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Crystallization and preliminary X-ray diffraction studies of the ISC-like [2Fe–2S] ferredoxin (FdxB) from *Pseudomonas putida* JCM 20004

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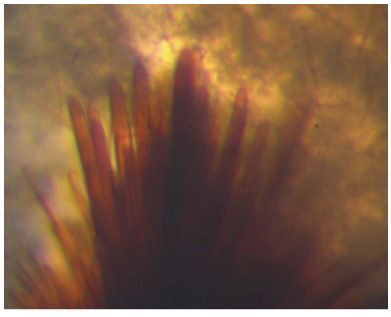


The iron–sulfur (Fe–S) cluster-biosynthesis (ISC) system of the γ-proteobacterium *Pseudomonas putida* JCM 20004 contains a constitutively expressed vertebrate-type [2Fe–2S] ferredoxin, FdxB, which lacks the conserved free cysteine residue near the Fe–S cluster site that has been proposed to function in the catalysis of biological Fe–S cluster assembly in other bacterial homologues. Recombinant FdxB was heterologously overproduced in *Escherichia coli*, purified and crystallized in its oxidized form by the hanging-drop vapour-diffusion and streak-seeding methods using 1.6 *M* trisodium citrate dihydrate pH 6.5. The thin needle-shaped crystals diffract to 1.90 Å resolution and belong to the hexagonal space group $P6_122$, with unit-cell parameters a = 87.58, c = 73.14 Å. The asymmetric unit contains one protein molecule.

1. Introduction

Biological iron–sulfur (Fe–S) clusters consisting of nonhaem Fe and inorganic S atoms have vital functions in electron transfer, substrate activation and environmental sensing and may be among the most ancient modular prosthetic groups (Beinert *et al.*, 1997; Beinert, 2000). A group of highly conserved proteins are responsible for directing the controlled assembly and maturation of Fe–S proteins under a reducing environment *in vivo* (Johnson *et al.*, 2005; Rouault & Tong, 2005). At least three types of Fe–S cluster-biosynthesis systems (NIF, ISC and SUF) are known and are unified by the involvement of cysteine desulfurases (such as IscS) and Fe–S cluster-scaffold proteins (such as IscU and IscA) with the capacity to construct transient [2Fe–2S] or [4Fe–4S] clusters and then transfer Fe–S clusters to target apoproteins (Johnson *et al.*, 2005; Fig. 1). The ISC machinery has been closely studied and microbial whole-genome sequence analysis has revealed the conserved genetic context of *iscS*, *iscU*, *iscA*, *hscB*, *hscA* and *fdx* (Fig. 1). However, owing to the complicated network of their possible interactions in various combinations (Tokumoto *et al.*, 2002), the sequential association and possibly dissociation events among their products, especially those concerning Fdx (an ISC-like [2Fe–2S] ferredoxin), HscA and HscB (the heat-shock chaperone DnaK and cochaperone DnaJ homologues, respectively), remain elusive. A deeper understanding of their functionalities through specific protein–protein binding would require a knowledge of structural information at atomic resolution.

Pseudomonas putida JCM 20004 (formerly *P. ovalis* IAM 1002) is a nonpathogenic γ-proteobacterium and contains a bacterial-type ferredoxin, having one $[3Fe–4S]^{1+,0}$ cluster and one $[4Fe–4S]^{2+,1+}$ cluster as the dominant species in the cytoplasm (Ohmori, 1984). It also contains a constitutively produced vertebrate-type [2Fe–2S] ferredoxin, FdxB, as a minor species with a probable housekeeping role (Ohmori *et al.*, 1989). Recent genetic and biochemical analyses have established that FdxB is encoded by the *fdxB* gene as a constituent of the cognate *isc* gene cluster (DDBJ/EMBL/GenBank code AB109467), the products of which are probably involved in the ISC machinery (Johnson *et al.*, 2005; Nelson *et al.*, 2002; Fig. 1). This 13 kDa adrenodoxin-like protein (Ohmori *et al.*, 1989) is unusual in that it inherently lacks the conserved free cysteine residue near the cluster-binding site (Fig. 1) that has been proposed to provide, through its solvent-exposed thiol group, a potential ligand for an S

atom or an Fe^{3+} ion during Fe–S cluster assembly in other ISC-like homologues, *e.g.* those from *Escherichia coli* (PDB code 1i7h; Kakuta *et al.*, 2001) and *Rhodobacter capsulatus* (PDB codes 1e9m and 1uwm; Sainz *et al.*, 2006). Interestingly, the equivalent cysteine residue is also conserved in the *Saccharomyces cerevisiae* mitochondrial homologue YAH1/YPL252C (Barros & Nobrega, 1999), which is involved not only in Fe–S protein biogenesis but also in haem A biosynthesis (Lill & Mühlenhoff, 2005). Recombinant FdxB has been overproduced in *E. coli* and can be obtained in appropriate forms for stable-isotope (*e.g.* ^{2}H, ^{15}N and ^{57}Fe) labelling and mutagenesis in order to elucidate its mechanistic action in the ISC system by using advanced pulsed electron paramagnetic resonance (EPR) techniques (in preparation). In this regard, further structural characterization of *P. putida* FdxB is indispensable. Here, we present the crystallization of the recombinant FdxB in a form suitable for high-resolution X-ray studies and preliminary X-ray data analysis.

2. Methods and results

2.1. Protein preparation and characterization

The *fdxB* gene coding for the ISC-like [2Fe–2S] ferredoxin (FdxB) of *P. putida* JCM 20004 (formerly *P. ovalis* IAM 1002; Ohmori *et al.*, 1989) has been cloned and sequenced as a part of its *isc* gene cluster (DDBJ/EMBL/GenBank code AB109467; Fig. 1) and heterologously overexpressed in *E. coli* BL21-CodonPlus(DE3)-RIL strain (Stratagene) using a pET28aFDXB-SG vector (based on a pET28a His-tag expression vector, Novagen) harbouring the *fdxB* gene (D. Ohmori, A. Kounosu & T. Iwasaki, unpublished results). The transformants were grown overnight at 298 K in Luria–Bertani medium containing 50 µg ml^{-1} kanamycin and 0.2 m*M* $FeCl_3$ and the recombinant holoprotein was overproduced with 1 m*M* isopropyl β-D-1-thio-galactopyranoside for 24 h at 298 K. The cells were pelleted by centrifugation and the recombinant FdxB with a hexahistidine tag plus a thrombin cleavage site and an engineered extra Ser-Gly linker at the N-terminus was purified at 277 K essentially as reported previously for archaeal Rieske [2Fe–2S] proteins (Kounosu *et al.*, 2004; Iwasaki *et al.*, 2004), except that heat treatment of the crude cell lysate was omitted. After proteolytic removal of the hexahistidine tag from the purified recombinant FdxB for 16–22 h at 297 K using a Thrombin Cleavage Capture Kit (Novagen) according to the manufacturer's instructions, the sample was further purified by gel-filtration chromatography (Sephadex G-75; Amersham Pharmacia Biotech), eluted with 10 m*M* Tris–HCl, 350 m*M* NaCl pH 7.5, concentrated to ~2–3.5 m*M* with a Centriprep-10 apparatus (Amicon) and stored frozen (193 K) until use.

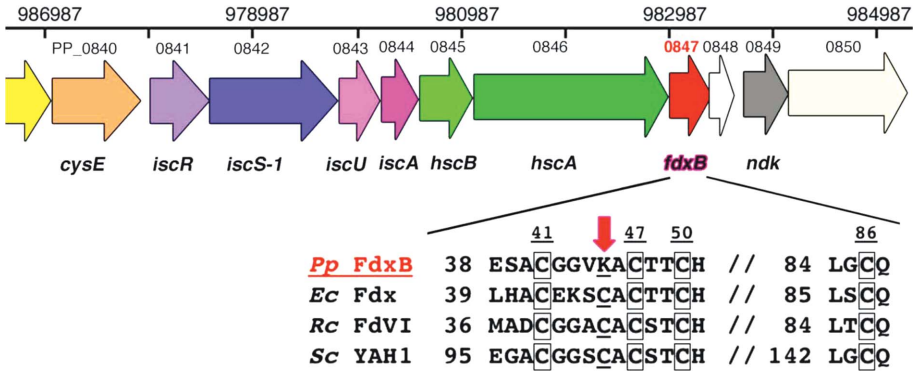
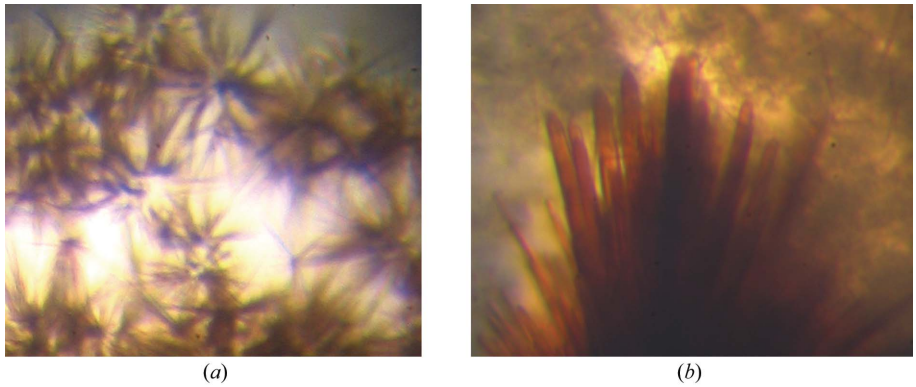


Figure 1
Schematic organization of the *isc* gene cluster, the products of which are involved in the Fe–S cluster-biosynthesis machinery, in the whole-genomic DNA sequence of *P. putida* KT2440 (Nelson *et al.*, 2002). Top, a close relative of strain JCM 20004 used in this work [the primary sequence of *P. putida* JCM 20004 FdxB (DDBJ/EMBL/GenBank code AB109467) is completely identical to that of *P. putida* KT2440 open reading frame PP_0847]. There is no direct information concerning the specific function of the products of the *hscB*, *hscA* and *fdx* genes in the Fe–S protein-maturation process (Johnson *et al.*, 2005), although protein–protein association analysis of the *E. coli* ISC components suggests direct interactions between Fdx and IscS as well as between Fdx and HscA (Tokumoto *et al.*, 2002). Multiple sequence alignment of selected ISC-like [2Fe–2S] ferredoxins (bottom) indicates that the cluster-binding site of FdxB (bottom) lacks the conserved cysteine residue (underlined) near the [2Fe–2S] cluster (indicated by a red arrow) that has been proposed to provide a potential ligand for an S atom or an Fe^{3+} ion during the action of the ISC machinery (Kakuta *et al.*, 2001). Accession Nos.: *P. putida* JCM 20004 (*Pp*) FdxB, DDBJ/EMBL/GenBank code BAD01054; *E. coli* (*Ec*) Fdx, PDB code 1i7h; *R. capsulatus* (*Rc*) FdVI, PDB codes 1e9m and 1uwm; *S. cerevisiae* (*Sc*) YAH1, GenBank code NP_015071. The metal-binding motifs are boxed.



(*a*) (*b*)

Figure 2
Typical crystals of recombinant FdxB obtained (*a*) before and (*b*) after streak-seeding. The maximum dimensions of the thin needle-shaped crystals (*b*, left) are approximately 0.025 × 0.025 × 0.3 mm.

Table 1
Data-processing statistics.

Values in parentheses are for the outer shell.

Space group	$P6_122$
Unit-cell parameters (Å)	a = 87.58, c = 73.14
Resolution range (Å)	75.81–1.90 (1.97–1.90)
No. of measured reflections	121620
No. of unique reflections	13390 (1285)
Completeness (%)	98.6 (97.8)
$R_{\rm merge}$† (%)	11.6 (35.0)
$\langle I/\sigma(I)\rangle$	8.7

† $R_{\rm merge} = \sum_{hkl}\sum_j |I_j(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_j \langle I(hkl)\rangle$, where $I_j(hkl)$ and $\langle I(hkl)\rangle$ are the intensity of measurement j and the mean intensity for the reflection with indices hkl, respectively.

The purified recombinant FdxB contains five extra residues (His-Met-Ser-Gly-Met) at the N-terminus, which were engineered to facilitate rapid purification and are absent in the native FdxB isolated from *P. putida* JCM 20004 (DDBJ/EMBL/GenBank code BAD01054; Ohmori *et al.*, 1989). The room-temperature visible absorption (measured using a Beckman DU-7400 spectrophotometer), visible–near-UV circular dichroism (CD; measured using a JASCO J720 spectropolarimeter with 0.5 cm cells) and low-temperature EPR ($g_{\|}$ = 2.02, $g_{\perp}$ = 1.94, measured at 9 K using a Jeol X-band JES-FA300 spectrometer equipped with an ES-CT470 Heli-Tran cryostat system and a Scientific Instruments digital temperature indicator/controller Model 9650) spectra of the recombinant FdxB (data not shown) were identical to those reported previously for the native protein (Ohmori *et al.*, 1989), showing the presence of a vertebrate-type $[2Fe–2S]^{2+,1+}$ cluster.

2.2. Crystallization

Preliminary screening was by standard hanging-drop vapour diffusion in Linbro plates at 277–293 K with 0.45 ml reservoirs of commercially available sparse-matrix screening kits (Hampton Research Crystal Screens I and II). Three conditions were found from which tiny sea urchin-like clusters of needle-shaped crystals could be obtained in 2–6 d at 293 K. For streak-seeding, the clusters of needles (Fig. 2*a*) obtained under aerobic conditions in 2–4 d at 293 K by combining 1.0–2.0 µl protein solution with 1.0–2.0 µl reservoir solution containing 1.6 *M* trisodium citrate dihydrate pH 6.5 were touched with a human hair and introduced into fresh protein crystallization drops (pre-equilibrated against the same reservoir solution for 1 d at 277 K) by streaking the hair in a straight line through the drops. Small needle-shaped crystals were obtained in 1–3 d at 277 K. Although the larger crystals, which grew to maximal dimensions of 0.04 × 0.04 × 0.5 mm in about one month at 277 K (Fig. 2*b*, middle), were highly sensitive to temperature change, thereby causing great difficulty in further manipulation, some of the resulting brown thin needle-shaped crystals with dimensions of ∼0.025 × 0.025 × 0.3 mm (Fig. 2*b*, left) could be transferred into a cryoprotective solution containing 10%(*v*/*v*) glycerol in the same reservoir solution for flash-cooling in liquid nitrogen.

2.3. Crystallographic data collection and processing

X-ray diffraction data of recombinant FdxB were collected from flash-frozen thin needle-shaped crystals using an ADSC Quantum 315 CCD detector installed on the BL41XU beamline at SPring-8, Japan. Data collection was performed with a total oscillation range of 120° and each diffraction image was obtained with an oscillation angle of 1.0° and an exposure time of 5 s. The thin needle-shaped crystals (Fig. 2*b*) were found to diffract to 1.90 Å resolution and to belong to the hexagonal space group $P6_122$, with unit-cell parameters a = 87.58, c = 73.14 Å (Table 1). Assuming the presence of one protein molecule per asymmetric unit, the Matthews coefficient is 3.3 Å^3 Da^{-1}, corresponding to a solvent content of 63% (Matthews, 1968).

Phase determination was successfully carried out by the molecular-replacement method from the atomic model of *E. coli* Fdx (PDB code 1i7h; Kakuta *et al.*, 2001) using the program *MOLREP* (Vagin & Teplyakov, 1997; Table 1). Construction, revision and analysis of atomic models using the FdxB sequence are currently in progress.

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