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Crystallization and preliminary X-ray diffraction analysis of a [2Fe–2S] ferredoxin (FdVI) from *Rhodobacter capsulatus*

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A [2Fe–2S] ferredoxin found in the photosynthetic bacterium *Rhodobacter capsulatus* has been purified in recombinant form from *Escherichia coli*. This protein, called FdVI, resembles ferredoxins involved in iron–sulfur cluster biosynthesis in various prokaryotic and eukaryotic cells. Purified recombinant FdVI was recovered in high yields and appeared to be indistinguishable from the genuine *R. capsulatus* ferredoxin based on UV–visible absorption and EPR spectroscopy and mass spectrometry. FdVI has been crystallized in the oxidized state by a sitting-drop vapour-diffusion technique using sodium formate as precipitant. Seeding larger drops from a previous hanging-drop-grown small crystal resulted in the formation of long red–brown prismatic needles. Preliminary X-ray diffraction analysis indicated that FdVI crystals are orthorhombic and belong to the space group $P2_12_12_1$, with unit-cell parameters a = 45.87, b = 49.83, c = 54.29 Å.

1. Introduction

Six different soluble ferredoxins have been identified from the purple non-sulfur bacterium *Rhodobacter capsulatus*. Genetic and biochemical studies indicated that four of these ferredoxins, designated FdI, FdIII, FdIV and FdV, are involved in nitrogen fixation (Armengaud *et al.*, 1994; Grabau *et al.*, 1991; Jouanneau *et al.*, 1993, 1995; Saeki *et al.*, 1991; Schatt *et al.*, 1989). Two additional ferredoxins, FdII and FdVI, have been found to play essential metabolic roles in *R. capsulatus* (Armengaud *et al.*, 1997; Saeki *et al.*, 1991). FdVI is a [2Fe–2S] ferredoxin (Naud *et al.*, 1994), sharing 40–60% amino-acid sequence identity with ferredoxins serving as electron carriers to monooxygenases (Nagy *et al.*, 1995; Peterson *et al.*, 1990, 1992; Wang *et al.*, 1995) or to ring-hydroxylating dioxygenases (Armengaud *et al.*, 2000; Armengaud & Timmis, 1997). These ferredoxins are generally involved in the degradation of aromatic or polycyclic hydrocarbons in *Pseudomonas* or *Sphingomonas* species. It appears unlikely that *R. capsulatus* FdVI participates in such a catabolic pathway, as this bacterium cannot utilize aromatic compounds such as benzoic acid as a carbon source. In addition, the gene encoding FdVI is not flanked by genes known to be responsible for aromatic hydrocarbon degradation (Armengaud *et al.*, 1997). Alternatively, FdVI might be involved in iron–sulfur cluster biosynthesis. Indeed, significant sequence similarities were observed between FdVI and ferredoxins found in *E. coli* (Takahashi & Nakamura, 1999), *Azotobacter vinelandii* (Jung *et al.*, 1999; Zheng *et al.*, 1998) and yeast (Lange *et al.*, 2000) whose common function is related to Fe–S cluster biogenesis. The yeast and *A. vinelandii* ferredoxins were found to be essential proteins for their respective host cells (Jung *et al.*, 1999; Lange *et al.*, 2000), as is the case for *R. capsulatus* FdVI. Also related to this subclass of ferredoxins are vertebrate adrenodoxins, which are implicated in the biosynthesis of steroid hormones (Coghlan & Vickery, 1989; Tanaka *et al.*, 1973).

Three-dimensional structures of several plant-type [2Fe–2S] ferredoxins have been described, including those from blue–green algae (Jacobson *et al.*, 1993; Tsukihara *et al.*, 1990) and from the archaebacterium *Haloarcula marismortui* (Frolow *et al.*, 1996). Structural models have been determined from NMR data for putidaredoxin (Pochapsky *et al.*, 1999; Pochapsky *et al.*, 1994) and terpredoxin (Mo *et al.*, 1999), two proteins which are phylogenetically distinct from plant ferredoxins. The crystal structure of a truncated version of bovine adrenodoxin (Adx4–108) has been solved (Müller *et al.*, 1998) and more recently the structure of the full-length protein has been reported (Pikuleva *et al.*, 2000). Current knowledge about this protein has recently been reviewed (Grinberg *et al.*, 2000).

There is currently no structural model for ferredoxins involved in the biosynthesis of Fe–S clusters. Although adrenodoxin is related to this group of ferredoxins, its structure may not constitute an appropriate model owing to its distinct function. In this context, we describe herein the purification and crystallization of *R. capsulatus* FdVI and a preli-

Table 1
Data-collection statistics.

Data set collected on the D2AM beamline at the ESRF. Numbers in parentheses correspond to data in the last resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	
a	45.87
b	49.83
c	54.29
Resolution range (Å)	15–2.30
Highest resolution shell (Å)	2.22–2.30
Asymmetric unit contents	Monomer
V_M (Å^3 Da^{-1})	2.68
No. of unique reflections	10050
Redundancy	2.7 (2.3)
Completeness (%)	90 (60)
R_{merge}† (all)	3.2 (21.4)
$\langle I\rangle/\sigma(I)$	18 (12)

† R_{merge} (all) = $\sum|I - \langle I\rangle|/\sum\langle I\rangle$.

minary X-ray diffraction analysis of the protein crystals.

2. Materials and methods

2.1. Expression of *fdxE* and purification of FdVI

The *fdxE* coding sequence specifying FdVI from *R. capsulatus* was isolated from plasmid pAJ65 (Armengaud *et al.*, 1997) as an *Nde*I–*Bam*HI fragment and cloned into pET9a (Novagen) to give plasmid pAJ66. The resulting plasmid was subjected to DNA sequencing in order to ascertain the integrity of the nucleotide sequence. Overexpression of *fdxE* was carried out essentially as described previously using *E. coli* BL21(DE3) transformed with pAJ66 (Armengaud *et al.*, 1994).

The purification of recombinant FdVI was performed from packed cells equivalent to either 2.5 or 20 l cultures. The pellet was thawed on ice and resuspended in 100 m*M* Tris–HCl buffer pH 8.0 containing 15 m*M* EDTA. The cells were treated with lysozyme (0.2 mg ml^{-1}) for 25 min at 310 K and broken by sonication. The cell extract was supplemented with 15 m*M* $MgCl_2$ and 2.5 units ml^{-1} of Benzonase (Merck) and then centrifuged at 19 000*g* for 45 min. Subsequent steps were performed at 277 K. The supernatant was diluted twofold in 25 m*M* Tris–HCl pH 8.0 containing 1 m*M* EDTA (buffer *A*) and loaded onto a DEAE-cellulose column (DE52, Whatman). The brown ferredoxin fraction was eluted with buffer *A* containing 170 m*M* NaCl and then concentrated to approximatively 10 ml or less by ultrafiltration through a YM10 membrane (Amicon). This fraction was applied to a 110 × 2.5 cm gel-filtration column of Ultrogel AcA54 (Biosepra) and eluted at a flow rate of 0.5 ml min^{-1} with buffer *A* containing 100 m*M* NaCl. The ferredoxin fraction was diluted threefold in buffer *A* and applied to a 1.6 × 10 cm Q-hyperD ion-exchange column (Biosepra). The column was eluted at a flow rate of 2 ml min^{-1} with a 60 min linear gradient of 0–0.4 *M* NaCl in buffer *A* using a Kontron HPLC system. FdVI eluted at approximately 110 m*M* NaCl and was desalted by overnight dialysis against buffer *A*. The purified protein was stored in liquid nitrogen.

2.2. Crystallization and X-ray conditions for data collection

After numerous trials varying critical factors such as temperature, drop size and crystallization techniques, the optimal crystallization condition consisted of 10 mg ml^{-1} protein solution buffered with 100 m*M* imidazole pH 7.6 plus sufficient concentrated sodium formate to make the drop initially 5.4 *M* formate. The drop was then equilibrated against 7.0 *M* sodium formate containing 100 m*M* imidazole pH 7.6 at 288 K. To increase the crystal size in the thinnest direction of the needle, sitting drops with an optimal volume of 40 µl were produced. Diffraction-quality crystals were obtained at 288 K by vapour equilibration (sitting drop) by seeding much larger drops from a previous hanging-drop-grown needle crystal. The seed needles, washed briefly in imidazole-buffered 3.5–4.2 *M* formate, produced long red–brown prismatic crystals.

After several trials, useful cryoprotected crystals were produced by placing selected fragments (0.2–0.3 mm) of the long needle crystals into a cryosolution composed of 6 *M* formate, 87 m*M* imidazole pH 7.6 and 13% glycerol. The frozen crystals, mounted on a cryoloop, were tested and preliminary diffraction data were obtained on beamline D2AM at the ESRF.

3. Results and discussion

FdVI is expressed at a low level in *R. capsulatus*; its cellular content was estimated to be between 0.002 and 0.02% of the soluble proteins (Naud *et al.*, 1994). We therefore overexpressed the structural gene encoding FdVI in *E. coli* as a means of obtaining amounts of ferredoxin sufficient for detailed biochemical and structural studies. Extracts from *E. coli* strain BL21(DE3)(pAJ66) revealed the presence of a pink protein, suggesting that FdVI was produced in the holo form. A three-step purification protocol was developed to purify the recombinant ferredoxin, yielding approximately 13 mg of purified protein per litre of culture. As shown by N-terminal sequencing, recombinant FdVI starts with an alanine residue, indicating that the initial methionine had been removed, as is the case for the genuine *R. capsulatus* FdVI. To make sure that the recombinant protein had not undergone some unexpected modification, it was subjected to mass-spectrometric analysis. The apoferredoxin exhibited an experimental mass of 11 401 ± 3 Da in the positive mode, which matches the expected value calculated from the amino-acid sequence of FdVI to within 2 Da (Fig. 1). The mass of purified holoferredoxin (11 575 ± 3 Da) obtained in the negative mode (Fig. 1) is in perfect agreement with the theoretical value expected if the polypeptide contained one [2Fe–2S] cluster. The sample appeared homogeneous as no peaks other than those corresponding to the multiprotonated apoFdVI (8–12 positive charges) were detected in the 900–1500 *M*/*z* range (data not shown).

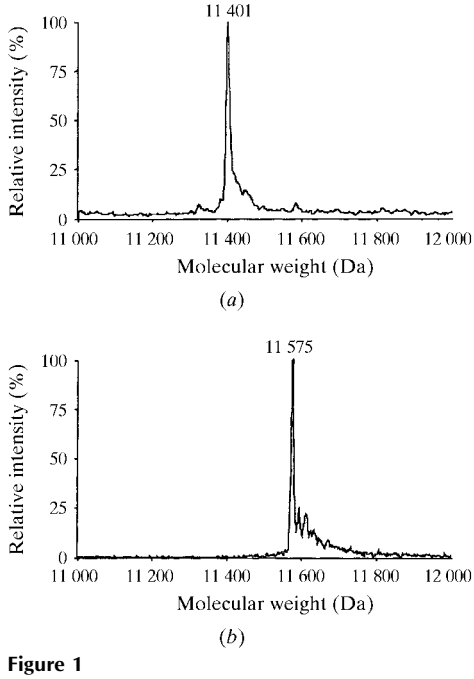

Figure 1
Molecular mass spectrum of recombinant FdVI obtained by electrospray-MS. The protein sample was dialyzed against 10 m*M* ammonium acetate pH 7.0. Mass-spectrometry analysis on apoferredoxin (*a*) and holoferredoxin (*b*) was carried out on a Perkin–Elmer Sciex API III triple-quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray source. The reconstructed molecular-mass profiles in the positive (*a*) and negative modes (*b*) were obtained by using a deconvolution algorithm by means of the *MacSpec* 3.3 *PE-Sciex API III* software.

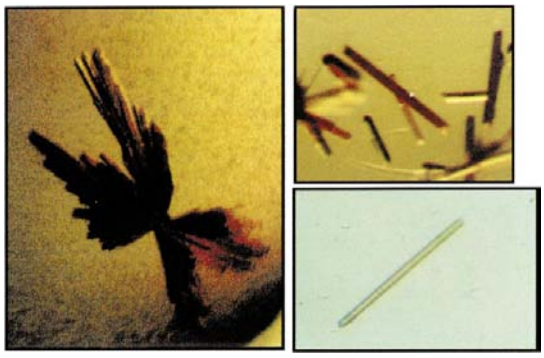

Figure 2
Recombinant FdVI crystals.

The absorbance spectrum of recombinant FdVI in the oxidized state exhibits three absorption peaks with maxima at 326, 414 and 456 nm, consistent with the presence of a [2Fe–2S] cluster in the ferredoxin (data not shown). An A_{414nm}/A_{280nm} value of 0.56 was obtained for the purest preparation. The EPR spectrum of reduced recombinant FdVI revealed an axial signal with g values at 1.936 and 2.022 (data not shown). These spectroscopic properties are identical to those previously reported for FdVI purified from *R. capsulatus* (Naud *et al.*, 1994).

To obtain 'good' diffracting crystals of FdVI, initial vapour-equilibration (hanging-drop) crystallization pH screens with ammonium sulfate and polyethylene glycols (PEGs) 4000 and 8000 were performed at different temperatures (281, 288 and 293 K). From these screens, the best temperature appeared to be 288 K over the pH range 6.5–8.5. Using a variety of salts and buffers within this pH range resulted in long thin hair-like crystals. The crystals obtained are red–brown in colour, indicating that the oxidized redox state of the protein is unmodified (Fig. 2). In many experiments, thousands of these needle crystals extended out from a single nucleating centre similar to the appearance of an artist's paint brush, indicating that the concentration of the protein should be reduced (Fig. 2). These experiments also indicated that some salts were better at producing crystals suitable for diffraction purposes. The experiments using PEG or 2-methyl-2,4-pentanediol (MPD) produced denatured-looking precipitate throughout the ranges examined. Finally, long crystals of recombinant FdVI (Fig. 2) were obtained using sodium formate as precipitant agent and using a seeding crystallization protocol to obtain much bigger crystals. To decrease radiation damage to the crystal during the data collection, the flash-freezing technique was used after brief soaking in a 13% glycerol cryosolution. Analysis of the frozen crystals mounted in a cryoloop was carried out using synchrotron radiation from beamline D2AM at the European Synchrotron Radiation Facility (ESRF, France). Data collection to 2.3 Å resolution was performed at the wavelength λ = 1 Å with a crystal-to-detector distance of 180 mm and an oscillation frame of 1° over an exposure time of 60 s. Data were processed using *DENZO* (Otwinoski & Minor, 1997). The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 45.87, b = 49.83, c = 54.29 Å. Relevant statistics of the data processing are summarized in Table 1. The ratio of the unit-cell volume to the molecular weight, V_M, is 2.68 Å^3 Da^{-1}, which is in the normal range for one molecule per asymmetric unit (Matthews, 1968).

Molecular-replacement trials using the program *AMoRe* (Navaza, 1994) were performed with different modifications of the adrenodoxin model (PDB code 1ayf). Despite several trials, the molecular replacement was unsuccessful. Additionally, ten different NMR models from putidaredoxin were used in *AMoRe* but unfortunately these produced no clear solution. Since FdVI contains a [2Fe–2S] cluster, a multi-wavelength anomalous dispersion (MAD) data collection on the iron edge will be scheduled.

Conditions for the crystallization of recombinant FdVI have been defined in the present study. The preliminary diffraction data indicate that this ferredoxin is amenable to three-dimensional structure determination.

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