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Crystallization and preliminary crystallographic investigations of cytochrome c_4 from *Pseudomonas stutzeri*.

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Abstract

Cytochrome c_4 from the bacterium *Pseudomonas stutzeri* has been crystallized and X-ray diffraction data have been collected to 2.2 Å resolution. The crystals belong to the monoclinic system, space group $P2_1$ with cell parameters $a = 49.49$, $b = 58.58$, $c = 63.51$ Å and $\beta = 96.96°$. The crystals contain two molecules in the asymmetric unit. Cytochrome c_4 is known to contain two covalently bound haem groups per molecule and positions of the four haem Fe atoms in the asymmetric unit were determined from native anomalous-dispersion differences. The shortest Fe–Fe distances found in the crystal were 15.8, 16.9 and 19.0 Å.

Introduction

Cytochromes c_4 are dihaem class I cytochromes c with $M_r \simeq 20$ kDa, found in a variety of bacteria (Pettigrew & Moore, 1987; Moore & Pettigrew, 1990). Each of the haem groups is covalently bound to the protein *via* two cysteines. The haem coordination is further characterized by having a methionine and a histidine as axial ligands to the central Fe atom, forming a hexacoordinated low-spin complex in its reduced state.

Most of the cytochrome c_4 isolated from cells of *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Azotobacter vinelandii* have been found to be tightly membrane bound (Pettigrew & Brown, 1988). The exact function of cytochrome c_4 in the cell is not known (Hunter, Brown & Pettigrew, 1989). However, it is assumed that it constitutes an important link in both aerobic and anaerobic respiration. It has been proposed that cytochrome c_4 might act as an electron conductor between oxidases and reductases in the membrane or that its dihaem arrangement is designed for cooperative delivery of a pair of electrons to a terminal oxidase (Leitch, Brown & Pettigrew, 1985). With a recent determination of the amino-acid sequence for *P. stutzeri* cytochrome c_4 by cDNA techniques (Christensen, 1994), sequences for three cytochrome c_4 variants are now known (Moore & Pettigrew, 1990, p. 140). Sequence alignments suggest that these dihaem proteins are composed of two similar halves. However, the sequence homology between half domains within one single species is less pronounced (typically around 30%) than the overall sequence homology between species (typically around 80%).

As early as 1981 crystallization of cytochrome c_4 from *P. aeruginosa* was reported together with a low-resolution X-ray electron-density map based on two heavy-atom derivatives (Sawyer, Jones, Damas, Harding, Gould & Ambler, 1981). There have been no further reports in the literature of a refined structure. Further details have been published in the book of Moore & Pettigrew (1990) (referred to as unpublished crystallographic data kindly provided by L. Sawyer). It is stated there that the structure of cytochrome c_4 from *P. aeruginosa* is organized in two helical domains, containing approximately 80 amino-acid residues (and one haem group) each, connected by a long extended polypeptide chain. The two haem groups are seen to be almost co-planar with the propionates pointing towards each other within hydrogen-bonding distance.

The purification procedure and electrochemical characterization in terms of reduction potentials of cytochrome c_4 from *P. stutzeri* have been reported by Conrad, Karlsson & Ulstrup (1995). Here we report the crystallization and preliminary crystallographic characterization of cytochrome c_4 from this organism.

Materials and methods

Crystallization

Cytochrome c_4 from *P. stutzeri* (ATCC 11607) used for crystallization was prepared by Conrad, Karlsson & Ulstrup (1995). Crystals were grown at 293 K by vapour diffusion using the hanging-drop method. Initial crystallization trials were set up with 0.7 ml reservoirs composed as described in the 'fast screen test' (Jancarik & Kim, 1991) using a protein concentration of approximately 20 mg ml^{-1} and drops composed of 2 µl protein solution and 2 µl reservoir. The protein solution was buffered with 5 m*M* Tris at pH = 7.5 containing 0.1 *M* NaCl. Within 2 d, several of the trial conditions gave small crystals or needle spherulites. One of the crystallization conditions was particularly successful. It had a reservoir composed of 0.2 *M* ammonium acetate and 30% PEG 4000 in a 0.1 *M* sodium citrate buffer at pH = 5.6. Within about 24 h, a few well shaped deep-red crystals were reproducibly grown under this condition (see Fig. 1). Crystals were typically elongated in one direction. The biggest crystals were approximately 0.4 × 0.4 × 0.8 mm. No further refinement of the crystallization conditions was needed.

The relatively easy and fast crystallization of the protein turned out to be a great advantage due to the very unstable nature of the protein sample. After about 14 d at 278 K the protein sample had decomposed to an extent that the crystallization could not be reproduced. However, crystals kept at room temperature proved to be very stable (at least for months) as estimated from their diffraction quality.

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Data collection

Data collection was performed with a R-AXIS II imaging-plate system mounted on a Rigaku Rotaflex RU 200 copper rotating anode operating at 50 kV and 180 mA as X-ray source with a graphite monochromator and a 0.5 mm collimator. For a 20 min still exposure with a crystal-to-detector distance of 100 mm, the diffraction pattern extended to at least 2.2 Å resolution. 60 oscillation images of 3° were recorded, each with an exposure time of 20 min, to cover 180° of reciprocal space. The crystal-to-detector distance was kept at 100 mm and the 2θ swing angle 0°. During data collection the crystal was cooled to 274 K with a gas-flow low-temperature system supplied by the Molecular Structure Corporation.

Preliminary auto-indexing to obtain cell parameters were performed with the software package *PROCESS* supplied with the imaging-plate system. Cell and setting parameters were further refined and integrated intensities obtained using the *HKL* package (Gewirth, 1994). Data reduction was performed with the programs *ROTAVATA* and *AGROVATA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

Results and discussion

Characterization of crystals and data quality

The crystal system is monoclinic with cell parameters $a = 49.49\,(0.03)$, $b = 58.58\,(0.04)$, $c = 63.51\,(0.02)$ Å and $\beta = 96.96\,(0.04)°$. From systematic extinctions for reflections $0k0$ with $k = 2n + 1$ the space group was determined to be $P2_1$. A total of 67 890 observations corresponding to 18 020 unique reflections were collected to 2.2 Å resolution with $R_{sym} = 3.3\%$ (12.1% for the outer most resolution shell: 2.32–2.20 Å), mean $I/\sigma(I) = 13.5$ (5.7 for 2.21–2.20 Å) resulting in a completeness of 98.1% (96.6% for 2.32–2.20 Å). No serious radiation damage to the crystal was detected during the data collection.

Fig. 1. Crystals of cytochrome c_4 from *Pseudomonas stutzeri* obtained by vapour diffusion in a hanging drop. The crystals are often elongated in the direction of the c axis. The biggest crystal obtained was approximately 0.4 × 0.4 × 0.8 mm.

Table 1. *Refined* Fe *positions in the asymmetric unit determined from native anomalous differences*

Site	Fractional coordinates x	y	z
1	0.0775	0.0000	0.0074
2	0.2949	0.0451	0.5347
3	0.8836	0.4859	0.7424
4	0.3240	0.0319	0.8022

Table 2. *Interpretation of the native anomalous difference Patterson map*

Peak	Generated interatomic vectors Fractional coordinates u	v	w	Originating sites*
1	0.0389	−0.0141	0.2502	(1,1)→(3,2)
	0.0291	−0.0132	0.2675	(2,1)→(4,1)
	0.0291	0.0132	0.2675	(4,2)→(2,2)
	0.0389	0.0141	0.2502	(3,1)→(1,2)
2	0.2465	0.0319	0.7948	(1,1)→(4,1)
3	0.2174	0.0451	0.5273	(1,1)→(2,1)
	0.2076	0.0460	0.5446	(3,2)→(4,1)
4	0.1785	0.0592	0.2771	(3,2)→(2,1)
5	0.4113	0.4408	0.7923	(2,2)→(3,2)
	0.4015	0.4681	0.8096	(4,2)→(1,1)
6	0.3724	0.4549	0.5421	(2,2)→(1,1)
7	0.4404	0.4540	0.0598	(4,2)→(3,2)
8	0.4102	0.5000	0.9306	(2,1)→(2,2)†
9	0.3811	0.4868	0.6631	(2,1)→(4,2)
	0.3811	0.5132	0.6631	(4,1)→(2,2)
10	0.2328	0.5000	0.5152	(3,1)→(3,2)†
11	0.3520	0.5000	0.3956	(4,1)→(4,2)†
12	0.1939	0.4859	0.2650	(1,2)→(3,2)
	0.1939	0.5141	0.2650	(3,1)→(1,1)
13	0.1550	0.5000	0.0148	(1,2)→(1,1)†

* In parentheses are given the site number in the asymmetric unit followed by the symmetry operation number. Atoms 1–4 are listed in Table 1. Symmetry operations are $1 = x, y, z$; $2 = -x, \frac{1}{2} + y, -z$ plus unit-cell translations.

† Harker plane.

In order to estimate the number of molecules in the unit cell and the solvent content, V_M (the crystal volume per unit of molecular mass; Matthews, 1968) was calculated with M_r = 22.9 kDa to be 3.99 and 2.00 Å^3 Da^{-1} assuming one or two molecules in the asymmetric unit, respectively. This corresponds to solvent contents of approximately 69 or 39%. Though several examples of solvent contents of even higher than 69% have been reported, the quality and the extent of the diffraction pattern indicate that a solvent content of 39% is the most probable, corresponding to two molecules in the asymmetric unit. As shown in the following section this was confirmed by using the anomalous dispersion to locate the haem Fe atoms.

Positions of the haem Fe *atoms from anomalous dispersion*

Friedel pairs were measured for a total of 14 489 reflections. The crystal had been mounted with the c axis parallel to the oscillation axis due to the crystal morphology which meant that collection of anomalous-difference data was not optimal. Nevertheless, we used reflections which fulfilled the conditions: $|\Delta F_{ano}| > 3.0\sigma(\Delta F_{ano})$ and $|\Delta F_{ano}|$ less than ten times the overall mean ($|\Delta F_{ano}|$) (2085 out of 5999 reflections measured in the resolution range 10–3.0 Å) to calculate a well defined anomalous Patterson map with peaks clearly distinguishable above noise level. Most of the reflections (3913) were rejected on the first 3σ criterion and one measurement was rejected on

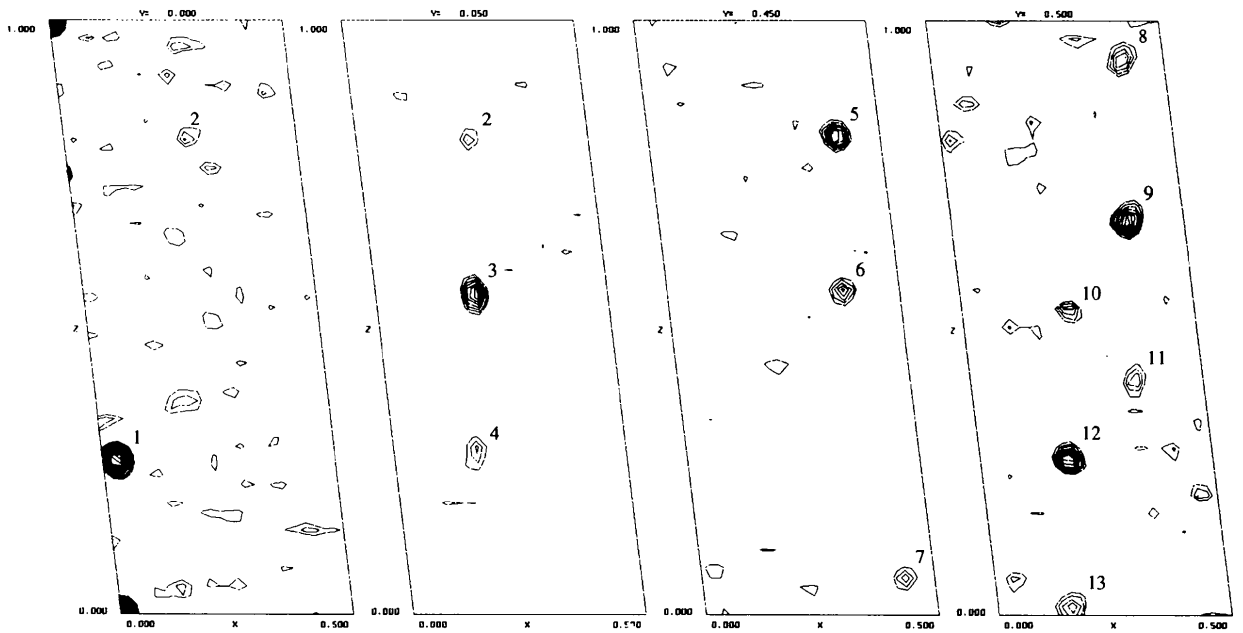

Fig. 2. Selected sections ($v = 0$, $v = 0.05$, $v = 0.45$ and $v = 0.5$) of the anomalous difference Patterson map (asymmetric unit) produced with the *PHASES* package (Furey, 1991). Contours are drawn for every σ starting at 2σ (*cf.* text and Table 2).

the second outlier criterion. Leaving out this latter reflection was crucial in order to obtain an interpretable anomalous Patterson map. This map could be interpreted by hand in terms of four Fe-atom sites in the asymmetric unit. Their positions were refined with the program *HEAVY* (Terwilliger & Eisenberg, 1983; Collaborative Computational Project, Number 4, 1994) using F_- and F_+ to simulate native and derivative data with the scattering factor for the imaginary part of the anomalous Fe added to the command file. The refined parameters are listed in Table 1. These four positions were used to generate interatomic Fe–Fe vectors. In Table 2 we list 16 interatomic vectors generated in the Patterson asymmetric unit together with four interatomic vectors lying just outside its border. All interatomic vectors could be assigned to the 13 highest peaks in the map (see Fig. 2) qualitatively explaining relative peak heights due to interatomic vector overlap.

The shortest Fe–Fe distances in the crystal calculated from the Fe positions in Table 1 are: 15.8, 16.9 and 19.0 Å. They must represent both intra- and intermolecular Fe–Fe distances. Considering the rather narrow range of these, it cannot be concluded which correspond to the intramolecular ones. However, it is noteworthy that they imply rather short intermolecular Fe–Fe contacts. In the structure solution we intend to make use of the anomalous-dispersion effects in the native data combined with isomorphous replacement.

References

Christensen, H. E. M. (1994). *Gene*, **144**, 139–140.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Conrad, L. S., Karlsson, J. J. & Ulstrup, J. (1995). In the press.

Furey, W. (1991). *PHASES, A Program for Phasing in Macromolecular Crystallography*. Univ. of Pittsburg, PA, USA.

Gewirth, D. (1994). *The HKL Manual: an Oscillation Data Processing Suite for Macromolecular Crystallography*. Department of Molecular Biophysics and Biochemistry, Yale Univ. New Haven, CT 06511, USA.

Hunter, D. J. B., Brown, K. R. & Pettigrew, G. W. (1989). *Biochem. J.* **262**, 233–240.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

Leitch, F. A., Brown, K. R. & Pettigrew, G. W. (1985). *Biochim. Biophys. Acta*, **808**, 213–218.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Moore, G. R. & Pettigrew, G. W. (1990). In *Cytochromes c: Evolutionary, Structural and Physicochemical Aspects*. Berlin: Springer-Verlag.

Pettigrew, W. & Brown, K. R. (1988). *Biochem. J.* **252**, 427–435.

Pettigrew, G. W. & Moore, G. R. (1987). In *Cytochromes c: Biological Aspects*. Berlin: Springer-Verlag.

Sawyer, L., Jones, C. L., Damas, A. M., Harding, M. M., Gould, R. O. & Ambler, R. P. (1981). *J. Mol. Biol.* **153**, 831–835.

Terwilliger, T. C. & Eisenberg, D. (1983). *Acta Cryst.* A**39**, 813–817.