Purification of Yeast Cytochrome c Oxidase with a Subunit Composition Resembling the Mammalian Enzyme*

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Yeast cytochrome c oxidase has been isolated by ion exchange chromatography using lauryl maltoside (n-dodecyl β-D-maltoside) as the solubilizing detergent. The enzyme prepared in this way has a heme ααα concentration of 8–9 nmol/mg of protein and a turnover number in the range of 180–210 s⁻¹ at pH 6.2 in 0.01% lauryl maltoside at 20 °C. Yeast cytochrome c oxidase prepared by any of several previously published methods which use Triton X-100 contains nine subunits. The enzyme isolated in lauryl maltoside contains these same nine different polypeptides and three others, including homologues of subunits Vla and Vlb of the mammalian enzyme.

Cytochrome c oxidase is the terminal enzyme of the electron transfer chain in aerobic bacteria as well as in the mitochondria of plants and animals (1–3). Bacterial cytochrome c oxidases are composed of three different subunits and include two hemes a and two copper atoms as prosthetic groups (1, 2). The enzyme from eukaryotes is more complex and includes three subunits encoded on mitochondrial DNA, which are the homologues of the subunits of the bacterial enzyme, and in addition contains a number of subunits encoded in the nucleus (2, 3). It is generally agreed that the mitochondrially coded subunits with their associated prosthetic groups are the functional core of the enzyme (3). The role of the nuclear coded subunits in cytochrome c oxidase function remains a matter of conjecture (3–5).

Attempts to define the roles of the nuclear coded subunits by their serial removal from the mammalian enzyme have had limited success (6, 7). The most promising approach to defining the functions of these subunits is through genetic approaches in yeast (e.g. 8–12). However, yeast cytochrome c oxidase has been less studied than its mammalian counterpart, and questions remain about the number of subunits in the enzyme from this source (13, 14). Thus, cytochrome c oxidase purified from mammals including bovine and, rat, and human has a total of 10 nuclear coded subunits (2, 3, 15), whereas the yeast enzyme has been found to contain maximally six (14). Experience with the mammalian enzyme has shown that the polypeptide composition of cytochrome c oxidase preparations depends on which detergents are used in the isolation procedure (3, 16). All of the published preparation methods for yeast cytochrome c oxidase use Triton X-100 at one step or other (14, 17, 18), which could account for the relatively low number of subunits. To test this possibility, we have purified yeast cytochrome c oxidase using only lauryl maltoside as the solubilizing detergent. We show that the enzyme isolated in this mild detergent contains more than the nine major polypeptides reported before and that two of the additional components present are homologues of mammalian cytochrome c oxidase subunits (Vla and Vlb) previously thought to be missing from the yeast enzyme.

EXPERIMENTAL PROCEDURES

Isolation of Cytochrome c Oxidase—The Saccharomyces cerevisiae strain JHRY1-2ca (leu2-3, leu2-112, ura3-52, his4-519, ade2, trpl) was grown aerobically at 30 °C in 8-liter batches of 1% (w/v) yeast extract, 2% (w/v) peptone supplemented with 1% (w/v) raffinose to midlogarithmic growth phase. Mitochondria were isolated according to Daum et al. (19), except that in the preparation of spheroplasts yeast lytic enzyme from Arthrobacter sp. (ICN) was used at 350 units/g, wet weight, of cells. After the preparation of spheroplasts all steps were performed between 0 and 4 °C. The final mitochondrial pellet was resuspended in 50 μl of 0.6 M mannitol, 20 mM Heps, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (Sigma) per original g, wet weight, of cells, frozen rapidly in liquid nitrogen, and stored at −80 °C.

Soluble proteins were removed from mitochondria by repeated washes in a solution of KCl in the presence of 1 mM phenylmethylsulfonyl fluoride as described (20) followed by a wash in 50 mM Tris-HCl, pH 8.0, 1 mM MgSO₄, 1 mM phenylmethylsulfonyl fluoride. Pelleted mitochondrial membranes were resuspended in the final wash buffer at a concentration of 10 mg of protein/ml. The amount of protein was estimated by dissociation of an aliquot in 0.6% (w/v) SDS at 95 °C for 4 min followed by a measurement of the absorbance at 280 nm against a solvent blank. The absorbance value was converted to mg of mitochondrial protein assuming that an absorbance of 1 is equivalent to 0.476 mg of protein (21).

Mitochondrial enzyme complexes were extracted from the resuspended membranes by adding a 40% (w/v) stock solution of lauryl maltoside (n-dodecyl β-D-maltoside; Sigma) to a final concentration of 0.8% (w/v) lauryl maltoside and incubating for 30 min. The mixture was centrifuged at 100,000 × g for 1.5 h. A 5 ml solution of NaCl was added to the clear supernatant to a final concentration of 100 mM NaCl, and this mixture was incubated for 1 h.

Cytochrome c oxidase was isolated from the lauryl maltoside extract by a single chromatographic step based on a method described by Ljungdahl et al. (22) for isolating cytochrome c reductase. The extract was applied to a DEAE-Bio-Gel A (Bio-Rad) column (1-cm diameter, 12 cm long) equilibrated with buffer A (50 mM Tris-HCl, pH 8.0, 1 mM MgSO₄, 0.01% (w/v) lauryl maltoside, 1 mM phenylmethylsulfonyl fluoride) containing 100 mM NaCl. The column with the absorbed sample was washed with 5 column volumes of buffer A, then eluted with 4 column volumes of a linear 100–400 mM NaCl gradient in buffer A. The flow rate was kept constant at 0.5 ml/min, the eluant was monitored at 280 nm, and 2-ml fractions were collected.

Oxidized versus reduced difference spectra were made of peak and shoulder fractions of the column to reveal the presence of the cytochromes. The presence of cytochrome c oxidase in the fractions was studied in detail by Western analysis. Aliquots of column fractions were resolved by SDS-polyacrylamide gel electrophoresis (see below) and transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore) as described (23). Protein binding sites on the blots were saturated with 10% (w/v) nonfat dry milk (Carnation) in phosphoric acid buffered saline and probed with polyclonal antibodies at 20 °C for at least 2 h. The blots were washed in 19 volumes of 1% (w/v) Nonidet P-40 in phosphate-buffered saline followed by washes in a solution of Tween 20 in the presence of 1 mM phenylmethanesulfonyl fluoride as described (20) followed by a wash in 50 mM Tris-HCl, pH 8.0, 1 mM MgSO₄, 1 mM phenylmethylsulfonyl fluoride. Pelleted mitochondrial membranes were resuspended in the final wash buffer at a concentration of 10 mg of protein/ml. The amount of protein was estimated by dissociation of an aliquot in 0.6% (w/v) SDS at 95 °C for 4 min followed by a measurement of the absorbance at 280 nm against a solvent blank. The absorbance value was converted to mg of mitochondrial protein assuming that an absorbance of 1 is equivalent to 0.476 mg of protein (21).

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phate-buffered saline. Biots were incubated with an antiserum against yeast cytochrome c oxidase raised in rabbits. Antigen-antibody complexes were visualized by incubation with alkaline phosphatase-conjugated goat anti-rabbit antibodies followed by color development (22). All incubations with antibodies and washes were performed in phosphate-buffered saline, 0.3% (v/v) Tween 20. To concentrate the preparation, column fractions containing immunodetectable cytochrome c oxidase were pooled, diluted with 1 volume of buffer A, and applied to a very small DEAE-Bio-Gel A column (0.5-cm diameter, 1 cm long, equilibrated with buffer A, 100 mM NaCl) with a flow rate of 0.5 ml/min. The absorbed sample was eluted with buffer A, 400 mM NaCl, and fractions of 0.5 ml were collected.

** SDS- Gel Electrophoresis:** Samples for SDS-polyacrylamide gel electrophoresis were dissociated at 37 °C in 50 mM Tris- HCl, pH 6.8, 4% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 12% (v/v) glycerol, 0.01% (w/v) bromphenol blue for 30 min and loaded, routinely, onto minigels. The stacking gel contained 7% (w/v) acrylamide, 6.0 M urea, and the separating gel contained 21% (w/v) acrylamide, 5.8 M urea with a ratio of acrylamide to bisacrylamide of 30:8 (25). To monitor the progress of the electrophoresis, precasted molecular mass protein standards from GIBCO-BRL (43, 29, 18, 14, 6, and 3 kDa) were used. When the 6-kDa marker had migrated to 1 cm from the end of the gel, electrophoresis was stopped. Gels were fixed and stained in 45% (v/v) methanol, 9% (v/v) acetic acid, 0.2% (w/v) Coomassie Brilliant Blue R for 2 h and destained with several washes in 45% (v/v) ethanol, 9% (v/v) acetic acid for 2 h. Finally, gels were subjected to light silver staining (positive image; 26).

**Protein Sequencing:** For N-terminal sequencing, purified cytochrome c oxidase, resolved by SDS-polyacrylamide gel electrophoresis on conventional slab gels (13 × 13 cm, 1.5 mm thick), was electroblotted onto polyvinylidene difluoride membranes in 10 mM CAPS/ KOH, pH 11.0, 10% (v/v) methanol using a Hoefer TE22 transblot apparatus at 250 mA for 4 h. Blots were stained with Coomassie Brilliant Blue and destained in a minimum amount of time (27). Bands were excised with a surgical blade and stored at −20 °C. For peptide mapping, cytochrome c oxidase was resolved by SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membranes, and stained with Ponceau S (28). The band of interest was excised, and the polypeptide was digested in situ with trypsin (28). Enzymatic cleavage fragments were separated on a C4 reverse-phase HPLC column (Brownlee Laboratories) using 0.1% trifluoroacetic acid in water and 0.08% trifluoroacetic acid, 70% acetonitrile in water as buffers. Sequence analysis was performed on a gas-phase protein sequenator (Applied Biosystems model 470A) equipped with an on-line phenylthiobodydantoin analyzer (Applied Biosystems model 120A).

**Analytical Assays:** Concentrations of heme aa3 were determined by air-reduced versus sodium dithionite-reduced difference spectra at room temperature, recorded on a Beckman DH-7 spectrophotometer, using the extinction coefficient at 605 nm of 24 mM−1·cm−1 (29). Mitochondrial fractions were solubilized in 100 mM potassium phosphate, pH 7.2, 25 mM NaCl, 1% (w/v) lauryl maltoside; column fractions were not diluted. The protein contents of the fractions were determined with the Pierce BCA protein assay kit. The amount of purified cytochrome c oxidase in the α-band region was determined by potassium ferricyanide-oxidized versus sodium dithionite-reduced difference spectra at liquid nitrogen temperatures, determined by potassium ferricyanide-oxidized versus sodium dithionite-reduced difference spectra.

A typical Eadie-Hofstee plot of the electron transfer activity of the purified cytochrome c oxidase as a function of cytochrome c oxidase activity was measured with an ATP-generating system as described (32).

**RESULTS**

**Purification of Cytochrome c Oxidase from Mitochondrial Membranes in Lauryl Maltoside:** As a first step in the purification of cytochrome c oxidase from yeast, isolated mitochondria were disrupted in a KCl solution of low ionic strength, and the membrane fraction was collected by centrifugation. This membrane fraction was then dissolved in lauryl maltoside for chromatography on DEAE-Bio-Gel A (Fig. 1) as detailed under "Experimental Procedures." A range of concentrations of lauryl maltoside was tested, with 0.8 g of detergent/ g of mitochondrial protein proving to dissolve the membrane optimally, and 0.01% detergent providing the best separation of the cytochromes bc, from aa3 on the column.

The single fractionation by ion exchange chromatography typically gave a 30–35-fold purification of cytochrome c oxidase by hemeb determination. As much as 2 mg of enzyme could be obtained from 200 mg of mitochondria (8 liters of yeast cells) by pooling the entire eluant containing immunodetectable cytochrome c oxidase, and 0.5 mg of highly purified oxidase was obtained when only peak fractions were pooled (with a heme aa3 concentration of 8–9 nmol/mg of protein). Peak fractions were free of succinate-cytochrome c reductase and ATPase activity.

**Spectral Features and Activity Measurements on Purified Enzyme:** Spectral analysis showed no contamination of peak samples with cytochromes b or c1. The purified oxidase had absorbance maxima of 422 nm (oxidized) and 441 nm (reduced) in the Soret region and a maximum of 605 nm in the α-band region for the potassium ferriyanide-oxidized versus sodium dithionite-reduced difference spectrum.

A typical Eadie-Hofstee plot of the electron transfer activity of the purified cytochrome c oxidase as a function of cytochrome c oxidase activity was measured with an ATP-generating system as described (32). The NaCl gradient is indicated by a dashed line.

**FIG. 1.** DEAE column profile obtained during the purification of yeast cytochrome c oxidase. The upper panel shows the absorbance at 280 nm (protein). The lower panel shows the reduced minus oxidized absorption difference at 605 nm (heme aa3) and 563 nm (heme b). The NaCl gradient is indicated by a dashed line.
chrome c concentration is shown in Fig. 2. The plot is biphasic with a $K_m$ of the initial high affinity phase of 0.06 μM and the second lower affinity phase of 5.8 μM. These values are similar to those obtained for the enzyme in yeast mitochondrial membranes prior to fractionation. The maximum turnover number of the purified enzyme in 50 mM potassium phosphate buffer, pH 6.2, 0.01% lauryl maltoside and at 20 °C was in the range of 180–210 s$^{-1}$.

**Subunit Structure of the Lauryl Maltoside-derived Yeast Cytochrome c Oxidase**—SDS-polyacrylamide gel electrophoresis in Fig. 3 shows the polypeptide composition of the enzyme purified in lauryl maltoside. A comparison of the number of polypeptides in mitochondria (lane 1) with the composition of the isolated enzyme (lane 2) confirms the considerable purification of cytochrome c oxidase achieved by the single column step. In all, 12 major bands were identified on the 21% polyacrylamide gel for the oxidase isolated in lauryl maltoside. This is three more than reported for enzyme purified in Triton X-100 (14). The polyacrylamide gel shown in Fig. 3 was stained twice, once with Coomassie Brilliant Blue and then a second time by light silver staining. This second staining step significantly increased the staining intensity of several of the bands (as shown in Fig. 3) including polypeptides labeled as IV, 3, and VII. Identification of the various bands with previously described subunits of the yeast oxidase was made by electrobloctting companion gels to that shown in Fig. 3 onto polyvinylidene difluoride membranes and then N-terminal sequencing these polypeptides according to the procedure of Matsudaira (27). Subunits II, IV, V, VI, and VIII were identified unequivocally in the polyacrylamide gel profile from their published sequences (e.g. 33–35). Subunits VII and VIIa co-migrate, and sequencing of the band containing these polypeptides gave the expected amino acids for the two sequences (36) in nearly equivalent amounts, with no other amino acids present to represent a third sequence. This leaves three additional polypeptides labeled 1, 2, and 3 in Fig. 3, migrating between subunits II and VII/VIIa, that have not been observed in previous preparations of the enzyme. These three polypeptides were sequenced twice, each from different enzyme preparations. Amino acid sequencing of polypeptide 1 gave a reproducible sequence for 16 residues following ambiguities in the first 3 cycles. This N-terminal sequence had very weak homology to subunit VIa of mammals but no similarity to other subunits of the mammalian enzyme. The mammalian subunit VIa is one of the three subunits present as isoforms (2, 15, 37). Comparison of the sequences of this polypeptide from different mammals shows most sequence conservation in the C-terminal half (37), and so peptide mapping and sequencing of polypeptide 1 were conducted to extend the sequence information. Ten major fragments were obtained from HPLC separation of a trypptic digest. Five of the fragments were sequenced. The sequence of each of these is given in Fig. 4, aligned to the sequence of the liver form of beef heart subunit VIa (37). The sequence identity within these fragments is 20 amino acids of 44. Sequence data for polypeptide 2 were not easily interpreted because of contamination from the co-migrating subunit VI, and other procedures such as HPLC or isoelectric focusing will be needed to separate this polypeptide for characterization. Fig. 4 shows that the N-terminal sequence of polypeptide 3 exhibits significant homology with the N-terminal sequence of subunit VIb in mammals (38, 39). In all, 5 of the 19 residues identified are identical with subunit VIb from beef heart, and there are conservative replacements at other positions.

**DISCUSSION**

It has been generally accepted that cytochrome c oxidase from yeast is simpler in subunit composition than its mammalian counterpart, with six nuclear coded subunits instead...
of the 10 found in higher eukaryotes (2, 3, 14). Experience in studying the multisubunit enzymes of mitochondrial oxidative phosphorylation has shown that the number of polypeptide components of a complex can be underestimated when less than optimally resolving gel conditions are used (15, 40), if subunits stain poorly or not at all with Coomassie Brilliant Blue (e.g. proteolipid proteins of the mitochondrial F_{F_{0}}-ATPase; 41) or if too high concentrations of detergents are used in the purification procedure (16, 42). Studies with mammalian cytochrome c oxidase have pointed to the lability of this complex, with several subunits released by certain detergent treatments (2, 3, 6, 16, 42). Even when the enzyme is isolated in bile salts, several of the polypeptides now considered to be bona fide subunits are depleted from the preparation, including subunits VIa and VIb (2, 40). Furthermore, when the oxidase is isolated with Triton X-100 or by treating the bile salt purified enzyme with Triton X-100, subunits VIa and VIb are removed completely along with III and VIIa (2, 42).

The fact that yeast cytochrome c oxidase has been isolated before only by methods that use Triton X-100 led us to reconsider the issue of the number of subunits in the fungal enzyme. We find that cytochrome c oxidase isolated from yeast in the mild detergent lauryl maltoside contains polypeptides in addition to those obtained when the enzyme is purified in Triton X-100, and these are in the molecular weight range of the four subunits present in mammals but thought to be missing from the fungal enzyme. One of the extra polypeptides is clearly the homologue of subunit VIa of mammals. Several protease-cleavage products were generated and isolated from polypeptide 1, and the sequences of each of these could be aligned with the sequence of subunit VIa from beef liver cytochrome c oxidase. The sequence identity between these peptides and the sequence of the mammalian subunit is greater than 40%. The N-terminal sequence of polypeptide 3 indicates that it is the homologue of subunit VIb in mammals. In recent work LaMarche et al. (44) have cloned and sequenced the gene encoding a polypeptide with the exact N-terminal sequence as our polypeptide 3. The full sequence of this polypeptide is 41% identical to bovine cytochrome c oxidase subunit VIb. LaMarche et al. (44) show that this gene product affects the assembly of a functional cytochrome c oxidase complex in yeast. Our data establish that the polypeptide co-purifies with the cytochrome c oxidase and is, therefore, an authentic subunit of the cytochrome c oxidase in yeast.

There is a third polypeptide present in the lauryl maltoside-purified yeast cytochrome c oxidase (band 2 in Fig. 3) which could be the fungal equivalent of the mammalian subunit VIIb or VIII. Unfortunately, it was not possible to obtain any sequence data on this polypeptide because of its close migration in gels with subunit VI.

An important aspect of the isolation procedure described here for yeast cytochrome c oxidase is its ease and rapidity. These features, along with the use of lauryl maltoside as a mild detergent to keep the complex intact, should make this procedure useful for isolating mutant forms cytochrome c oxidase, such as the one we described recently (12), with altered stability and enzyme function.

### REFERENCES


**Fig. 4.** Amino acid similarities between polypeptides present in isolated yeast cytochrome c oxidase and subunits of bovine cytochrome c oxidase. Panel A compares yeast polypeptide 1 (Y 1) with bovine liver subunit VIa (BL VIa). The full sequence of BL VIa: SSGARKERGFWKQVGVSWLGNWMKSVRHNKRHESSFRPFPQDPNQK~CWQNYLDFHRCE~~~GGDVSVCEWYRRVYKS~PIS~ST~K~D~EG~FPGKI.
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