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# Purification, crystallization and preliminary X-ray analysis of adenylylsulfate reductase from *Desulfovibrio vulgaris* Miyazaki F

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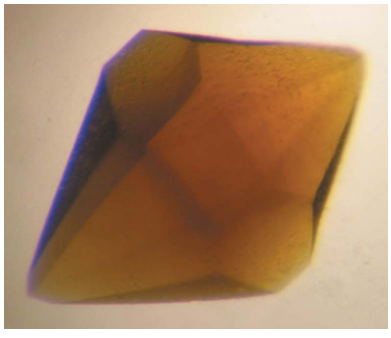
Sulfur in its various oxidation states is used for energy conservation in many microorganisms. Adenylylsulfate reductase is a key enzyme in the sulfur-reduction pathway of sulfate-reducing bacteria. The adenylylsulfate reductase from *Desulfovibrio vulgaris* Miyazaki F has been purified and crystallized at 277 K using the vapour-diffusion method with ammonium sulfate as the precipitating agent. A data set was collected to 1.7 Å resolution from a single crystal at 100 K using synchrotron radiation. The crystal belonged to space group $P3_1$, with unit-cell parameters $a = b = 125.93$, $c = 164.24$ Å. The crystal contained two molecules per asymmetric unit, with a Matthews coefficient ($V_M$) of 4.02 Å$^3$ Da$^{-1}$; the solvent content was estimated to be 69.4%.

## 1. Introduction

Sulfur is used for energy conservation by many bacteria, archaea and plants. Sulfate respiration was developed 2.8–3.1 billion years ago (Schidlowski, 1979). Adenylylsulfate reductase (APS reductase; EC 1.8.99.2) plays a key role in the sulfur metabolism of many sulfate-reducing bacteria. Sulfate is used as a terminal electron acceptor and the final product forms hydrogen sulfide in a dissimilatory sulfate reduction (Postgate, 1959; Peck *et al.*, 1965; Odom & Peck, 1981). There are three major steps in sulfate reduction (for a review, see Fritz *et al.*, 2005). In the first step, sulfate is reduced by ATP sulfurylase to adenosine 5′-phosphosulfate (APS) and pyrophosphate at the expense of two equivalents of ATP. The reduction potential $E^{\circ\prime}(SO_4^{2-}/HSO_3^-)$ is −516 mV, whereas when sulfate is activated by ATP sulfurylase the redox potential $E^{\circ\prime}(\mathrm{APS/AMP} + HSO_3^-)$ is shifted to −60 mV (Thauer *et al.*, 1977). In the next step, APS reductase catalyzes a two-electron reduction of APS to AMP and sulfite. The electron donor of APS reductase is not yet known. The reduction of APS by APS reductase is inhibited by sulfite (Yagi & Ogata, 1996). Finally, dissimilatory sulfite reductase (Dsr, formerly desulfoviridin; EC 1.8.99.3) catalyzes the six-electron reduction of sulfite to sulfide. The assimilatory sulfate reductions in the biosynthesis of cysteine and methionine use the same pathway (Saito, 2004). Recently, the crystal structure of the assimilatory APS reductase from *Pseudomonas aeruginosa* has been reported. The assimilatory APS reductase is composed of two subunits (31 kDa), forming a homodimer containing two Fe–S clusters (Chartron *et al.*, 2006). In sulfur-oxidizing organisms the reaction occurs in the opposite direction, starting from sulfide, inorganic sulfur and thiosulfate.

The dissimilatory APS reductase from *Desulfovibrio vulgaris* Miyazaki F is a heterodimeric complex composed of an $\alpha$-subunit (75 kDa) that contains a noncovalently bound flavin adenine dinucleotide (FAD) and a $\beta$-subunit (18 kDa) containing two Fe–S clusters. Recently, the crystal structure of APS reductase from the hyperthermophilic archaeon *Archaeoglobus fulgidus* has been determined (Fritz, Roth *et al.*, 2002; Schiffer *et al.*, 2006). The two Fe–S clusters have different redox potentials. Fe–S cluster I, which is located proximal to FAD, has a high redox potential (−60 mV). On the other hand, Fe–S cluster II, which is located at the distal position



to FAD, has a very negative potential (∼−500 mV). This difference was explained by polar interaction with the backbone amides surrounding the Fe–S cluster (Fritz, Roth *et al.*, 2002). The reduction of APS occurs at the nucleophilic N5 atom of FAD (Fritz, Roth *et al.*, 2002; Fritz, Büchert *et al.*, 2002).

Alignment of the amino-acid sequences of the $\alpha$-subunits of APS reductase from *D. vulgaris* Miyazaki F and from *A. fulgidus* shows 48% identity. Alignment of the equivalent $\beta$-subunits shows 65% identity. To date, no crystal structure of an APS reductase from a sulfate-reducing bacterium has been reported. In order to understand the dissimilatory sulfate-reduction mechanism, which plays a central role in sulfate-reducing bacteria, we purified APS reductase from the Gram-negative sulfate-reducing bacterium *D. vulgaris* Miyazaki F. In this paper, we report the purification, crystallization and preliminary X-ray analysis of the Fe–S flavoenzyme APS reductase from *D. vulgaris* Miyazaki F.

## 2. Materials and methods

### 2.1. Sequence analysis

N-terminal sequencing and the MALDI-generated peptide sequences of the protein were used to design degenerate primers for the $\alpha$-subunit of the APS reductase of *D. vulgaris* Miyazaki F (Kaur *et al.*, unpublished work; NCBI submission code EU127913). The nucleotide sequencing was completed from a cosmid clone of a genomic library of *D. vulgaris* Miyazaki F (unpublished work). The positive clone was identified by Southern hybridization of the cosmid DNA using a nonradioactive digoxygenin-labelled dUTP probe (Roche).

### 2.2. Protein purification

The protein was purified from the sulfate-reducing bacterium *D. vulgaris* Miyazaki F. Cell growth in a 50 l fermenter was carried out using the method described previously (Yagi *et al.*, 1968). Cells were lysed by sonication on ice using a buffer containing 10 m*M* NaCl, 25 m*M* Tris–HCl pH 7.4, which was followed by centrifugation at 184 000*g* at 277 K for 90 min. All purification steps were performed at 277 K under aerobic conditions. The cytoplasmic fraction was collected and 0.25 n*M* Pefabloc SC (Biomol GmbH, Germany) was added as a serine protease inhibitor. The proteins were loaded onto a DEAE-Toyopearl 650S (Tosoh, Japan) anion-exchange column (10–25% linear gradient of 1 *M* NaCl). The eluted APS reductase fractions from the DEAE column were concentrated to 5 ml and passed through a Sephacryl S200HR (GE Healthcare, Uppsala, Sweden) gel-filtration column. The eluted APS reductase fractions were again loaded without concentration onto a DEAE-Toyopearl 650S (Tosoh, Japan) anion-exchange column (0–22.5% linear gradient of 1 *M* NaCl). As the final step in the purification, a HiLoad 26/60 Superdex 200 prep-grade (GE Healthcare, Uppsala, Sweden) gel-filtration column was used with 25 m*M* Tris–HCl buffer pH 7.4. After purification, the samples were sealed in an anaerobic chamber and stored in liquid nitrogen. The quality of the samples was determined by SDS–PAGE.

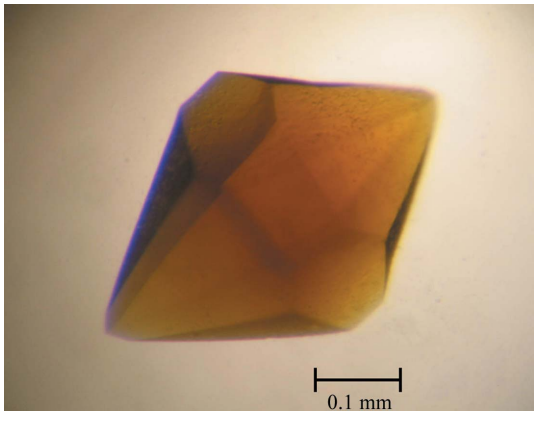



**Figure 1**
Crystal of APS reductase from *D. vulgaris* Miyazaki F.

**Table 1**
X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| X-ray source | BL14.1 at BESSY II |
| Wavelength (Å) | 0.91841 |
| Space group | $P3_1$ |
| Unit-cell parameters (Å) | $a = b = 125.93$, $c = 164.24$ |
| Resolution range (Å) | 51.78–1.70 (1.79–1.70) |
| Observed reflections | 1499388 |
| Unique reflections | 317895 |
| $R_{\rm merge}$† | 0.100 (0.402) |
| Completeness (%) | 99.8 (98.9) |
| $\langle I/\sigma(I)\rangle$ | 4.6 (1.8) |
| $V_{\rm M}$ (Å$^3$ Da$^{-1}$) | 4.02 |

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the $i$th observation and $\langle I(hkl)\rangle$ is the mean intensity of the reflections.

### 2.3. Crystallization

The purified protein solution was concentrated to 15 mg ml$^{-1}$ by centrifugation using an Amicon Ultra Centrifugal filter device (30 kDa molecular-weight cutoff; Millipore). Initial crystal screening of APS reductase from *D. vulgaris* Miyazaki F was carried out using the sitting-drop vapour-diffusion method at 277 K under anaerobic conditions. Crystal Screen Cryo, Crystal Screen 2 (Hampton Research, California, USA), Wizard 1, Wizard 2, Cryo 1 and Cryo 2 (Emerald Biosystems Inc., Washington, USA) were used for initial screening. The protein droplets were prepared by mixing 10 µl APS reductase solution and 10 µl reservoir buffer solution and were set up in a Cryschem plate (Hampton Research, California, USA) with 1 ml reservoir solution in an anaerobic chamber (95% $N_2$, 5% $H_2$). Crystals were obtained after three weeks. After optimization, crystals suitable for diffraction experiments (Fig. 1) were obtained using the hanging-drop vapour-diffusion method with the following conditions: 0.1 *M* HEPES pH 7.5, 0.1 *M* NaCl, 2.0 *M* ammonium sulfate and 15%(*v*/*v*) glycerol. The crystal dimensions were typically 0.5 × 0.5 × 0.5 mm.

### 2.4. Data collection and analysis

A preliminary X-ray diffraction experiment was carried out using a CCD detector (Bruker AXS X8 Proteum) and a Cu $K\alpha$ rotating-anode source with Montel mirror optics (2.4 kW). Diffraction images were indexed and integrated with the *PROTEUM* program and subsequently scaled and truncated using the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). A complete native data set was collected at 100 K using beamline BL14.1 at BESSY II (Berlin, Germany). The detector was a MAR Mosaic 225. 843 frames were collected with 10 s exposure time and 0.2° oscillation. The distance between the crystal and the detector was maintained at 190 mm. In order to collect the data at cryogenic temperature, the crystal was frozen in liquid nitrogen and mounted on the goniostat under a nitrogen-gas stream at 100 K. The data set was indexed and integrated using the program *MOSFLM* (Leslie, 1992). Scaling was

carried out with *SCALA* (Collaborative Computational Project, Number 4, 1994). The conditions of the data collection and the results obtained are summarized in Table 1.

## 3. Results

The protein sequence of the APS reductase from *D. vulgaris* Miyazaki F was deduced by translating the operon sequenced from a cosmid library and confirmed by N-terminal sequencing. APS reductase was successfully purified and crystallized. Single crystals were obtained at 277 K using the hanging-drop vapour-diffusion method with ammonium sulfate as a precipitating agent. The crystals diffracted to 1.7 Å resolution and belonged to space group $P3_1$, with unit-cell parameters $a = b = 125.93$, $c = 164.24$ Å. The calculated Matthews coefficient ($V_M$) of 4.02 Å$^3$ Da$^{-1}$, with a solvent content of 69.4%, indicates the presence of two molecules in the asymmetric unit. The crystal structure of APS reductase from *D. vulgaris* Miyazaki F was solved by the molecular-replacement method using the program *CNS* (Brünger *et al.*, 1998). As coordinates for the search model, the data for APS reductase from *A. fulgidus* (PDB code 1jnr) were used, which has 48% ($\alpha$-subunit) and 65% ($\beta$-subunit) amino-acid sequence identity to APS reductase from *D. vulgaris* Miyazaki F. After the calculation of the electron-density map using the molecular-replacement solution, two heterodimers in the asymmetric unit that form an $\alpha_2\beta_2$ heterotetramer were observed. The electron-density map shows one FAD in the $\alpha$-subunit and two 4Fe–4S clusters in the $\beta$-subunit. Model building and refinement are now in progress.

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