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Crystallization and preliminary X-ray crystallographic analysis of a periplasmic tetrahaem flavocytochrome c_3 from *Shewanella frigidimarina* NCIMB400 which has fumarate reductase activity

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The fumarate reductase of *Escherichia coli* and other bacteria is a membrane-bound enzyme consisting of four subunits. A soluble periplasmic 64 kDa tetrahaem flavocytochrome c_3 from *Shewanella frigidimarina* NCIMB400 which possesses a catalytic fumarate reductase activity has been crystallized. The crystals belong to space group $P2_12_12_1$ with unit-cell parameters $a = 72.4$, $b = 110.1$, $c = 230.2$ Å. Assuming a molecular dimer in the asymmetric unit, the crystals contain 65% solvent and, when cryocooled to 100 K, the crystals diffract to at least 3.0 Å resolution. The crystals, however, display an inherent lack of isomorphism and the plausibility of a MAD phasing experiment has therefore been investigated by measuring the iron *K* absorption edge from a single crystal.

1. Introduction

Fumarate may act as the terminal electron acceptor in the respiratory pathways of many facultative anaerobes. The fumarate reductase of *Escherichia coli* and other bacteria is a membrane-bound enzyme of four subunits encoded by the *frd* operon (Cole *et al.*, 1982). A 69 kDa subunit encoded by *frdA* contains covalently bound flavin and provides the active site for fumarate reduction (Cole, 1982). The product of the *frdB* gene is a 27 kDa iron–sulfur protein which together with the flavoprotein forms a catalytic complex anchored to the membrane by the remaining two smaller subunits. *Shewanelda frigidimarina* (formerly *S. putrefaciens*) NCIMB400 is a Gram-negative bacterium which produces numerous periplasmic *c*-type cytochromes when grown under anaerobic conditions (Morris *et al.*, 1990). One of these is a 64 kDa tetrahaem flavocytochrome *c*3, Fcc_3, which contains bis-His-ligated c_3-haems and non-covalently bound FAD (Pealing *et al.*, 1995). Fcc_3 has been demonstrated to be the only physiological fumarate reductase present in *S. frigidimarina* NCIMB400 (Gordon *et al.*, 1998). The sequence of the gene encoding Fcc_3 reveals that this soluble enzyme is related to the catalytic subunit of membrane-bound fumarate reductases from other bacteria (Pealing *et al.*, 1992).

Recently, a second periplasmic tetrahaem flavocytochrome c_3, Ifc_3, has been purified from *S. frigidimarina* NCIMB400 grown anaerobically with soluble Fe^{III} as the sole available terminal electron acceptor (Dobbin *et al.*, 1999). Ifc_3 also features bis-His-ligated c_3-haems and non-covalently bound FAD, and moreover possesses a fumarate reductase activity equivalent to that of the physiological enzyme Fcc_3. However, Ifc_3 is not expressed during anaerobic growth of the bacterium on fumarate. Primary structure analysis for Fcc_3 and Ifc_3 reveals 43% identity and 53% similarity. Ifc_3 is thus also homologous to the membrane-bound *E. coli* fumarate reductase catalytic subunit, and the protein provides an alternative target for structural studies of fumarate reduction.

In this paper, we report the crystallization and preliminary X-ray crystallographic analysis of the periplasmic Fe^{III}-induced flavocytochrome c_3, Ifc_3, from *S. frigidimarina* NCIMB400 and demonstrate the suitability of this crystal form for structure determination by MAD phasing by measuring the X-ray fluorescence spectrum around the iron *K* edge.

2. Materials and methods

2.1. Protein purification

Periplasmic Fe^{III}-induced flavocytochrome c_3, Ifc_3, was produced by *S. frigidimarina* during anaerobic growth in the presence of 50 m*M* Fe^{III} citrate. The protein was purifed according to the protocol developed by Dobbin *et al.* (1999).

2.2. Crystallization and crystal characterization

Protein solution in 50 m*M* Na HEPES pH 7.5 and 100 m*M* NaCl was concentrated to 10–15 mg ml^{-1} using an Amicon diafiltration device at 277 K. Initial crystallization screening experiments were performed at 277 K and 291 K using the screens of Jancarik & Kim (1991) and Cudney *et al.* (1994), using hanging-

drop vapour diffusion in 4 µl drops containing equal volumes of concentrated protein solution and screen solution. Each hanging drop was equilibrated against 1 ml reservoir solution. Of the several screen solutions which gave growth of single crystals, that containing 12%(*w*/*v*) PEG 20K and 100 m*M* Na MES pH 6.5 at 291 K was chosen for further optimization. As reproducibility of crystallization using PEG 20K rapidly became obvious, a further screening of precipitant molecular weight was carried out. Consequently, hanging-drop vapour-diffusion experiments were performed using 6–15%(*w*/*v*) PEG in the average molecular-weight range 4–17.5K. These experiments were buffered using Na MES in the pH range 5.5–7.5. Attempts were made to improve crystal size and quality by growth in agarose gels according to the protocol described by Robert *et al.* (1992). Agarose (Sigma) of gelling temperatures in the range 290–309 K and of gel strengths in the range 100–650 g cm^2 [for 1%(*w*/*v*) gels] was used as an additive in these experiments at concentrations of 0.05–0.2%(*w*/*v*). For cryoprotection experiments, crystals were soaked in solutions containing 25%(*v*/*v*) ethylene glycol, 100 m*M* Na MES pH 6.5 and a concentration of the appropriate PEG which was 2%(*w*/*v*) greater than that used in the reservoir of the crystallization experiment in which the crystals grew. Cryocooling was attempted by mounting single crystals in cryo-loops (Hampton Research, CA) followed by either immediate rapid immersion in a nitrogen-gas stream at 100 K, by prior immersion in liquid nitrogen or by immersing crystals in liquid-nitrogen-cooled liquid propane and then in liquid nitrogen. Preliminary diffraction trials using either room-temperature or cryocooled crystals were carried out using a Rigaku R-AXIS IV area detector and an Oxford Cryosystems Cryostream cryocooler operated at 100 K on an RU-200HB rotating copper-anode generator with focusing mirror optics operated at 50 kV, 100 mA. Room-temperature experiments were performed using crystals mounted in 0.7 mm diameter glass capillaries and sealed with wax.

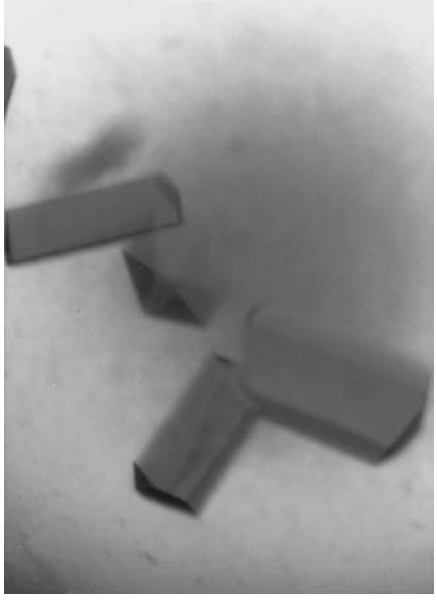


Figure 1
Crystals of native periplasmic Ifc$_3$ from *S. frigidimarina*. These crystals were grown using PEG 20K as precipitant. The largest crystal shown has approximate dimensions 400 × 200 × 100 µm. Crystals of similar morphology grew from solutions containing PEG 8, 10, 12 and 17.5K as precipitant.

2.3. Heavy-atom soaks and EDAX

Soaking experiments were carried out by placing single crystals of Ifc$_3$ in 20 µl solutions of heavy-atom salts at varying concentrations in sitting-drop vapour-diffusion experiments at 291 K. Energy-dispersive analysis of X-rays (EDAX) experiments were undertaken using a Jeol 2000 EX transmission electron microscope operated at 100 kV fitted with an Oxford Microanalytical SATW energy dispersive X-ray detector (136 eV resolution). A single crystal of native or heavy-atom treated Ifc$_3$ was washed three times with heavy-atom-free well solution and placed on a graphite-coated grid using a cryo-loop before being placed in the electron microscope.

2.4. X-ray fluorescence spectra and diffraction data collection

X-ray fluorescence spectra and diffraction data were measured on station BM14 at the ESRF (Grenoble, France) using a single crystal of Ifc$_3$ grown from 6%(*w*/*v*) PEG 17.5K, 100 m*M* Na MES pH 6.5 and soaked in 8%(*w*/*v*) PEG 17.5K, 100 m*M* Na MES pH 6.5 and 25%(*v*/*v*) ethylene glycol. Calculation of anomalous scattering factors from the raw fluorescence spectrum was performed using the *CHOOCH* program (Evans, 1998). For diffraction data collection, the crystal was preoriented to place the crystallographic *a** axis approximately parallel to the camera φ axis, and non-overlapping 0.5° oscillations were recorded on a 345 mm MAR imaging-plate system at an X-ray energy of 12 keV (1.033 Å) and a crystal-to-detector distance of 480 mm. Indexing of images and data processing were performed using the *HKL* program suite (Otwinowski & Minor, 1997). Programs from the *CCP*4 program library were used for the preparation of reflection files (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Crystallization trials using standard screens in vapour-diffusion experiments yielded an appreciable number of conditions producing Ifc$_3$ crystals of varying quality. The most promising of these yielded large red single crystals at 291 K using 12%(*w*/*v*) PEG 20K as precipitant buffered with 100 m*M* Na MES at pH 6.5 (Fig. 1). SDS–PAGE analysis of serially washed crystals showed a single band of the expected apparent molecular mass (data not shown). The enzyme thus appears to be stable to proteolytic degradation during the course of crystallization. Preliminary X-ray crystallographic characterization was performed using this crystal form at room temperature. The Bravais lattice is primitive orthorhombic and has unit-cell parameters a = 72.4, b = 110.1, c = 230.2 Å. Assuming the usual crystal solvent-content ranges, there may be two or three molecules in the asymmetric unit. This corresponds to 65 or 38% solvent content, respectively. However, the crystals displayed sensitivity to radiation damage, allowing only approximately 15° of data to be collected from any one crystal. Subsequent attempts to reproduce crystals from PEG 20K were unsuccessful and so further crystallization screening was performed, varying the PEG average molecular weight in the range 4–17.5K. Large single crystals grew from all the PEG solutions used (4, 8, 10, 12 and 17.5K). Growth in agarose gels did not result in any significant improvement in crystal quality.

Cryoprotection experiments were performed on crystals grown from each of the PEG solutions using various cryoprotectants. Interestingly, good-quality diffraction could only be obtained from crystals grown from PEG 17.5K and then only when these crystals were directly immersed in the nitrogen-gas stream at 100 K. The optimal conditions involved presoaking crystals in 25%(*v*/*v*) ethylene glycol and a concentration of PEG 17.5K 2%(*w*/*v*) greater than that used in the reservoir solution. It was not possible to freeze crystals by direct immersion in liquid nitrogen. Attempts to achieve useful diffraction from crystals grown from other average molecular-weight PEG solutions by prior immersion in a liquid-nitrogen-cooled liquid propane solution prior to immersion in liquid nitrogen were also unsuccessful. From the results of these experiments, it appears that crystals of Ifc$_3$

are sensitive in the chosen cryoprotectant solution to liquid-nitrogen temperatures (88 K). Cryoprotection extended the lifetime of the crystals when exposed to X-ray radiation on our rotating-anode X-ray source, allowing us to collect complete diffraction data sets. It rapidly became clear, however, that the crystals grown from PEG 17.5K displayed an inherent lack of isomorphism when cryocooled to 100 K. The *c* cell parameter was particularly variable, and tests with a large number of native protein crystals gave lengths for this edge in the range 214–227 Å.

Given that protein crystals could only be cryoprotected reproducibly from solutions containing PEG 17.5K as precipitant and that the frozen cells were non-isomorphous, it was apparent that a MAD experiment would be essential to obtain experimental phases. However, we were concerned as to the likelihood of success of a MAD experiment based on the known iron content of the crystals (four haem groups per monomer, giving eight or 12 iron sites per asymmetric unit based on estimates of two or three monomers per asymmetric unit, respectively). This was a consequence of the large unit-cell size and the known absorption problems at X-ray wavelengths corresponding to the iron *K* edge. Therefore, we began to screen for conventional derivatives by soaking crystals in heavy-atom salt solutions in the hope of incorporating a suitable anomalous scatterer which could be used in a MAD phasing experiment. Consequently, a number of salt solutions were used in soaks and screened by EDAX. For example, soaking a crystal in a 10% saturated solution of MeHgCl for 4 h at 291 K gave an EDAX spectrum which showed clear evidence of incorporation of mercury (Fig. 2). From this figure, it is also evident that the EDAX method was sufficiently sensitive to detect the iron present in crystals of Ifc_3, where the concentration of iron is approximately 0.16%(*w*/*w*) of the crystal asymmetric unit (the presence of a characteristic iron emission line is indicated). Soaking a single crystal in a solution of 20 m*M* $KAuCl_4$ in 25%(*v*/*v*) ethylene glycol, 10%(*w*/*v*) PEG 17.5K, 100 m*M* Na MES pH 6.5 at 291 K for 24 h gave a unit cell of dimensions $a = 71.5$, $b = 108.5$, $c = 191.2$ Å at 100 K. This represented a contraction of the *c* cell edge of 17%. Subsequent analysis by EDAX revealed that no gold was detectable in the crystal (data not shown). We assume that the presence of the heavy-atom salt may have contributed to this very large change in the longest unit-cell dimension. However, given this smaller unit cell, we consider that a packing involving two molecules in the crystalline asymmetric unit is most likely. This would result in a solvent content of 58%.

Although quantification of the extent of heavy-atom incorporation into the crystals of Ifc_3 by EDAX will be difficult and we have not yet had an opportunity to verify the existence of suitably diffracting heavy atoms in the crystal lattice by collection of anomalous differences, we believe that this method may prove to be generally useful for preliminary screening of heavy-atom soak conditions. This may be particularly true where an inherent lack of isomorphism in cell parameters is encountered and conventional methods for assessing the degree of derivitization (for example, by measurement of isomorphous differences) are not applicable, as is the case for crystals of Ifc_3.

We subsequently had an opportunity to measure the X-ray fluorescence spectrum at the iron *K* edge of a single crystal of Ifc_3 on station BM14 at the ESRF. The measured spectrum and calculated anomalous scattering factors as a function of X-ray energy are presented as Fig. 3. The anomalous scattering factors, f' (inflection point) and f'' (peak) calculated from the raw fluorescence spectrum were −9.07 e at 7123.6 eV and 5.16 e at 7127.4 eV, respectively. We were also able to measure a complete diffraction data set at an X-ray energy of 12 keV

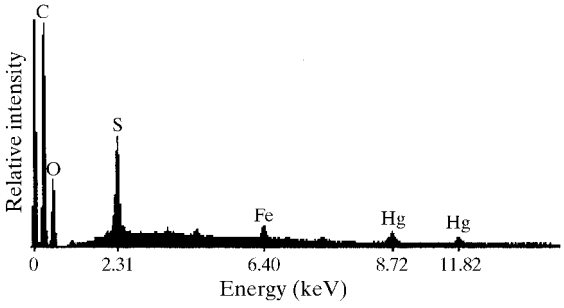
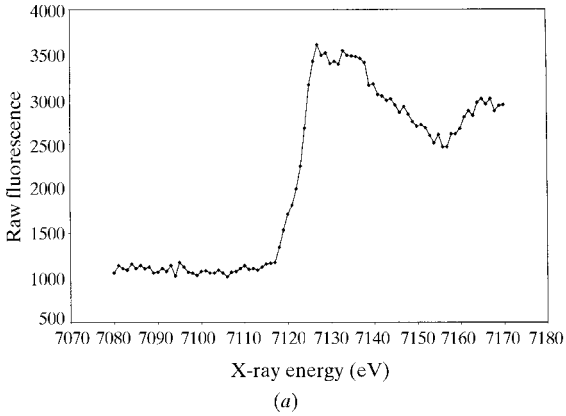


Figure 2
EDAX spectra of crystals of Ifc_3 soaked in 10%(*w*/*v*) MeHgCl for 4 h at 291 K.



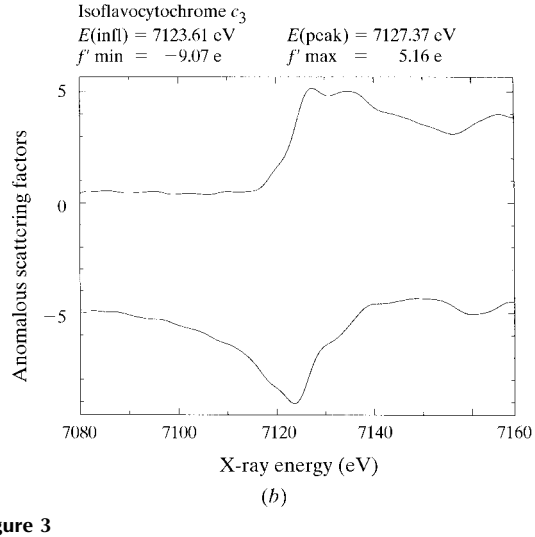


Figure 3
(*a*) X-ray fluorescence spectrum of a single crystal of Ifc_3 at 100 K. The crystal had already been exposed to X-rays for approximately 24 h before this spectrum was measured. (*b*) Anomalous scattering factors determined from the spectrum in (*a*) as a function of X-ray energy about the iron *K* edge.

Table 1
X-ray diffraction data-collection statistics.

Figures in parentheses refer to data in the highest resolution shell.

Wavelength (Å)	1.033
Temperature (K)	100
Collected at	ESRF BM14
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 71.7$l, $b = 109.7$, $c = 225.3$; $\alpha = \beta = \gamma = 90$
Resolution (Å)	3.0
Completeness (%)	99.3 (99.0)
$R_{\rm merge}$ (%)	5.6 (14.2)
$\langle I\rangle/\langle\sigma(I)\rangle$	10.0 (4.2)
Independent reflections	35842

(1.033 Å) to a resolution of 3.0 Å. The statistics for this data set are presented as Table 1. The clearly resolved iron *K* edge apparent from the fluorescence spectrum together with the quality of the diffraction to medium resolution give us confidence as to the likely success of an iron MAD phasing experiment for this crystal form.

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