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Biochemistry

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

(cytochromes *a* and a_3)

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ABSTRACT **The two-subunit cytochrome *c* oxidase from *Paracoccus denitrificans* has been sequentially digested with chymotrypsin and *Staphylococcus aureus* V8 protease. The smaller subunit of the enzyme (apparent M_r 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 M_r 45,000 to M_r 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 α 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 a_3. The enzymatic activity with reduced cytochrome *c* as substrate in the presence of Triton X-100 proceeded with equal affinity (apparent K_m = 0.5–1.0 μM) and with a V_{max} 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 K_m became 2 μM for both enzymes and the V_{max} 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 *Microsporidium* (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 $NaDodSO_4$/polyacrylamide gel electrophoresis) have homologous primary structures and exhibit immune crossreactivity. The cytochrome *c* oxidase of *Thermus thermophylus* (6) is, however, composed of two subunits, homologous to subunit I and cytochrome c_1.

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 (Cu_A), 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 Cu_A (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*(-$^{\alpha}$*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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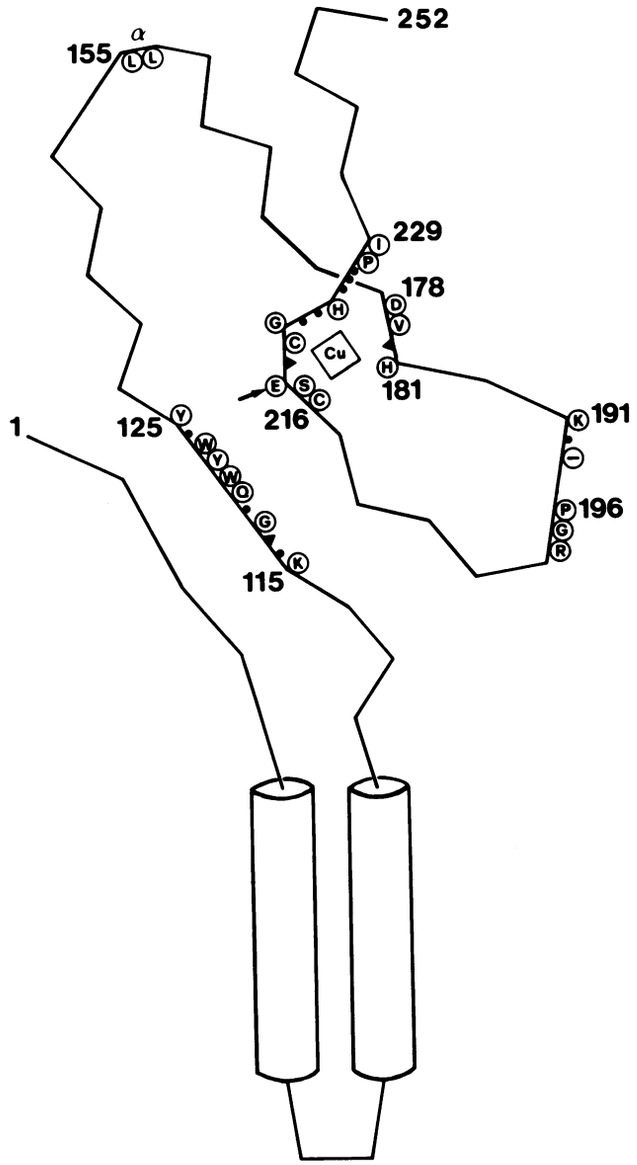

FIG. 1. Schematic model of cytochrome *c* oxidase subunit II. The numbers in the diagram correspond to the amino acid residues in the sequence of cytochrome *c* oxidase subunit II from *P. denitrificans*. The open circles represent evolutionarily conserved amino acids (one-letter code) or charges (+ and −). Triangles represent conserved hydrophobic amino acids. The dots represent evolutionarily conserved positions without any apparent special function. The two cylinders represent two evolutionarily conserved α-helices spanning the hydrophobic region of a membrane. The arrow represents the conserved negatively charged amino acid that was protected by cytochrome *c* against modification by water-soluble carbodiimide (15).

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 *S. aureus* V8 protease (final concentration of 50 units/ml) to cytochrome oxidase (≈10 μM). After 20 min of incubation at 20°C in the dark, the reaction was stopped by the addition of 1 mM diisopropyl fluorophosphate, 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 *S. aureus* V8 protease) from subunit I by loading the solution on a (polyethyleneimine Baker Bond, widebore) HPLC column (4.6 × 250 mm) equilibrated with 20 mM Hepes/0.1% Triton X-100R, pH 7.2.

The main fragment of subunit I (M_r, 43,000) 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 × *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 (M_r, 43,000) by other polypeptides was estimated at ≈5%, as judged from silver-stained $NaDodSO_4$ gels.

The concentration of cytochrome *a* was calculated from difference spectra (dithionite-reduced minus air-oxidized) taken with an Aminco model DW-2a or a Hewlett–Packard model HP 8541A diode array spectrophotometer with $\varepsilon_{605} - \varepsilon_{630} = 11.7\ cm^{-1} \cdot mM^{-1}$ (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 $\varepsilon_{550} - \varepsilon_{540} = 19.4\ cm^{-1} \cdot mM^{-1}$. 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 Laemmli (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 *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 ≈57% when the activity was assayed in the presence of Triton X-100 and soybean phospholipids. Heme purification after the proteolytic treatments

Table 1. Characterization of cytochrome *c* oxidase from *P. denitrificans* after treatment with proteases

	M_r	$A_{280-310}/A_{424-480}$	K_m, M*	V_{max}, s^{-1}	Heme A yield, %
Native enzyme					
Subunit I	45,000	1.8–2.1			
Subunit II	32,000		2.1	731	100
Enzyme treated with chymotrypsin					
Subunit I	43,000	1.6–1.7	1.7	310	—
Subunit II	24,000				
Enzyme treated with *S. aureus* V8 protease					
Subunit I	43,000	1.2–1.5	2.8	168	70
Subunit II	—				

*Activity was measured in the presence of Triton X-100 supplemented with soybean phospholipids.

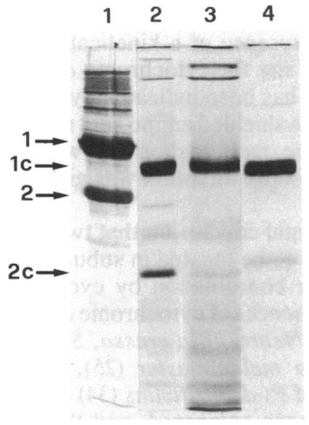

FIG. 2. $NaDodSO_4$/polyacrylamide (15%) gel electrophoresis of *P. denitrificans* cytochrome *c* oxidase before and after proteolytic treatment. Lanes: 1, native enzyme; 2, chymotrypsin-treated enzyme; 3, enzyme after chymotrypsin and *S. aureus* V8 protease treatment; 4, same sample as lane 3 purified 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 I; 1c, 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 $NaDodSO_4$/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 M_r 43,000 and M_r 24,000, respectively.

Subsequently, the chymotrypsin-treated enzyme was digested with *S. aureus* V8 protease, an enzyme that cleaves 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 a_3 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 K_m for cytochrome *c* was nearly identical to that of the native oxidase, whereas V_{max} 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 Cu_A, and for cytochrome *c* (a negatively charged region). Sequence

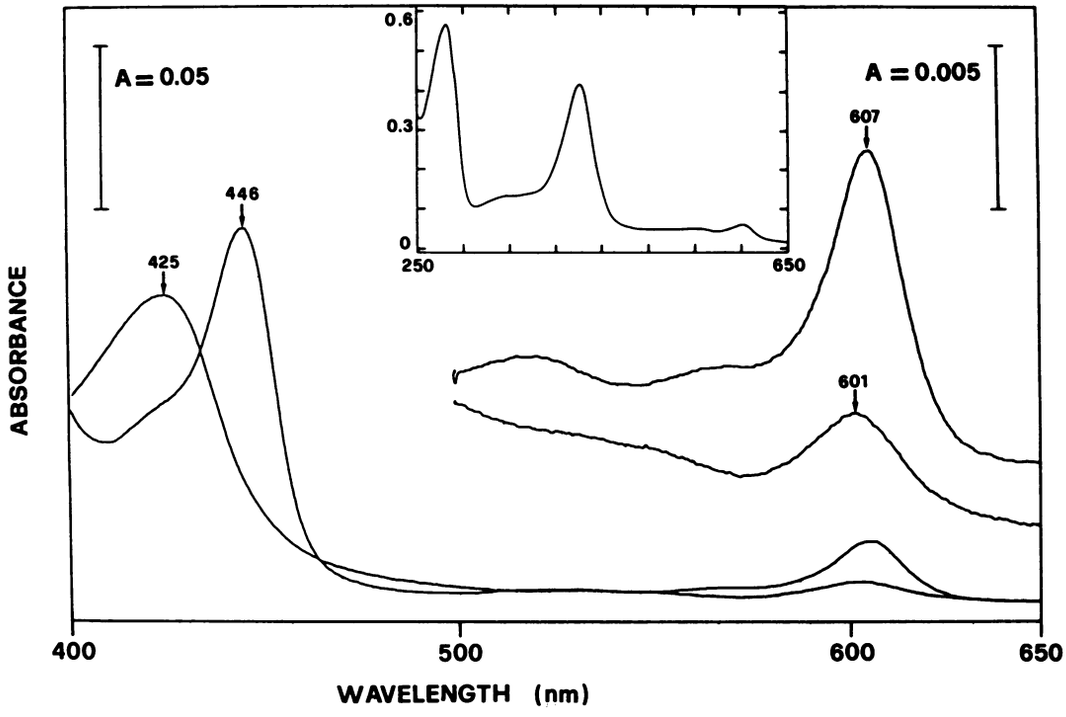

FIG. 3. Spectral analysis of *P. denitrificans* oxidase after digestion with chymotrypsin and *S. aureus* V8 protease. The air-oxidized spectrum of *P. denitrificans* oxidase after digestion with chymotrypsin and *S. aureus* V8 protease has maxima at 425 and 601 nm, and the dithionite-reduced spectrum 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

Wavelength pair	$\Delta\varepsilon$, $mM^{-1}\cdot cm^{-1}$	Native enzyme ΔA	Native enzyme Heme A, μM	Subunit Ic ΔA	Subunit Ic Heme A, μM
447–480	90.5	0.1520	1.68	0.0910	1.007
605–630	15.5	0.0260	1.68	0.0150	0.970
592–608	3.5	0.0052	1.57	0.0039	1.100
Soret/CO*		1.07		0.915	
α-band/CO†		1.07		0.880	

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 α-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/a_3 ratio. ΔA, absorbance difference at the given wavelength pair; $\Delta\varepsilon$, extinction coefficient at the first wavelength of the pair minus the extinction coefficient at the second wavelength.

*Ratio between the concentration of the heme A [measured in the Soret band (447–480 nm)] after dithionite reduction and that of the (dithionite reduced + CO) minus (dithionite reduced) enzyme measured at the α-band.

†Ratio between the concentration of the heme A [measured in the α-band (605–630 nm)] after dithionite reduction and that of the (dithionite reduced + CO) minus (dithionite reduced) enzyme measured at the α-band.

analyses in fact show the absence of conserved histidines (the heme A ligand) other than those needed for complexing Cu_A; the number of conserved negatively charged amino acids residues is also reduced to only three. Two sequences, the first from aspartate-178 to histidine-181 (where histidine is a putative copper ligand) and the second containing three more putative copper ligands, cysteine-216, cysteine-220 and histidine-224, were confirmed to be highly conserved, in agreement with Millet *et al.* (15).

By using two proteolytic enzymes, chymotrypsin and *S. aureus* V8 protease, in sequence it was possible to reduce the protein mass of cytochrome *c* oxidase subunit I to M_r 43,000 without losing at least three of the metal centers of the enzyme. The presence of cytochrome *a* and cytochrome a_3 in equal quantities was demonstrated by spectroscopic analysis in presence and absence of CO as reported in Table 2 by using the specific extinction coefficients calculated by Ludwig and Schatz (4) for the *P. denitrificans* oxidase. These coefficients are slightly different from the classical values reported for the bovine enzyme by Vanneste (28). Since Cu_B is strongly associated with cytochrome a_3 (29) and since its spectral properties are not modified, it can be concluded that Cu_B is also present in the one-subunit enzyme.

It has been suggested that subunit II is the binding site of Cu_A; however, the evidence is not definitive and, in fact, Cu_A may be located in subunit I. Since this center is detected spectroscopically and by electron paramagnetic resonance (12, 29), it should be possible to confirm this hypothesis by using our one-subunit preparation.

Gelles and Chan (30) reported that a *p*-hydroxymercuribenzoate modification of the Cu_A binding site in bovine heart cytochrome *c* oxidase resulted in a reduction of its electron transfer activity to ≈25%, which compares favorably with the activity remaining (20%) after removal of subunit II by proteolytic digestion. The experiment of Gelles and Chan (30), the evidence for a one-subunit cytochrome *c* oxidase (6), and our present results indicate that Cu_A may not be absolutely necessary for the reduction of oxygen by cytochrome *c* oxidases. It has been also suggested by Nicholls and Chanady (31) that electrons can be delivered directly to cytochrome a_3 from cytochrome *c* without passing through Cu_A.

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 hydrophobic 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 (36).

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.

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