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FOR THE RECORD

Preliminary crystallographic analysis of the oxidized form of a two mono-nuclear iron centres protein from *Desulfovibrio desulfuricans* ATCC 27774

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Abstract: Crystals of the fully oxidized form of desulfoferrodoxin were obtained by vapor diffusion from a solution containing 20% PEG 4000, 0.1 M HEPES buffer, pH 7.5, and 0.2 M CaCl₂. Trigonal and/or rectangular prisms could be obtained, depending on the temperature used for the crystal growth. Trigonal prisms belong to the rhombohedral space group R32, with a = 112.5 Å and c = 63.2 Å; rectangular prisms belong to the monoclinic space group C2, with a = 77.7 Å, b = 80.9 Å, c =53.9 Å, and $\beta = 98.1^{\circ}$. The crystallographic asymmetric unit of the rhombohedral crystal form contains one molecule. There are two molecules in the asymmetric unit of the monoclinic form, in agreement with the self-rotation function.

Keywords: crystallization; desulfoferrodoxin; non-heme iron proteins; sulfate reducing bacteria; X-ray diffraction

Desulfoferrodoxin (DFX), isolated from *Desulfovibrio desulfuricans* ATCC 27774 (Moura et al., 1990) is a monomeric protein with two mono-nuclear iron centers: one (center I) as in *D. gigas* desulforedoxin (DX), coordinated by four cysteinyl sulfur atoms in a distorted tetrahedral geometry of rubredoxin type, and another octahedrally coordinated (center II) with predominantly nitrogen/oxygen-containing ligands and probably one or two cysteinyl residues (Tavares et al., 1994). This protein, with molecular mass 13,383 Da, has 125 amino acid residues. Its primary sequence was determined recently (Devreese et al., 1995), confirming the existence of five cysteines. The first four cysteines, in positions 9, 12, 28, and 29 are in identical po-

sitions when compared with the amino acid sequence of DX. Two of those are consecutive residues, Cys 28 and Cys 29, and are believed to coordinate the iron atom of center I, as was found in the structure of *D. gigas* desulforedoxin (Archer et al., 1995). The structure determination of this protein has shown that, due to this fact, the distortion from a tetrahedral coordination geometry at that iron atom is more pronounced in DX than in rubredoxin (Dauter et al., 1992). A similar distortion is anticipated for the structure of DFX.

The physiological function of this protein is still unknown; one of the major drawbacks for that determination is that the redox potentials of its redox centers (+4 mV and +240 mV forcenters I and II, respectively) are barely compatible with the low redox potentials that are usually found in *Desulfovibrio* metabolic pathways. However, analogies of DFX center II with centers found in other enzymes such as nitrile hydratase (Tavares et al., 1994) have been noted. It is hoped that the establishment of DFX structure will help to clarify these relationships and allow the finding of new guidelines for the determination of its function.

The difference between the midpoint redox potentials of DFX centers I and II is such that it allows the separation of the protein in three oxidation states. In the fully oxidized state (grey form), both irons are in the ferric state. Upon one-electron reduction, a semi-reduced form (pink form) is obtained, corresponding to the reduction of center II. Finally, in the fully reduced state (colorless form), the two irons are in the ferrous state. It is possible to separate the two first forms by aerobic anionic exchange chromatography, which implies different isoelectric points for the grey and pink forms of DFX.

In this paper, we describe the crystallization conditions for the fully oxidized DFX, which crystallizes in two different crystal forms. Space group and cell dimensions are reported.

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Protein purification and crystallization: Cells were grown and the non-heme iron protein purified following the protocol described (Moura et al., 1990). The purity of the protein solution was checked by SDS-PAGE. The protein solution obtained is very stable, because it could be kept for several months at -20 °C.

Crystallization conditions were screened using the hanging drop vapor diffusion method, but large crystals, as described below, could only be grown using the sitting drop technique. The composition of the crystallization solution is: PEG 4000, 20% (w/v), 0.1 M HEPES buffer, pH 7.5, calcium chloride 0.2 M. Each drop was prepared by mixing 10 μ L of the protein solution with 5 μ L of the crystallization solution on a polystyrene microbridge (Harlos, 1992), and was equilibrated by vapor diffusion against 700 μ L of the solution contained in the reservoir of a Linbro tissue-culture plate. The well was covered with a plastic coverslip and sealed with silicone grease.

Single crystals could be grown at different temperatures (4, 10, 16, and 20 °C). However, if the temperature was not kept stable during the crystallization assays, only multiple crystals could be obtained. Different crystal forms appeared at different temperatures: trigonal prisms mainly at low temperatures (4 and 10 °C), rectangular prisms at 20 °C, and a mixture of both forms at the intermediate temperature of 16 °C.

For all crystal forms, 4–5 days after setting up the crystallization assays, a crystalline precipitate appears. After 2 more days, small crystals become visible and grow about a total of 15 days to reach their maximum size; meanwhile, the crystalline precipitate dissolves. If, however, the early process of crystal growth is disturbed (as, for example, by removing the coverslip and touching the drop with a needle), irregular thick plates are formed. These forms have been characterized, and the space group and cell dimensions are found be similar to those of the rectangular prisms obtained at 20 °C.

The quality and size of the trigonal prisms was systematically better than that of all other forms obtained. Thus, the subsequent X-ray analysis after the crystal characterization and data collection now reported will be concentrated only in the trigonal prisms grown at 4 °C.

The larger trigonal crystals were obtained using a concentration of the protein solution of 45 mg·mL⁻¹ ($0.4 \times 0.4 \times 1.0$ mm). However, smaller crystals could be obtained with a lower protein concentration ($16 \text{ mg} \cdot \text{mL}^{-1}$). The presence of CaCl₂ is essential for the crystal formation, and the increase of salt concentration up to 0.2 M corresponds to an increase in the crystal size, which is not continued if the salt concentration is further increased.

The morphology of the trigonal and rectangular crystal forms is illustrated in Figure 1.

X-ray analysis: The diffraction data for the rhombohedral crystals were collected at station 9.5 at DRAL in Daresbury using synchrotron radiation of wavelength 0.994 Å and a small MAR-Research image-plate area detector. For the monoclinic crystals, the diffraction data were collected at the Laboratory of Molecular Biophysics (Oxford, UK), using a Rigaku rotating anode, operated at 4.2 kW (60 kV/70 mA) and equipped with a graphite monochromator, using Cu-K α ($\lambda = 1.5418$ Å). A small MAR-Research image-plate detector was also used. Data were processed with DENZO and its companion program SCALE-PACK (Otwinowski, 1993); further processing was done with the CCP4 package (CCP4, 1994).

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Fig. 1. Crystals of desulfoferrodoxin from *D. desulfuricans* ATCC 27774. A: C2 form. B: R32 form. Approximate length of the largest R32 crystal is 1.0 mm.

Trigonal prism crystals diffracted up to 1.7 Å resolution, but data was statistically significant only to 1.8 Å resolution ($R_{sym} =$ 4.7%, completeness = 94.8%). They belong to the rhombohedral space group R32, with a = 112.5 Å and c = 63.2 Å. Assuming one molecule per asymmetric unit, gives a volume per unit of molecular weight (Vm) of 2.75 Å³/Da, corresponding to 55% solvent content. This is within the normal range observed for protein crystals (Matthews, 1968). The rectangular prisms belong to the monoclinic space group C2, with a = 77.7 Å, b = 80.9 Å, c = 53.9 Å, and $\beta = 98.1^{\circ}$, and diffracted significantly only to 2.2 Å resolution (R_{sym} 8.7%, completeness = 90.6%). To obtain a Vm value for the C2 crystals within the normal range, two or three molecules can be assumed per asymmetric unit. A self-rotation function analysis (Rossmann & Blow, 1962) shows a strong peak, with a height of 6.0σ above background, in section $\kappa = 180^\circ$, at position $\psi = 50.0^\circ$ and $\phi = 40.9^\circ$. This is in agreement with a content of two molecules per asymmetric unit.

To obtain the phases for the calculation of the electronic density, several experiments involving the preparation of heavy atoms derivatives were performed. Although a great number of compounds with heavy atoms and different soaking conditions were tried, it was impossible to obtain satisfactory results. To overcome this problem, the multiple wavelength dispersion method of phase determination has been tried successfully. The determination of the iron atoms' positions and the calculation of electron density maps is currently underway.

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Crystallization of desulfoferrodoxin

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