STRUCTURE, FUNCTION AND DYNAMICS OF METAL-CONTAINING REDOX ENZYMES	Experiment number: LS-1027
Beamline: BM14, ID14	**Date of experiment:** from: 1 July 1998 to: 30 June 2000	**Date of report:** 5 April 2000
Shifts: 72 shifts	**Local contact(s):** A. Thompson, V. Stojanoff, G. Leonard, W. Burmeister, S. Arzt, S. Wakatsuki et al.	*Received at ESRF:*

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Report: The project is a long-term project comprising two laboratories and several groups in Uppsala. In the following report, the major results of each project are presented.

STRUCTURAL STUDIES ON RIBONUCLEOTIDE REDUCTASES (RNR)

RNR is an essential enzyme responsible for all new synthesis of deoxyribonucleotides for DNA synthesis. The reaction is initiated by an organic free radical. This is done in several ways, and at least three different classes have been characterised. Most of the RNR enzymes use an iron containing protein for generation of the radical (classIa and Ib). The classI enzymes are composed of two homodimeric proteins denoted R1 and R2. The R2 protein harbours the iron centre and he R1 protein has a substrate pocket and nucleotide binding sites for allosteric regulation. The classIa and Ib differ in the allosteric regulation.

Aerobic ribonucleotide reductase RNR class I holoenzyme: Ribonucleotide reductases of class I are present in eukaryotes and some bacteria. The crystal structure of the separate subunits of the *E. coli* and *Salmonella typhimurium* enzymes have been determined as well as the small subunit of the mouse enzyme. Presently crystals of the R1 subunit and the R1-R2 complex from *Salmonella typhimurium* and the complex of *E. coli* R1-R2 have been obtained. Data were collected at Grenoble station ID14-4 in may 1999 on two R1 mutants to 3 Å resolution and on R1-R2 (*E. coli*) to 4.0 Å resolution, space group I4 a=155.8 Å, c=220.4 Å. Preliminary crystallographic studies were performed. Further data collection on these projects on the ESRF synchrotron is planned to be performed in April. In addition crystals of the yeast heterodimer Rnr2p-Rnr4p, where one subunit binds iron and forms active enzyme only in a dimer with the iron-free homolog Rnr4p, which probably acts as a chaperonin have been obtained. Also, crystallization experiments of the complex between

yeast R1 and the inhibitor Sml1 have been started. The structures of heterodimer Rnr2p-Rnr4p, the R1 subunit and the R1-R2 complex of *Salmonella typhimurium* and the *E. coli* R1-R2 complex are planned to be determined within the application period

Anaeobic Ribonucletide Reductase (RNR classIII) from *E.coli*: In anaerobic organisms the radical reaction is initiated using a glycyl radical which is formed using a 4Fe-4S iron-sulfur activase. The [4Fe-4S](+) form is shown to be extremely sensitive to oxygen requiring anaerobic crystallisation. Trials to crystallise several components of the native and mutant RNRIII are under way.

FERREDOXIN THIOREDOXIN REDUCTASE

Light generates reducing equivalents in chloroplasts which are not only used for carbon reduction but also for the regulation of the activity of chloroplast enzymes by reduction of regulatory disulfides via the ferredoxin:thioredoxin reductase (FTR) system. FTR, the key electron/thiol transducer enzyme in this pathway, reduces disulfides via an iron-sulfur cluster, by forming a tight contact of its active site disulfide and the iron-sulfur center. The thin, flat FTR molecule achieves the two-electron reduction by forming a mixed disulfide with thioredoxin on one side while on the opposite side providing access to ferredoxin for delivering electrons. Crystals belonging to to space group $P4_32_12$ with cell constants a=b=45.2 Å and c=172.6 Å were obtained. MAD data at three wavelengths were collected on beamline BM14 at the ESRF. The four Fe sites and heavy atom sites from three derivatives were refined together. The structure has now been refined to a present R-value for all reflections in the resolution range 15-1.6Å of 23%.

STRUCTURAL STUDIES ON OXYGENASES/ DIOXYGENASES

The reactions catalysed by the class of enzymes are very diverse, and most of them are synthetically impossible at present. Therefore an understanding of the basic mechanisms of these enzymes may lead to the invention of new chemical processes by altering substrate and product specificities.

Structural studies on bacterial dioxygenases and their ferredoxin-type redox partners: Bacterial dioxygenases catalyse a wide range of chemical reactions involved in biosynthetic and detoxification processes. Our work focuses on mononuclear ferrous enzymes involved in bioremediation through the degradation of polycyclic aromatic hydrocarbons typically found in hazardous waste sites, crude oil, and in areas where organic materials are burned.

During the last year and a half we have solved a number of high resolution X-ray structures of NDO with bound ligands. The aim of these structural studies is to propose a reaction mechanism for this group of dioxygenases. Studies of reduction upon exposure of X-rays of the 2FE2S-center in NDO has also been made. Planned experiments in this field includes production of an intermediate with both substrate and O_2/NO bound to NDO. Mutants of NDO has also been expressed and crystallized. The reductase component of benzoate dioxygenase (BenDo) has recently been crystallized and both MAD and MIR data has been collected. Crystallization of anthranylene Dioxygenase(AntDO), 2-nitrotoluenedioxygease (2-NTDO) and biphenyl dioxygenase(BiPDO) are also under way.

Mechanistic studies on deacetoxycephalosporin C synthase (DAOCS): The penicillin and cephalosporin antibiotics are amongst the most important therapeutic agents in current use. Their commercial production is complicated, requiring semi-synthesis from fermented products. Rational engineering of substrate and product selectivities requires knowledge of

the structure and mechanism of these enzymes. DAOCS is a mononuclear ferrous enzyme that catalyses the expansion of the penicillin nucleus into the six-membered core of the cephalosporins by using a ferryl intermediate produced by initial splitting of the cosubstrate a-ketoglutarate and dioxygen. Following the successful determination of the structure of the enzyme from merohedrally twinned crystals, our aim is to probe the mechanism by studying complexes with substrates, products and intermediates. These experiments turned out to be extremely difficult for a number of reasons: (i) Crystal manipulations have to be performed in an anaerobic box. (ii) Since the twinning ratio varies from crystal to crystal and a high ratio reduces map quality substantially, careful monitoring of the twinning ratio is cruicial and discarding highly twinned crystals is necessary. (iii) Soaking of big ligands tend to disrupt the crystal packing and often results in improductive binding. Therefore from hundreds of soaking experiments more than 30 data sets were collected mainly from BM14 and ID14. Around 10 of these were found to be useful according to the selection criteria outlined above. These structures, representing structures of various complexes with substrates, product, and intermediates are currently being refined.

TIME RESOLVED STRUCTURAL STUDIES ON HEME PROTEINS

Cytochrome *cd*1 nitrite reductase is a key enzyme in the denitrification pathways in the denitrifying bacteria such as *P. pantotrophus* and can also perform the reduction of O_2 to H_2O and has thus been known as a cytochrome oxidase. Upon reduction with dithionite of oxidised crystals the protein undergoes large conformational rearrangements which includes haem ligand switching at both haems. The unique combination of time-resolved crystallographic techniques with single crystal microspectroscopy allows the correlation of electronic transitions with structural transitions. By freeze quenching techniques it has been possible to trap high resolution reaction intermediates from both nitrogen reduction (Williams *et al* 1997) and in this period, the first half of the oxygen reduction (Sjögren & Hajdu, manuscript in preparation). The binding and release of CO to the active site have also been studied and crystallographic studies have been combined with pre-steady state studies in solution (Sjögren *et al.*, manucript submitted).

The crystal structures of horseradish peroxidase isoenzyme C (HRPC) compound I, compound II, compound III and resting state enzyme in complex with acetate have determined at high resolution (1.6-1.7 Å). Initially, a full X-ray data set was collected on one frozen crystal of compound III. The resulting structure did not show compound III and the absorbance spectrum recorded from the crystal after data collection showed that it had become reduced, and that the resulting time-averaged structure thus represented a mixture of species. In order to obtain a homogenous population of compound III, it was necessary to collect data from a number of crystals with different start orientations, and merging the first non-reduced parts from each data set to give a final complete composite data set. A similar data collection stragegy was also applied in order to get the compound I, compound II and resting state structures. We have further exploited the phenomenon that the enzyme is reduced by X-rays to follow the reduction of the dioxygen in compound III (oxyperoxidase) in 3D. In this case, full data sets were collected on several crystals, each systematically starting at a different position in reciprocal space. Composite data sets were then created by splitting each data set into a set of 'time slots' and merging corresponding time slots into a series of new data sets each showing different stages in the reduction of compound III, thus creating a movie of the process. The results show that the oxygen-oxygen bond breaks first. A water molecule is released, while the other oxygen atom remains bound to the iron. This oxygen is then also reduced to water. We have recently collected a 1.1 Å resolution data set

on a compound II crystal at the ESRF. Structural characterisation of the highly oxidized intermediates at this resolution will give atomic details, leading to an understanding of heme catalysis.

STRUCTURAL STUDIES ON MEMBRANE PROTEINS

The following main results have been obtained:

(1) Cytochrome *bc1* complex from bovine heart
1. Complete structure of the *bc*1 complex has been revealed at 2.5 Å resolution.
2. Molecular mechanism of "Q-cycle" was proposed based on the Rieske FeS protein motion.
3. Quinone binding site stuctures have been revealed.
(a) The cause of a genetic disease, exercise intolerance, has been revealed.
(b) Development of new fungicides based on the structure.
4. The *bc*1 complex is bifunctional; the core subunits are involved in protein transport to mitochondria.

(2) Cytochrome c oxidase from *Rhodobactor sphaeroides*
1. High Resolution Structure of cytochrome c oxidase *Rhodobacter spaeroides*
(a) Two proton pathways were confirmed. Water molecules in the pathway were assigned.
(b) Oxygen channel was determined.
(c) First evidence of Glu286 motion which could be the key of proton gating.

(3) Ubiquinol oxidase from *E. coli*
1. First structure of Ubiquinol Oxidase
(a) Subunits and most of the helices are assigned.
(b) Quinone binding site was revealed.

Publications

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