Cytochrome c oxidase from Paracoccus denitrificans: Both hemes are located in subunit I

cytochromes a and a1)

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ABSTRACT The two-subunit cytochrome c oxidase from Paracoccus denitrificans has been successively digested with chymotrypsin and Staphylococcus aureus V8 protease. The smaller subunit of the enzyme (apparent Mr 32,000) was split into numerous peptides that were removed by anion-exchange HPLC. The larger subunit was only digested to a limited extent (from an apparent Mr 45,000 to Mr 43,000), and the spectral properties were preserved relative to the native enzyme (a reduced minus oxidized difference spectrum with maxima at 447 and 607 nm in the Soret and a region, respectively). As judged from CO-reduced spectra this proteolytically digested, one-fragment oxidase was found to contain an equal amount of cytochromes a and a1. The enzymatic activity with reduced cytochrome c as substrate in the presence of Triton X-100 proceeded with equal affinity (Km = 0.5-1.0 µM) and with a Vmax of ~20% (40 s⁻¹) of that found with the native enzyme (200 s⁻¹). When the assay system was supplemented with soybean phospholipids, the Km became 2 µM for both enzymes and the Vmax became 730 and 170 s⁻¹ for the native and the digested enzyme, respectively. Thus subunit I of P. denitrificans oxidase, and most probably of the other cytochrome c oxidases as well, contains both hemes and at least one Cu atom and has significant enzymatic activity.

Cytochrome c oxidase, the terminal enzyme of the oxidative chains of many prokaryotes and in all eukaryotes [with the exception of Microsporium (1)] is composed of multiple polypeptide subunits (2, 3). The two-subunit oxidase isolated from Paracoccus denitrificans (4), Rhodopseudomonas sphaeroides (5), and other prokaryotes, despite their structural simplicity, are qualitatively similar in proton and electron transfer to the 12-subunit mammalian enzyme. The two largest subunits of all cytochrome c oxidases (based on apparent molecular weights estimated by NaDodSO4/polyacrylamide gel electrophoresis) have homologous primary structures and exhibit immune crossreactivity. The cytochrome c oxidase of Thermus thermophilus (6) is, however, composed of two subunits, homologous to subunit I and cytochrome c2.

The active centers of cytochrome c oxidase contain two porphyrin iron and two copper atoms, but their location and the time sequence by which these metals participate in the catalytic process have not been resolved. The fact that the metal content and the spectral and kinetic properties of isolated bacterial oxidases are indistinguishable from the mammalian enzymes supports the hypothesis that the two largest subunits play a central role in binding the reactive metals of the enzyme.

Subunit II of cytochrome c oxidase contains one binding site for copper (Cuα) based on its primary structural similarities with the blue copper proteins (7). One heme (cytochrome a) binding site was also assigned to this subunit on the basis of controlled denaturation studies of the bovine heart enzyme (8, 9). However, the discovery that histidine-24 of bovine heart enzyme is not conserved in the oxidases from Leishmania tarentolae, Trypanosoma brucei, and Crithidia fasciculata (10) is not compatible with subunit II having a heme binding site. In fact, the assignment of precise ligands to the heme of cytochrome a (two histidine residues and Cuα) (two histidine and two cysteine residues), obtained by electron nuclear double resonance (ENDOR) and extended x-ray absorption fine structure (EXAFS) (11-14) for bovine heart oxidase, has made it impossible to find enough conserved residues to coordinate both metal centers in subunit II of P. denitrificans oxidase (Fig. 1).

Since the copper center assignment is supported by rather convincing evidence, the remaining alternative, which is consistent with reports in the literature, is that subunit I contains two hemes and one copper. Such a conclusion may be important, since it would mean that subunit II needed to bind only copper and cytochrome c. The cytochrome c binding property has been confirmed by several approaches that indicate that subunit II participates with other subunits to create the cytochrome c binding domain (15-17). The evolutionary variability of subunit II does not negate its involvement in cytochrome c binding, since the sequence of the binding site may have undergone evolutionary changes to compensate for changes in subunit II.

In the present study, we have proteolytically digested the two-subunit cytochrome c oxidase of P. denitrificans and purified a single polypeptide with a molecular weight slightly less than subunit I. This polypeptide contains two hemes and has spectral and ligand properties indistinguishable from the native enzyme as well as significant enzymatic activity.

METHODS AND MATERIALS

P. denitrificans (strain ATCC 13543) cells were grown on a succinate medium and cytochrome c oxidase was purified as described by Ludwig (18). For enzymatic digestion the oxidase stock solution (160 µM cytochrome a) was diluted to 23 µM cytochrome a in a medium composed of 25 mM Tris Cl/0.5% dodecyl maltoside, pH 8.3, and chymotrypsin (8 mg/ml) in 25 mM Tris Cl (pH 8.3) containing 0.5 mM N-[(p-tosyl)-l-lysine chloromethyl ketone) was added to the diluted enzyme solution at a 1:1 ratio (wt/wt), and digestion was carried out for 60 min in the dark at 23°C. The digestion was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride and the solution was chromatographed on a Whatman DE 52 cellulose column equilibrated with digestion buffer (see above). Cytochrome oxidase was retained by the column, whereas chymotrypsin and fragments produced by the digestion were eluted. The enzyme was eluted with 0.5 M Tris Cl/0.5% dodecyl maltoside, pH 7.8, and then desalted on

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a Sephadex G-25 column equilibrated with 10 mM sodium phosphate/2 mM EDTA/0.1% dodecyl maltoside, pH 7.8. Further proteolytic fragmentation was carried out by adding \textit{S. aureus} V8 protease (final concentration of 50 units/ml) to cytochrome \(c\) oxidase (\(\approx 10 \mu\text{M}\)). After 20 min of incubation at 20°C in the dark, the reaction was stopped by the addition of 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride, and the mixture was chromatographed on a Sephadex G-100 column equilibrated with 10 mM sodium phosphate/2 mM EDTA/0.1% dodecyl maltoside, pH 7.8. Alternatively, the mixture was absorbed on a DE 52 column and the enzyme was eluted with 0.5 M Tris Cl/2 mM EDTA/0.1% dodecyl maltoside, pH 7.8.

Further purification of the enzyme was achieved by removing the fragments of subunit II (produced by treatment with \textit{S. aureus} V8 protease) from subunit I by loading the solution on a (polyethyleneimine Baker Bond, widebore) HPLC column (4.6 \(\times\) 250 mm) equilibrated with 20 mM Hepes/0.1% Triton X-100R, pH 7.2. The main fragment of subunit I (55,400) was eluted by a linear salt gradient (0–1 M NaCl) in 20 mM Hepes/0.1% Triton X-100R, pH 7.2 at a rate of 1.5 ml/min by using a Varian model 5000 liquid chromatograph and a Hewlett-Packard model HP 8541A diode array spectrophotometer equipped with an HPLC flow cell. The enzymatically active fractions were pooled and concentrated by ammonium sulfate precipitation (50% saturation, 4°C). After centrifugation (10 min, 10,000 \(\times\) g), the green floating layer was collected, resuspended in 20 mM Hepes/0.1% dodecyl maltoside, pH 7.2, and passed through a Sephadex G-25 column equilibrated with 20 mM Hepes/0.1% dodecyl maltoside, pH 7.2. Contamination of the main fragment of subunit I (55,400) by other polypeptides was estimated at \(\pm\)5%, as judged from silver-stained NaDodSO\(_4\) gels.

The concentration of cytochrome \(c\) was calculated from difference spectra (dithionite-reduced minus air-oxidized) taken with an Amino model DW-2a or a Hewlett-Packard model HP 8541A diode array spectrophotometer with \(\epsilon_{605} - \epsilon_{530} = 11.7 \text{ cm}^{-1}\text{mM}^{-1}\) (4). Horse heart ferrocytochrome \(c\) was prepared by reduction with dithionite and chromatography on a Sephadex G-25 column. Cytochrome \(c\) oxidase activity was measured spectrophotometrically at 550–540 nm and calculated by using the difference extinction coefficient \(\epsilon_{550} - \epsilon_{540} = 19.4 \text{ cm}^{-1}\text{mM}^{-1}\). The protein concentration was determined as described by Gornall et al. (19), Lowry et al. (20), or Smith et al. (21) with bovine serum albumin as standard. NaDodSO\(_4\)/polyacrylamide gel electrophoresis was performed by the slightly modified version of the procedure of Laemmlli (22) as described by Müller and Azzi (23) without the addition of polymerized 0.5% polyacrylamide. Slab gels were conventionally stained with Coomassie blue or by silver as described by Bio-Rad.

**RESULTS**

The enzymatic characteristics of \textit{P. denitrificans} cytochrome \(c\) oxidase are reported in Table 1. Treatment with chymotrypsin resulted in the production of a lower molecular weight fragment from each subunit (called here subunit Ic and Iic, where c stands for chymotrypsin treated; Fig. 2). The chymotrypsin treatment did not change the \(K_m\) for cytochrome \(c\), but the \(V_{max}\) was reduced by \(\approx\)57% when the activity was assayed in the presence of Triton X-100 and soybean phospholipids. Heme purification after the proteolytic treatments

<table>
<thead>
<tr>
<th>Protein</th>
<th>(M_r)</th>
<th>(K_m) (\text{M}^{-1})</th>
<th>(V_{max}) (\text{cm}^{-1}\text{mM}^{-1})</th>
<th>Heme A yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit I</td>
<td>45,000</td>
<td>1.8–2.1</td>
<td>2.1</td>
<td>731</td>
</tr>
<tr>
<td>Subunit II</td>
<td>32,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme treated with chymotrypsin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit I</td>
<td>43,000</td>
<td>1.6–1.7</td>
<td>1.7</td>
<td>310</td>
</tr>
<tr>
<td>Subunit II</td>
<td>24,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme treated with (S. aureus) V8 protease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit I</td>
<td>43,000</td>
<td>1.2–1.5</td>
<td>2.8</td>
<td>168</td>
</tr>
<tr>
<td>Subunit II</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Activity was measured in the presence of Triton X-100 supplemented with soybean phospholipids.
specifically at aspartic and glutamic residues (24). Subunit Ic was not modified by this protease (Fig. 2, lane 3) whereas subunit IIC was almost completely fragmented. Gel filtration through a Sephadex G-100 removed most of the small fragments derived from subunit IIC. A better result was obtained by ion-exchange HPLC and is shown in Fig. 2, lane 4. The small contamination observed was probably a fragment of subunit Ic.

The removal of subunit IIC did not affect the spectral properties of the reduced minus oxidized or the CO-reduced minus reduced enzyme, although the absorption ratio at 280 nm versus 424 nm decreased further after treatment with S. aureus V8 protease (Fig. 3). Although some loss of heme occurred after both proteolytic treatments (<30%) the cytochrome a/cytochrome a₃ ratio (calculated from CO-dithionite reduced minus dithionite reduced spectra) remained unchanged to the native enzyme and thus was close to 1 (Table 2).

The kinetic analysis of the single-fragment enzyme (subunit Ic) indicated that the Kₘ for cytochrome c was nearly identical to that of the native oxidase, whereas Vₘₐₓ was attenuated to ~54% relative to the chymotrypsin-treated preparation (Table 1).

**DISCUSSION**

The comparison of the amino acid sequences of cytochrome c oxidase subunit II from human, bovine, and mouse heart, Drosophila melanogaster, Oenothera, maize, and yeast (25), Leishmania tarantolae, Trypanosoma brucei, and Crithidia fasciculata (10), and P. denitrificans (26, 27) identifies various evolutionarily conserved amino acids (Fig. 1), which is inconsistent with the model proposed by Millet et al. (15) and Wikström et al. (3), suggesting that subunit II of cytochrome oxidase has binding sites for the heme, for Cuₐ, and for cytochrome c (a negatively charged region). Sequence

![Figure 2](image1.png)

**FIG. 2.** NaDodSO₄/polyacrylamide (15%) gel electrophoresis of *P. denitrificans* cytochrome c oxidase before and after proteolytic treatment. Lanes: 1, native enzyme; 2, chymotrypsin-treated enzyme; 3, enzyme after chymotrypsin and *S. aureus* V8 protease treatment; 4, same sample as lane 3 purified by HPLC. The one-fragment enzyme migrated at the same level as the ovalbumin used as standard. The gel was stained by silver. 1, subunit Ic; 2, subunit II; 2c, subunit IIC.

was confirmed by the diminution of the absorption ratio at 280 nm versus 424 nm. To permit the determination of the protein concentration at 280 nm, in this experiment, the detergent used was 0.1% dodecyl maltoside (Fig. 3 Inset).

Examination of the polypeptide pattern from NaDodSO₄/polyacrylamide gel electrophoresis (Fig. 2, lane 2) showed that the digestion of the oxidase by chymotrypsin did not proceed beyond the formation of two polypeptides of Mr 43,000 and Mr 24,000, respectively.

Subsequently, the chymotrypsin-treated enzyme was digested with *S. aureus* V8 protease, an enzyme that cleaves

![Figure 3](image2.png)

**FIG. 3.** Spectral analysis of *P. denitrificans* oxidase after digestion with chymotrypsin and *S. aureus* V8 protease. The air-oxidized spectrum of *P. denitrificans* oxidase after digestion with chymotrypsin and *S. aureus* V8 protease has maxima at 425 and 601 nm, and the dithionite-reduced spectrum has maxima at 446 and 607 nm. These values are identical to the native enzyme (cf. ref. 4). The air-oxidized spectrum, extended to the UV region (Inset), shows a protein absorption band/Soret band ratio of 1.2, comparable to that of the native enzyme (1.8–2.1), indicating protein loss not associated to heme loss after the proteolytic treatments. Soret band, maximum at 425 nm. A = 0.05 refers to the spectra traces starting at 400 nm; A = 0.005 refers to the magnified recording of the spectra starting at 500 nm.
Table 2. Reduced and CO difference spectral data of *P. denitrificans* oxidase

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Δε</th>
<th>Heme A</th>
<th>Subunit Ic</th>
</tr>
</thead>
<tbody>
<tr>
<td>447-480</td>
<td>90.5</td>
<td>0.1520</td>
<td>1.68</td>
</tr>
<tr>
<td>605-630</td>
<td>15.5</td>
<td>0.0260</td>
<td>1.68</td>
</tr>
<tr>
<td>592-600</td>
<td>3.5</td>
<td>0.0052</td>
<td>3.57</td>
</tr>
</tbody>
</table>

The experiments were carried out in 50 mM sodium phosphate (pH 7.4) and 0.1% dodecyl maltoside. The extinction coefficients for *P. denitrificans* are from Ludwig and Schatz (4). The ratios Soret/CO and a-band/CO show that the native and *S. aureus* V8 protease-treated enzyme have essentially the same values indicating no change in the cytochrome a/a3 ratio. Δε, absorbance difference at the given wavelength pair; ΔA, extinction coefficient at the first wavelength of the pair minus the extinction coefficient at the second wavelength.


Another important feature conserved by the one-subunit enzyme is the presence of a kinetically demonstrable high-affinity reaction site for cytochrome c. The location of this site on subunit I has been indicated by experiments in which cytochrome c has shielded amino acid residues located in this subunit from the modification by water-soluble carbodiimides (although little attention was given to it; cf. refs. 15–17).

The experimental conclusion that two heme A and at least one copper have to be located in subunit I cannot ignore the problem of their coordination by evolutionarily conserved ligands. The sequences of cytochrome c oxidase from human, bovine, mouse, *Neurospora crassa*, Saccharomyces cerevisiae, *Drosophila melanogaster* (25), *Oenothera* (32), and maize (33) and of *P. denitrificans* (34) cytochrome c oxidase subunit I have been compared, and the evolutionarily conserved amino acids were identified. The limits of the hydrophilic transmembrane segments are those calculated by Lundeen *et al.* (35). It was observed that enough ligands are present in this subunit for two hemes and one copper atom, and possibly for a second copper atom as well, whose existence was proposed (35).

With the publication of the gene sequences of *P. denitrificans* oxidase genes (34), a tentative model of the oxidase active sites was proposed (37), consistent with the present experimental evidence indicating that two hemes and one copper can be bound to subunit I and also consistent with the somewhat different model proposed by Lundeen *et al.* (35).

In conclusion, the data presented here are consistent with a great deal of available information on cytochrome c oxidases and with a model in which subunit I of the enzyme contains two hemes and at least one copper and is able to catalyze the reduction of oxygen to water.