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Three-dimensional crystals of cytochrome-*c* oxidase from *Thermus thermophilus* diffracting to 3.8 Å resolution

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Abstract The ba_3-type cytochrome-*c* oxidase from *Thermus thermophilus* has been crystallized in its native form. Crystallization was achieved by the batch and the vapour diffusion sitting drop methods using polyethylene glycol monomethyl ether 2000 as precipitating agent in the presence of octyl-β-D-thioglucoside as detergent. The crystals diffract to 3.8 Å, belong to the space group P2 or $P2_1$ and have unit cell dimensions of $a = 80.7$ Å; $b = 116.0$ Å; $c = 156.9$ Å and $\beta = 104.4°$. The asymmetric unit contains two ba_3-type oxidase molecules.

Key words: Membrane protein crystallization; X-ray diffraction; cytochrome-*c* oxidase; ba_3-oxidase; *Thermus thermophilus*

1. Introduction

Cytochrome-*c* oxidase (EC 1.9.3.1) is the terminal respiratory enzyme of prokaryotic and eukaryotic organisms. It transfers electrons from cytochrome-*c* to O_2 and functions as an electrogenic H^+-pump according to the equation:

$$4\ \text{Cyt}\ c^{2+} + 8\ H_i + O_2 = 4\ \text{Cyt}\ c^{3+} + 4\ H_o^+ + 2\ H_2O$$

where H_i and H_o represent protons at the inner (i) and outer (o) side of the membrane respectively (reviews [1]). In some aerobic bacteria homologous terminal enzymes using quinol as substrate (quinol oxidases) have been described (review [2]). All members of this superfamily of terminal oxidases represent integral membrane protein complexes composed of 2 to 13 subunits.

Although many structural and functional aspects have been elucidated in recent years, mechanisms of e^--transfer and coupled proton translocation are not yet understood. Several attempts have been made to obtain three-dimensional crystals of this membrane protein [3–8], but so far only weakly diffracting species and a resolution insufficient for structure determination have been published.

Well-ordered membrane protein crystals sufficient for high-resolution X-ray analysis and structure determination have so far only been obtained from the reaction centres of *Rhodopseudomonas viridis* [9,10] and *Rhodobacter sphaeroides* [11–13], from the porins from *Escherichia coli* and *Rhodobacter capsulatus* [14,15], and from the prostaglandin H-synthase from sheep [16].

We have analysed the terminal oxidases from the thermophilic eubacterium *Thermus thermophilus* HB8 (ATCC 27634). Depending on the fermentation conditions and the O_2-supplies, this organism expresses two different oxidases known as caa_3- and ba_3-types according to the hemes bound [17]. A detailed protein chemical description of these enzymes will be presented elsewhere (Bresser, Soulimane, Steffens and Buse, in preparation). The ba_3-oxidase was originally described as a single-subunit enzyme [18]. However, a new preparation protocol and detailed protein chemical investigation showed it to be a two-subunit complex [19], containing a subunit I with the O_2-activating heme a_3Cu_B center, heme *b* instead of heme *a*, and a subunit II with the electron accepting Cu_A-center, recently recognized as a homodinuclear copper site [20].

2. Materials and methods

2.1. Chemicals

Detergents and buffers were purchased from BIOMOL (Hamburg, Germany). Polyethylene glycol monomethyl ether 2000(PEG-2000) was obtained from Merck (Darmstadt, Germany). All chemicals were of the highest available purity.

2.2. Isolation and characterization of cytochrome-c oxidase

The ba_3-type cytochrome-*c* oxidase of *Thermus thermophilus* was isolated and purified according to Bresser and Buse [19].

Heme A concentration was obtained spectrophotometrically from the reduced-minus-oxidized spectrum at 613 nm with a molar absorption coefficient of 6300 M^{-1} cm^{-1} [18].

Protein concentration was determined by amino acid analysis, performed on a Biotronik LC 5001 equipped with fluorescence monitor for detection of *o*-phtalaldehyde-derivatives.

Purity of the enzyme was checked by SDS--polyacrylamide gel electrophoresis according to Lämmli [21] and N-terminal sequence analysis [22] on a Knauer 816 protein sequencer with autoconversion and online HPLC identification of the phenylthiohydantoin amino acids.

2.3. Crystallization

The crystallization experiments were performed at 20°C using the sitting drop vapour diffusion in MVD/24 crystal growth chambers (Charles Supper Co., USA) and batch methods [23]. In total, ten nonionic detergents were tried in these crystallization experiments. Crystals, which had been grown in octyl-β-D-glucoside and in *N*-lauryl-*N*,*N*-dimethyl-amine-*N*-oxide diffracted at low resolution. However, with crystals grown in octyl-β-D-thioglucoside, we achieved the results reported.

2.4. Microscopy and X-ray diffraction

Crystals were observed under polarized light.

X-Ray diffraction pattern of a single cytochrome-*c* oxidase crystal was recorded on an imaging plate detector (Mar-Research) at the MPI für Biochemie, Martinsried, Germany. Graphite monochromatized CuK_α radiation was used, delivered from an RU200 rotating anode generator (Rigaku, Tokyo, Japan). The generator was operated at 5.4 kW with an apparent focal spot size of 0.4×0.4 mm^2. Data were recorded in frames of 1.0° and processed using IMSTILLS and REFIX from the MOSFLM v5.23 program package [24].

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3. Results and discussion

The purified enzyme (12.4 nmol heme *a*/mg protein) – representing the smallest cytochrome-*c* oxidase known – was concentrated with Centricon-30 concentrators (Amicon) to 10 mg protein/ml for the crystallization in batch and 4 mg protein/ml for the sitting drop method. In both cases the buffer was 10 mM Tris-HCl (pH 7.6) and the octyl-β-D-thioglucoside concentration was 0.55% (twice the CMC).

For the crystallization in batch 0.5 ml of the protein solution was transfered into a 6 ml glass tube, adjusted to 16% polyethylene glycol monomethyl ether 2000 as precipitant and closed with Parafilm (Laboratory film; Greenwich, CT, USA). After 20 h the solution became turbid and crystals appeared after five days. Crystals were grown also by vapour diffusion in sitting drops containing 8 μl protein solution and 2 μl from the 1 ml reservoir solution containing 14% polyethylene glycol monomethyl ether 2000 as precipitant in 20 mM Bis-Tris-propane buffer (pH 7.6) at 20°C (Fig. 1). Crystals were harvested after one week, mounted in 1 mm glass capillaries and analysed using X-ray rotation photography as described in Section 2. Both crystallization methods lead to crystals of about equal quality, however, differing in size and form (data not shown). The largest crystals were 1 mm × 0.7 mm × 0.1 mm in size. The investigated, brownish tetragonal and pentagonal crystals (0.5 mm × 0.3 mm × 0.1 mm) diffract to a resolution of about 3.8 Å (Fig. 2). Autoindexing of the diffraction pattern revealed the space group P2 or $P2_1$ with unit cell dimensions of $a = 80.7$ Å; $b = 116.0$ Å; $c = 156.9$ Å; $\alpha = \gamma = 90°$ and $\beta = 104.4°$. Assuming a molecular mass of 80 561 Da for the two subunit monomer and two monomers per asymmetric unit, the volume-to-mass ratio turns out to be $V_m = 4.56$ Å^3/Da (Matthews' coefficient [25]), which results in a solvent and detergent content of 73% of the crystal volume. This value is within the acceptable range for membrane proteins as shown in several other cases, e.g. $V_m = 4.9$ Å^3/Da for the reaction center from *Rhodopseudomonas viridis* [9] and $V_m = 4.69$ Å^3/Da for the porin from *Escherichia coli* [14]. Several crystals were collected, washed with the reservoir solution, dissolved in 0.1 M Tris-HCl (pH 6.8) and 4% SDS in the presence of 1% β-mercaptoethanol and applied to the SDS-polyacrylamide gel: only two bands corresponding to subunits I and II of the ba_3-oxidase are observed (data not shown).

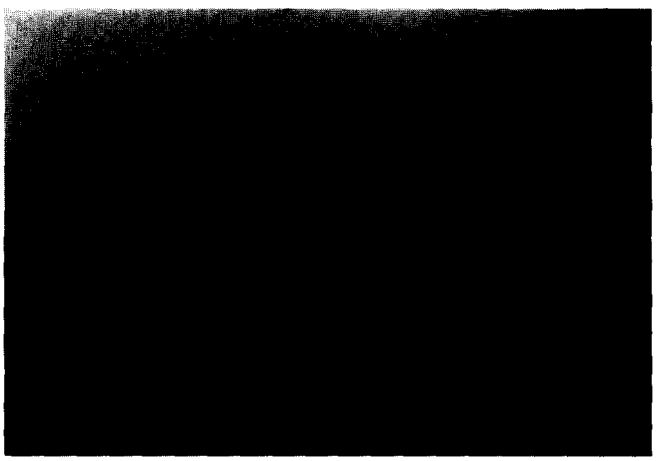

Fig. 1. Crystals from the ba_3-type *Thermus thermophilus* cytochrome-*c* oxidase observed under polarized light. Scale bar = 0.1 mm.

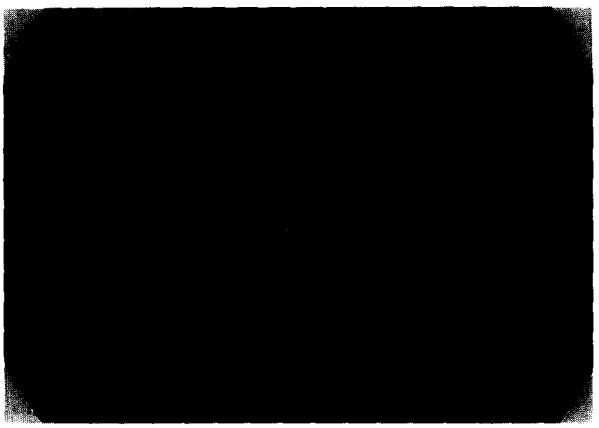

Fig. 2. X-Ray diffraction pattern of a ba_3-type cytochrome-*c* oxidase crystal. A 1.0° rotation image of an unaligned crystal is shown, with a crystal to detector distance of 250 mm. Diffraction spots were obtained to 3.8 Å.

Improvements of the crystal quality by variation of detergent concentration and temperature, advanced data collection – like cryo-crystallography and synchrotron radiation – are under way.

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