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Cross-crystallization method used for the crystallization and preliminary diffraction analysis of a novel di-haem cytochrome c_4

Ivana Tomčová,[a,b]* Rui Miguel Mamede Branca,[c] Gabriella Bodó,[c] Csaba Bagyinka[c] and Ivana Kutá Smatanová[a,b]

[a]Institute of Physical Biology, University of South Bohemia in České Budějovice, Zámek 136, CZ-373 33 Nové Hrady, Czech Republic, [b]Institute of Systems Biology and Ecology, Academy of Sciences of the Czech Republic, Zámek 136, CZ-373 33 Nové Hrady, Czech Republic, and [c]Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, Temesvári krt. 62, HU-6726 Szeged, Hungary

Correspondence e-mail: tomcova@ufb.jcu.cz

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The newly discovered di-haem cytochrome c_4 from the purple sulfur photosynthetic bacterium *Thiocapsa roseopersicina* is the first cytochrome c_4 to be crystallized from an anaerobic organism. It was crystallized using the addition of metal-ion salts to the standard vapour-diffusion method. Coloured well shaped three-dimensional crystals with dimensions of approximately 0.6 × 0.05 × 0.02 mm grew within 3–4 d at pH 5 and diffracted to 1.72 Å without radiation damage. Cytochrome c_4 crystallized in space group $P4_12_12$ as a primitive tetragonal system with unit-cell parameters $a = b = 75.29$, $c = 37.12$ Å, $\alpha = \beta = \gamma = 90^\circ$.

1. Introduction

Cytochromes are members of a larger class of proteins known as haemoproteins (Yamanaka, 1992; Pettigrew & Brown, 1988). The haemoproteins derive their name from the presence of one or more iron-porphyrin prosthetic groups (called haems). In addition to cellular bioenergetics, haemoproteins are also engaged in ligand-binding reactions necessary for oxygen transport (Hunter *et al.*, 1989; Moore & Pettigrew, 1990; Pettigrew & Moore, 1987). Cytochromes are ubiquitous proteins involved in a variety of intracellular processes essential for life. Most notable is their participation in electron-transfer reactions, usually as components of a complex reaction pathway necessary for the production of energy, either through the oxidation of metabolites or *via* photosynthesis (Pettigrew & Moore, 1987).

Cytochromes c_4 are di-haem or mono-haem proteins (Brown *et al.*, 1999) bound at the periplasmic side of the cytoplasmic membrane (Pettigrew & Brown, 1988; Hunter *et al.*, 1989; Wood, 1983). The haem-binding sites are of the classical Cys-*X*-*Y*-Cys-His type (Van Beeumen, 1991). They are characterized by His and Met axial coordination, a relatively high redox potential (above ∼200 mV; Leitch *et al.*, 1985), a split α absorption band (at ∼553 nm) and a low α/β intensity ratio in the reduced form (β is at ∼523 nm; Pettigrew & Brown, 1988; Hunter *et al.*, 1989; Leitch *et al.*, 1985). Their high redox potential is indicative of a position close to the terminal oxidase in the electron-transport chain.

The function of this class of cytochromes is a matter of question. They have been purified from quite diverse bacterial sources. This diverse distribution indicates that cytochrome c_4 can be located in different electron-transport chains or distinct enzyme systems in various organisms (Ambler *et al.*, 1987; Kadziola & Larsen, 1997).

Several cytochromes have already been purified from *Thiocapsa roseopersicina*. The newly isolated cytochrome shows all the characteristics of a cytochrome c_4. It also shows very peculiar thermodynamic and redox properties (Branca *et al.*, 2006). Similarly to the hydrogenase from *T. roseopersicina* (Gogotov *et al.*, 1976; Zorin & Gogotov, 1982; Keszthelyi *et al.*, 1986), the cytochrome c_4 is a heat-tolerant protein if maintained under anaerobic conditions, even though the bacterium itself does not grow above 303 K.

One of the first successful crystallizations of c_4-type di-haem cytochromes was reported by Sawyer *et al.* (1981). This cytochrome c_4 from *Pseudomonas aeruginosa* was precipitated by adding saturated

$(NH_4)_2SO_4$ to the protein solution and spinning down. The crystals grew from this suspension. The other cytochromes *c* (Kadziola & Larsen, 1995; Benini *et al.*, 2000; Chen *et al.*, 1994) were crystallized by standard vapour-diffusion methods using various precipitation agents (polyethyleneglycol, sodium acetate, ammonium acetate).

We used the precipitating agent $(NH_4)_2SO_4$ and the vapour-diffusion method for our initial crystallization experiments on cytochrome c_4 from *T. roseopersicina*. Here, we report the newly developed cross-crystallization method and the preliminary diffraction analysis of di-haem cytochrome c_4 from *T. roseopersicina.*

2. Materials and methods

T. roseopersicina strain BBS was isolated from an estuary of the White Sea (Bogorov, 1974) and was a kind gift from E. N. Kondratieva (Moscow State University, Russia). The cells were grown under standard photosynthetic conditions as described previously (Bagyinka *et al.*, 2003). Cells were harvested in the logarithmic phase of growth and were stored at 293 K.

2.1. Cytochrome purification

The wet cell paste was resuspended in 90% cold (253 K) acetone–water mixture and filtered. The acetone removed the lipophilic cell components. The procedure was repeated until the filtrated acetone became colourless. The pellet was finally washed with pure (100%) acetone, dried and stored at 253 K. For purification, 15 g of dried pellet was resuspended in distilled water and stirred overnight at 277 K. The slurry was centrifuged at 20 000*g* for 2 h and the supernatant was used. The first purification step was a batch chromatography with 60 g Whatman DEAE (diethylaminoethyl) DE-52 in 20 m*M* Tris–HCl pH 7.5. Cytochrome c_4 was washed off with 450 m*M* NaCl.

Ammonium sulfate $(NH_4)_2SO_4$ [final concentration 10%(*w*/*w*)] was added to the fraction and it was loaded onto a Butyl-Sepharose column (Amersham XK 50, 5 cm diameter, 6 cm length). Fractions were eluted by a gradient from 10 to 0% $(NH_4)_2SO_4$ in 1 m*M* Tris–HCl pH 7.5 using the Amersham Biosciences FPLC (fast protein liquid chromatography) system. Cytochrome c_4 eluted at around 1% $(NH_4)_2SO_4$ (Fig. 1*a*).

The next purification step was ion-exchange chromatography with 23 ml Q-Sepharose Fast Flow in an Amersham HR 16/20 column using 20 m*M* Tris–HCl pH 7.5 and 0–1 *M* NaCl gradient for elution. The cytochrome c_4 eluted at 100 m*M* NaCl (Fig. 1*b*). Another hydrophobic chromatography [1 m*M* Tris–HCl pH 7.5, 10–0% $(NH_4)_2SO_4$] followed using 8 ml ToyoPearl Phenyl-650S in an Amersham HR 10/15 column. The cytochrome eluted in the 3–0% $(NH_4)_2SO_4$ interval (Fig. 1*c*). Finally, 1 ml Q-Sepharose Fast Flow in an Amersham HR 5/10 column was used with a 20 m*M* Tris–HCl pH 8.5 buffer system and a 0–1 *M* NaCl gradient for elution. The cytochrome c_4 eluted in pure form at around 50 m*M* NaCl (Fig. 1*d*). The purity and molecular weight of around 22 kDa were determined by

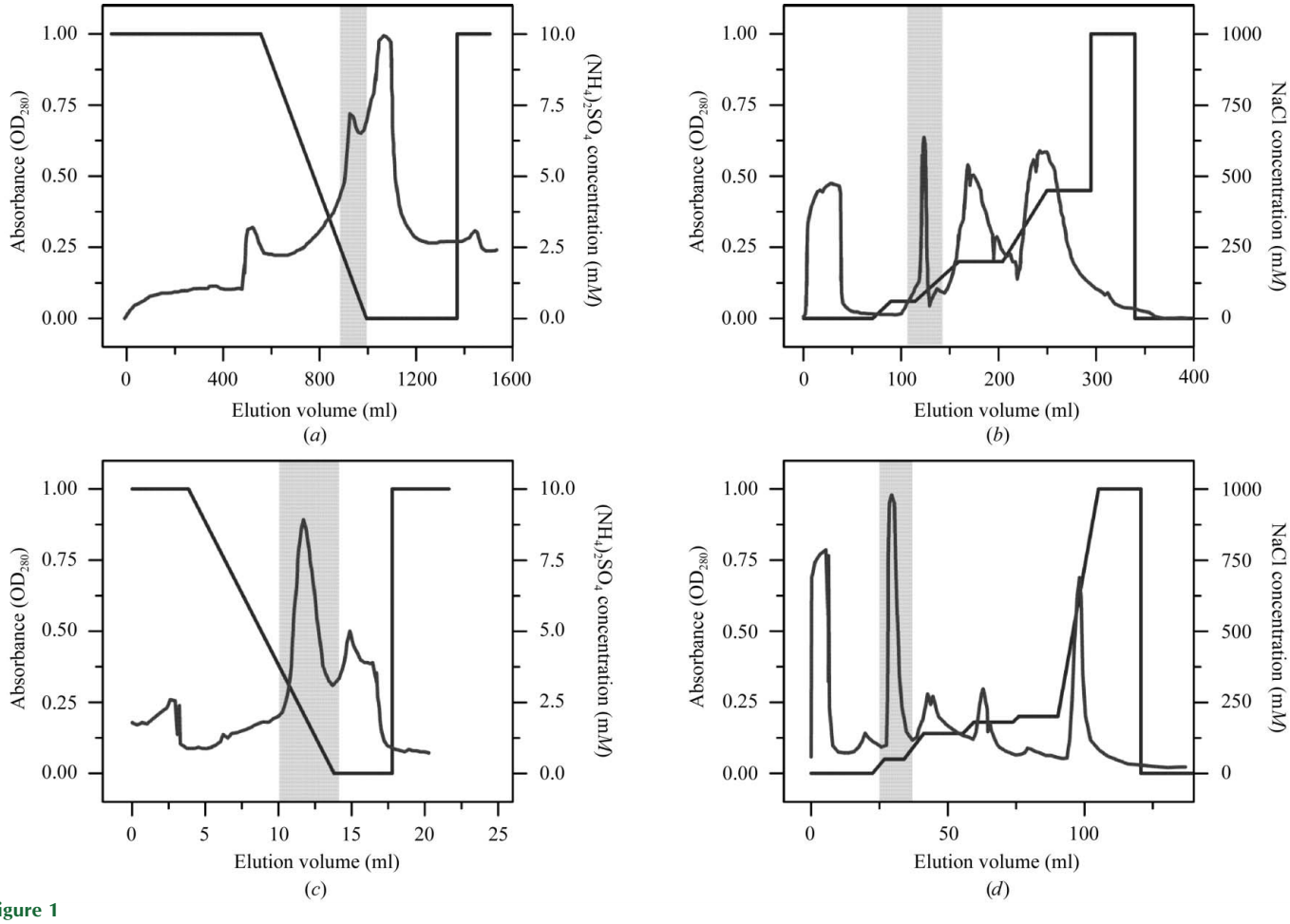

Figure 1
Purification of cytochrome c_4 from the purple photosynthetic bacteria *T. roseopersicina*. (*a*) Butyl-Sepharose chromatography. (*b*) Q-Sepharose chromatography. (*c*) Phenyl-Sepharose chromatography. (*d*) Q-Sepharose chromatography. Shaded areas represent cytochrome-containing fractions. For experimental details see text.

SDS–PAGE. A 10–20% polyacrylamide gel gradient was used (Maniatis *et al.*, 1982).

The protein concentration was determined both by measuring the optical density of the 280 nm absorption peak and by using the Bradford method (Bradford, 1976). In both cases BSA (bovine serum albumin, Sigma) was used as standard.

2.2. Initial screening of crystallization conditions

After purification, the protein was concentrated with a Microcon YM-3 Centrifugal Filter Unit (Millipore, Czech Republic) and the buffer was changed to 20 m*M* Tris–HCl pH 8. Initial screening experiments were performed as described previously (Jancarik & Kim, 1991; Jancarik *et al.*, 2004; Bergfors, 1999) using the Wizard III crystal screen kit (Emerald Biosystems, Washington State, USA) in Cryschem plates (Hampton Research, California, USA). A protein concentration of 10 mg ml^{-1}, a reservoir solution volume of 0.7 ml and drops consisting of 1 µl protein solution plus 1 µl of reservoir solution were used in each crystallization trial. These experiments yielded thin red plate-shaped pseudocrystals (Fig. 2) in the presence of 1.7 *M* $(NH_4)_2SO_4$ and 100 m*M* NaCl in 100 m*M* sodium citrate buffer pH 6. Protein phase separation (Asherie, 2004) occurred at pH values higher than 7.5. Subsequently, crystals of similar shape were also obtained with higher concentrations of $(NH_4)_2SO_4$ regardless of the pH and NaCl concentration. Similarly shaped but poorly diffracting crystals (Fig. 3) were also grown in capillaries using the advanced crystallization method based on counter-diffusion (López-Jaramillo *et al.*, 2001).

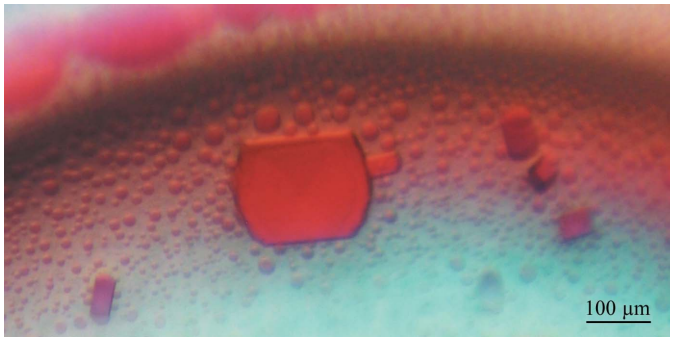

Figure 2
Pseudocrystals of cytochrome c_4 grown from solution containing 1.7 *M* $(NH_4)_2SO_4$, 100 m*M* sodium citrate pH 6.0 and 100 m*M* NaCl.

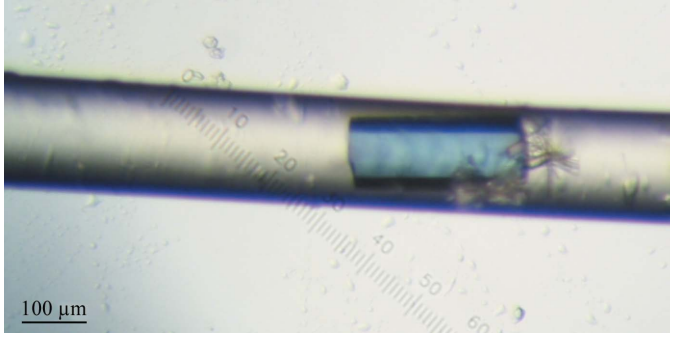

Figure 3
Crystal of cytochrome c_4 grown inside the 0.1 mm capillary using the advanced crystallization method based on counter-diffusion.

2.3. Optimization of conditions

Crystallization conditions were further optimized to improve the quality and especially the stability of crystals. Standard vapour-diffusion methods were applied with combinations of additives (see §3.1; McPherson, 1999; Sigel & Sigel, 1990) and by screening of pH values (5–6), NaCl concentration (0–0.1 *M*) and $(NH_4)_2SO_4$ concentration (1.7–3.2 *M*). Under optimized conditions, the protein crystal grows without defects or fluctuation effects (Figs. 4*a* and 4*b*).

2.4. Data collection and processing

Coloured well shaped protein crystals of cytochrome c_4 with dimensions of approximately 0.6 × 0.05 × 0.02 mm (Figs. 3, 4*a* and 4*b*) were tested at the home source diffractometer at LEC (Laboratorio de Estudios Cristalográficos, University of Granada, Spain) and at the DESY/EMBL synchrotron (Deutsches Elektronen Synchrotron at European Molecular Biology Laboratory, Hamburg, Germany) in loops or directly in the capillaries. Complete data-set collection was executed at the EMBL Hamburg Outstation at beamline X11 with fixed wavelength λ = 0.81 Å using Oxford Cryosystem magnets for crystal mounting. Diffraction data were collected to 1.72 Å resolution (resolution range 15.23–1.72 Å; Fig. 5) using a MAR CCD 165 mm detector at the DORIS storage ring with triangular monochromator and bent mirror beam. Crystals grown without any cryoprotectant were fished out from the crystallization drops and flash-frozen in a stream of nitrogen (Oxford Cryosystem) at 100 K. Crystals were centred manually at the goniometer. A crystal-to-detector distance of 150 mm was used to collect 360 frames. The exposure time for each image was 30 s. The oscillation increment was 1°. The data were processed and refined using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

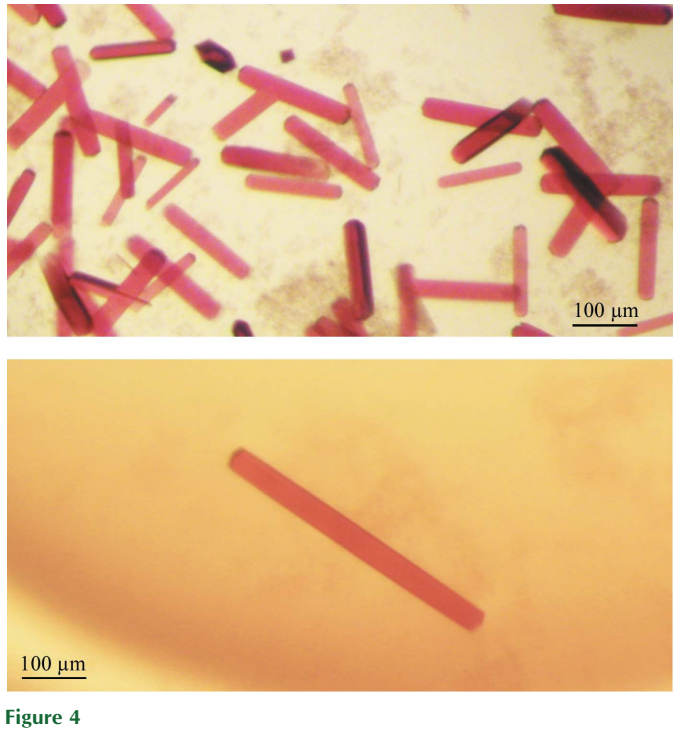

Figure 4
Colored well shaped three-dimensional crystals of cytochrome c_4 with maximal dimensions of approximately 0.6 × 0.05 × 0.02 mm grown within 3–4 d in the presence of 5 m*M* $CuCl_2$, 3.2 *M* $(NH_4)_2SO_4$ and 0.1 *M* citric acid pH 5 in the protein drop and 3.2 *M* $(NH_4)_2SO_4$, 0.1 *M* citric acid pH 5 in the reservoir and with the influence of the additives 5 m*M* $CdCl_2$, 5 m*M* $CoCl_2$, 5 m*M* $BaCl_2$ in the other wells (see also §3.1).

3. Results and discussion

3.1. Cross-crystallization method

Starting with the crystals grown under the optimized initial conditions, we used a new crystallization procedure, which we named cross crystallization, that further optimized the crystals by the addition of additives. The Emerald Biosystems plate (EBS plate, JenaBioscience, Germany) with one central reservoir surrounded by four drop wells (Fig. 6) is used in this procedure. Initial trials conducted with the chloride salts of copper, cadmium, cobalt and barium (as a multivalent cations) from Hampton Research Additive Screen (Hampton Research, California, USA) yielded protein crystals only in the presence of $CuCl_2$. Crystals grew slowly within 3–4 d to maximum dimensions of ~0.6 × 0.05 × 0.02 mm (Figs. 4*a* and 4*b*) at 293 K. Unexpectedly, these crystals could not be reproduced unless the other metal salts were present in the remaining drop wells. Only metal salts and reservoir solution, and not protein, were required in the three remaining wells to promote crystallization in the fourth well. We assume these metal ions influence evaporation in the protein drop even if they are absent from that drop. If so, this effect may be nonspecific, but this has not yet been tested. However, the effect of copper on crystal growth appears to be specific, because no other successful combination of ion salts with protein was found among these four salts singly or in pairs.

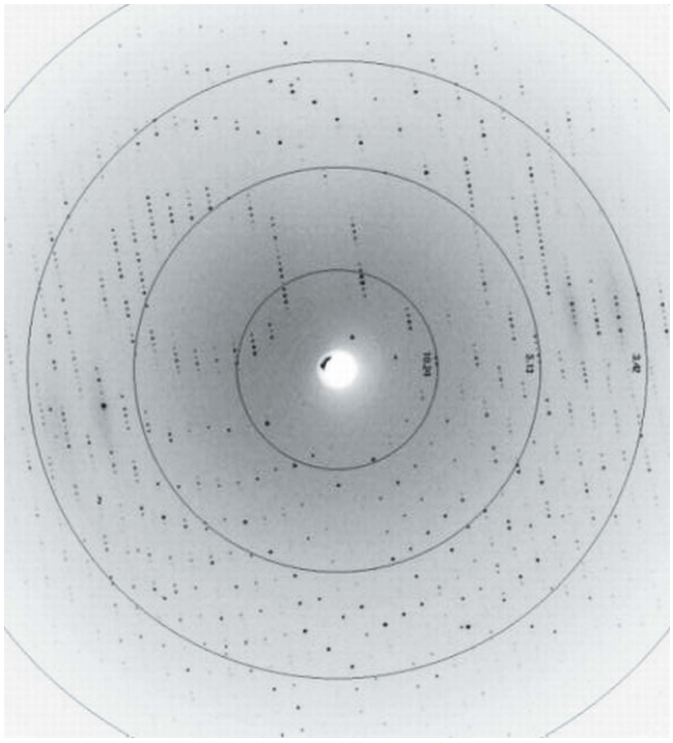

Figure 5
Diffraction image of a cytochrome c_4 crystal collected at the EMBL Hamburg Outstation at beamline X11 to a resolution of 1.72 Å.

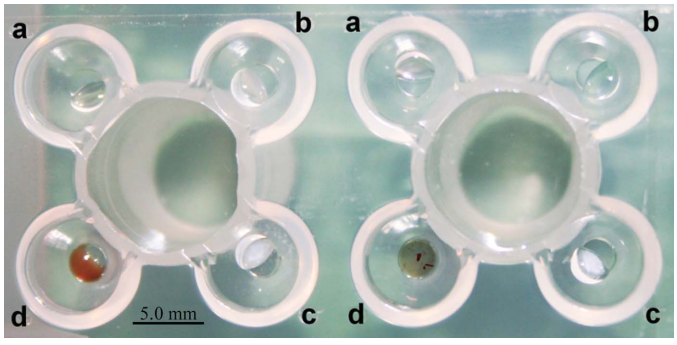

Figure 6
Emerald Biosystems crystallization plate (JenaBioscience, Germany) used to perform the cross-crystallization procedure. Drop wells *a*, *b*, *c* and *d* were each filled with 0.5 µl of various 5 m*M* chloride salts (see text) and 0.5 µl precipitant. Protein at a concentration of 15 mg ml^{-1} (1 µl) was only added into drop well *d*, containing 5 m*M* $CuCl_2$.

Table 1
X-ray data-collection statistics for *T. roseopersicina* BBS cytochrome c_4.

Space group	$P4_12_12$
Unit-cell parameters (Å, °)	$a = b = 75.29$, $c = 37.12$, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	15.227–1.722
Oscillation range (°)	1.0
Solvent content (%)	26
No. of unique reflections	11770
No. of collected frames (images)	360
Overall completeness (%)	99.69
R_{free} (%)	24.3
R_{factor} (%)	20.8
R_{merge}† (%)	8.0
$I/\sigma(I)$ in highest shell	13.82
Mosaicity	0.8
Crystal system	Primitive tetragonal

† $R_{merge}\dagger = \sum|I_i - \langle I\rangle|/\sum\langle I\rangle$, where I_i is an individual intensity measurement and $\langle I\rangle$ is the average intensity for this reflection with summation over all data.

3.2. Characterization of crystals and data quality

The cytochrome c_4 crystallized as a primitive tetragonal system, with unit-cell parameters $a = b = 75.29$, $c = 37.12$ Å, $\alpha = \beta = \gamma = 90°$. Evaluation of the crystal-packing parameters indicated that the lattice could accommodate one cytochrome c_4 monomer in the asymmetric unit, with a solvent content of approximately 26%. From a fourfold rotation, the space group was determined (Otwinowski & Minor, 2000; Matthews, 1968; Brünger *et al.*, 1998) to be $P4_12_12$. A total of 11 770 unique reflections were collected to 1.72 Å resolution (Fig. 5) with an R_{sym} of 6.6% and a mean $I/\sigma(I)$ of 13.82 for the highest resolution shell, resulting in a completeness of 99.69%. The data-collection and processing parameters are listed in Table 1. No detectable radiation damage to the crystal was noted during the data collection. Phasing was performed by the molecular-replacement method using similarly constructed di-haem and mono-haem cytochromes type *c* (Van Beeumen *et al.*, 1991; Kadziola & Larsen, 1995; Benini *et al.*, 2000; Chen *et al.*, 1994) as templates. The electron-density map of our cytochrome c_4 clearly showed the two haem groups and several Cu atoms, water molecules and sulfate anions derived from crystallization. It was found that both vinyl groups of the haem are present in thioether bonds and the haem iron is coordinated by His18/123 from the left side and Met80/167 from the right side. This characteristic grouping of histidine and methionine around the haem groups confirmed the prototypical c_4-type of our cytochrome.

Model building and refinement of *T. roseopersicina* cytochrome c_4 using *REFMAC*5 (Murshudov *et al.*, 1997) are under way.

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Crystallography for access to their facility. We would like to thank Paul Tucker, Santhos Pajinkar and Victor Lamzin (EMBL Hamburg) for help with access to EMBL and processing at the X11 beamline and Jose A. Gavira for his help with crystal testing at the LEC diffractometer. We also thank the Hungarian Science Foundation (OTKAT049276, OTKAT049207) and AUTOESKORT Ltd for financial support. The travel grant TeT CZ-2/4 made the cooperation possible. RMMB acknowledges the financial support of the Portuguese Science and Technology Foundation under the PhD fellowship of POCTI, SFRH/BD/13128/2003.

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