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Crystallization and preliminary X-ray analysis of the selenate reductase from *Thauera selenatis*

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Selenate reductase from *Thauera selenatis* was crystallized using ammonium sulfate as a precipitant. Crystals of selenate reductase belong to the space group *C*2, with unit-cell parameters a = 116.9, b = 67.5, c = 186.7 Å, $\beta = 90°$. Native data to 2.1 Å resolution have been collected and a heavy-atom derivative has been identified following soaking of the crystals in a solution of trimethyl lead acetate.

1. Introduction

Thauera selenatis, a member of the β subclass of the Proteobacteria, was isolated from selenate-contaminated waste water in the San Joaquin Valley, California, USA (Macy *et al.*, 1989, 1993). This bacterium conserves energy *via* respiration, using oxygen as the terminal electron acceptor or, in the absence of oxygen, selenate and nitrate. *T. selenatis* is the only organism which has been found to reduce selenate using the respiratory substrate, acetate, as its source of electrons and carbon. Because of its unique metabolism *T. selenatis* is particularly well suited for use in bioremediation.

The reduction of selenate to selenite is catalyzed by a soluble periplasmic selenate reductase consisting of three heterologous subunits α (99 kDa), β (37 kDa) and γ (23 kDa). It is a heterotrimer $\alpha_1\beta_1\gamma_1$, with a native molecular mass of 159 kDa. The enzyme contains one molybdenum, approximately three iron–sulfur centres (probably [4Fe–4S] clusters) and a single cytochrome *b* prosthetic group per $\alpha_1\beta_1\gamma_1$ complex (Schröder *et al.*, 1997; Krafft *et al.*, 2000).

Selenate reductase is the only dissimilatory selenate reductase known and is thus of considerable interest both in its own right and as a novel member of the molybdenum enzyme family. Mononuclear molybdoenzymes can be divided into three groups based on the structure of their molybdenum centres: (i) the xanthine oxidase family (the molybdenum hydroxylases) which catalyze the hydroxylation of a broad range of aldehydes and aromatic heterocyclics; (ii) the sulfite oxidase family (the eukaryotic oxo transferases) including sulfite oxidase and the assimilatory nitrate reductases, which catalyze O-atom transfer to or from a substrate; and (iii) the DMSO reductase family, which is a diverse group of prokaryotic enzymes that catalyze either O-atom transfer or other oxidation/reduction reactions. Enzymes of this group have a common molybdenum centre in which the metal is coordinated by a pair of dithiolene ligands contributed by two equivalents of the molybdopterin cofactor (Hille *et al.*, 1999).

This third family can be further divided into two subclasses: (i) those which are monomeric and have the molybdenum centre as their sole redox-active centre (*Rhodobacter sphaeroides* and *R. capsulatus* DMSO reductases) and (ii) the often multisubunit enzymes which contain additional redox-active cofactors such as iron–sulfur clusters and *b*-type cytochromes. Based on sequence similarities and its cofactor content, selenate reductase belongs to the second of these subclasses, which includes enzymes such as the *Escherichia coli* DMSO reductase, respiratory nitrate reductases and formate dehydrogenases (Hanlon *et al.*, 1996; Hille *et al.*, 1999; Krafft *et al.*, 2000; Romão *et al.*, 1997). In addition, a preliminary study of the molybdenum site of selenate reductase by X-ray absorption spectroscopy has shown it to have an active-site structure similar to that of the DMSO reductases, consisting of an Mo atom coordinated by two dithiolene groups, a single oxo group and possibly an oxygen or nitrogen ligand provided by the polypeptide chain (M. J. Maher, J. M. Santini, I. J. Pickering, R. C. Prince, J. M. Macy & G. N. George, unpublished results).

In order to gain structural information on this enzyme and to understand its mechanism, we have initiated an X-ray structure analysis. We report here the crystallization and preliminary X-ray characterization of the selenate reductase from *T. selenatis*.

2. Experimental procedures

2.1. Purification

T. selenatis selenate reductase was purified as previously described (Schröder *et al.*, 1997),

with the following modifications which were designed to yield a greater amount of enzyme.

T. selenatis was grown anaerobically at 291 K in a minimal salt medium containing 0.1% yeast extract, 20 m*M* sodium selenate and 10 m*M* sodium acetate as previously described (Macy *et al.*, 1989). For enzyme purification, *T. selenatis* was grown in either 20 or 40 l batch cultures. Cultures were harvested after 16 h growth at a final optical density (A_{600nm}) of 0.7–0.8. Cells were harvested using a Pellicon Tangential Flow Filtration System (Millipore, USA). The periplasmic fraction was prepared by suspending cells in 30 m*M* Tris–HCl buffer pH 8.0 containing 0.75 *M* sucrose at a ratio of 0.5 g of cells per millilitre of buffer. Following incubation of the cell suspension for 5–10 min at 273 K, lysozyme was added to a final concentration of 3.5 mg ml^{-1} and incubation continued for a further 30 min. Two volumes of an ice-cold solution of EDTA (15 m*M*, pH 8.0) were then added slowly over a 10 min period. The suspension was stirred on ice for an additional 20 min and then placed at room temperature for 15–20 min to permit the formation of spheroplasts. The spheroplasts were separated from the periplasmic fraction by centrifugation (25 000*g*; 20 min). The proteins in the periplasmic fraction were concentrated by ammonium sulfate precipitation (50–80% saturation). The precipitated material was collected by centrifugation (25 000*g*; 20 min) and resuspended in 50 m*M* piperazine buffer pH 6.0 containing 2.0 *M* ammonium sulfate. The suspension was loaded onto a phenyl Sepharose high-performance hydrophobic interaction column (HiLoad 16/10; Pharmacia Biotech). The fractions containing selenate reductase were pooled, concentrated and loaded onto a Superose 12 gel-filtration column (Pharmacia Biotech). The hydrophobic interaction step was repeated and fractions containing the selenate reductase were collected, concentrated and stored at 193 K until required.

2.2. Crystal growth and analysis

Crystallization conditions were screened according to the sparse-matrix method (Jancarik & Kim, 1991) using commercially available buffers (Hampton Research, Laguna Hills, California, USA) and the hanging-drop vapour-diffusion technique (McPherson, 1992). Ammonium sulfate was found to be the best crystallization precipitant. Other parameters such as buffer composition, pH, temperature and protein concentration were varied in order to improve crystal quality.

The best crystals grew under conditions where hanging drops were prepared by mixing 2.0 µl of protein solution (10 mg ml^{-1}, 0.3–0.5 *M* ammonium sulfate, 50 m*M* piperazine pH 6.0) with 2.0 µl of reservoir solution (1.8–2.2 *M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.0–8.9) and were equilibrated against 1.0 ml reservoir solution at 293 K. Small crystals of selenate reductase (100 × 50 × 50 µm) grew in the presence of a large amount of orange–brown precipitate in 2–4 weeks. The crystals were cryoprotected by successive soaking in reservoir solution containing increasing quantities of glycerol (the final concentration of glycerol was 25%, which was achieved in 5% steps) and flash-frozen in liquid nitrogen for data collection. A native data set to 2.1 Å resolution (Table 1) was recorded at 110 K on BioCARS beamline 14C at the Advanced Photon Source, Argonne National Laboratory.

In order to determine the subunit composition of the selenate reductase crystals, several crystals were washed with mother liquor and subsequently dissolved in water. The resulting protein sample was prepared in 2.5% SDS, 5% β-mercaptoethanol and 0.005% bromophenol blue and after heating (363 K, 5 min) was applied to a 10–15% gradient polyacrylamide SDS gel (Phastsystem, Pharmacia Biotech).

A preliminary search for heavy-atom derivatives was performed by preparing crystals under cryoprotective conditions (2.5 *M* ammonium sulfate, 25% glycerol, 100 m*M* Tris–HCl pH 8.5) and transferring them to drops of the same solution containing heavy-atom compounds (5 m*M*). The crystals were soaked at 293 K overnight. X-ray data were collected at 110 K on an R-AXIS IIc imaging-plate detector mounted on a Rigaku RU-200 rotating-anode generator using a 0.2 × 0.2 mm focus. X-rays (Cu $K\alpha$, 1.5418 Å) were collimated and focused with mirror optics.

All data were integrated, scaled and merged using the *HKL* suite (Otwinowski & Minor, 1997). Isomorphous difference Patterson functions were calculated and interpreted using *CNS* (Brünger *et al.*, 1998). *MLPHARE* from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994) was used to refine the heavy-atom positions.

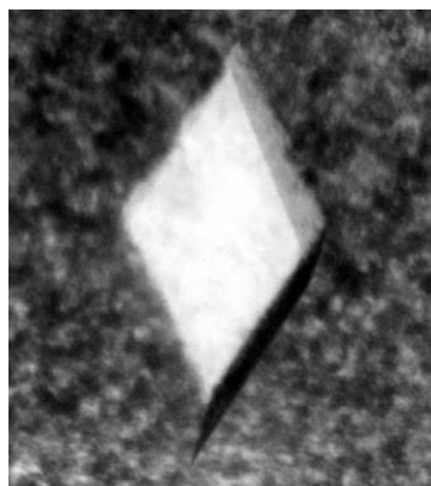

Figure 1
Monoclinic crystal of *T. selenatis* selenate reductase. Typical dimensions are approximately 100 × 50 × 50 µm.

Table 1
Data-collection statistics.

Values for the highest shell are given in parentheses.

	Native	Pb derivative
Resolution (Å)	2.1	2.6
Mosaicity (°)	0.37	0.55
Observations	166518	77795
Unique reflections	75165	40056
Redundancy	2.2 (1.9)	1.9 (1.8)
Completeness (%)	87.9 (86.1)	90.9 (94.4)
$I/\sigma(I)$	9.6 (1.4)	11.0 (3.1)
R_{merge}	0.055 (0.315)	0.051 (0.174)

3. Results and discussion

Small (100 × 50 × 50 µm) bipyramidal crystals of selenate reductase formed in 2–4 weeks using 1.8–2.2 *M* ammonium sulfate as a precipitant (Fig. 1). Unsuccessful experiments have been carried out to increase the size of these crystals. These have been hampered by the difficulty in controlling

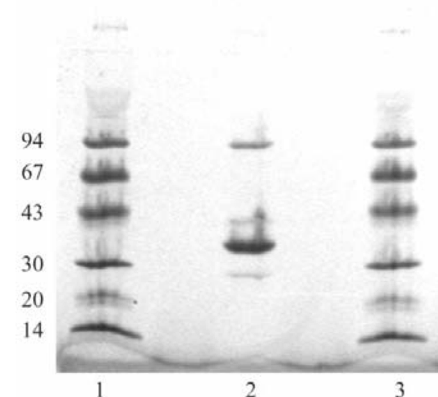

Figure 2
SDS–polyacrylamide gel electrophoresis of dissolved crystals of the selenate reductase enzyme. Lanes 1 and 3, molecular-weight standards in kDa; lane 2, dissolved crystals of selenate reductase.

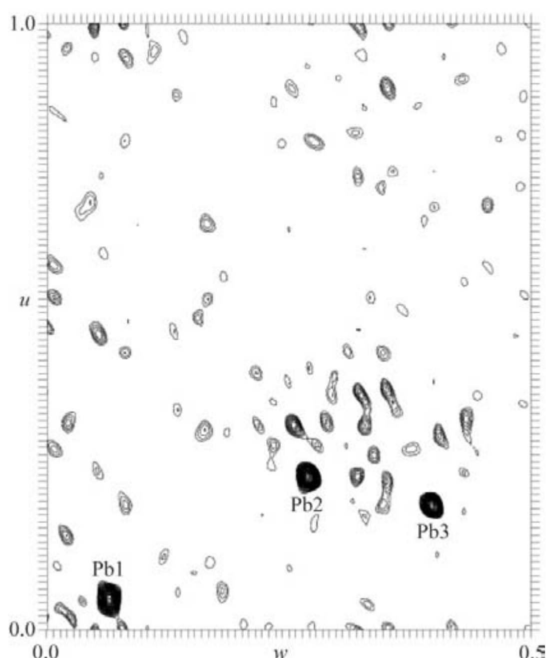

Figure 3
Heavy-atom difference Patterson map of the lead derivative of selenate reductase at 4.0 Å resolution. The asymmetric unit of the Harker section ($y = 0$) is shown. The three strong Harker peaks are labelled Pb1, Pb2 and Pb3.

nucleation, as the crystals grow in the presence of a large amount of protein precipitate. Since selenate reductase is an oxygen-sensitive enzyme and the crystallization experiments were carried out in the presence of air, it is possible that this large amount of protein precipitate results from oxidative damage.

Despite the small size of these crystals, native data to 2.1 Å resolution have been recorded. The symmetry in the diffraction pattern showed that the crystals were monoclinic, space group *C*2, with unit-cell parameters $a = 116.9$, $b = 67.5$, $c = 186.7$ Å, $\beta = 90^\circ$. Given that $\beta = 90^\circ$, the possibility that the crystals were really orthorhombic was eliminated by merging the data in an orthorhombic cell. This gave $R_{\rm merge} > 0.4$, compared with $R_{\rm merge} = 0.06$ in a monoclinic cell, confirming the space group as *C*2. An SDS–PAGE analysis of dissolved crystals showed two major bands corresponding to the α and β subunits (99 and 37 kDa, respectively; Fig. 2). The third (γ) subunit (23 kDa) was not visible. With this in mind and using Matthews' formula (Matthews, 1968), it is possible that one heterodimeric molecule ($\alpha_1\beta_1$, 54% solvent, $V_{\rm M}$ = 2.7 Å^3 Da^{-1}) rather than one heterotrimeric molecule ($\alpha_1\beta_1\gamma_1$, 47% solvent, $V_{\rm M}$ = 2.3 Å^3 Da^{-1}) of selenate reductase is present per asymmetric unit. Confirmation of the subunit composition of the crystals will have to await solution of the structure.

A putative heavy-atom derivative was identified following soaking of a crystal in a solution of trimethyl lead acetate. Data were collected from the Pb-derivatized crystal to 2.6 Å resolution. An isomorphous difference Patterson map calculated at 4.0 Å resolution showed three strong Harker peaks ($\sim$14–16σ; Fig. 3). The positions of the three Pb sites were determined and refined (the phasing powers after refinement for acentric and centric reflections were 1.81 and 1.27, respectively, to 3.0 Å resolution). Currently, we are searching for additional heavy-atom derivatives to determine the three-dimensional structure of selenate reductase by multiple isomorphous replacement. In addition, we are planning experiments so that the structure may be solved by the multiple-wavelength anomalous dispersion (MAD) method using the Pb derivative.

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