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. This article must therefore be by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 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 beef, rat, and human has a total of 10 nuclear coded subunits (2, 3, 15), whereas the yeast enzyme has been found to contain maximally 14 (16). Experience with the mammalian enzyme has shown that the polyepitope 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 (VIA and VIB) 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, ade6, 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 Hepes, pH 7.4, 1 mM phenylmethanesulfonyl 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 phenylmethanesulfonyl fluoride as described (20) followed by a wash in 50 mM Tris-HCl, pH 8.0, 1 mM MgSO4, 1 mM phenylmethanesulfonyl 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 mM 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 phenylmethanesulfonyl fluoride) containing 100 mM NaCl. The column with the absorbed sample was washed with 5 column volumes of buffer A, 100 mM NaCl and 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 phos-
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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 hemeprotein 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 ferricyanide-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 cyto-

The abbreviations used are: CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; HPLC, high performance liquid chromatography.
chrome concentration is shown in Fig. 2. The plot is biphasic with a $K_m$ of the initial high affinity phase of 0.06 $\mu$M and the second lower affinity phase of 5.8 $\mu$M. 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 VIII. Identification of the various bands with previously described subunits of the yeast oxidase was made by electroblotting 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 I, 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 tryptic 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 VIIb in mammals (38, 39). In all, 5 of the 19 residues identified are identical with subunit VIIb 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...
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(A)

\[
\begin{array}{cccccccc}
Y & 1 & : & S71LPAKELAFGPPQFKV & DTSMNEV & HNPOLGQPER & DTFMNR336KPP7GDQ & TLFNWPH\hline
\end{array}
\]


(B)

\[
\begin{array}{cccccccc}
Y & 3 & : & AQENSP25LKTGFDAYFPQ & \ldots & \ldots
\end{array}
\]


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 Vla (BL VIa). Panel B compares yeast polypeptide 3 (Y 3) with bovine subunit Vlb (B Vlb). Solid lines indicate amino acid sequence data obtained by N-terminal sequencing; dashed lines indicate amino acid sequence data obtained by tryptic cleavage followed by N-terminal sequencing. Question marks depict sequence ambiguities. Identical amino acid residues are indicated by circles; conservative replacements are indicated by dots. Asterisks denote amino acid residues conserved in subunit Vla from bovine heart and liver, rat heart and liver, and human heart and liver (37).

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 VIII or VIIb. 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 malside 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

Purification of yeast cytochrome c oxidase with a subunit composition resembling the mammalian enzyme.
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