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Purification, crystallization and preliminary crystallographic studies of an integral membrane protein, cytochrome bo_3 ubiquinol oxidase from *Escherichia coli*

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Cytochrome bo_3 ubiquinol oxidase has been successfully purified for crystallization. Single crystals of this integral membrane protein diffract X-rays to 3.5 Å resolution and belong to the orthorhombic space group $C222_1$. From the diffraction data, the unit-cell parameters were determined to be a = 91.3, b = 370.3, c = 232.4 Å. The crystals have a solvent content of 59% and contain two molecules per asymmetric unit. A search model generated from the structures of cytochrome *c* oxidase from *Paracoccus denitrificans* and the extrinsic domain of cytochrome bo_3 ubiquinol oxidase from *Escherichia coli* was used for molecular-replacement studies, resulting in a solution with sensible molecular packing.

1. Introduction

Haem-copper oxidases are integral membrane proteins located in the mitochondrial inner membrane or the bacterial inner cell membrane. These enzymes are the final electron acceptors in the respiratory chain and catalyze the four-electron reduction of molecular oxygen to water. The free energy liberated from this exergonic reaction is directly coupled to proton translocation across the membrane. The proton gradient generated by the oxidase and the other members of the aerobic respiratory chain can then be converted to a more useful energy form *via* energy-conserving systems such as ATPase (Mitchell, 1979; Saraste, 1999). Assignment to the haem-copper oxidase superfamily is based on two criteria: (i) a high sequence homology within the largest subunit (subunit I) and (ii) a binuclear centre forming the oxygen-binding site, coupled with a low-spin haem that facilitates electron transfer to O_2 (Babcock & Wikström, 1992). This enzyme superfamily is divided into two branches based on substrate specificity. Oxidases from mitochondria and many bacteria use cytochrome *c* as a substrate and are called cytochrome *c* oxidases, whereas some prokaryotic respiratory oxidases utilize membrane-bound quinol as a substrate and are thus called quinol oxidases.

Both cytochrome *c* and quinol oxidases have been extensively studied by spectroscopy and site-directed mutagenesis (Wikström, 1998; Iwata, 1998). In recent years, there has been a great deal of progress in functional studies on terminal oxidases, mainly as a consequence of the determination of atomic resolution structures of cytochrome *c* oxidase (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995). Based on these structures, several new models for proton pumping by the terminal oxidases have been proposed. However, because of a lack of resolution and the invisible nature of protons to X-ray techniques, no definite mechanism for the proton pumping has been reached. Comparative studies of cytochrome *c* oxidase and ubiquinol oxidase should resolve this question, as the two enzymes catalyze quite different reactions but should share a similar proton-pumping mechanism.

Unfortunately, ubiquinol oxidase has not been investigated to the same extent as cytochrome *c* oxidase, most likely because of the limited structural information available. Only the structure of the extrinsic domain of subunit II has been reported (Wilmanns *et al.*, 1995). Here, we report the purification and crystallization of the entire cytochrome bo_3 ubiquinol oxidase. We have obtained a molecular-replacement solution that shows sensible molecular packing using a search model generated from the structures of cytochrome *c* oxidase from *P. denitrificans* and the extrinsic domain of cytochrome bo_3 ubiquinol oxidase from *E. coli*.

2. Materials and methods

2.1. Cell culture

Bacterial growth conditions and the preparation of membranes from *E. coli* cells were as described previously (Morgan *et al.*, 1995). The bacterial strain used (GO105) contains the plasmid pJRHisA, which encodes for a genetically modified cytochrome bo_3 oxidase with a carboxyl-terminus histidine tag on subunit II (Rumbley *et al.*, 1997).

2.2. Purification

A two-step purification method using affinity and anion-exchange chromatography was necessary in order to obtain enzyme which was pure enough for crystallographic purposes. The initial chromatographic step was an affinity column using Ni^{2+}–NTA as a medium (Qiagen, Chatsworth, CA, USA). After ultracentrifugation, solubilized membranes were applied to a 65 ml bed volume that had been equilibrated with 20 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 5 m*M* imidazole, 0.03% dodecyl maltoside (Anatrace). The column was washed with three bed volumes of the equilibration buffer to remove any non-specific binding proteins and the sample was then eluted with a linear imidazole gradient (5–150 m*M*). The resulting chromatograms showed two clear components that were termed 'low' and 'high' based on the elution concentration of the imidazole. These components were pooled separately and washed in an Amicon cell (30 kDa cutoff) using 20 m*M* Tris–HCl pH 7.5, 0.03% dodecyl maltoside in order to lower the salt concentration.

The following chromatographic step was performed in the presence of 1 m*M* potassium ferricyanide. The protein fractions from the affinity column were independently applied to an anion-exchange column (MonoQ 10/10; Pharamacia Biotech, Sweden) equilibrated with 20 m*M* Tris–HCl pH 7.5, 1% octyl glucoside (Anatrace). The sample was slowly washed with six bed volumes of the above equilibration buffer and was then eluted using the equilibration buffer with the addition of 600 m*M* NaCl in a linear gradient over 20 column volumes. Fractions containing homogenous enzyme were pooled and washed in a Centricon cell (100 kDa cutoff) with 20 m*M* Tris–HCl pH 7.5, 1% octyl glucoside and were then concentrated to 20 mg ml^{-1}.

2.3. Crystallization

Crystals were obtained by the hanging-drop vapour-diffusion technique. The enzyme solution contained 20 m*M* Tris–HCl pH 7.5, 1% octyl glucoside and 20 mg ml^{-1} protein. A reservoir solution of 9–10%(*w*/*v*) PEG 1500, 100 m*M* HEPES pH 7.0, 100 m*M* NaCl, 100 m*M* $MgCl_2$, 5% ethanol was used. The protein solution was mixed in a 1:1 ratio with the reservoir solution and left to equilibrate at 277 K. The 'high' imidazole portion was not used for further crystallization trials as the crystals were not reproducible.

2.4. Data collection, processing and molecular replacement

All data were collected from frozen crystals (100 K). Before freezing the crystals in liquid nitrogen, they were soaked in cryosolutions in which both the glycerol (5–20%) and PEG 1500 (10–13%) concentrations were gradually increased. Two data sets were collected at the ESRF on beamlines ID14/EH3 and ID14/EH4. Image data were processed using the *HKL* program suite (Otwinowski, 1993) and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Molecular replacement was performed using the program *AMoRe* (Navaza, 1994).

3. Results and discussion

Optimization of the protein purification protocol was crucial for successful crystallization. The Ni^{2+}–NTA column chromatography always gives two clear components that were termed 'low' and 'high'. The difference between these two components is likely to be related to a partial degradation of the carboxy terminus of subunit II including the His_6 tag (Rumbley *et al.*, 1997); however, both components show the same quinol-oxidation/proton-pumping activities. Although the components only differ by a small degradation of subunit II, the 'high' imidazole component did not give consistent crystals and only the 'low' imidazole component was used for crystallization.

An additional anion-exchange chromatography step was needed to isolate a homogenous sample and proved essential to obtain crystals. However, initial trials with the anion-exchange column proved to be erratic and irreproducible. We relate this to the oxidation states of free cysteines within the protein molecule. The sample is naturally in an oxidized state, but after reduction with dithiothreitol or complete oxidation with potassium ferricyanide we were able to obtain single products. The potassium ferricyanide treated sample was used for further experiments, since only the oxidized sample produced crystals. From this sample, rod-like crystals grew within one week with average crystal dimensions of 0.6 × 0.2 × 0.1 mm (Fig. 1).

We have collected two data sets from crystals which diffract X-rays beyond 3.5 Å. These crystals belong to the space group $C222_1$, with unit-cell parameters a = 91.3, b = 370.3, c = 232.4 Å. The first data set was processed to 3.5 Å resolution with an overall completeness of 87% (78% in the outer shell) using 141 448 reflections (44 359 unique). The $R_{\rm merge}$ values in the lowest (40.0–7.53 Å) and highest resolution shells (3.64–3.5 Å) are 0.07 and 0.40, respectively, with the overall $R_{\rm merge}$ value being 0.12. The average $I/\sigma(I)$ is 7.9. Although $I/\sigma(I)$ falls to 1.5 in the highest resolution shell (3.64–3.5 Å), in the 4.14–3.94 Å shell it is 3.3. After careful examination, including a test for merohedral twinning, we concluded that the somewhat high $R_{\rm merge}$ value arises from the diffused spot shape and is not caused by

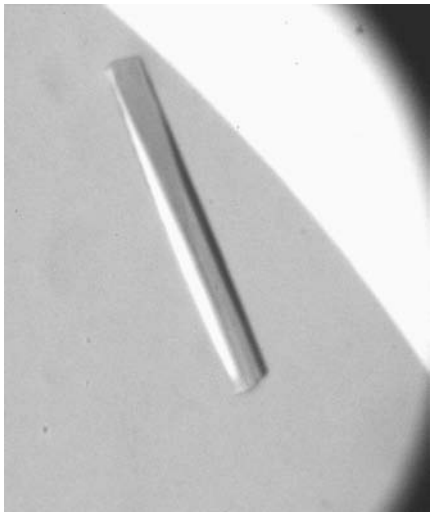

Figure 1
Crystal of cytochrome bo_3 ubiquinol oxidase from *E. coli*. The dimensions of the crystal are 0.6 × 0.2 × 0.1 mm.

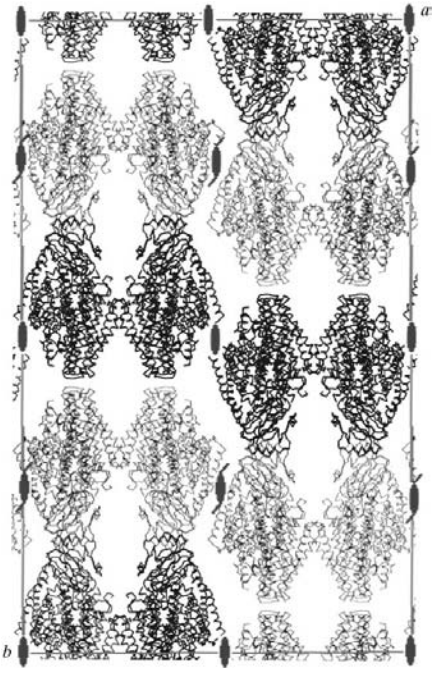

Figure 2
The crystal packing of cytochrome bo_3 ubiquinol oxidase. The molecules shown in two different shades represent the two independent molecules in the asymmetric unit.

misindexing. The correctness of the data processing was also supported by the molecular-replacement study.

The second data set had a higher resolution of 3.2 Å, but the completeness was considerably less at 77.3%; the $R_{\rm merge}$ value was higher at 0.15. Assuming a dimer of ubiquinol oxidase (MW = 288 kDa) per asymmetric unit, the ratio of the volume to unit protein mass, V_m, and the solvent content of the crystal are 3.4 Å^3 Da^{-1} and 59%, respectively. The V_m and solvent contents are higher than for ordinary soluble protein crystals but similar to those of other membrane-protein crystals (Ostermeier *et al.*, 1995; Iwata *et al.*, 1998), which typically show a solvent content of 65–75%.

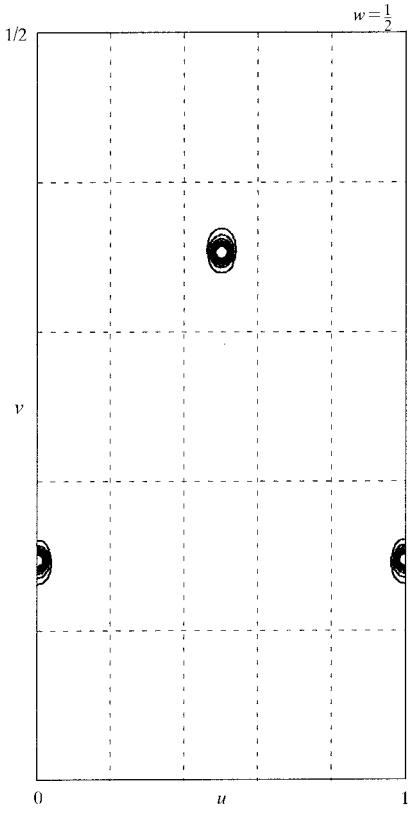

Figure 3
The $w = \frac{1}{2}$ section of 15.0–4.0 Å native Patterson map. Contouring starts at 5σ with intervals of 5σ. The peak at u = 0.50, v = 0.35, w = 0.50 is the only significant peak in the native Patterson map.

The first data set at 3.5 Å resolution has been used for a molecular-replacement study because of its higher completeness and lower $R_{\rm merge}$ value. The coordinates of cytochrome *c* oxidase from *P. denitrificans* were modified and used as a search model for molecular replacement. The following alterations were made when constructing the model. The previously solved hydrophilic domain of subunit II (PDB code 1cyw) was aligned with and used to replace the corresponding region in cytochrome *c* oxidase. All conserved residues between cytochrome *c* oxidase and ubiquinol oxidase were maintained, as well as common atoms from similar residues, while those remaining were mutated to alanine. The level of sequence identity between the largest subunit (subunit I, making up 45% of the entire protein) of cytochrome *c* oxidase from *P. denitrificans* and cytochrome bo_3 from *E. coli* is 31.8%. Using the program *AMoRe* (Navaza, 1994) with a data resolution range 15–5 Å gave a solution with a correlation coefficient of 59.6% and an *R* factor of 44.6%. This solution was very clear; the correlation coefficient and *R* factor for the next best solution were only 39.3 and 48.2%, respectively. The two molecules in the asymmetric unit have exactly the same orientation and are related by only a translation (x = 0.50, y = 0.35, z = 0.50). As seen in Fig. 2, a layer composed of one molecule in the asymmetric unit and a second layer composed of the other molecule are stacked along the *b* axis of the crystal. The two layers have the same orientation, with a shift in the *a* and *c* axis plane. The following observations also support the solution obtained. (i) We could observe only one strong peak in the cross-rotation function at Euler angles 13.8, 24.6 and 88.8°. (ii) There are no clear peaks in the self-rotation function other than those representing crystallographic symmetry. (iii) There is a strong peak in the native Patterson map at u = 0.50, v = 0.35, w = 0.50 (Fig. 3). The packing diagram was checked in the program *O* (Jones *et al.*, 1990) and seemed reasonable as there is no overlap between molecules and only the hydrophilic surfaces of the molecule have crystal contacts. The absence of crystal contacts along the transmembrane helices, which is consistent with crystal packing from integral membrane proteins, is a consequence of this region being covered with detergent micelles. Structural refinement and remodeling is currently in progress based on the solution obtained.

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