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# Preliminary X-ray crystallographic analysis of sulfide:quinone oxidoreductase from *Acidithiobacillus ferrooxidans*

**Yanfei Zhang,[a,b] Maia M. Cherney,[a] Matthew Solomonson,[a] Jianshe Liu,[b] Michael N. G. James[a] and Joel H. Weiner[a]***

[a]Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, and [b]School of Minerals Processing and Bioengineering, Central South University, Changsha, Hunan 410083, People's Republic of China

Correspondence e-mail: joel.weiner@ualberta.ca

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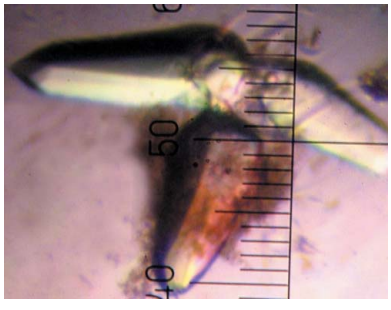



The gene product of open reading frame AFE_1293 from *Acidithiobacillus ferrooxidans* ATCC 23270 is annotated as encoding a sulfide:quinone oxidoreductase, an enzyme that catalyses electron transfer from sulfide to quinone. Following overexpression in *Escherichia coli*, the enzyme was purified and crystallized using the hanging-drop vapour-diffusion method. The native crystals belonged to the tetragonal space group $P4_22_12$, with unit-cell parameters $a = b = 131.7$, $c = 208.8$ Å, and diffracted to 2.3 Å resolution. Preliminary crystallographic analysis indicated the presence of a dimer in the asymmetric unit, with an extreme value of the Matthews coefficient ($V_M$) of 4.53 Å$^3$ Da$^{-1}$ and a solvent content of 72.9%.

## 1. Introduction

*Acidithiobacillus ferrooxidans* is a motile Gram-negative acidophilic chemolithotrophic bacterium that utilizes $Fe^{II}$, $H_2S$, $S^0$, reduced inorganic sulfur compounds and molecular hydrogen as energy sources (Temple & Delchamps, 1953). The oxidation of sulfur produces sulfuric acid, which is responsible for some of the interesting characteristics of *A. ferrooxidans*. It is generally found in acidic environments such as mining dumps and acid mine drainages. This organism plays one of the key roles in microbial communities by taking part in the bacterial chemical processes of bioleaching under mesophilic conditions (Olson *et al.*, 2003).

Sulfur can exist in various oxidation states from −2 to +6, complicating studies of the enzymatic steps involved. Based on comparative genomic analysis, sulfur oxidation in *A. ferrooxidans* is catalyzed by the sulfide:quinone oxidoreductase (SQR) system and the initial step of the SQR system is the oxidation of sulfide to elemental sulfur by SQR. The sulfur may be further oxidized *via* $S_2O_3^{2-}$ and $SO_3^{2-}$ to the final oxidation state +6 in $SO_4^{2-}$ by other enzymes of the SQR system (Valdes *et al.*, 2008). The mechanism of SQR catalysis includes electron transfer from sulfide anions to quinone, resulting in the reduction of the latter. SQRs are ancient flavoproteins that are present in many organisms from archaea to humans. Several SQRs have been characterized by biochemical methods (Griesbeck *et al.*, 2002; Theissen & Martin, 2008; Arieli *et al.*, 1994; Shibata & Kobayashi, 2006; Wakai *et al.*, 2007; Shibata *et al.*, 2007; Vande Weghe & Ow, 1999). Recently, two structures of SQR from the hyperthermoacidophilic archaeon *Acidianus ambivalens* (PDB code 3h8l; Brito *et al.*, 2009) and from the hyperthermophilic bacterium *Aquifex aeolicus* (PDB codes 3h27, 3h28 and 3h29; Marcia *et al.*, 2009) have been reported. The structure of a related protein, flavocytochrome *c* sulfide dehydrogenase (FCC) from *Chromatium vinosum*, was published almost 15 years ago (PDB code 1fcd; Chen *et al.*, 1994). It has been found that Cys160 and Cys356 (in the *A. ferrooxidans* numbering) are the active-site residues (Fig. 1). Interestingly, all reported structures had the flavin adenine dinucleotide (FAD) cofactor molecules covalently bound to the protein

through a linkage of the C8M group of FAD to a Cys128 residue that is different from the active-site cysteines. Several other SQRs, including that presented here, have noncovalently bound FAD (Griesbeck *et al.*, 2002) that can sometimes be lost during purification steps. However, the exact mechanism of the electron transfer between the sulfides and quinone is not entirely clear. Further structural and mutagenic studies are needed.

In this study, we report the molecular cloning, protein purification and preliminary X-ray crystallographic analysis of the AFE_1293 gene product from *A. ferrooxidans*, which has been annotated as a sulfide:quinone oxidoreductase (SQR). It has a bright yellow colour arising from the nonconvalently bound cofactor FAD (data on FAD binding are not presented here).

## 2. Materials and methods

### 2.1. Cloning, expression and purification

Genomic template DNA from *A. ferrooxidans* ATCC 23270 was prepared using the EZ-10 spin-column genomic DNA-isolation kit

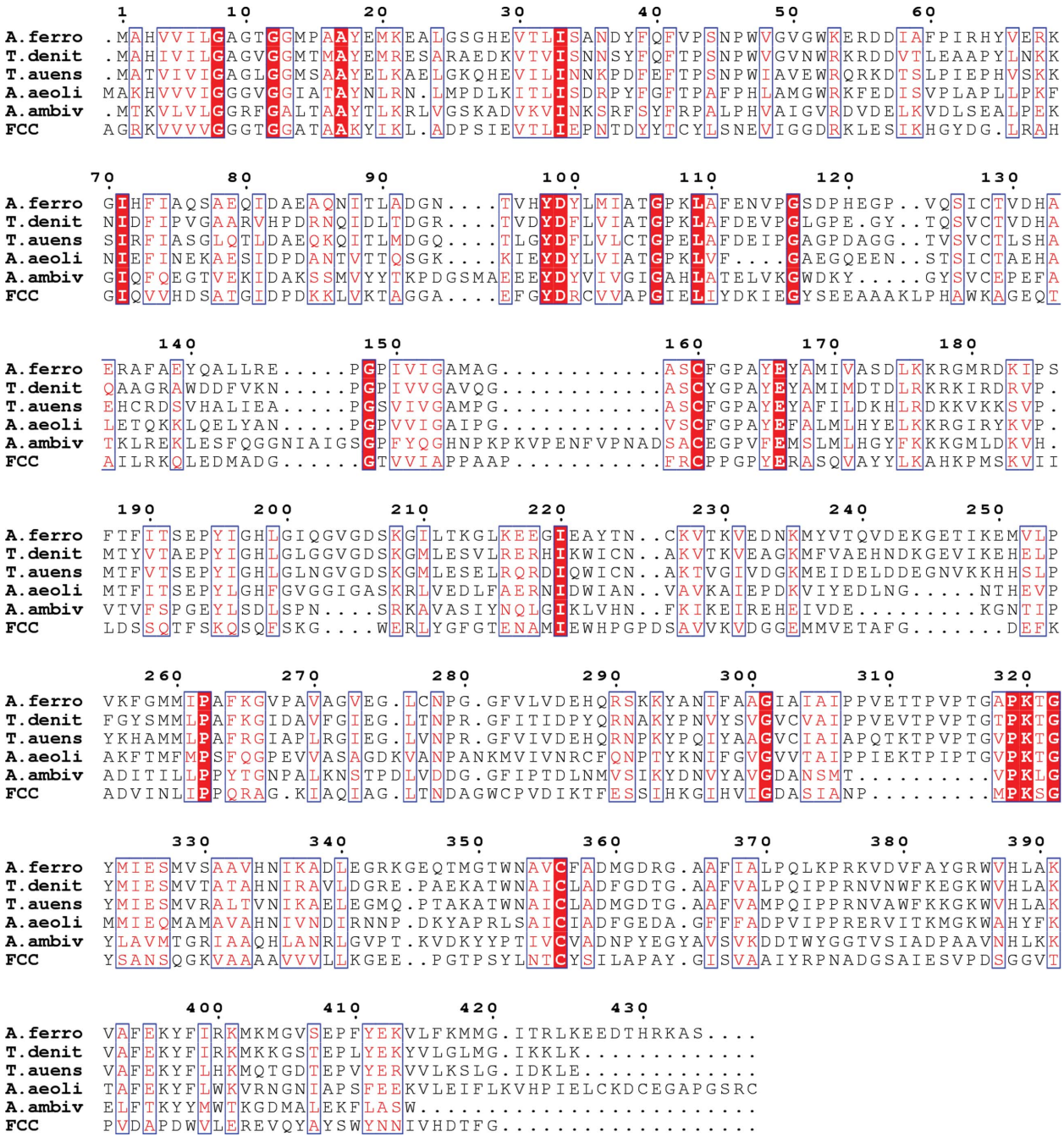


**Figure 1**
Sequence alignment of SQR from *A. ferrooxidans* (A.ferro) with SQRs from *Thiobacillus denitrificans* (T.denit), *Tolumonas auensis* (T.auens), *Aquifex aeolicus* (A.aeoli) and *Acidianus ambivalens* (A.ambiv) and FCC (flavocytochrome *c* sulfide dehydrogenase) from *Chromatium vinosum*. Conserved residues are shown on a red background, whereas residues with similar chemical properties are shown in red. The sequence identities between A.ferro and T.denit, T.auens, A.aeoli, A.ambiv and FCC are 57, 51, 40, 27 and 25%, respectively. Sequence alignment was performed using the program *ClustalW* (Thompson *et al.*, 1994) and the figure was generated with the program *ESPript* (Gouet *et al.*, 1999).

from Bio Basic Inc. according to the manufacturer's instructions for bacterial DNA extraction. The SQR open reading frame (ORF) was amplified by the polymerase chain reaction (PCR) using primers that were designed to add six continuous histidine codons to the 3′ primer. The sequence of the forward primer was 5′-CGCGCGGATCCAGGAGGAATTTAAAATGAGAGGATCGGCACATGTGGTAATTTTGGGTG-3′, containing a *Bam*HI site (GGATCC), a ribosome-binding site (AGGAGGA), codons for the amino-acid sequence MRGS (start codon) and codons for amino acids 2–8 of SQR. The sequence of the reverse primer was 5′-CTGCAGGTCGACTCAGTGATGGTGATGGTGATGGGAGGCCTTACGATGGGTATCTTCTT-3′, containing a *Sal*I site (GTCGAC), a stop anticodon (TTA) and anticodons for the hexa-His tag and the last eight amino acids of SQR. The resulting PCR product was gel purified, doubly digested and ligated into a pLM1 (Sodeoka *et al.*, 1993) expression vector, resulting in the pLM1::SQR plasmid.

The positive plasmid was confirmed by DNA-sequencing analysis (ABI23270) and transformed into *Escherichia coli* strain BL21 (DE3) competent cells for expression purposes. An overnight culture from a single colony was used to inoculate 2 l Terrific Broth (TB) supplemented with 100 mg ml$^{-1}$ ampicillin. Shaking of the culture at 310 K was continued until its $OD_{600\,nm}$ reached 0.8. Subsequently, the temperature of the culture was shifted to 298 K and protein overexpression was induced with 0.3 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) for 22 h. Production of the selenomethionine-substituted protein followed a published procedure (Van Duyne *et al.*, 1993). Cells were harvested by centrifugation and resuspended in 40 ml buffer *A* (50 m*M* MOPS pH 7.0, 300 m*M* NaCl, 5 m*M* *β*-mercaptoethanol, 200 µ*M* PMSF). The cells were ruptured in an EmulsiFlex high-pressure cell homogenizer (Avestin) at ∼103 MPa and ultracentrifuged at 40 000 rev min$^{-1}$ for 90 min at 277 K. The supernatant was filtered through a 0.45 µm filter (Sartorius) and loaded onto a 5 ml Hi-Trap column (GE Healthcare) pre-equilibrated with buffer *A*. The column was washed with wash buffer (50 m*M* MOPS pH 7.0, 300 m*M* NaCl, 5 m*M* *β*-mercaptoethanol, 200 µ*M* PMSF, 150 m*M* imidazole) and the His-tagged protein was eluted in the same buffer with the inclusion of 300 m*M* imidazole and was precipitated by the addition of ammonium sulfate to 70% saturation. Dissolved precipitate was loaded onto a Superdex 200 gel-filtration column (GE Healthcare) and eluted in a suitable buffer for crystallization (5 m*M* Tris–HCl pH 7.0, 300 m*M* NaCl, 1 m*M* DTT). The resulting solution was concentrated to 5 mg ml$^{-1}$ using a YM-30 Centricon tube (Amicon). All steps of protein purification subsequent to the initial thawing of cells were performed at 277 K and the result of each step was monitored by 12% SDS–PAGE (Laemmli, 1970).

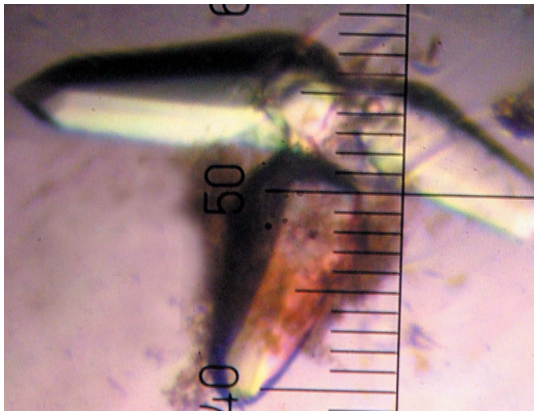

**Figure 2**
Tetragonal crystals of SQR from *A. ferrooxidans*.

### 2.2. Crystallization, data collection and preliminary crystallographic analysis

The search for crystallization conditions for SQR from *A. ferrooxidans* was performed by the sitting-drop vapour-diffusion method in Intelli-Plates (Hampton Research) using a crystallization robot (Hydra96 Plus One, Robbins Scientific) and the commercially available Crystal Screens I and II and Index Screen (Hampton Research). Equal volumes (0.3 µl) of protein and reservoir solutions were mixed and equilibrated against the reservoir solutions at room temperature. Several crystallization conditions were found in the initial screens. After optimization of the best screening conditions, X-ray diffraction-quality tetragonal crystals grew in hanging drops from 30% PEG 600, 0.1 *M* bis-tris pH 5.5, 0.1 *M* ammonium sulfate in 1–2 d (Fig. 2). For data collection, the crystals were flash-cooled in liquid nitrogen. Native data were collected on beamline 9-2 at the Stanford Synchrotron Radiation Laboratory (SSRL) using a MAR 325 image-plate detector (Fig. 3). The raw data were processed using the *HKL*2000 program suite (Otwinowski & Minor, 1997). The crystal structure was solved by molecular replacement using *MOLREP* (Vagin & Teplyakov, 1997; Collaborative Computational Project, Number 4, 1994) and *Phaser* (McCoy *et al.*, 2005). A truncated molecule *A* (residues 2–375) of the SQR trimer from *Aquifex aeolicus* (PDB code 3h27; 40% sequence identity) was used as a search model.

## 3. Results

A complete X-ray diffraction data set was collected from a single crystal at 100 K without additional cryoprotection. The crystals belonged to space group $P4_22_12$, with unit-cell parameters $a = b = 131.7$, $c = 208.8$ Å. The crystallographic statistics of the native data are summarized in Table 1. The data set was 100% complete to 2.3 Å resolution. The full-length SQR molecule contains 434 amino-

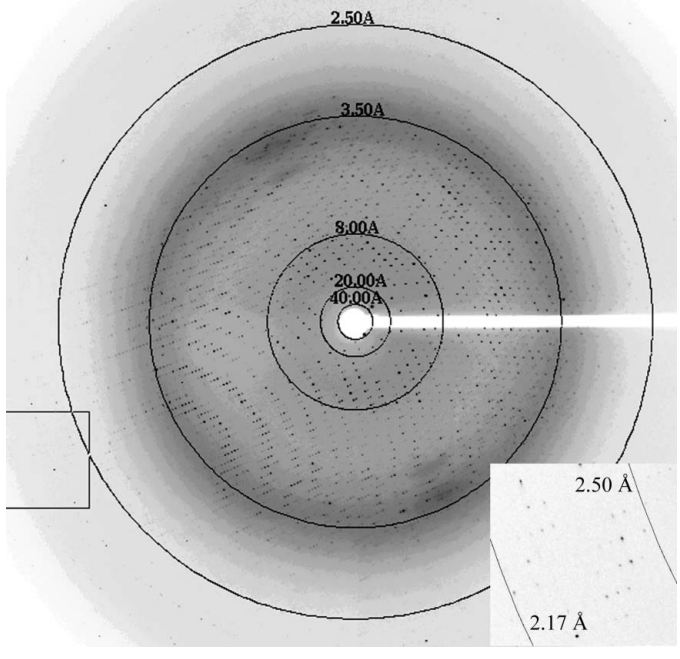


**Figure 3**
A 0.5° oscillation image of diffraction from the SQR crystal. Details of the boxed region are shown in the inset.

**Table 1**
Crystal parameters and data-collection statistics.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Space group | $P4_22_12$ |
| Unit-cell parameters (Å) | $a = b = 131.73$, $c = 208.86$ |
| No. of molecules in the unit cell ($Z$) | 16 |
| Solvent content (%) | 72.9 |
| Temperature (K) | 100 |
| Detector | MAR Q325 |
| Wavelength (Å) | 0.97946 |
| Resolution (Å) | 50.00–2.30 (2.38–2.30) |
| No. of unique reflections | 82166 (8046) |
| Multiplicity | 8.0 (7.6) |
| $\langle I/\sigma(I)\rangle$ | 19.5 (1.94) |
| Completeness (%) | 100 (100) |
| $R_{\rm merge}$† | 0.097 (0.986) |

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

acid residues and has a molecular mass of approximately 50 kDa. Calculation of the Matthews coefficient $V_{\rm M}$ (Matthews, 1968) suggested the presence of four or three molecules in the asymmetric unit, with $V_{\rm M}$ values of 2.26 Å$^3$ Da$^{-1}$ (45.7% solvent) or 3.02 Å$^3$ Da$^{-1}$ (59.2% solvent), respectively. However, the molecular-replacement programs *MOLREP* and *Phaser* were only able to find two molecules in the asymmetric unit. Attempts to find a third or fourth molecule in the asymmetric unit failed. The correlation score calculated by *MOLREP* for the dimer had a value of 0.200. Similarly, the log-likelihood gain *Phaser*) for the dimer had a value of 354. The solution containing a dimer in the asymmetric unit has been accepted. As a result, the Matthews coefficient $V_{\rm M}$ is 4.53 Å$^3$ Da$^{-1}$ and the solvent content is 72.9%. These values are outside the usual values for the Matthews coefficient of between 1.62 and 3.53 Å$^3$ Da$^{-1}$. However, the high solvent content does not prevent the SQR crystals from diffracting to 2.3 Å resolution. Structure refinement and detailed structural analysis of SQR are in progress.

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