| ESRF | **Experiment title:** Crystal structure analysis of the isoflavocytochrome c from *Shewanella putrefaciens* | **Experiment number:** LS-924 |
|---|---|---|
| **Beamline:** BM14 | **Date of experiment:** from: 18.6.98 to: 21.6.98 | **Date of report:** 20.2.99 |
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**Report**:

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. A 69 kDa subunit encoded by *frdA* contains covalently bound flavin and provides the active site for fumarate reduction. 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. *Shewanella frigidimarina* (formerly *S. putrefaciens*) NCIMB400 is a Gram-negative bacterium which produces numerous periplasmic *c*-type cytochromes when grown under anaerobic conditions. 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, Cheesman, Reid, Thomson, Ward & Chapman, 1995). $Fcc_3$ has been demonstrated to be the only physiological fumarate reductase present in *S. figidimarina* NCIMB400 (Gordon, Pealing, Chapman, Ward & Reid, 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, Black, Manson, Ward, Chapman & Reid, 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 terminal electron acceptor available (Dobbin, Powell, Reid & Richardson, 1998). $Ifc_3$ 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.

**Sample preparation**

Protein solution in 50 mM NaHEPES pH 7.5 and 100 mM NaCl was concentrated to 10 - 15 mg/ml using an Amicon diafiltration device at 4 °C. Initial crystallization screening experiments were performed at 4 °C and 18 °C . Crystals could be grown from PEG solutions of average molecular weights between 4K and 20K but that containing 12% (w/v) PEG 17.5K and 100 mM NaMES pH 6.5 at 18 °C gave crystals of the best quality. For cryoprotection experiments, crystals were soaked in solutions containing 25 % (v/v) ethylene glycol, 100mM NaMES pH 6.5 and 14% (w/v) PEG 17.5K. Cryocooling was performed by mounting single crystals in cryoloops (Hampton Research, CA) followed by either immediate rapid immersion in a nitrogen gas stream at 100K. The crystallographic cell was found to be $P2_12_12_1$ with cell parameters a=71.68Å, b=110.66Å, c=216.70Å. There are two molecules of the enzyme in the asymmetric unit.

**X-ray Fluorescence Spectra and Diffraction Data Collection**

X-ray fluorescence spectra and diffraction data were measured on station BM14 at the ESRF using a single crystal of $Ifc_3$ grown from 6%(w/v) PEG 17.5K, 100mM NaMES pH 6.5 and soaked in 8% (w/v) PEG 17.5K, 100mM NaMES pH 6.5 and 25% (v/v) ethylene glycol. Calculation of anomalous scattering factors from the raw fluorescence spectrum was performed using the CHOOCH programme of Gwyndaf Evans (1998).

For diffraction data collection, the crystal was preoriented to place the crystallographic $a^*$ axis approximately parallel to the camera phi axis and non-overlapping 0.5° oscillations were recorded on a 345 mm MAR imaging plate system at three X-ray energies around the iron K-edge. Indexing of images and data processing were performed using the HKL program suite. Programmes of the CCP4 program library were used for the preparation of reflection files (Collaborative Computational Project, Number 4, 1994). The crystallographic cell was $P2_12_12_1$ with cell parameters a=71.68Å, b=110.66Å, c=216.70Å. Statistics for the 3 datasets collected are presented below (table 1)

| | e1 Peak | e2 Inflection Point | e3 Remote |
|---|---|---|---|
| **Wavelength/Å** | 1.739 | 1.741 | 1.033 |
| **Resolution/Å** | 2.95 | 2.95 | 3.00 |
| **$R_{merge}$/%** | 2.6 (7.2) | 2.3 (7.2) | 1.9 (9.8) |
| **$R_{anon}$/%** | 3.3 (5.6) | 2.5 (4.9) | 1.6 (5.8) |
| **$< I > / < \sigma I >$** | 21.6 (9.1) | 25.5 (9.1) | 29 (5.8) |
| **Independent Reflections** | 32819 | 32607 | 27068 |
| **Completeness/%** | 88.7 (73.1) | 88.1 (70.9) | 72.9 (40.8) |
| **Multiplicity** | 3.4 | 3.3 | 3.2 |

**Table 1 MAD data collection statistics**

The positions of 8 iron sites were determined from inspection of isomorphous and anomalous difference patterson maps and the heavy atom parameters refined using SHARP. The resulting phase set has a figure of merit of 0.6 at 2.95Å resolution. The phases were then modified using the CCP4 program DM by solvent flattening and non-crystallographic symmetry averaging to give a clearly interpretable final electron density map. Interpretation of the map is in progress.

**References**


Dobbin, P.S.et al (1998) Submitted to *Biochem. J.*

Gordon, E.H.J.et al (1998) *Microbiology* **144**, 937-945.

Pealing, S.L. et al (1992) *Biochemistry* **31**, 12132-12140.

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