Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary crystallographic study of an extremophile cytochrome c_4 from *Thiobacillus ferrooxidans*

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Received 19 June 2000
Accepted 18 July 2000

Soluble periplasmic dihaemic cytochrome c_4, of 21 293 Da molecular mass, has been characterized from *Thiobacillus ferrooxidans*, an acidophilic bacteria. The native cytochrome has been purified from the bacteria using ion-exchange chromatography and crystallized using solution 27 of the Hampton Research Crystal Screen II. The crystals belong to the hexagonal space group $P6_222$ or $P6_422$, with unit-cell parameters a = 101.59, b = 101.59, c = 151.59 Å. Frozen crystals diffract to 2.17 Å resolution. The MAD method is currently being used (four Fe atoms per asymmetric unit) to solve the protein structure.

1. Introduction

Extremophilic bacteria are able to grow in adverse environments such as high temperature, high salt concentrations or extreme pH values. The study of extreme acidophilic bacteria is of fundamental biological interest, since functioning at pH values below 2 implies special structure–function relationships. In addition, there is an economic interest, as these acidic conditions are those under which various metals are attacked (bioleaching) with a concomitant solubilization of metals in the medium. *T. ferrooxidans* is the main agent used in bioleaching of ores to extract metals. These chemolithotrophic bacteria have developed an unusual bioenergetic metabolism since they live in acidic medium at the expense of Fe^{2+} and/or sulfide/sulfur oxidation using oxygen as an exogenous electron acceptor.

Most of the metalloproteins involved in the respiration electron-transfer chain are therefore soluble acid-stable proteins that exhibit high redox potentials.

Although some evidence exists for the role of acid-stable cytochromes in ferrous ion oxidation in *T. ferroxidans* (Ingledew & Cobley, 1980; Mansch & Sand, 1992; Valkova-Valchanova & Chan, 1994), various membrane and soluble cytochromes described to date by several authors are the subject of controversy. Furthermore, several hypotheses have been proposed concerning their functional role in the iron electron-transport chain (Blake & Shute, 1994).

We have characterized a soluble periplasmic cytochrome of 21 293 Da molecular mass containing two haems exhibiting redox potentials of 385 and 480 mV (Cavazza *et al.*, 1996) and named cytochrome c_4 (Mr 21000). The gene of this cytochrome has been cloned and its sequence established (Appia-Ayme *et al.*, 1998). This sequence clearly shows an ancestral gene duplication and the two cytochrome c_4 domains characteristic of the c_4-type cytochrome. These results demonstrate that the previously described monohaemic soluble cytochrome c_{552} and the membrane-bound c_{552}(m) could be related to the cytochrome c_4. However, the molecular mass and the number of haems have been underestimated.

Furthermore, we have purified and characterized a new cytochrome c_4 of 26 567 Da (Giudici-Orticoni *et al.*, 2000) named c_4 (Mr 26000). The determined N-terminal sequence was found to be present in the genome of *T. ferrooxidans*; the corresponding full-length protein shows 34.5% homology with cytochrome c_4 (Mr 21000), which is much higher than the sequence homology to other c_4-type cytochromes. They have been compared with the three previously studied c_4-type cytochromes from *Pseudomonas stutzeri* and *Azotobacter vinelandii* as well as the *P. nautica* cytochrome c_{552}, a dimerizing monohaem cytochrome showing homology to cytochrome c_4 (Giudici-Orticoni *et al.*, 2000).

To our knowledge, *T. ferroxidans* is the only organism reported so far with two different representatives of this family of dihaemic cytochromes. This raises the question of the structural and functional characteristics which favour the c_4-type dihaem over the use of standard monohaem cytochromes. Possible explanations could be an involvement of the dihaemic structure in the stabilization of the molecule in extremely acidic pH, a better efficiency of electron transfer and/or the maintainance of high redox potential haems (+365 and +480 mV).

The elucidation of the three-dimensional structure of the cytochrome c_4 (Mr 21000) may help to answer these questions.

2. Results and discussion

2.1. Purification of the cytochrome c_4 (Mr 21000)

All purification steps were performed at 277 K and pH 4.8. Cells (100 g of wet cells) were resuspended in 0.5 m*M* sulfuric acid, 1 m*M* phenylmethylsulfonyl fluoride ($PhMeSO_2F$) and 0.1%(*w*/*v*) Zwittergent 3-12. Cells were subsequently broken by passing them several times through a Menton–Gaulin press; the cells were then centrifuged at 180 000*g* for 1 h to remove the membranes. The supernatant was adjusted to pH 4.8, 20 m*M* ammonium acetate, 1 m*M* $PhMeSO_2F$, 0.1%(*w*/*v*) Zwittergent 3-12 (buffer *A*) and applied to a carboxymethylcellulose (CMC Waters; 16 × 5 cm) column equilibrated in buffer *A*. The column was eluted with a gradient of 50 m*M* to 1 *M* ammonium acetate, 1 m*M* $PhMeSO_2F$, 0.1%(*w*/*v*) Zwittergent 3-12 pH 4.8. Rusticyanin and cytochrome c_4 were eluted with 220 m*M* ammonium acetate, 1 m*M* $PhMeSO_2F$, 0.1%(*w*/*v*) Zwittergent 3-12 pH 4.8. The resulting fraction was dialysed against 20 m*M* ammonium acetate pH 4.8. The subsequent chromatography steps were carried out on an FPLC apparatus. The dialysed solution was applied to an S Sepharose (HiLoad 16/10 Pharmacia) column equilibrated with 50 m*M* ammonium acetate pH 4.8. The proteins were eluted with a gradient of 50 m*M* to 1 *M* ammonium acetate pH 4.8. Rusticyanin eluted at 300 m*M* ammonium acetate pH 4.8. The fraction containing cytochrome c_4 eluted at 600 m*M* ammonium acetate pH 4.8 and was concentrated and dialysed by ultrafiltration through an Amicon Centriprep 10 membrane concentrator and then applied to a mono S column (HR/S Pharmacia) equilibrated with 50 m*M* ammonium acetate pH 4.8. Cytochrome c_4 eluted at 800 m*M* ammonium acetate and was pure based on SDS–PAGE analysis and tetramethylbenzidine colouration tests. The cytochrome was basic with an isoelectric point around 9. The native cytochrome was produced and extracted from the bacteria periplasm. The final yield was of 9 mg pure cytochrome c_4 from 100 g bacteria. The protein was oxidized by addition of an excess of hexachloroiridate in 20 m*M* glycine–HCl pH 2.9. The oxidized cytochrome was then loaded onto a PD-10 column (Sephadex G25) equilibrated in the same buffer in order to remove the potassium hexachloroiridate. Here, we report crystallization conditions and preliminary data on the crystals.

2.2. Crystallization

The cytochrome c_4 protein was concentrated to 10 mg ml^{-1} in 20 m*M* glycine–HCl buffer pH 2.9, 1 m*M* ascorbate under argon using a centrifugal filter device (Ultrafree Biomax 10K, Millipore, Bedford, MA, USA). Precipitation experiments were carried out on the c_4 protein at acidic pH using different precipitating agents [*i.e.* $(NH_4)_2SO_4$, PEG, NaCl, MPD, ethanol] at four pH values (3.5, 4.5, 5.5 and 6.5). We obtained promising precipitates when using polyethylene glycol (PEG) 8000 as a precipitating agent. The acidic solutions of the Hampton Research crystal screens were then used and crystallization trials were performed at 293 K by hanging-drop vapour diffusion using 24-well culture plates. Each hanging drop was prepared by mixing 0.5 µl of the 10 mg ml^{-1} cytochrome c_4 with an equal volume of the reservoir solution. The hanging drop on the cover glass was vapour-equilibrated against 500 µl of the reservoir solution in each well of the tissue-culture plate. The wells were saturated with argon. Crystals appeared after one week; the best crystals were obtained at 0.1 *M* MES 25–30% PEG MME, 10 m*M* zinc sulfate, 1 m*M* ascorbate pH 6.5. Subsequently, 5% glycerol was added to the crystallization medium as a cryoprotectant agent.

2.3. Data collection and processing

One crystal was collected in a Hampton Research 0.5 mm^3 loop, flash-frozen in liquid nitrogen and subjected to X-ray diffraction. This data set was collected on a MAR CCD camera at the ESRF radiation synchrotron facility (ID14 EH2) at a wavelength of 0.9326 Å. Data collection was carried out with an oscillation angle of 1.0° and a crystal-to-detector distance of 200 mm. The total oscillation range collected was 90°. Space-group determination was performed using the autoindexing option in *DENZO* (Otwinowski, 1993). The crystals belong to the hexagonal space group $P6_222$ or $P6_422$, with unit-cell parameters $a = b = 101.59$, $c = 151.59$ Å. The packing density for two monomers of c_4 (21 kDa) in the asymmetric unit of these crystals (volume = 1 337 516.8 Å^3) is 2.77 Å^3 Da^{-1}, a reasonable value for globular proteins, indicating an approximate solvent content of 55.62% (Matthews, 1968).

The data set was processed using the *MOSFLM* package (Kabsch, 1993; Campbell, 1995; Steller *et al.*, 1998); the *SCALA* program from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) was used for the scaling and data reduction of the native data set. The crystal diffracted to 2.17 Å and 494 740 reflections were measured in the resolution range 2.17–28.8 Å. This was reduced to a data set of 27 007 unique reflections with an R_{sym} value of 4.1. This represents a completeness of 99.6%, with a multiplicity of 4.7 and an average $I/\sigma(I)$ of 8.6. For the highest resolution shell 12 349 reflections were measured in the resolution range 2.17–2.26 Å, corresponding to 2578 unique *hkl*, an R_{sym} value of 26.7 and an average $I/\sigma(I)$ of 2.9, a completeness of 100% and a multiplicity of 4.8.

No molecular-replacement solution was found using the dihaemic cytochrome c_4 structure (PDB code 1etp) and the *AMoRe* software (Navaza, 1994). In order to solve the cytochrome c_4 structure, three wavelength data sets were collected at the ESRF radiation synchrotron facility (BM30) in order to use the MAD method (Hendrickson *et al.*, 1990). These three data sets were processed using the *DENZO* package (Otwinowski, 1993) and programs from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) were used for scaling. This work is currently in progress.

We thank Dr C. Cambillau for access to X-ray diffraction equipment.

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