Purification and two-dimensional crystallization of bacterial cytochrome oxidases

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(Received 3 August 1995) — EJB 95 1290/4

A novel strategy which employs chromatography on an immobilized metal ion has been developed for the purification of bacterial cytochrome c and quinol oxidases. Many bacterial oxidase complexes appear to have a natural affinity to bind to the chelated copper ion. A combination of three different chromatographic principles (anion exchange, metal-affinity and gel filtration) makes an effective tool chest for the preparation of homogeneous and protein-chemically pure bacterial oxidases. These preparations have been used for two-dimensional crystallization. Until now, crystals have been obtained using the Paracoccus denitrificans and Rhodobacter sphaeroides cytochrome aa₃ and the Escherichia coli cytochrome bo. The crystals diffract to approximately 2.5 nm in negative stain and have potential for further structural studies.

Keywords: cytochrome-c oxidase; quinol oxidase; purification; metal affinity chromatography; two-dimensional crystals.

Cytochrome oxidase (cytochrome aa₃) is the terminal catalyst of the mitochondrial respiratory chain. This enzyme oxidizes dioxygen to water with electrons derived from cytochrome c and, coupled to the redox reaction, translocates protons across the membrane building an electrochemical gradient that is used for synthesis of ATP (Babcock and Wikström, 1992). The mitochondrial enzyme has homologs in eubacteria and archaea that are structurally simpler than their mitochondrial counterpart. The latter is assembled by two pools of subunits. The functionally central subunits I, II and III are encoded by the organellar genome. Depending on species, 5-10 additional nuclear-coded proteins assemble with the three mitochondrial subunits into the active complex. The prokaryotic oxidases are comprised of the three key subunits homologous to the mitochondrial subunits plus one or two additional proteins without a eukaryotic homolog (Capaldi, 1990; Saraste, 1990; Calhoun et al., 1994; Garcia-Horsman et al., 1994a; Trumpower and Gennis, 1994).

The bacterial and archaean oxidases are divided into two main branches within the superfamiliy. One branch uses cytochrome c as the electron donor whereas the other one uses quinols. The active site in both is a bimetallic haem iron/copper centre. Electrons are transferred to this centre via a low-spin haem. All these metal centres reside in subunit I (Capaldi, 1990; Saraste, 1990; Garcia-Horsman et al., 1994a; Trumpower and Gennis, 1994). The fourth redox-active metal centre is only present in the cytochrome-c-oxidizing enzymes. It is a dinuclear copper site bound to the membrane-exposed domain of subunit II. In the quinol-oxidizing enzymes such as the Escherichia coli cytochrome bo, this copper centre is not present (van der Oost et al., 1992; Castresana et al., 1994). Recently, a novel oxidase complex has been characterized in rhizobia and purple bacteria (Preisig et al., 1993; de Gier et al., 1994; Garcia-Horsman et al., 1994b; Gray et al., 1994). This enzyme which is thought to function under a very low oxygen tension (Preisig et al., 1993), contains a homolog of subunit I but the two other proteins of the complex are membrane-bound c-type cytochromes.

Along with many other laboratories, we have been engaged with attempts to obtain high-resolution structural information on cytochrome oxidases. These efforts necessarily require highly purified, homogeneous preparations. Several improved purification methods have recently been published both for the bacterial and mitochondrial oxidases (Steiffens et al., 1990; Hosler et al., 1992; Minghetti et al., 1992; Steffens et al., 1993; Hallia et al., 1994; Geier et al., 1995; Henning et al., 1995; Kleymann et al., 1995; Sontimane and Buse, 1995). Here we describe the use of immobilised-metal-ion affinity chromatography (IMAC) with copper in the purification of untagged cytochrome-c oxidases from Paracoccus denitrificans and Rhodobacter sphaeroides as well as of the E. coli cytochrome bo quinol oxidase. These oxidases have a natural affinity to bind to immobilised Cu²⁺, and IMAC forms an integral part of procedures which facilitate the rapid preparation of highly purified samples. They have further been crystallized in two dimensions by the removal of detergent by dialysis after the addition of lipids.

MATERIALS AND METHODS

Materials. Dodecyl β-D-maltoside (DodGlc₃) was obtained from Anatrace. Lauryl(dimethyl)amine-N-oxide (LDAO) was purchased from Fluka, and octyl β-D-glucoside (OctGlc), decyl β-D-maltoside (DecGlc₃) and Triton X-100 from Calbiochem. All chromatographic materials and prepacked columns were obtained from Pharmacia. The following lipids (with catalogue numbers) were obtained from Sigma: dimyristoylglycerophosphocholine (Myr,GroPC, P6392), egg phosphatidylcholine...
(PtdCho) type III-E (P7318), soybean PtdCho (P7443), and brain extract type III (Folch fraction III, B1627) which is a source of crude phosphatidylserine (PtdSer).

**Bacterial strains and growth.** Bacterial cells were grown in a 150-L fermenter. *P. denitrificans* MR-3, a strain in which the gene encoding an isoform of subunit I has been deleted (Raio et al., 1990), was grown at 30°C in the succinate medium described by Ludwig (1986) with 50 µg/ml kanamycin. The *E. coli* strain RG145 which is an overproducer of the wild-type cytochrome bo (Au and Gennis, 1987), and the strain RG129/prCO1 which overproduces an engineered cytochrome bo with a genetic fusion between cyaA and cyaB encoding subunits II and I (Ma et al., 1993), were kind gifts of Dr Robert Gennis (University of Illinois). They were grown at 37°C in Luria-Bertani medium with 50 µg/ml ampicillin. The *R. sphaeroides* strain CY91 (Dosler et al., 1992) was obtained from Dr Shelagh Ferguson-Miller (Michigan State University). It was grown in medium A of Sistrom (1960) with 50 µg/ml kanamycin. Bacterial cells were harvested in the exponential phase by concentration of the media by ultrafiltration and subsequent centrifugation, and stored at −80°C. Yields were typically 5 g wet cells.

**Preparation and solubilization of membranes.** Frozen cells of *P. denitrificans* (200 g), *R. sphaeroides* (400 g) or *E. coli* (50 g) were thawed and suspended in 1–2 ml/g cell paste of 20 mM Tris/HCl, pH 8, 1 mM MgCl₂, 50 µg/ml DNaseI and 1 mM. Cells were broken by a single passage through a French pressure cell fitted with a rapid-fill facility. EDTA was added to 10 mM, and membranes were pelleted by centrifugation at 20000 g for 2 h. Only the upper layer of cytoplasmic membranes was recovered and resuspended using an Ultra Turrax homogenizer. Membranes from *P. denitrificans* were washed with 20 mM Tris/HCl, pH 8, containing 0.4 M KCl, 1 mM deoxycholate, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride as described by Ludwig (1986), then with 20 mM Tris/HCl, pH 8, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride before final resuspension in the same buffer at a protein concentration of approximately 20 mg/ml. The membranes from *E. coli* and *R. sphaeroides* were washed with 20 mM Tris/HCl, pH 8, 1.5 M NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, then in 20 mM Tris/HCl, pH 8, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, and finally resuspended in this buffer at a protein concentration of approximately 20 mg/ml. All prior operations were carried out at 4°C. Membranes were stored at −80°C.

Membranes were solubilized with DdGlc₂, which was added to thawed membrane suspensions at protein concentration 15–20 mg/ml in the proportion of 2 g detergent/g protein. The suspensions were centrifuged at 200000 g for 1 h at 4°C. Supernatants were recovered and applied to the first column.

**Purification of bacterial cytochrome oxidases.** All chromatographic steps were carried out with Pharmacia GradiFrac or FPLC systems with dual-wavelength monitoring at 280 nm and 405 nm at room temperature. Buffers for the initial anion-exchange step were (A) 20 mM Tris/HCl, pH 8, 0.2 mM EDTA and 0.1% DdGlc₂, and (B) 20 mM Tris/HCl, pH 8, 1 M NaCl and 0.1% DdGlc₂ (note, there was no EDTA in buffer B). 0.5 mM EDTA was included in buffers A and B in the anion-exchange chromatography following IMAC.

A self-packed 40-ml XK26/20 Chelating Sepharose Fast Flow column was used for IMAC. The IMAC buffer A contained 20 mM Tris/HCl, pH 8, 0.5 M NaCl and 0.1% DdGlc₂, and buffer B contained in addition 125 mM imidazole. The Chelating Sepharose was prepared for use by passing CuCl₂ through the column until the development of a light blue colour indicated saturation with Cu²⁺. It was equilibrated with buffer A until the column had developed a deep blue colour.

For the final gel filtration, a buffer containing 20 mM Tris/HCl, pH 8, 50 mM NaCl, 0.5 mM EDTA, and 0.1% DdGlc₂ (or another desired detergent) and 2 mM sodium azide (to prevent bacterial growth during the subsequent crystallization experiments) was used.

Protein samples were concentrated by ultrafiltration in a 50-ml Amicon chamber with Filtron 50-kDa-cut-off Omega membranes or 50-kDa-cut-off Filtron spin-concentrators to 10–20 mg/ml and stored at −80°C with 20% (mass/vol.) glycerol.

**P. denitrificans cytochrome aa₃**, 0.4–0.6 g of solubilized membrane protein in a buffer solution at pH 8 (see above) containing DdGlc₂, were applied to a 50-ml self-packed Q-Sepharose High Performance column. Successive gradients of 0 to 20% buffer B (20 ml), 20% to 45% buffer B (450 ml) and 45% to 100% buffer B (25 ml) were applied at 4 ml/min. The fractions containing cytochrome-c oxidase eluted at approximately 0.4 M NaCl.

Prior to the application to the IMAC column, 0.08% (final concentration) l-auryl dimethylamine-N-oxide was added to the oxidase fractions, and they were briefly centrifuged at 1000 g. Cytochrome oxidase bound as a tight coloured band to the top of the IMAC column. Successive gradients of 0 to 12% buffer B (400 ml) and 12% to 100% buffer B (50 ml) were applied at 5 ml/min, and the elution was then continued with 100% buffer B. The fractions containing cytochrome oxidase eluted with the second gradient. They were concentrated and stored frozen.

Further purification was carried out on a reduced scale. Thawed oxidase samples from the IMAC column were first incubated with 0.08% l-auryl dimethylamine-N-oxide for 10 min, briskly centrifuged, and diluted fivefold in buffer A containing 0.2% DecGlc₂, before the application to a HR16/10 (20 ml) Q-Sepharose HP column. After washing the bound sample with 50 ml of buffer A with 0.2% DecGlc₂, a gradient of 20% to 50% buffer B with 0.1% DecGlc₂ (250 ml) was applied at 2 ml/min. The oxidase fractions eluted from the column were concentrated to 10–20 mg/ml and applied in 0.1 ml aliquots to an equilibrated Superose 6 HR10/30 gel-filtration column which was run at 0.25 ml/min.

**R. sphaeroides cytochrome aa₃**, 3–4 g membrane protein were solubilized and applied to a 200-ml DEAE-Sepharose Fast Flow column. A gradient of 10% to 55% buffer B (2600 ml) was applied at 12 ml/min. Green fractions containing cytochrome oxidase eluted as a shoulder on the tail end of a major peak containing b-type and c-type cytochromes. These fractions were directly applied to the IMAC column, and a gradient of 2.5% to 5% buffer B (250 ml) at 4 ml/min was developed. Elution was maintained with 2.5% buffer B until the first peak had been eluted from the column. A steep gradient of 2.5% to 25% buffer B (40 ml) was then applied in order to elute cytochrome oxidase. The cytochrome oxidase fractions from the copper column were concentrated and stored frozen.

Further purification was carried out by a double Q-Sepharose chromatography. Thawed fractions were diluted fivefold in anion-exchange buffer A containing 0.2% DecGlc₂, before application to a HR16/10 (20 ml) Q-Sepharose HP column. After washing the bound sample with the starting buffer, a gradient of 10% to 50% buffer B with 0.1% DecGlc₂ (275 ml) was applied at 2 ml/min. Four variants of the cytochrome oxidase were partially resolved with this column (Fig. 5). Further individual purification of these variants was carried out by application of fractions from peak 1, 2, 3 or 4 to a HR10/10 (6 ml) Q-Sepharose HP column after fivefold dilution in buffer A. A gradient of 10% to 50% buffer B (85 ml) was applied at 1 ml/min. This resulted in the elution of single sharp peaks of variants 1–3. These were concentrated to approximately 0.2 ml before application in 0.1-ml aliquots to a Superose HR10/30 column which was eluted.
at 0.25 ml/min before final concentration and storage. Sharp symmetrical peaks eluted in each case. Variant 4 could not be further purified.

**E. coli cytochrome bo.** The detergent for the first anion exchange and the following IMAC steps was DodGlc2, and for the latter anion-exchange and gel-filtration columns DecGlc2. 0.5 g solubilised membrane protein was applied to a 200-ml XK50/20 DEAE Sepharose FF column. A gradient from 0 to 40% buffer B with 0.1% DodGlc2 (3 l) was applied at 12 ml/min. The fractions containing cytochromes eluted in a single major peak at approximately 0.25 M NaCl, and were applied directly to the 40 ml IMAC column. The cytochromes bound as a tight band to the top of the column. A yellowish-brown peak with a high A280 / A600 ratio eluted during the initial gradient of 0 to 15% buffer B (400 ml). A second gradient of 15% to 100% buffer B (50 ml) was then used to elute cytochrome bo.

The fractions from the IMAC column were diluted fivefold in anion-exchange buffer A containing 0.2% DecGlc2, and applied to an XK26/20 (50 ml) Q-Sepharose HP column. After washing the bound sample with 100 ml buffer A containing 0.2% DecGlc2, a gradient of 0–40% buffer B (750 ml) was applied at 4 ml/min. The oxidative fractions which eluted as a single peak at approximately 0.25 M NaCl were concentrated to 2 ml and applied to a 26/60 Sephacryl 300 HR gel-filtration column equilibrated with the gel-filtration buffer containing 0.1% DecGlc2, operated at 1 ml/min. Material from the single sharp peak was concentrated and stored frozen.

**Protein and haem determinations.** Protein concentrations were estimated during the purification procedures with the Pierce BCA (bicinchoninic acid) method using bovine serum albumin as a standard. For the final preparations, they were measured using the method of Gill and von Hippel (1989) and molar absorption coefficients calculated from the predicted concentrations of aromatic amino acids (Chepuri et al., 1990; Raitio et al., 1987, 1990; Steinru!cke et al., 1987). These are 300 mM cm−1 for the four-subunit *E. coli* cytochrome bo and 190 mM cm−1 for the two-subunit *Paracoccus* cytochrome aa3 at 280 nm. Dithionite-reduced minus ferricyanide-oxidised pyridine haemochrome spectra were recorded after the addition of pyridine to 20% (by vol.) and NaOH to 50 mM. Haem contents were determined using the molar absorptivities determined by Berry and Trumpower (1987). The haem content of the cytochrome bo was calculated with the absorbance coefficient of the haemochrom bo using the wavelength pair 553–590 nm of the reduced minus oxidized spectrum.

**Polyacrylamide gel electrophoresis.** SDS/PAGE was carried out as described by Laemmli (1970) with 12% acrylamide gels. Gels were stained by two cycles of silver staining following the first Coomassie staining and a complete destaining to maximize sensitivity.

**Enzymatic activity.** Cytochrome oxidase activity was determined at 20°C spectrophotometrically using either N,N,N′,N′-tetramethyl-p-phenylenediamine [Ph(NMe2)2] or reduced horse heart cytochrome c as the electron donor for quinol and cytochrome c oxidases, respectively. The assays were carried out in order to follow the stability of cytochrome oxidases in the different detergent solutions employed for crystallisation experiments. Highly concentrated cytochrome oxidases in buffers containing DecGlc2 were diluted to various detergent solutions. The free detergent concentrations were kept at the level of twice the critical micelle concentration, and if no precipitation was observed, the activity was determined at daily intervals storing the protein solutions at room temperature. Only those detergents which maintained a high proportion of activity over several days were used for crystallization.

**Two-dimensional crystallization.** Microdialysis of protein/lipid detergent mixtures was carried out using bent glass tubes with a 3-mm internal diameter, as described by Kühnbrandt (1992). These were fitted with 12–14-kDa cut-off dialysis membranes. 50 µl of solutions with a protein concentration of 0.1–1.5 mg/ml (as determined by the bicinchoninic acid method) were added to each tube and dialysed against buffer without detergent. 2 mM sodium azide was always included in the dialysis buffer. Lignins were dissolved at 2–4 mg/ml in 1% detergent prior to the addition to crystallization mixtures. The final purification of the oxidases was carried out in 0.1% DecGlc2, but this was subsequently diluted by a factor of 25–100 with the detergent for the subsequent crystallization experiment.

Aliquots were withdrawn from dialysis tubes to monitor the progress of crystallization. 1–2 µl was applied to glow-discharged carbon-coated grids and negatively stained with uranyl acetate. For routine checking, Phillips 400 or 301 microscopes were used. The crystalline structure was observed with an optical diffractometer.

**RESULTS**

**Purification of bacterial oxidases.** An intrinsic difficulty in purification of membrane protein complexes is their tendency to fragment in detergent solutions. Detergents dissociate protein contacts in different ways. Non-ionic detergents such as decyl and dodecyl maltosides are generally more gentle, preserving intact complexes. In contrast, lauryl dimethylamineoxide has a tendency to split weak protein contacts (Kühnbrandt, 1988). The *P. denitrificans* cytochrome-c oxidase tends to loose the third subunit during purification (Ludwig and Schatz, 1980; Haltia et
Fig. 2. Reduced-minus-oxidized spectra of the *P. denitrificans* cytochromes during purification. The spectra of solubilized membrane proteins (A), the fractions containing cytochrome *aa*, pooled from Q-Sepharose (B), and the pooled fractions from the Chelating-Sepharose column (C) are shown.

Fig. 3. SDS/PAGE of the preparations during the purification and at the final stage. (A) Polypeptide composition of the *P. denitrificans* cytochrome *aa*, preparations after membrane solubilization (lane 1), in the Q-Sepharose peak 4 (lane 2, see Fig. 1A), and after the Chelating-Sepharose column (lane 3). (B) Polypeptide composition of the *E. coli* cytochrome *bo* preparations after membrane solubilization (lane 1), DEAE-Sepharose column (lane 2) and Chelating-Sepharose column (lane 3). (C) The final preparations of the two-subunit *P. denitrificans* oxidase (lane 1), the two-subunit and three-subunit *R. sphaeroides* oxidase with the longer version of subunit II (lanes 2 and 3), and the four-subunit *E. coli* cytochrome *bo* (lane 4). The molecular-mass markers are shown on the left.

Fig. 4. The gel-filtration profiles of purified oxidase complexes. The profile for the two-subunit *P. denitrificans* cytochrome *aa*, is shown with a dashed line, and the profile for the four-subunit *E. coli* cytochrome *bo* with a solid line. Both proteins are complexes with DecGlC. The column is Superose 6. The numbers refer to the elution positions of the marker proteins ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa) are numbered 1–4.

Fig. 5. Elution profile of the *R. sphaeroides* cytochrome *aa*, from Q-Sepharose. The figure shows the third stage in the purification of the *R. sphaeroides* cytochrome *aa*. The preparation is fractioned into four components. These contain (1) a two-subunit complex with the long version of subunit II, (2) a two-subunit complex with the short version of subunit II, (3) a three-subunit complex with the long version of subunit II, and (4) a three-subunit complex with the short version of subunit II, shown by SDS/PAGE on the side of the elution profile.

The purification protocols of the bacterial cytochrome oxidases involve three chromatographic techniques with different fractionation principles. The combination of anion-exchange chromatography, IMAC and gel filtration has enabled us to design purification schemes that rapidly and with a high yield re-
Table 1. Purification of cytochrome aa, from P. denitrificans. The table shows a typical purification experiment. The yield is based on total haem A. Note that the haemA/protein ratio of the final preparation is erroneously high due to the underestimation of protein concentration by the bicinchoninic acid method (see text).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein</th>
<th>Haem A</th>
<th>Haem A/ protein</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td>565</td>
<td>500</td>
<td>0.88</td>
<td>100</td>
</tr>
<tr>
<td>Solubilised membranes</td>
<td>465</td>
<td>470</td>
<td>1.0</td>
<td>94</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>32</td>
<td>405</td>
<td>12.6</td>
<td>81</td>
</tr>
<tr>
<td>IMAC</td>
<td>18.5</td>
<td>335</td>
<td>18.0</td>
<td>67</td>
</tr>
<tr>
<td>Final</td>
<td>2-5</td>
<td></td>
<td>39.0</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Purification of cytochrome bo from E. coli. The table shows a typical purification experiment. The yield is estimated from the protohaem and haem O content (see Materials and Methods) which includes a contribution of haemns extracted from other enzymes such as succinate dehydrogenase that are present during the initial phases of purification.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein</th>
<th>Haem</th>
<th>Haem/protein</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td>840</td>
<td>1430</td>
<td>1.7</td>
<td>100</td>
</tr>
<tr>
<td>Solubilised membranes</td>
<td>500</td>
<td>1100</td>
<td>2.2</td>
<td>77</td>
</tr>
<tr>
<td>DEAE</td>
<td>260</td>
<td>990</td>
<td>3.8</td>
<td>69</td>
</tr>
<tr>
<td>IMAC</td>
<td>55</td>
<td>760</td>
<td>13.8</td>
<td>53</td>
</tr>
<tr>
<td>Final</td>
<td>30</td>
<td>645</td>
<td>21.5</td>
<td>45</td>
</tr>
</tbody>
</table>

The final purification was carried out with a smaller anion-exchange column (Materials and Methods). Cytochrome oxidase usually elutes as a single sharp peak from this column. However, multiple peaks were observed when a large amount of material was loaded to this column. This may be due to a tendency to oligomerisation at a high concentration. The homogeneity of our final (monomeric) preparation of the two-subunit complex is shown by SDS/PAGE in Fig. 3C (lane 1) and gel filtration in Fig. 4. The haem/protein ratio was determined to be 23.0 nmol/mg using the method of Gill and von Hippel (1989) for protein quantitation (the bicinchoninic acid method gives a much too low protein concentration). This is close to the theoretical value (21.7 nmol/mg) for the 1:1 subunit I1I complex. The typical activity of the horse heart cytochrome c was 200 s⁻¹ at room temperature. The purification is summarized in Table 1.

Purification of the R. sphaeroides cytochrome c oxidase can be carried out by a modification of the procedure outlined above in the presence of 0.1% DodGlc₂. However, the affinity of this oxidase to the copper column is lower than that of the P. denitrificans oxidase. Therefore, the gradient used in IMAC was modified to, the partial processing of subunit II which is present in two forms (short and long), and to the partial dissociation of subunit III from the complex. Four types of complexes can be separated on Q-Sepharose HP (Fig. 5). These are two-subunit or three-subunit complexes either with the short and long form of the subunit II. The two-subunit and three-subunit complexes containing the longer form of subunit II are present in larger amounts and can be purified to homogeneity by repeated anion exchange (Fig. 3C, lanes 2 and 3), but this makes the overall yields low (data not shown).

Table 3. Two-dimensional crystallization of bacterial cytochrome oxidases. The P. denitrificans enzyme is a two-subunit complex, the E. coli enzyme is a four-subunit complex, and the R. sphaeroides enzyme is a three-subunit complex. Cyt. cytchrome.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Protein</th>
<th>Detergent</th>
<th>Lipid</th>
<th>Morphology</th>
<th>Layer group</th>
<th>Lattice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cyt aa₁</td>
<td>DecGlc₂</td>
<td>egg PtdCho + brain PtdSer</td>
<td>tubular</td>
<td>c12</td>
<td>a = 20.0 nm, b = 13.6 nm, γ = 90°</td>
</tr>
<tr>
<td>2</td>
<td>Cyt bo (E. coli)</td>
<td>DecGlc₂</td>
<td>Myr₂,GroPCh</td>
<td>tubular</td>
<td>p2</td>
<td>a = 13.7 nm, b = 13.0 nm, γ = 118°</td>
</tr>
<tr>
<td>3</td>
<td>Cyt bo (E. coli)</td>
<td>Lauryl dimethylamine-N-oxide</td>
<td>soybean PtdCho + brain PtdSer</td>
<td>tubular</td>
<td>p22,2</td>
<td>a = 21.0 nm, b = 9.0 nm, γ = 90°</td>
</tr>
<tr>
<td>4</td>
<td>Cyt bo (E. coli)</td>
<td>Triton X-100</td>
<td>egg PtdCho + brain PtdSer</td>
<td>sheet</td>
<td>p22,2</td>
<td>a = 21.0 nm, b = 9.0 nm, γ = 90°</td>
</tr>
<tr>
<td>5</td>
<td>Cyt aa₁ (R. sphaeroides)</td>
<td>DecGlc₂</td>
<td>egg PtdCho + brain PtdSer</td>
<td>vesicular or sheet</td>
<td>p22,2</td>
<td>a = 16.3 nm, b = 10.7 nm, γ = 90°</td>
</tr>
</tbody>
</table>
Fig. 6. Two-dimensional crystals of bacterial cytochrome oxidases. (A) Tubular crystals of the *P. denitrificans* cytochrome *aa* and egg PtdCho plus brain extract in 2.6:1 ratio grown by dialysis against 20 mM Bistris-propane (pH 7) at 12°C starting with a protein concentration of 0.2 mg/ml, a lipid/protein of 0.85:1 and 0.2% DecGlc. Optical diffraction of the micrograph is shown in the insert. (B) Tubular crystals of the *E. coli* cytochrome *bo* with Myr,GroPCho after the removal of DecGlc, by dialysis against 20 mM Tris/HCl, pH 8, containing 10% isopropanol at 24°C. The initial conditions were 1.5 mg/ml protein, lipid/protein ratio of 1.25:1 and 0.2% DecGlc. (C) Tubular crystals of the *E. coli* cytochrome *bo* with a 2:1 mixture of soybean PtdCho and brain extract. The initial lipid/protein was 0.65 with 0.35 mg/ml protein, and the lauryl dimethylamine-N-oxide concentration was 0.2%. The crystals were grown by dialysis against 20 mM Bistris-propane, pH 6.9, containing 10% isopropanol. Optical diffraction of the micrograph is shown in the insert. (D) Crystalline sheets of the *E. coli* cytochrome *bo* with a 2.6:1 mixture of egg PtdCho and brain extract which was used with a lipid/protein of 0.7. The initial protein concentration was 0.35 mg/ml and that of Triton X-100 0.2% (mass/vol.). (E) Vesicular crystals of the 3-subunit complex of the *Rhodobacter* cytochrome *aa*, grown under the following conditions: 0.1–0.2 mg/ml protein, 1:1 lipid/protein, 2.6:1 egg PtdCho/brain extract, dialysis against 20 mM Bistris-propane at pH 7 at 24°C. Crystals appeared within 24 h. (F) The crystals formed under the same conditions as (E) but 2.5% isopropanol was added to the dialysis buffer. The bar in (B) is 0.25 μm, and the magnification is the same in figures (A–F).
The cytochrome bo quinol oxidase. The cytochromes eluting from the first column (DEAE-Sepharose) were directly applied to the copper ion column. The initial gradient separates yellowish-brown cytochromes which have an absorbance maximum at 559 nm in the reduced-minus-oxidized spectrum, from the cytochrome bo which elutes as a single sharp peak with the second gradient. The elution profile is very similar to that shown in Fig. 1B. Cytochrome bo is already rather pure after the copper-ion-column step (Fig. 3B). Further purification was carried out in DecGlc, using anion exchange on a Q-Sepharose HP and gel filtration on a Sephacryl 300 HR column (see Materials and Methods).

The preparation was homogeneous as judged by the gel-filtration profile, and monomeric (Fig. 4). The haem/protein ratio was measured as 9.5 nmol/mg by means of the Gill and von Hippel method for protein determination. This value is lower than the theoretical value for the four-subunit complex (11.9 nmol/mg). This difference may be due to a background of impurities which are seen in the SDS/PAGE (Fig. 3C, lane 4), or to the loss of haem. The turnover activity was 175 s⁻¹ using Ph(NMe₂)₂ as the electron donor at room temperature. The purification is summarized in Table 2.

Two-dimensional crystallization. We have found that the bacterial cytochrome oxidases readily form membrane crystals. Table 3 describes four conditions for two-dimensional crystallization of cytochromes aa₃ purified from P. denitrificans and R. sphaeroides as well as cytochrome bo purified from E. coli with the methods outlined above. We have found crystals with two different morphologies, namely sheets and tubular crystals. Their layer groups and lattices are shown in Table 3. The resolution refers to negatively stained specimens.

Initial attempts to crystallize Paracoccus cytochrome aa₃, were carried out with Myr₃GroPCho by dialysis of DecGlc. The dialysis against a Tris buffer (pH 8.5) containing 5% isopropanol resulted in the formation of short tubular crystals with maximal dimensions of 0.3 μm × 0.7 μm within 48 h at 24°C. The addition of isopropanol appears to favor the growth of tubes rather than vesicles. The optical diffraction of tubular crystals was strong, but the distortions in the lattice resulted in a blurred diffraction pattern. Experiments with Myr₃GroPCho alone were limited due to the aggregation at low pH and in the presence of additives such as CaCl₂ and MgCl₂. Therefore, a mixture of egg PtdCho and bovine brain extract (crude PtdSer) was used as it allows experimentation over a wider pH range. Using the experimental conditions 1 in Table 3, small well-ordered tubes could be grown by dialysis against 20 mM Bistris-propane, pH 7, at 12°C. The largest dimensions of these tubes were 0.12 μm × 0.6 μm (Fig. 6A). Despite their small size, a strong optical diffraction was observed with the resolution down to 2.5 nm. Similar results could be obtained at other pH values but only with the addition of 10 mM CaCl₂. Tubular crystals were also formed by dialysis from Triton X-100 but these were even smaller than those obtained from DecGlc. All crystalline material was stable for several weeks at 4°C.

Similarly to the Paracoccus cytochrome aa₃, the E. coli cytochrome bo formed tubular crystals with Myr₃GroPCho after the removal of DecGlc (experiment 2 in Table 3). Under these conditions, multilayered crystals also formed at pH 6–7.5 whereas two-dimensional crystals grew at pH 5.5 and 8 when the lipid/protein ratio was 1.25:1 (Fig. 6B). With a lower lipid/protein ratio (1:1), formation of crystalline membranes was preferred. The crystals with Myr₃GroPCho were unstable, existing for only a few hours, and showed poor optical diffraction that was at its best when free protein was still present. This and the high inclusion of negative stain indicated the involvement of detergent in these crystals. The experiments were continued with other lipids because the same observation was made when lauryl dimethylamine-N-oxide and Triton X-100 were used. Using a mixture of soybean PtdCho and brain extract at the lipid/protein ratio 0.65:1, the dialysis of lauryl dimethylamine-N-oxide resulted in the formation of tubular crystals at pH 6.9 with dimensions of 0.35 μm × 1.0 μm (experiment 3 in Table 3, Fig. 6C). Mainly vesicles were formed, but the number of tubes increased with the addition of isopropanol to the dialysis buffer. These tubes provide an easily recognizable feature for the further experiments with cryo-electron microscopy. A mixture of egg PtdCho and brain extract in the lipid/protein ratio of 0.7 gave stable membrane- and vesicular two-dimensional crystals after dialysis of either OctGlc, DecGlc, or Triton X-100 at pH 7. The best crystals diffracted to 2.5 nm (experiment 4 in Table 3, Fig. 6D).

Initial crystallization trials of the cytochrome aa₃, from R. sphaeroides were carried out using the variant 1 with the long version of subunit II and no subunit III (Fig. 5) as this was available in the largest quantities. With 0.15 mg/ml protein and a lipid/protein ratio of 3:1 stacked two-dimensional crystals formed with Myr₂GroPCho by dialysis of DecGlc, at pH 8. Reduction in the lipid/protein ratio resulted in the incorporation of protein into vesicles and membranes with no crystalline arrays. With variant 3 (three-subunit complex), small vesicles with crystalline patches were obtained by dialysis of 0.1–0.2 mg/ml protein with egg PtdCho/brain extract at pH 7.5 (experiment 5 in Table 3, Fig. 6E). These were maximally 0.5 μm in diameter. The same starting conditions resulted in the formation of larger (1–2 μm diameter) vesicles and membranes with crystalline patches when dialysis was against buffer supplemented with 2.5% isopropanol (Fig. 6F).

DISCUSSION

The implementation of IMAC as a step in the purification of bacterial cytochrome oxidases provides a method which is complementary to the recently devised protocols (Sieffens et al., 1990; Hosler et al., 1992; Minghetti et al., 1992; Haltia et al., 1994; Geier et al., 1995; Henning et al., 1995; Kleyemann et al., 1995). The IMAC step allows the omission of lengthy membrane washing steps with urca and cholate that have been previously employed in the case of the E. coli cytochrome bo (see Minghetti et al., 1992, and references therein), or with chaotropic salts in the case of R. sphaeroides cytochrome aa₃ (Hosler et al., 1992). In the cytochrome bo complex, one of the binding sites for the copper ion column appears to be in the membrane-exposed part of subunit II (CyoA) because this domain can also be purified with an IMAC column after it has been separately expressed (van der Oost et al., 1992).

The P. denitrificans cytochrome c oxidase was purified as a two-subunit complex. The removal of subunit III by means of incubations with lauryl dimethylamine-N-oxide was carried out because it is less stable than the other core subunits (Haltia et al., 1994). Its removal was considered important in view of the necessity of carrying out crystallizations with Myr₃GroPCho above the lipid-phase-transition temperature at 23°C. However, it is also possible to purify the three-subunit version of the P. denitrificans cytochrome aa₃, with Chelating Sepharose using a mild detergent such as DodGlc (Haltia et al., 1994). The three-subunit form of the R. sphaeroides cytochrome c oxidase binds more strongly to the copper column than the two-subunit form.

IMAC utilising copper has additionally been used in the purification of cbb₃-type cytochrome oxidase of P. denitrificans characterized by de Gier et al. (1994). This may indicate the
general applicability of this method. Its failure in the cases of the terminal oxidase complexes of *Sulfolobus acidocaldarius* (Lübben et al., 1994) may result from the lability of the complexes at the high pH required for IMAC (A. Warne, unpublished results). Two-dimensional crystals of cytochrome oxidase have been studied for a long time (e.g. Henderson et al., 1977; Frey et al., 1978; Fuller et al., 1979; Deatherage et al., 1982; Valpuesta et al., 1990) but all these studies have been carried out with the bovine mitochondrial enzyme. These studies have lead to low-resolution three-dimensional models of the enzyme as well as mapping of some subunits (Frey et al., 1978; Crum et al., 1994) and cytochrome-c-bridging site (Frey and Murray, 1994) on the complex. Until now, two-dimensional crystals of the bacterial oxidases have not been prepared, although these have the advantage of being structurally simpler than the eukaryotic enzyme. We anticipate that electron crystallography of the bacterial oxidases will offer a way to map their basic structural features as well as substrate(cytochrome c, quinol)-binding sites. These studies will be greatly aided by the determination of crystal structures of the membrane-exposed domain (van der Oost et al., 1993; Wilmanns et al., 1995) and the entire bacterial (Iwata et al., 1995) and mitochondrial (Tsuikihara et al., 1995) oxidase complexes. The preparation of crystalline samples will be helpful for spectroscopic studies with oriented membrane cytochrome oxidase (e.g. George et al., 1993). The readiness of the oxidases studied here to form two-dimensional crystals indicates that our procedures yield homogeneous preparations. The need for advanced purification methods for bacterial oxidases is reinforced by the increasing number of mutagenesis studies that will be performed as a result of emerging structural information.

We are grateful to Dr Werner Kühlbrandt for advice and discussions, and to Karoline Pimlilkar for her assistance with experiments. This work has been supported by a European Union grant SC1-CT91-0698 (CAESAR project).

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