	Experiment title: Structure determination of Sulfolobus solfataricus poly(ADP-ribose) polymerase	**Experiment nur** **LS1803**
Beamline: ID14-1	**Date of experiment**: from 9-02-2001 to 10-02-2001	**Date of repo** 10-06-01
Shifts to BAG: 9	**Local contact(s)**: Hassan BELRHALI	*Received at ES*

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Poly(ADP-ribose)polymerases are a family of eukaryote nuclear enzymes, using NAD+ as a substrate to transfer the ADP-ribose moiety onto a numer of protein targets. PARPs play a pivotal role in maintaining genome integrity through modulation of multiple cellular responses, in reaction to genotoxic stress. The pharmaceutical interest in PARPs is very high, since PARPs inhibitors could represent promising drugs against a variety of deseases, including stroke, diabetes, and cancer. Very recently, the identification of an enzyme displaying PARP activity has been reported in Sulfolobus solfataricus (1). The exact biological function of PARP in S. solfataricus remain obscure. However, a role of PARP in genome protection seems plausible, considering the extreme enviromental conditions experienced by this organisms. The structure of the catalytic domain of chicken PARP1 was previsouly determined (2). However, the structures of either the DNA-binding binding domain or the internal automodification domain, are still unknown. Interestingly, the S. solfataricus enzyme displays both DNA binding and auto-ADP-ribosylation activities, therefore representing a good model for the eukaryote counterpart. Morever, the crystals we have obtained have been proved to diffract near to atomic resolution, giving the possibility to provide a highly detailed picture of the active site in complex with substrates. Infact, the enzyme is totally inactive at room temperature, allowing the determination of the structure in complex with intact NAD+ Such a result could be of relevance for the rational design of inhibitors. The structure determination of both the native enzyme and complex with a protein target are in progress.

(1) Faraone Mennella MR et at. FEMS Microbiol Lett., 2000, 192, 9-14

(2) Ruf et al. Proc. Natl. Acad. Sci. USA, 1996, 93, 7481-7485

Table: Summary of poly(ADP-ribose) polymerase data collection:

Crystal	Native
Space Group	P21
Unit cell	a=41.5 Å, b=80.4 Å, c=54.5 Å β= 93.2°
Resolution	2.0 Å
N° measurements	83601
N° reflections	23923
Completeness	98.0%
Rsym	6.0%

Experiment title:	**Experiment number**:
Structure determination of *E. coli* L-aspartate oxidase in the holoform	**LS1803**

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Beamline: ID14-1	**Date of experiment**: from 9-02-2001 to 12-02-2001	**Date of report**: 10-08-01
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L-aspartate oxidase catalyses the conversion of L-Asp to iminoaspartate, using either oxygen or fumarate as electron donor. The reaction catalysed by L-aspartate oxidase is the first step in the *de novo* biosynthesis of NAD+. This pathway is currently being subjected to considerable research because it is present only in bacteria, thus being a potential target for new antibiotics.

In 1999, our group solved (ref. 1 and 2) the three-dimensional structure of the *E. coli* L-aspartate oxidase in the FAD-free apoform, revealing the overall enzyme architecture that consists of three distinct domains. Despite very considerable efforts, the wild-type enzyme could be never crystallised in the active FAD-bound form. However, based on the apoenzyme structure, a protein engineering project was undertaken (ref. 3). One mutant (R386L) affecting an Arg side chain supposed to be involved in substrate binding, turned out to crystallise in a new crystal form, containinig the enzyme in the FAD-bound state. This feature was totally unexpected since the mutant exhibited reduced(!) affinity for FAD when compared to the wild-type protein. This new crystal form allowed us to characterise the enzyme structure in the active holoform.

In the course of the previous round of BAG beam time, we measured a 2.7 Å resolution data set, which was used for structure determination by molecular replacement. We have now continued the structural analysis of the holo form of L-aspartate oxidase by measuring data on the complexes with the iminoaspartate product and the substrate analogue mercaptosuccinate, respectively (see the table). The analysis of these complexes and their comparison with the apoenzyme structure (ref. 2) has revealed a few important features concerning enzyme function:

-The so-called capping domain is flexible being able to rotate up to 30°. In fact, this domain acts as an active site gate that, in the open conformation, allows admission of the substrate in the catalytic centre whereas, in its closed conformation, it makes the active site solvent inaccessible.

-The binding of FAD significantly affects the protein structure. Upon FAD-binding, two loops (for a total of about 50 amino acids) become ordered.

-The holoenzyme structure supports a mechanism for substrate α,β dehydrogenation in which an Arg side chain is unusual in that it functions as active site base.

These data will be the subject of a manuscript, which is currently in preparation and will be soon submitted for publication.

References

1. Bacchella, L., Lina, C., Todone, C., Negri, A., Tedeschi, G., Ronchi, S., Mattevi, A. (1999). Crystallization of L-aspartate oxidase, the first enzyme in the bacterial de novo biosynthesis of NAD. *Acta Cryst*. D**55**, 549-551.

2. Mattevi, A., Tedeschi, G., Bacchella, L., Coda, L., Negri, A., Ronchi, S. (1999). Structure of L-aspartate oxidase: implications for the succinate dehydrogenase/fumarate reductase oxidoreductase family. *Structure* **7**, 745-756.

3. Tedeschi, G., Ronchi, S., Simonic, T., Treu, C., Mattevi, Negri, A. (2001) Probing the active site of l-aspartate oxidase by site-directed mutagenesis: role of basic residues in fumarate reduction. *Biochemistry* **40**, 4738-4744.

Table: Summary of L-aspartate oxidase data collection

Crystal	Iminoaspartate complex	Mercaptosuccinate complex
Space Group	$P4_32_12$	$P4_32_12$
Unit cell	a=b=73.3 Å, c=313.9 Å	a=b=73.0 Å, c=311.6 Å
Resolution	2.4 Å	2.6 Å
N° measurements	71001	79106
N° reflections	31432	21692
Completeness	94.2%	98.5%
Rsym	10.8%	12.5%

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	Experiment title: Structure determination of *M. tuberculosis* FprA protein	**Experiment number:** **LS1803, LS1933**
Beamline: ID14-1 ID14-2	**Date of experiment:** from 9-02-2001 to 12-02-2001 From 11-06-2001 to 13-06-2001	**Date of report:** 10-08-01
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The fprA gene of the pathogen *Mycobacterium tuberculosis* codes for an FAD-containing enzyme, whose exact role has still to be established. FprA shows significant sequence identity with the adrenodoxin reductase enzyme of mammals and with its yeast homologue. This information suggests a possible involvement of FprA either in the iron metabolism or in cytochrome P450 reduction. Given the major role of both processes for *M. tuberculosis* survival, studies on the FprA protein could be of interest for a better understanding of the metabolism of this organism, possibly resulting in the development of new therapeutic agents against the pathogen. This notion finds support in the observation that knocking out the gene encoding for the FprA homologue protein in yeast is lethal (ref. 1).

The fprA gene has been cloned and overexpressed in *E. coli*, and the enzyme has been purified to homogeneity. Extensive biochemical characterisation demonstrated that fprA functions as a reductase, using NADPH as the physiological electron donor (manuscript in preparation). We have crystallised *M. tuberculosis* FprA in complex with $NADP^+$. Crystals belong to space group $P2_12_12_1$ and have maximum dimensions of 0.2 x 0.3 x 0.2 mm. A first complete data set has been collected to 2 Å resolution at the beamline ID14-EH1. Based on this data, the structure has been solved through molecular replacement. In a subsequent experiment, a 1.5 Å data set on the NADPH complex has been measured. The crystallographic refinement is now in progress. The most intriguing observation is that the bound NADP+ displays a covalent modification on the C4 atom nicotinamide ring. Such covalent adduct is unprecedented and raises the question of its relevance in catalysis.

Our laboratory at the University of Pavia is member of the consortium on Structural Genomics of *M. tuberculosis* directed by T. Terwilliger and the FprA project is being carried in the context of this membership.

References

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Table: Summary of *M. tuberculosis* fprA data collection

Crystal	$NADP^+$ complex	NADPH complex
Space Group	$P2_12_12_1$	$P2_12_12_1$
Unit cell	a= 69.4 Å	a=69.6
	b=90.1 Å	c=89.6 Å
	c=161.9 Å	c=161.7 Å
Resolution	2.0 Å	1.5 Å
N° measurements	54292	365596
N° reflections	27459	109222
Completeness	99.2%	98.1%
Rsym	3.8%	12.2%

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ESRF	**Experiment title:** Structure determination of human monoamine oxidase	**Experiment number:** **LS1803, LS1933**
Beamline: ID14-3 ID14-4 ID14-1 ID14-1 ID14-3 ID14-2	**Date of experiment:** from 07-09-2000 to 09-09-2000 from 06-10-2000 to 07-10-2000 from 23-11-2000 to 25-11-2000 from 09-02-2001 to 12-02-2001 from 28-04-2001 to 30-04-2001 From 11-06-2001 to 13-06-2001	**Date of report:** 10-08-01
Shifts to BAG: **9** **3** **9** **9** **12** **6**	**Local contact(s):** Steffi ARZT Raimond RAVELLI Hassan BELRHALI Hassan BELRHALI Edward MITCHELL St phanie MONACO	*Received at ESRF:*

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Monoamine oxidase (MAO) is a FAD-dependent enzyme that is inserted in the outer mitochondrial membrane of eukaryotic cells. It is expressed in two forms, MAO-A and MAO-B, which are both homodimers of about 60 kDa (70% sequence identity) that catalyse the oxidative deamination of aromatic amines. In particular, both isoforms are involved in the catabolism of neurotransmitters, such as dopamine and serotonin, and also in the biodegradation of exogenous amines. MAO-A and MAO-B play a role in many neurological disorders such as Parkinson's disease and depression. Some MAO inhibitors are currently employed in the treatment of these pathological conditions and few others are in advanced clinical trials. The structure determination of MAO could lead to the rational design of new effective drugs against such diseases.

As integral membrane proteins, MAOs have been intrinsically difficult to handle. Crystals of MAO-B were obtained which belong to space group P1. In the course of the previous round of BAG beam time a data set up to 3.3 Å resolution was measured. Unfortunately, these MAO-B crystals have been difficult to reproduce and a limited number of them showed detectable diffraction. However, we have now collected other data sets on these native crystals and, in particular, one complete data set has been measured to 3.1 Å with a crystal soaked in Hg-acetate, which is know to bind to the protein (see Table 1).

Moreover, during the experiments carried out at ESRF, we have identified a new crystal form (centered orthorombic space group) obtained by using a different detergent in crystallization trials and two data sets up to 3.3 Å resolution were measured (see Table 2). The orthorombic symmetry of these new crystals make them very attractive for the employment of methods for the solution of the phase problem. In fact, these crystals grow in the presence of a covalent inhibitor that contains a bromine atom. We are planning a MAD experiment at the Br edge that we hope to carry out on ID29 during the next round of BAG beam time.

Table 1: Summary of monoamine oxidase data collection (space group P1):

Crystal	native	native	native	Hg soaking
Space Group	P1	P1	P1	P1
Unit cell	a=109.0 Å	a=110.6 Å	a=109.4 Å	a=108.4 Å
	b=132.1 Å	b=132.7 Å,	b=132.9 Å	b=132.4 Å
	c=155.7 Å	c=156.0 Å	c=155.5 Å	c=154.9 Å
	α=90.4°	α=91.1	α=89.9°	α=90.1°
	β=90.9°	β=88.6	β=91.0°	β=90.7°
	γ=114.3°	γ=114.7	γ=114.6°	γ=114.1°
Resolution	4.0 Å	5.0 Å	3.0 Å	3.0 Å
N° measurements	115621	56502	259640	532558
N° reflections	63029	30864	150781	140447
Completeness	94.3%	89.5%	94.6%	98.7
Rsym	16.5%	15.7%	11.8%	8.8%

Table 2: Summary of monoamine oxidase data collection (centered orthorombic space group):

Crystal	inhibitor complex	inhibitor complex	inhibitor complex
Space Group	C222	C222	C222
Unit cell	a=139.4 Å	a=137.6 Å	a=139.8 Å
	b=222.6 Å	b=224.0 Å	a=224.3 Å
	c=86.6 Å	c=86.5 Å	a=87.1 Å
Resolution	3.7 Å	3.7 Å	3.0 Å
N° measurements	53535	54317	195509
N° reflections	12962	13718	24034
Completeness	89.7%	98.1%	95.3
Rsym	17.7%	16.1%	12.8%

ESRF	**Experiment title:** Towards the structure of the ferredoxin-dependent glutamate synthase from *Synechocystis* sp.	**Experiment number**: **LS1803**
Beamline: ID14-1	**Date of experiment**: From 06-10-200 to 07-10-2000	**Date of report**: 14-06-01
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Glutamate synthase (GltS) is an iron-sulphur flavoprotein whose function requires the channelling of ammonia and electrons among distinct catalytic sites. GltS catalyses the reductive transfer of the amide group of Gln to the C2 carbon of 2-oxoglutarate yielding two molecules of Glu. Recently, the crystal structure of the alpha-subunit of the NADPH-dependent glutamate synthase from *Azospirillum brasilense* was determined using the weak anomalous signal of the single 3Fe-4S present in the 1479 amino acid subunit (ref. 1; data measured at BM14). The structure of the 163 kDa alpha-subunit of *A. brasilense* GltS consists of an unexpected combination of four domain topologies. Furthermore, the three-dimensional structure has revealed a 31 Ang. tunnel that goes across the protein and connects the Gln site on the N-terminal domain to the 2-oxoglutarate binding site in proximity of the FMN. Such a tunnel is perfectly suited to allow diffusion of the ammonia resulting from Gln hydrolysis to 2-oxoglutarate in order to generate the 2-iminoglutarate intermediate.

In order to shed light into the mechanism of active site cross-talk and glutaminase site activation, we have undertaken the X-ray analysis of GltS from *Synechocystis sp*. PCC6803. This protein utilizes ferredoxin as electron donor and consists of a single polypetide chain (150 kDa), a FMN cofactor and a 3Fe-4S cluster (ref. 2). *Synechocystis* GltS has been crystallized in the substrate-free state and in complex with substrate analogues and diffraction data have been measured to 2.8 Ang. resolution. Structure determination is in progress by means of molecular replacement using the coordinates of the *A. brasilense* enzyme. From the current partly refined model, it appears that the conformation of a few catalytic residues in the glutaminase site of the *Synechocystis* GltS differs from that observed in *A. brasilense* GltS. This implies that the *Synechocystis* protein crystallises in a different functional state with respect to the *A. brasilense* protein.

The structure determination of *Synechocystis* GltS is the first step for further in-depth studies of this complex enzymatic system. Indeed, a very attractive property of the *Synechocystis* GltS crystals is that the crystalline protein retains catalytic activity. This feature has been proven by crystal microspectrophotometry. The crystalline enzyme can be reduced using an artificial electron donor such as dithionite and, then, it can be reoxidized through soaking in the substrates 2-oxoglutarate and Gln. Most important, the microspectrophotometric studies have led to the identification of conditions for the stabilization in the crystal of reaction intermediates involved in the overall catalytic cycle. Knowledge of these reaction intermediate structures will be of the utmost importance for the understanding the mechanism of GltS function and regulation.

References

1. Binda, C., Bossi, R.T., Wakatsuki, S., Arzt, S, Coda, A., Curti, B., Vanoni, M.A., Mattevi, A. (2000) Cross-talk and ammonia channeling between active centres in the unexpected domain arrangement of glutamate synthase. Structure 8, 1299-1308.

2. Navarro F, Martin-Figueroa E, Candau P, Florencio FJ. (2000) Ferredoxin-dependent iron-sulfur flavoprotein glutamate synthase (GlsF) from the cyanobacterium synechocystis sp. PCC 6803: expression and assembly in escherichia coli. Arch Biochem Biophys. 379, 267-76.

Table: Summary of GltS data collection

Crystal	Native	Substrate analogue complex
Space group	$P4_32_12$	$P4_32_12$
Unit cell	a=b=166.7 Å	a=b=166.1 Å
	c=220.8 Å	c=221.5 Å
Resolution	2.73 Å	2.79 Å
N° measurements	214,451	434,388
N° unique reflections	75,282	76,589
Completeness	92.6 %	99.4 %
Rsym	8.8 %	12.0 %

	Experiment title: Pathological mutation in human erythrocyte pyruvate kinase	**Experiment number:** **LS1803, LS1933**
Beamline: ID14-1 ID14-3	**Date of experiment:** From 09-02-2001 to 12-02-2001 From 11-06-2001 to 13-06-2001	**Date of report:** 14-08-01
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Pyruvate kinase (PK) deficiency is the most frequent enzyme abnormality of the glycolytic pathway, and, together with class I glucose-6-phosphate dehydrogenase deficiency, is the most common cause of the hereditary non-spherocytic haemolytic anaemia (ref.1). Clinical manifestations vary from very mild to life-threatening neonatal anaemia and jaundice necessitating transfusion therapy and, occasionally, splenectomy.
PK catalyses the conversion of phosphoenolpyruvate to pyruvate coupled to the synthesis of one ATP molecule. The reaction occupies a nodal point of the cellular metabolism since it represents the last step of glycolysis (ref.2). In patients affected by non-spherocytic haemolytic anaemia, impairment of the glycolytic pathway caused by PK deficiency leads to ATP depletion, that is responsible for the haemolytic process ultimately resulting in the onset of the disease. So far, 147 mutations in the human gene coding for erythrocyte PK have been described (ref.3) and a continuously updated review is reported on the World Wide Web at http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?266200. This project investigates abnormalities in erythrocyte PK, through mutagenesis, crystallographic and enzymological methods. The main aim is to correlate the levels of alteration in the regulatory responsiveness and stability of PK with the degree of anaemia and the severity of its symptoms. For this purpose, we are carrying out characterisation of the structural and functional properties of PK mutants found in patients who are being or have been followed by the group of prof. Zanella at the Haematology Department of the “Ospedale Maggiore” in Milano (Italy). The ultimate goal will be a better definition of the prognosis and therapeutic intervention.

In the framework of this project, we are performing the structural analysis of pathological mutants of human erytrhocyte PK. In the period October 2000-July 2001, we have measured diffraction data for crystals of the R479H and R489W mutants (see Table). TheR479H mutation affects an Arg residue close to the binding for the allosteric activator fructose-1,6-bisphosphate. The crystallographic analysis shows that the mutation does not induce any conformational change with respect to the wild-type enzyme. This suggests that the main effect of this mutation is mainly due to the replacement of the charged and long Arg side chain, which interacts with the activator. This observation is in keeping with the biochemical analysis of the mutant, which shows a reduced responsiveness towards fructose-1,6-bisphosphate.

The second data set collected at ESRF was measured on a crystal of the R489W mutant. This mutation is particularly interesting in that it leads to a tenfold decrease in the catalytic efficiency (K_{cat}/K_m) coupled to a higher (!) enzyme stability as indicated by an increase on the Tm of 10° C. The crystallographic refinement for this mutant is still in progress. The mutation affects Arg489, which is not part of the catalytic or allosteric site. Rather, this residue is located at a domain interface within the PK subunit. In particular, the R->W mutation induces a local conformational change in this area. Our interpretation of these data is that the perturbation at the domain interface affects the mechanism of relay of the allosteric signal from the allosteric site to the catalytic centre. In this way, the R-state/T-state equilibrium is shifted towards the T-state, leading to a decrease in the enzyme activity.

References

1. Zanella, A., Bianchi, P. (2000) Red cell pyruvate kinase deficiency: from genetics to clinical manifestations *Baillieres Best Pract. Res. Clin. Haematol.* **13**, 57-81.
2. Mattevi, A., Valentini, G., Speranza, M.L., Rizzi, M., Bolognesi, M., Coda, A. (1995) Crystal structure of the allosteric pyruvate kinase type-I from *Escherichia coli*. *Structure* ***3,*** 729-741.
3. Bianchi, P., Zanella, A. (2000) Hematologically important mutations: red cell pyruvate kinase *Blood Cells Mol. Dis.* **26**, 47-53.

Table: Summary of human pyruvate kinase data collection

Crystal	R479H	R489W
Space group	$P2_1$	$P2_1$
Unit cell	a=74.0 Å	a=73.6 Å
	b=171.8 Å	b=171.2 Å
	c=85.1 Å	c=85.1 Å
	β =91.2°	β=91.7°
Resolution	2.60 Å	2.86 Å
N° measurements	113348	92400
N° unique reflections	60016	44671
Completeness	92.2 %	92.7 %
Rsym	9.8 %	9.9 %