A two-subunit cytochrome c oxidase (cytochrome aa₃) from Paracoccus denitrificans

(bacterial respiration/respiratory control/immunoprecipitation/copper/mitochondrial evolution)

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ABSTRACT Cytochrome c oxidase (ferrocytochrome c: oxygen oxidoreductase, EC 1.9.3.1) was purified from the cytoplasmic membrane of the bacterium Paracoccus denitrificans. The enzyme contains two heme groups (a and a₂) and two copper atoms per minimal unit, oxidizes mammalian cytochrome c at a high rate, and, when incorporated into liposomes, generates an electrochemical proton gradient during cytochrome c oxidation. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis reveals only two subunits of apparent molecular weights 45,000 and 28,000; they appear to correspond to the two largest mitochondrially made subunits of the seven-subunit cytochrome c oxidase isolated from yeast mitochondria. Because of its structural simplicity, Paracoccus cytochrome c oxidase offers new possibilities for exploring the mechanism of cytochrome c oxidase function.

Cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1) from mitochondria of eukaryotic cells is one of the most complex enzymes known (1, 2). As the terminal member of the electron transport chain, it mediates the transfer of electrons from reduced cytochrome c to oxygen and conserves the free energy of this reaction as an electrochemical proton gradient across the mitochondrial inner membrane (3). Earlier experiments suggested that this proton gradient results only from the consumption of protons on the matrix side of the mitochondrial inner membrane (4). More recent studies indicate, however, that cytochrome c oxidase also acts as an outwardly directed proton pump and that this activity may generate part, or even all, of the proton gradient (2).

Cytochrome c oxidase from mitochondria of yeast (5, 6) and Neurospora crassa (7) consists of seven polypeptide subunits, two hemes (a and a₂), and two copper atoms. The enzyme from mammalian sources appears to have a similar subunit structure, but contains several additional polypeptides whose significance is still open (8). The large number of polypeptide subunits has made it difficult to decide which subunits bind the heme and copper moieties or, indeed, which subunits are required for enzyme activity. Also, the structural complexity of the eukaryotic oxidase is a serious obstacle in current efforts to determine the three-dimensional structure of the enzyme by electron diffraction of unstained crystalline sheets (9).

The present study was undertaken in the hope that cytochrome c oxidase from a bacterial source might have a different, and perhaps simpler, subunit structure. Although several bacterial "cytochrome oxidases" have been previously isolated (10), none of them is a cytochrome aa₃-type oxidase that could be considered analogous to the mitochondrial enzyme. We decided to isolate the cytochrome aa₃-type oxidase from Paracoccus denitrificans because the respiratory chain and the oxidative phosphorylation system of this organism are strikingly similar to those of mitochondria (11). For example, the cytochrome c oxidase of Paracoccus can function with mammalian cytochrome c as a substrate (12), which indicates a high degree of relatedness at the molecular level.

Here we report that purified Paracoccus cytochrome c oxidase is functionally analogous to its mitochondrial counterpart, yet has a much simpler polypeptide composition. We suggest that this bacterial oxidase might help to answer some structural and functional questions that could not be solved through studies of the enzyme from mitochondria.

MATERIALS AND METHODS

Preparation of Cytoplasmic Membranes. A. P. denitrificans strain (ATCC 13545) was grown to the mid-log phase (13) at 30°C in 10- to 1500-liter batches with 1% succinate as the major carbon source as described in ref. 14 with the following modifications: (i) the P₆ concentration was raised to 100 mM; (ii) a pH control during growth was omitted; (iii) a silicon-based antifoam emulsion was used. After the cells were harvested by centrifugation, they were immediately frozen at -30°C. For the preparation of membranes, the cells were thawed, suspended to 400 g/liter (weight) in 100 mM KPi, pH 7.6, and disrupted with glass beads (0.1-mm diameter) in a Dyno Mill (15). The temperature was kept below 10°C. Large debris was removed by centrifugation for 30 min at 7000 × g and the supernatant was centrifuged for 4 hr at 50,000 × g. The sedimented membranes were resuspended in the same buffer, centrifuged again, and stored in the buffer at -80°C at 30-50 mg of protein per ml.

Purification of Cytochrome c Oxidase. All steps were performed at 0-4°C. (The capital letters refer to the purification scheme given in Table 1.) Cytoplasmic membranes (A; 20-30 mg of protein per ml in 100 mM KPi, pH 7.6/0.4 M KCl/1 mM EDTA) were preextracted with deoxycholate (0.1 mg/mg of protein) and resolated by centrifugation (4 hr at 50,000 × g). The pellet (B) was suspended at 10 mg of protein per ml in the KPi/EDTA buffer supplemented with 1% Triton X-100 and 1 M KCl and sedimented by centrifugation as above. The supernatant (C) was adjusted to 1.5% Na cholate and fractionated with ammonium sulfate saturated at 4°C. The material precipitating between 30 and 40% of saturation (D) was suspended in 50 mM KPi, pH 7.6/1 mM EDTA/0.1% sodium cholate and refractionated with ammonium sulfate as above. The precipitate (E) was dissolved in a minimal volume of 50 mM KPi (pH 7.6), dialyzed for 1 hr against 0.2% Triton X-100/50 mM KPi, pH 7.6/0.2 M KCl/1 mM EDTA, and passed through a column of Ultrogel AcA 34 (LKB; 100 ml of gel per g of cytoplasmic membranes) that had been equilibrated with dialysis buffer. Fractions with the highest heme a-to-protein ratio were pooled and supplemented with 3 mg of sodium cholate per ml, and the oxidase was precipitated with

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Abbreviation: NaDodSO₄, sodium dodecyl sulfate.
ammonium sulfate between 30 and 40% of saturation. The precipitate (F) was dialyzed overnight against 10 mM KF, pH 8.0/0.2% Triton X-100 and applied to a DEAE-cellulose column (Whatman DE 52; 5 ml per g of cytoplasmic membrane). The column was washed with 2 bed vol of 10 mM KF, pH 8.0/0.5% Triton X-100 and the enzyme was eluted with a linear salt gradient (2 bed vol each of 10 mM KF, pH 8.0/0.2% Triton X-100 and 200 mM KF, pH 8.0/0.2% Triton X-100). The enzyme started to elute at about 40 mM KF. Fractions with the highest heme a-to-protein ratio were pooled, adjusted to 0.3% cholate, and precipitated between 30 and 40% ammonium sulfate as above. The pellet (G) was dissolved in a minimal volume of the buffer used for the first Ultrogel step and again passed through an Ultrogel AAc 34 column as above (20 ml of gel per g of cytoplasmic membrane). The fractions with the highest heme a-to-protein ratio were pooled, adjusted to 0.3% cholate, and precipitated with ammonium sulfate between 30 and 40% of saturation. The resulting pellet (H) was dissolved in 50 mM Tris-HCl, pH 7.6, and stored in small aliquots at –30°C.

As an optional step, fraction H was subjected to preparative electrophoresis in a 5% polyacrylamide gel in a discontinuous buffer system (16) supplemented with 0.1% Triton X-100 (17). Although this step raised the heme a-to-protein ratio only slightly, it removed trace contaminants.

Isolation of Subunits. The enzyme was electrophoresed on a 15% polyacrylamide gel slab in the presence of sodium dodecyl sulfate (NaDodSO4), and the bands were located by staining and cut out. Protein was electroeluted from the slices (18), excess NaDodSO4 was removed by dialyzing first against water and then against 95% ethanol, and the precipitated protein was recovered by centrifugation.

Electrophoresis. NaDodSO4/polyacrylamide gel electrophoresis was performed in three different systems: system I, 10% or 15% gels (acylamide-to-crosslinker ratio = 30) containing 8 M urea (19); system II, 15% gels as above, but without urea; system III, 15% gels in a discontinuous buffer system (20). Samples were dissociated in NaDodSO4 for 30 min without heating. Gels were calibrated with a mixture of proteins of known molecular weight.

Miscellaneous. Absorption spectroscopy was done with an Amino DW-2 split beam spectrophotometer at a band pass of 1 nm. The wavelength scale was calibrated with a holmium filter. The pyridine hemochromogen of heme a was measured by using a Δε (587 – 620 nm) of 21.7 cm−1 mM−1 (21). Copper was measured by flameless atomic absorption (22) on three different enzyme preparations that had been dialyzed overnight against 10 mM Tris-HCl, pH 7.5/5 mM EDTA. Five 1-μg aliquots of each preparation were measured; the values were averaged and corrected for a reagent blank. This correction amounted to less than 20%. Enzyme activity was assayed polarographically at 25°C in the presence of 50 mM KF, pH 7.4/1 mM EDTA/25 μM horse heart cytochrome c/30 mM ascorbate. Solubilized preparations were preincubated for 10 min in the assay buffer supplemented with 0.5 mg of sonicated soybean phospholipids (asolcetin). Oxygen uptake was corrected for that insensitive to 1 mM KCN. Published methods were used for measuring protein (23) and raising rabbit antisera (17).

RESULTS

Cytochrome c oxidase can be purified in good yield from the cytoplasmic membrane of P. denitrificans as an active enzyme in a spectrally pure form (Table 1). Depending on the cytochrome aa3 content of the cytoplasmic membranes, overall purification was 100- to 200-fold. The purified enzyme has a heme a content of about 27 nmol/mg of protein; when purified further by gel electrophoresis under nondissociating conditions, this value rises slightly to 28–29 nmol/mg. In NaDodSO4/polyacrylamide gels, which resolve the yeast mitochondrial cytochrome c oxidase into seven subunits, the bacterial enzyme shows only two subunits (Fig. 1). In order to exclude the possibility that this two-subunit preparation is an artifact reflecting loss of smaller subunits during the lengthy isolation procedure, cells were grown in 15SO42− in order to uniformly label all proteins; the isolated membranes were then solubilized in Triton X-100 and subjected to immunoprecipitation with an antiserum that had been raised against the active two-subunit enzyme. The immunoprecipitate was separated on a NaDodSO4/polyacrylamide gel, which was then stained and autoradiographed (Fig. 2). The autoradiogram reveals only two labeled bands; these comigrate with the two major stained bands. The diffuse stained band below subunit II is probably the light immunoglobulin subunit. This result supports the view that the bacterial oxidase contains only two polypeptide subunits.

Molecular weights for the subunits were determined in three different gel systems, which gave closely similar results. Values for the apparent molecular weights of subunits I and II were 45,000 (range: 43,000–47,000) and 28,000 (range: 27,000–30,000), respectively.

The amino acid compositions (Table 2) of the separated subunits (Fig. 3) show that both subunits are rather hydrophobic. Their "polarity indices" (26) resemble those determined for subunits I and II of the yeast oxidase (27).

The native enzyme shows a typical cytochrome aa3 spectrum (Fig. 4A and Table 3). No other cytochromes can be detected. The presence of heme a3 is documented by the absolute and difference absorption spectra of the CO-saturated enzyme (Fig. 4B).

The copper content of the purified oxidase ranged from 31.0 to 34.6 nmol/mg of protein. Because the heme a content was 26.4–29.1 nmol/mg, the oxidase appears to contain one copper atom per heme group.

The isolated cytochrome c oxidase was active with reduced horse heart cytochrome c, but showed a strong dependency on
added phospholipid which could not be replaced by 0.5% Tween 80, Triton X-100, or bile salts (not shown). In the presence of aseptin, specific activities were usually between 9 and 16 μmol of O2 reduced per min/mg of oxidase protein; activity in membranes ranged from 0.2 to 0.4 μmol of O2 reduced per min/mg of membrane protein. In terms of activity per heme a, the purified enzyme has thus retained up to 40% of its activity in membranes.

When the enzyme was incorporated into phospholipid vesicles, its activity was stimulated about 3.6-fold on addition of both an uncoupler and K+ plus valinomycin (Fig. 5). In analogy to mitochondrial cytochrome c oxidase, the bacterial oxidase can thus couple electron flow to the generation of an electrochemical proton gradient across the membrane in which it is embedded. This result further strengthens the view that the two-subunit enzyme described here has all the functional attributes of the cytochrome c oxidase operating in vitro.

**DISCUSSION**

The main conclusion of this study is that cytochrome c oxidase from *Paracoccus* is functionally analogous to, but structurally much simpler than, the corresponding enzyme from mitochondria. How valid is this contention? There can be little doubt about the functional similarity; absorption properties, copper content, specific activity, lipid requirement, and ability to generate an electrochemical proton gradient are all identical or very similar for both types of enzymes. It is more difficult to prove that the two-subunit enzyme is the "native" enzymic unit operating in *vivo*. Three arguments support this view: (i) the turnover number of the purified enzyme is only moderately lower than that of the membrane-bound one; a similar drop in turnover number upon purification is observed with mitochondrial cytochrome c oxidase (29); (ii) the same subunit composition is found regardless of whether the enzyme is isolated by conventional multistep purification or by immunoprecipitation under gentle conditions; (iii) the purified enzyme can be reconstituted into liposomes and shown to generate a proton gradient during cytochrome c oxidation.

We suggest that the simplest enzyme complex possible consists of one copy of each subunit (total molecular weight, 73,000), two hemes (a and a3), and two copper atoms. The calculated heme a content of such a unit is 27.4 nmol of heme a per mg of protein, in good agreement with the experimentally determined values of 26–28 nmol of heme a per mg.

What is the relationship of subunits I and II of the *Paracoccus* enzyme to the seven subunits of the mitochondrial cytochrome c oxidases from various sources? Subunits I and II of the *Paracoccus* enzyme are only slightly larger than subunits I and II

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**FIG. 1.** Purified *Paracoccus* cytochrome c oxidase contains only two subunits. *Paracoccus* enzyme (10 μg) and yeast enzyme (30 μg; ref. 5) were electrophoresed on a 15% NaDodSO4/polyacrylamide gel (system III) and stained with Coomassie blue.

**FIG. 2.** Immunoprecipitated *Paracoccus* cytochrome c oxidase contains only two subunits. Cells were grown in 50 ml of medium, the SO42− content of which had been decreased to 100 μM (14). After growth had stopped (100 Klett units), 2 mCi (1 Ci = 3.7 × 1010 becquerels) of carrier-free 35SO42− plus 5 μmol of unlabeled sulfate were added. After 4 hr, the cells had incorporated 38% of the 35S. Cells were isolated and membranes were prepared by lysozyme treatment (24), diluted with a 10-fold weight excess of unlabeled membranes, and solubilized in 1% Triton X-100/0.4 M KCl/50 mM KP+, pH 7.6/1 mM EDTA at 3 mg of protein per ml. The oxidase was then immunoprecipitated with a specific antiserum raised against the active enzyme. The immunoprecipitate was washed once in the same medium and once in a medium in which Triton X-100 had been replaced by 0.5% cholate; an aliquot of the washed precipitate was electrophoresed on a 15% NaDodSO4 gel in system II. Lanes: center, Coomassie blue stain of immunoprecipitate; left, autoradiogram after 16 hr of exposure; right, purified enzyme stained with Coomassie blue.
Table 2. Amino acid composition of purified subunits

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Subunit I</th>
<th>Subunit II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>6.9</td>
<td>10.3</td>
</tr>
<tr>
<td>Thr</td>
<td>6.1</td>
<td>4.0</td>
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<td>Ser</td>
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<td>10.6</td>
</tr>
<tr>
<td>Met</td>
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</tr>
<tr>
<td>Ile</td>
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<td>6.3</td>
</tr>
<tr>
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<td>10.8</td>
</tr>
<tr>
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</tr>
<tr>
<td>His</td>
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<td>2.5</td>
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<tr>
<td>Ýs</td>
<td>2.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Arg</td>
<td>3.1</td>
<td>2.8</td>
</tr>
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</table>

Polarity index 33.5% 39.3%

Twenty micrograms of each subunit was hydrolyzed for 24 hr., and the amino acids (except Cys and Trp) were determined (25) on a Durrum D500 amino acid analyzer. The polarity index represents the sum of mole percentages of Asp, Thr, Ser, Glu, His, Lys, and Arg (26).

of the oxidases from yeast (27) and bovine heart (8). Moreover, polarity indices derived from the amino acid composition of subunits I and II are very similar for the three enzymes: the polarity of subunit I is always very low (Paracoccus, 33.5%; yeast, 34.7%; beef heart, 35.7%) and that of subunit II, moderately low (39.3%, 42.1%, and 44.7%, respectively). Subunit I of Paracoccus also shares two additional characteristic features with cytochrome c oxidase subunit I of beef heart and yeast: a "fuzzy" appearance on NaDODSO₄ gels not containing urea (see Fig. 1) and a tendency to aggregate irreversibly upon heating to 100°C in NaDODSO₄/buffer before electrophoresis.

Recently, Sone and Kagawa (30) described a cytochrome c oxidase from a thermophilic bacterium. The enzyme contained not only heme a, but also equimolar amounts of heme c, yet gave only a single band of apparent molecular weight 38,000 upon NaDODSO₄/polyacrylamide gel electrophoresis. Surprisingly, the heme a content was only 13.5 nmol/mg of protein; to account for this low value the authors postulated the existence of two 38,000-dalton subunits; however, these two polypeptides would have to be nonidentical in order to carry the three ligands (heme a, heme c, and copper). No comparison can be made with the Paracoccus enzyme until this discrepancy

Table 3. Optical extinction coefficients of Paracoccus cytochrome c oxidase

<table>
<thead>
<tr>
<th>Wavelength pair, nm</th>
<th>Δε</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized</td>
<td>602 - 630</td>
</tr>
<tr>
<td></td>
<td>424 - 480</td>
</tr>
<tr>
<td>Dithionite-reduced</td>
<td>605 - 630</td>
</tr>
<tr>
<td></td>
<td>445 - 480</td>
</tr>
<tr>
<td>Reduced minus oxidized</td>
<td>605 - 630</td>
</tr>
<tr>
<td>CO-reduced minus reduced</td>
<td>592 - 608</td>
</tr>
</tbody>
</table>

Values are taken from Fig. 4 and are based on the pyridine hemochromogen difference spectrum of heme a [Δε (687 - 620 nm) = 21.7 cm⁻¹ mM⁻¹ (ref. 21)].
has been resolved; still, the properties of the thermophilic enzyme suggest that a simple subunit structure may be a property of many and perhaps most bacterial cytochrome c oxidases.

What, then, is the function of the additional subunits of the cytochrome c oxidases from mitochondria? One intriguing possibility would be that they are necessary for the proton-pumping activity of the enzyme and that the bacterial enzymes cannot perform this function, even though quantitative measurements with Paracoccus cells suggest that the $H^+/e^-$ ratio in this region of the chain is higher than 1 (31).

Because one or both of the two subunits of the Paracoccus enzyme must obviously carry the heme $a$ and copper moieties, it appears very likely that these moieties are associated with subunits I or II of the cytochrome c oxidase from mitochondria. This agrees with the finding that the amino acid sequences of subunit II from bovine (32) and yeast (33) cytochrome c oxidase show homology to several copper-binding proteins from bacterial sources. The association of heme $a$ with subunit I of the yeast oxidase is also suggested by biochemical (34) and genetic (35) data.

The observation that the two subunits of Paracoccus cytochrome c oxidase appear to correspond to two mitochondrially made subunits of eukaryotic cytochrome c oxidases adds yet another suggestive argument to the view that mitochondria have evolved from respiring prokaryotes (36).

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