Cytochrome c Oxidase from Bakers’ Yeast

III. PHYSICAL CHARACTERIZATION OF ISOLATED SUBUNITS AND CHEMICAL EVIDENCE FOR TWO DIFFERENT CLASSES OF POLYPEPTIDES*

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Earlier studies have shown that cytochrome c oxidase from bakers’ yeast is an oligomeric enzyme which contains three polypeptides (I to III) synthesized on mitochondrial ribosomes and four polypeptides (IV to VII) synthesized on cytoplasmic ribosomes. These polypeptide subunits have now been isolated by a simple protocol which utilizes differences in polypeptide charge, solubility, and size. Their molecular weights determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, gel filtration in the presence of guanidine hydrochloride, and amino acid analysis were: I, 40,000; II, 33,000; III, 22,000; IV, 14,500; V, 12,700; VI, 12,700; and VII, 4,600. All seven polypeptide subunits exhibited acidic isoelectric points; cytoplasmically made subunits were more acidic than mitochondrially made ones.

The amino acid composition of two mitochondrially made subunits and two cytoplasmically made subunits was determined. The two mitochondrial translation products, I and II, contained only 34.7% and 42.1% polar amino acids, respectively, whereas the two cytoplasmic translation products, IV and VI, contained 48.3% and 49.3%, respectively. This agreed with the observation that Subunits I and II are very insoluble, requiring detergents for solubility, whereas Subunits IV and VI are water-soluble in the absence of any added detergent.

These results indicate that the cytochrome c oxidase subunits synthesized on mitochondrial and cytoplasmic ribosomes are fundamentally different in size, isoelectric properties, and hydrophobicity. They also suggest the possibility that at least some of the mitochondrially made subunits are buried in the lipid phase of the mitochondrial inner membrane.

Recent studies have shown that cytochrome c oxidase from bakers’ yeast and Neurospora crassa consists of seven polypeptides (2-4), whose biosynthesis and assembly results from the coordinated functioning of cytoplasmic and mitochondrial protein synthesis (1, 4, 5). Although a great deal of information has been obtained concerning the sites of synthesis of these polypeptides and the interplay between mitochondrial and cytoplasmic protein synthesis in the assembly of this enzyme (6-10), there is still relatively little known about the function and physicochemical properties of these polypeptides and their arrangement within the mitochondrial membrane.

In view of the oligomeric nature of cytochrome c oxidase, information concerning the properties and arrangement of polypeptide subunits within the membrane would be particularly pertinent for understanding the function of each subunit and the assembly of the holoenzyme in vivo. In this paper, we describe the isolation and characterization of the seven polypeptide subunits of yeast cytochrome c oxidase. We have found that the polypeptide subunits translated on mitochondrial ribosomes are larger, less acidic, and more hydrophobic than the subunits translated on cytoplasmic ribosomes. Subsequent communications will deal with the use of subunit-specific antisera to study the function of each subunit (11) and the use of chemical probes to study the spatial arrangements of subunits in the solubilized and membrane-bound enzyme (11a).

METHODS

Preparation of Cytochrome c Oxidase

Cytochrome c oxidase was prepared from commercially grown bakers’ yeast (Fleischmann’s yeast, 1) obtained from Standard Brands.

1 For unknown reasons, the quality of commercially grown Fleischmann’s yeast available to us changed around the spring of 1973. This was reflected in a lower yield and a lower purity of cytochrome c oxidase prepared from it. In subsequent studies we switched to Red Star Yeast and modified the isolation procedure slightly (cf. Ref. 11a).
Inc., New York, N.Y.) as described previously (2) except that the last two ammonium sulfate fractionations (P1, P2) were omitted and ammonium sulfate was added to 30% saturation instead of 34% saturation in the fractionation immediately preceding the 30% saturation cut which produces the P4 pellet. All of the ammonium sulfate fractionations were performed at a pH of 7.2 to 7.4. The P2 was dissolved in a minimal volume of 20 mM sodium phosphate (pH 7.0) containing 1% Triton X-100, centrifuged at 40,000 rpm for 15 min in a Spinco No. 40 rotor for clarification, desalted by gel filtration, and applied to either a DEAE-cellulose column, a hydroxylapatite column, or a sucrose gradient. Desalting, chromatography on a DEAE-cellulose column, and sucrose gradient centrifugation were performed as previously described (2). For chromatography on hydroxylapatite, an elution solvent containing 30 to 50 mg of protein was used, and 10 mM sodium phosphate (pH 6.8)-0.1% sodium dodecyl sulfate was used for the hydroxylapatite column (0.9 x 25 cm; Bio-Gel HTP) equilibrated with 10 mM sodium phosphate (pH 6.8)-0.5% Triton X-100. The column was eluted with 150 ml of a gradient ranging from 0 to 300 mM sodium phosphate ranging from 0.2 M to 0.7 M sodium phosphate (pH 6.0-1% sodium dodecyl sulfate-1 mM diethanol). After loading, the column was washed with 1 bed volume of starting buffer and eluted with 100 ml of a linear gradient ranging from 0.2 M to 0.7 M sodium phosphate (pH 6.4)-1% sodium dodecyl sulfate-1 mM diethanol. Fractions of 1 ml were collected and monitored by their absorbance at 280 nm, protein content, and polypeptide composition. Fractions from the major peak, which eluted at a conductivity of 15 to 16 mMHO were pooled, concentrated by ultrafiltration with a PM-10 membrane filter and dialyzed extensively against 10 mM sodium phosphate (pH 7.0-1% sodium dodecyl sulfate-1% 6-mercaptoethanol). The column was poured over a 0.5-cm layer of Sephadex G-25 (coarse) and equilibrated with 10 mM sodium phosphate (pH 6.8)-0.1% sodium dodecyl sulfate-1 mM diethanol. An aliquot containing 1.5 to 2.0 mg of protein was applied to a slab gel of Composition c and subjected to preparative gel electrophoresis as described below.

Isolation of Polypeptide Subunits

The usual starting material was a partially purified preparation (P2) of yeast cytochrome c oxidase with 6.0 to 7.5 nMol of heme a per mg of protein. The P2 was resuspended in 20 mM sodium phosphate (pH 7.0)-1% Triton X-100, clarified by centrifugation, and desalted as above.

Step 1: Dissociation of Enzyme—An aliquot containing 50 to 100 mg of enzyme protein (10 to 20 mg per ml) was dialyzed against 1 liter of 50 mM Tris-Cl (pH 8.5)-10 mM dithiothreitol-2 mM EDTA at 4°C overnight. The sample was then warmed to room temperature and solid guanidine HCl was added to a final concentration of 6 M. All subsequent steps were performed at room temperature unless otherwise stated. The solution was then dialyzed (Spectrapor No. 3 dialysis tubing) against 2 liters of distilled water for 1 hour. The dialysate was replaced with fresh water and dialysis was continued for additional 2 hours. By the end of the 3rd hour, noticeable turbidity had developed as the concentration of guanidine and reducing agent fell, and by the end of the 3rd hour, a flocculent precipitate containing an aggregate of Subunits I, II, III, V, and VII had formed. Subunits IV and VI remained soluble. Soluble and insoluble fractions were separated by centrifugation at 35,000 rpm for 15 min in a Spinco No. 40 rotor. The supernatant, containing Subunits IV and VI, was processed immediately as described below. The pellet, containing the other five subunits and much of the non-cytochrome c oxidase protein present in the partially purified starting material, was washed twice with distilled water and frozen.

Step 2: Isolation of Subunits IV and VI—The supernatant from Step 1 was added 7/8 volumes of 500 mM Tris-Cl (pH 9.0)-1 mM EDTA and sufficient solid urea to give a final concentration of 40%. The solution was then dialyzed against 30 mM Tris-Cl (pH 8.0)-0.1 mM EDTA-6 M urea (Tris-EDTA-urea buffer) for 4 hours at 4°C to remove any residual guanidine HCl. An aliquot containing up to 40 mg of protein was applied to a DEAE-cellulose column (0.9 x 24 cm; Whatman DE52) which had been equilibrated with Tris-EDTA-urea buffer under a positive pressure maintained by a pump. The column was washed with 30 ml of Tris-EDTA-urea buffer and eluted at a flow rate of 20 ml per hour with 200 ml of a linear gradient ranging from 0 to 0.2 M NaCl in the same buffer. Polypeptide peaks were localized by their absorbance at 280 nm and by protein determination as described below.

Step 3: Agarose Gel Filtration of Subunits I, II, III, V, and VII—The frozen pelleted obtained in Step 1 (40 to 60 mg of protein) was warmed to room temperature, suspended in 4 to 10 ml of 10 mM sodium phosphate (pH 7.0)-1% 6-mercaptoethanol-5% sodium dodecyl sulfate, and solubilized by boiling for 2 min. The sample was then dialyzed overnight against 1 liter of 50 mM Tris-Cl (pH 7.4)-0.5% sodium dodecyl sulfate-1 mM EDTA at room temperature, concentrated to 1.5 ml by dialysis against solid sucrose and subjected to gel filtration in a 6% agarose gel column (2.5 x 90 cm; Bio-Gel A-5m) equilibrated with 50 mM Tris-Cl (pH 7.4)-0.5% sodium dodecyl sulfate-1 mM EDTA. Fractions of 3 ml were collected and monitored for absorbance at 280 nm and polypeptide composition by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fractions from each of the two major protein peaks, 1 and 2 (cf. "Results") were pooled and concentrated by ultrafiltration with a PM-10 membrane filter.

Step 4: Isolation of Subunits I and II—The combined Peak 1 fractions from the agarose column (Step 3) were mixed with 1/3 volume of 1 M sodium dodecyl sulfate and 1% sodium phosphate (pH 6.4)-0.1% sodium dodecyl sulfate-1 mM dithioethanol overnight at room temperature. The sample, containing up to 15 mg of protein, was then chromatographed on a hydroxylapatite column (0.9 x 25 cm; Bio-Gel HTP) in the presence of sodium dodecyl sulfate as described by Moss and Rosenthal (12). The column was poured over a 0.5-cm layer of Sephadex G-25 (coarse) and equilibrated with 10 mM sodium phosphate (pH 6.8)-0.1% sodium dodecyl sulfate-1 mM diethanol. An aliquot containing 1.5 to 2.0 mg of protein was applied to a slab gel of Composition c and subjected to preparative gel electrophoresis as described below.

Analytical Gel Electrophoresis

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed in a discontinuous buffer system (13), chosen for its improved resolution. This system was a modified form of System A of Ugel et al. (14) and contained the following buffer components: upper and lower reservoir buffer, pH 8.94, 0.052 M Tris-0.052 M glycine-0.03% sodium dodecyl sulfate; upper gel buffer, pH 6.96, 0.032 M H3PO4-0.059 M Tris-0.5% sodium dodecyl sulfate; lower gel buffer, pH 8.8, 0.048 M Tris-0.32% sodium dodecyl sulfate. A gel matrix of 14% acrylamide-0.31% bisacrylamide was carried out in either cylindrical gels (0.6 x 10 cm) or slab gels (cf. 13). A 1-cm stacking gel which contained 2.5% acrylamide, 0.15% bisacrylamide, 0.025% N,N,N',N'-tetramethylethylenediamine, and 0.005% riboflavin and which was polymerized by light was routinely used. Three different types of lower gel were employed: gels of Composition a were step gels with 15% acrylamide-0.47% bisacrylamide for the bottom 7 cm and 10% acrylamide-0.31% bisacrylamide for the top 3 cm; gels of Composition b were 14% acrylamide-0.78% bisacrylamide; and gels of Composition c were 12% acrylamide-0.375% bisacrylamide. Gels were loaded with protein, subjected to electrophoresis, fixed, and stained as described earlier (2).

Molecular weights were determined by subjecting to electrophoresis preparations of either the holoenzyme or the isolated subunits on a slab gel with adjacent mixtures of several standard proteins: bovine serum albumin (mol wt, 68,000); ovalbumin (45,000); 2-aminobutyric acid oxidase (37,000); glyceraldehyde phosphate dehydrogenase (36,000); trypsin dissolved in fluorophore (23,300); β-lactuglobulin (18,400); ribonuclease (13,700); cytochrome c (12,300); pancreatic trypsin inhibitor (6,200); adrenocorticotropic hormone (4,550); gluca- gon (3,480); and insulin B chain (3,400). Molecular weights were determined relative to bromophenol blue as described by Weber and Osborn (15).

"Ferguson plots," employed to check "anomalous migration," were obtained from gels of varying acrylamide concentrations but with a constant ratio of bisacrylamide to acrylamide (1:32) (16). The split gel technique of Dunker and Runne (16) was used to compare cyto- chrome c oxidase polypeptides and marker proteins on the same gel. Relative mobilities of polypeptides in these studies were calculated as described by Neville (17).

Samples (0.5 mg per ml) were routinely dissociated in 10 mM sodium phosphate (pH 7.0)-1% sodium dodecyl sulfate-1% β-mercaptoethanol-10% sucrose by boiling for 2 min. Protein samples to be reduced and reacted with iodoacetamide were dissolved at a concentration of 2 mg per ml in 50 mM Tris-Cl (pH 8.0)-2 mM EDTA-50 mM dieth-
threitol-8 M urea, incubated for 4 hours and reacted with 0.2 M iodoacetamide for 20 min in the dark. The reaction mixture was diluted 1.5 with 10 mM sodium phosphate (pH 7.0), brought to 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol and dissociated as above. Protein samples were oxidized with performic acid as described by Burgess (18). After oxidation, the lyophilized reaction mixture was suspended in sodium dodecyl sulfate dissociation buffer and dissociated as above.

Preparative Gel Electrophoresis

Preparative gel electrophoresis in sodium dodecyl sulfate was performed on a vertical slab gel (0.3 x 10 x 10 cm) in a two-slab Plexiglas apparatus. The buffer system and the upper and lower gel compositions were the same as described above for analytical gel electrophoresis. Protein bands were monitored directly in the gel slabs during electrophoresis by observing fluorescence (366-nm peak output) of dansylated aliquots (50 μg) of each sample which were applied to small slots at each edge of the slab. Protein-sodium dodecyl sulfate complexes were dansylated as described by Talbot and Yphantis (19). The bulk of the protein (1.5 to 2.0 mg) was applied to the middle portion of each slab bracketed on both sides by the fluorescent aliquots. Electrophoresis was initiated at 10 ma per slab and continued until the tracking dye had reached the bottom of the gel. Following electrophoresis, the desired protein was cut off the gel using the fluorescent samples on each edge of the slab as markers. Gel slices 1 cm thick were cut from the gel tube and dialyzed at room temperature against 50 mM Tris-Cl (pH 8.0) to remove excess sodium dodecyl sulfate. Reservoir buffers used for elution were the same as those used for electrophoresis.

Gel Chromatography in 6 M Guanidine Hydrochloride

Gel chromatography in 6 M guanidine HCl was carried out on a column (1.5 x 84 cm) of Bio-Gel A-5m (200 to 400 mesh) equilibrated and run with sample buffer (pH 6.0)-0.1 M guanidine HCl (as described by Fish et al. (21). The proteins were reduced and reacted with iodoacetamide as described above except that guanidine HCl replaced urea in the dissociation buffer and the reaction with iodoacetamide was stopped by lowering the pH to 6.0. Before being exposed to iodoacetamide, the holoenzyme was partially dissociated by incubating in 6 M guanidine HCl at 25°C for 4 hours or completely dissociated by incubating in 6 M guanidine HCl at 50°C for 4 hours. After reaction with iodoacetamide, the sample was mixed with 0.5 ml of column buffer containing 0.6% blue dextran, 0.1% dinitrophenylalanine, and 100 mM of sucrose. A 0.6 ml aliquot of this solution was loaded on the column. Bovine serum albumin, ovalbumin, chymotrypsinogen, β-lactoglobulin, ribonuclease, cytochrome c, and insulin (A and B chains) were reacted with iodoacetamide as above in 6 M guanidine HCl and used as molecular weight markers. The column was monitored for protein by absorbance at 280 nm, for blue dextran by absorbance at 630 nm, and for dinitrophenylalanine by absorbance at 360 nm.

Isoelectric Focusing

Isoelectric focusing was performed in cylindrical polyacrylamide gels (0.6 x 8 cm) containing 4.0% acrylamide, 0.136% bisacrylamide, 1 mM dithiothreitol, 8 M urea, and 0.4% Ampholine (pH 3 to 10). Gel polymerization, focusing, fixation, staining, and pH determination were carried out as described by Finlayson and Chrambach (22). Protein samples (5 to 10 mg per ml) were dialyzed against 30 mm Tris-Cl (pH 7.0)-0.1 mM EDTA-0.1 mM dithiothreitol-0.5 M urea for 4 hours at room temperature. Solid sucrose was added to a final concentration of 10% and a small aliquot (25 to 35 μl) of protein was loaded on the top of each gel underneath a 1-mm layer of 0.4% Ampholine (pH 3 to 10)-5% sucrose.

Amino Acid Analysis

Protein samples of 0.5 to 1.0 mg were dialyzed extensively against three changes of distilled water and hydrolyzed in 6 N HCl at 107°C in evacuated tubes for 22, 48, and 72 hours. The hydrolysates were taken to dryness in a vacuum desiccator containing pellets of KOH, lyophilized, then dissolved in citrate buffer, and analyzed as described by Spackman et al. (23). Amino acid compositions were determined with a Spino Instruments 120-C amino acid analyzer equipped for determination of amino acids in the range of 2.5 to 30 nmol (24). The amino acid contents reported are the average of duplicate analyses for each sample. Valine, isoleucine, and leucine contents were taken from 72-hour hydrolysates. Values for threonine, tyrosine, and serine were taken from 22-hour hydrolysates. The half-cystine and methionine contents were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation (25). Tryptophan was determined with the amino acid analyzer using a 20-hour hydrolysate containing 3% (v/v) thioglycolic acid (26).

Analytical Procedures

Absorption spectra of yeast cytochrome c oxidase were obtained on a Cary 14 recording spectrophotometer. Heme a content was determined from the difference in absorbance at 633 and 630 nm of dithionite-reduced enzyme dissolved in 20 mM sodium phosphate (pH 7.0)-1% Triton X-100 as described earlier (2). Protein concentrations were estimated by the method of Lowry et al. (27) in the presence of 0.4% sodium deoxycholate. Conductivities on column fractions were determined at room temperature with a Radiometer conductivity meter (type CDM 2).

Materials

Electrophoresis grade acrylamide and bisacrylamide, Bio-Gel HTP, Bio-Gel A-5m, and AG 501-X8(D) were obtained from Bio-Rad Laboratories (Richmond, Calif.). Ammonium (carrier amphotolyte), pH 3 to 10, was purchased from LKB-Produkter AB (Sweden). 5-Dimethylaminonaphthalene-1-sulfonyle chloride (as a 10% solution in aceton) was obtained from Pierce Chemical Co. (Rockford, Ill.) and ultrapure urea, guanidine hydrochloride, ammonium sulfate, and dinitrophenylalanine were obtained from Schwarz-Mann (Orangeburg, N.Y.). All urea stock solutions were deionized just before use by passage through a mixed bed ion-exchange resin (AG 501-X8(D). Sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, Mo.) was recrystallized from 95% ethanol (18) and iodacetamide (Eastman Organic Chemicals, Rochester, N.Y.) was recrystallized twice from water (28). Spectrocou 3 dialysis membranes (mol wt cutoff of 3500) were purchased from Spectrum Medical Industries, Inc. (New York, N.Y.) and PM 10 membrane filters from Amicon Corp. (Lexington, Mass.).

Proteins used as standards in gel electrophoresis and gel filtration were from the following sources: bovine serum albumin, cytochrome c (horse heart), trypsin inhibitor (bovine pancreas), adrenocorticotropic hormone (porcine), and glucagon (bovine pancreas) from Sigma; ovalbumin (chicken), β-amino acid oxidase (porcine kidney), glycerol-dehyde phosphate dehydrogenase (rabbit muscle), trypsin-diisopropyl fluorophosphate (bovine pancreas), ribonuclease (bovine pancreas), and chymotrypsinogen A (bovine pancreas) from Worthington; and g-globulin and insulin (A and B chains) from Schwarz-Mann.

RESULTS

Number of Polypeptide Subunits in Yeast Cytochrome c Oxidase

In earlier studies, electrophoresis of purified yeast cytochrome c oxidase on 12% polyacrylamide gel containing sodium dodecyl sulfate at an alkaline pH (Composition c) revealed the presence of six major polypeptide components (2, 29). It was noted, however, that at acrylamide concentrations between 14 and 17% one of the polypeptide bands (Component V) splits into two bands (Fig. 1A) suggesting the presence of a seventh polypeptide. Other laboratories have reported that yeast cytochrome c oxidase contains from four to seven polypeptides as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3, 30, 31). All of these studies (including our own) were limited by the resolution inherent in a one-dimensional separation procedure as well as by possible anomalous migration of polypeptides in sodium dodecyl sulfate-acrylamide gels (32-35). These uncertainties prompted us
The aggregation and incomplete dissociation that has occurred in sodium dodecyl sulfate-polyacrylamide gels can be prevented by heating the sample to 100°C for a few minutes in the presence of 1% 2-mercaptoethanol (36). To exclude the reformation of disulfide bonds during electrophoresis, cytochrome c oxidase was first dissociated in 8 M urea and either reduced with dithiothreitol, carboxymethylated with iodoacetamide, or oxidized with performic acid before it was denatured in sodium dodecyl sulfate and subjected to electrophoresis. Fig. 1, A to D shows that the gel electrophoretic patterns of untreated enzyme, reduced enzyme, and enzyme reacted with iodoacetamide or performic acid are identical. This result makes it very unlikely that the polypeptide subunits of this enzyme are linked to each other by disulfide bridges or that the polypeptide bands observed on sodium dodecyl sulfate gels are aggregates or incompletely dissociated polypeptide subunits.

Although most proteins migrate according to their molecular weight in sodium dodecyl sulfate gels, some proteins with unusual charge (32), conformation (34), unreduced sulfhydryl groups (35), and carbohydrate moieties (33) do not. As a prelude to an accurate estimate of molecular weight by sodium dodecyl sulfate gel electrophoresis and an approach to detecting anomalous migration, the enzyme was dissociated in sodium dodecyl sulfate and run at different acrylamide concentrations to determine free electrophoretic mobilities and retardation coefficients (37). A plot of log $R_e$ versus acrylamide concentration (38) for the sodium dodecyl sulfate-polypeptide complexes is shown in Fig. 2. For all polypeptides, the plot is essentially linear. The free electrophoretic mobilities (intercept at acrylamide concentration = 0) for Subunits II to VII are similar and identical with those of a number of standard proteins subjected to co-electrophoresis on the same gels. However, the free electrophoretic mobility of Subunit I is extremely high (about 50% higher than the average). This anomalous migration makes it virtually impossible to determine the molecular weight of Subunit I by sodium dodecyl sulfate gel electrophoresis and may have contributed to the variance in subunit number noted above. Because Subunits I and II have identical relative mobilities on 8.5% acrylamide gels (see Fig. 2), electrophoresis at this gel concentration would have underestimated the number of subunits. At gel concentrations between 15 and 17% (not shown), Subunits V and VI become visible as distinct bands. Below 14% acrylamide, Subunit V co-migrates with Subunit VI and above 17% it co-migrates with Subunit IV. The reason for this anomalous migration of Subunit V is uncertain.

**Determination of Number of Subunits by Other Methods**

Isoelectric focusing of a urea-dissociated enzyme in a gel containing urea and Triton X-100 and ampholytes ranging from pH 3 to 10 revealed seven major bands (Fig. 3). Initial attempts to identify these bands combined sodium dodecyl sulfate gel electrophoresis with gel isoelectric focusing. After isoelectric focusing, the cylindrical gels were laid across a sodium dodecyl sulfate-polyacrylamide slab gel containing 12% acrylamide and subjected to electrophoresis in the second dimension. Unequivocal results could not be obtained from this system because Subunits I, II, and III precipitated at their isoelectric points and were thus retained by the electrofocusing gel.
Fig. 3. Isoelectric focusing of yeast cytochrome c oxidase in an urea-polyacrylamide gel. Gels (4%) with pH 3 to 10 ampholytes were used. The gels were stained, scanned, and sliced for pH determination as described under “Methods.”

However, Subunits IV to VII did enter the second dimension where they formed four discrete spots. Unequivocal results for all seven subunits were obtained by slicing an isoelectric focusing gel, extracting each slice with boiling sodium dodecyl sulfate dissociation buffer and subjecting to electrophoresis each extract on a separate sodium dodecyl sulfate gel. Each protein peak obtained by isoelectric focusing yielded only a single protein band by sodium dodecyl sulfate gel electrophoresis. A two-dimensional analysis using size fractionation by guanidine HCl gel filtration in the first dimension and charge fractionation by gel isoelectric focusing in the second dimension gave similar results. Each polypeptide peak from the gel filtration column (cf. below) gave only one major polypeptide band upon gel isoelectric focusing.

Thus, by the criteria of charge and size, applied separately in one-dimensional systems and together in two-dimensional systems, purified yeast cytochrome c oxidase appears to contain seven polypeptide subunits.

Isolation of Subunits

The seven subunits were isolated by the protocol shown in Fig. 4. This scheme permits the isolation of all seven polypeptides from both crude (3 to 6 nmol of heme a per mg of protein) and purified (9 to 10.4 nmol of heme a per mg of protein) preparations of yeast cytochrome c oxidase. A partially purified enzyme preparation which has not been carried through HCl to partially dissociate the enzyme. Denaturation of yeast cytochrome c oxidase in 6 M guanidine HCl and reducing agent at room temperature selectively removes Subunits IV and VI from an aggregate of the other five subunits. Subunits IV and VI can be separated from the aggregate by either of two methods: (a) gel filtration on a 6% agarose column in 6 M guanidine HCl; (b) rapid removal of guanidine HCl by dialysis followed by centrifugation.

Method a reveals a large peak of absorbance at 280 nm in the excluded volume and a smaller double peak (corresponding to a mol wt of 14,000) which contains Subunits IV and VI. Dissociation of the enzyme by higher concentrations of guanidine HCl and at a higher temperature completely dissociates all seven subunits as discrete peaks (Fig. 5). The large peak of absorbance in the excluded volume of the gel filtration column probably represents solely Triton X-100. Method b leads to the quantitative precipitation of an aggregate containing Subunits I, II, III, V, and VII within 1 to 2 hours. Subunits IV and VI remain in solution and can be conveniently recovered by centrifugation. Because Method b proved to be reproducible and easy, it was adopted as the first step in our purification procedure.

Purification of Subunits IV and VI—The supernatant resulting from the centrifugation of the flocculent precipitate (Method b) contains subunits IV and VI and 75% of the Triton X-100 present in the starting material (as assessed by using [3H]Triton X-100). Subunits IV and VI are separated from each other by DEAE-cellulose chromatography in the presence of 6 M urea (Fig. 6). Omission of urea leads to aggregation of Subunit IV and VI and incomplete separation. Typical recoveries of Subunits IV and VI are 50% and 30%, respectively, of the amount of each subunit loaded on the column. More than 95% of the [3H]Triton X-100 applied to the column emerges in the flow-through (Fig. 6). The remaining [3H]Triton X-100 is more or less randomly distributed over all of the column fractions. Counts which appear in fractions containing Subunits IV and VI do not increase across the peak and amounts to less than 0.4 mol of Triton X-100 per 1 mol of protein, indicating that there is little, if any, Triton X-100 bound to these subunits. Because these subunits appeared to be free of bound detergent, we tested their solubility in water by dialyzing them extensively against distilled water and centrifuging them at 200,000 × g for 3 hours. Under these conditions, insoluble proteins or large aggregates of subunits would be expected to form a measurable turbidity or even a pellet. There was no turbidity, no pellet, and no loss of protein for either purified subunit. This finding, in combination with the observation that these subunits do not bind detergent, shows that they are water-soluble under these conditions.

Purification of Subunits I and II—Preliminary fractionation of the flocculent precipitate remaining after removal of guanidine HCl and dithiothreitol was achieved by denaturation in sodium dodecyl sulfate and gel filtration on 6% agarose in the
FIG. 5. Gel filtration of reduced and carboxamidomethylated subunits of yeast cytochrome c oxidase (9.4 nmol of heme a per mg of protein) on 6% agarose in 6 M guanidine HCl after complete dissociation of the enzyme. The Roman numerals indicate the subunits present in each peak as determined by sodium dodecyl sulfate gel electrophoresis. The arrow denotes the position of the void volume. Complete dissociation was achieved by incubating the enzyme at 50°C in 8 M guanidine HCl for 4 hours prior to treatment with iodoacetamide. Absorbance of protein was measured at 280 nm (O—O) and absorbance of dinitrophenyl-alanine at 360 nm (O—O).

FIG. 6. Separation of subunits IV and VI from [3H]Triton X-100 on a DEAE-cellulose column. The subunits were eluted with a linear NaCl gradient (0 to 0.2 M) in Tris-EDTA-urea buffer (cf. “Methods”). The partially purified cytochrome c oxidase used as the starting material in the subunit isolation procedure was prepared as described under “Methods” except that [3H]Triton X-100 (7.6 x 10^6 cpm per mg of protein) was used. The Roman numerals denote the subunits present in the two major peaks. Protein was measured at 750 nm according to Lowry et al. (27). The counts per min. given in the figure are corrected for background (about 20 cpm).

FIG. 7. Separation of the polypeptide subunits precipitated by Method b (see text) by gel filtration on a 6% agarose column in the presence of 0.5% sodium dodecyl sulfate. Fractions were pooled as indicated and analyzed by electrophoresis on acrylamide gels. The arrow designates the position of the void volume. Roman numerals designate the subunits present in each peak.

FIG. 8. Hydroxylapatite chromatography of Peak 1 material from a sodium dodecyl sulfate-agarose column. The column was equilibrated and eluted with a linear sodium phosphate gradient (0.2 to 0.7 M) in a buffer containing 0.1% sodium dodecyl sulfate and 1 mM dithiothreitol as described under “Methods.” The Roman numerals designate the position of Subunits I and II.

Anomalous migration on sodium dodecyl sulfate gels, the use of high acrylamide concentrations maximizes differences in mobilities of these two subunits and thus facilitates their separation. Between 60% and 80% of Subunits I and II found in the flocculent precipitate are recovered as purified subunits.

Purification of Subunits III, V, and VII—Peak 2 from the 6% agarose-0.5% sodium dodecyl sulfate column is essentially free of non-cytochrome c oxidase protein. Subunits III, V, and VII present in this peak are purified by preparative electrophoresis on 10% or 12% polyacrylamide gels. Recoveries for these subunits vary from 60% to 90% of the amount of each subunit estimated to be in the flocculent precipitate.

Characterization of Isolated Subunits

Homogeneity—The purified subunits move as single bands of protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 9) and gel isoelectric focusing (not shown).

Molecular Weights—The molecular weights of the isolated subunits were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, by gel filtration in
FIG. 9. Densitometric tracings of yeast cytochrome c oxidase (top) and purified subunits (I to VII) analyzed by electrophoresis on polyacrylamide gels (12%) containing sodium dodecyl sulfate. The gels were stained with Coomassie blue R-250 and scanned as described under "Methods." The arrow marks the position of the tracking dye (bromphenol blue). The holoenzyme used for the top tracing contained 10.0 nmol of heme a per mg of protein, and the subunits displayed in each of the lower tracings were isolated as described in Fig. 4.

In order to improve the accuracy of the molecular weights determined by gel electrophoresis, all of the samples were run on a slab gel in slots adjacent to a mixture of standard proteins. In addition, gels of three different compositions were used. The values presented in Table I are the average of duplicate samples of three enzyme preparations (9.5 to 10.3 nmol of heme a per mg of protein) which had been obtained by using three different procedures for the final step in the purification protocol. These were DEAE-cellulose chromatography, hydroxylapatite chromatography, and sucrose gradient centrifugation.

As mentioned earlier, Subunits I and V exhibit anomalous migration on sodium dodecyl sulfate gels. Any anomalies resulting from conformation can be assessed by determining molecular weights by gel filtration in 6 M guanidine HCl, because protein conformation in this denaturant is quite different from that in sodium dodecyl sulfate (39, 40). As can be seen from Fig. 5 and Table I, there is remarkably good agreement between the molecular weights determined by these two procedures for all seven subunits. The over-all agreement between the values for molecular weight obtained by amino acid analysis and gel filtration in guanidine HCl with those determined by sodium dodecyl sulfate gel electrophoresis suggests that the high percent acrylamide gels chosen for these studies give reasonably accurate estimates of molecular weight even for Subunits I and V.

Isoelectric Properties—The isoelectric points of the subunits were determined in the presence of 8 M urea and reducing agent. Under these conditions the value obtained for each subunit should approach that of a completely denatured polypeptide in which all charged groups are accessible and capable of contributing to the migration of the polypeptide in a pH gradient. The total charge exhibited by these denatured polypeptides would be expected to mirror that which could be predicted from their amino acid composition. As seen in Table II, all of the subunits of yeast cytochrome c oxidase have acidic isoelectric points. The three large subunits which are synthesized on mitochondrial ribosomes have less acidic isoelectric points than the four smaller subunits which are synthesized on cytoplasmic ribosomes. With those four subunits for which the amino acid composition is known (cf., below), the isoelectric point decreases with an increase in the ratio of acidic to basic amino acid residues.

Amino Acid Composition—Amino acid analyses of four subunits of yeast cytochrome c oxidase and of the native enzyme were performed on samples of the preparations shown in Fig. 9. The amino acid composition of the holoenzyme (Table III) is very similar to that of cytochrome c oxidase from beef heart or Neurospora crassa (3, 41). All three enzymes have a low content of hydrophilic amino acids. The polarities (42) of the holoenzymes from yeast, Neurospora, and beef heart are 39.2%, 38.5%, and 40.4%, respectively.

Two subunits (I and II) synthesized on mitochondrial ribosomes and two subunits (IV and VI) synthesized on cytoplasmic ribosomes were chosen for a comparison in order to see if any major differences in amino acid composition could be detected between the two types of translation products. The data of Table III indicate that each of the four subunits possesses a distinct amino acid composition. However, common to the two mitochondrially made subunits is a high content of apolar amino acids. Subunits I and II have polarities (42) of 34.7% and 42.1%, respectively. The two cytoplasmically made subunits have a low content of apolar amino acids and a polarity of approximately 49%, characteristic of soluble cytoplasmic proteins. Although all four subunits analyzed exhibit a high content of acidic amino acids, the cytoplasmically made subunits are distinctly more acidic than the mitochondrially made ones. Thus, although the holoenzyme itself has a low polarity typical of many membrane proteins, it is composed of...
TABLE I
Molecular weights of subunits of yeast cytochrome c oxidase

Three different preparations of cytochrome c oxidase isolated by using either DEAE-cellulose chromatography, hydroxylapatite chromatography, or sucrose gradient centrifugation as the final purification step were analyzed in duplicate on gels of Composition a to c and by gel filtration in the presence of 6 M guanidine HCl as described under "Methods". The minimal molecular weights given for Subunits I, IV, and VI are taken from Table III.

| Analytical Method                  | Subunit I | Subunit II | Subunit III | Subunit IV | Subunit V | Subunit VI | Subunit VII |
|-----------------------------------|-----------|------------|-------------|------------|-----------|------------|-------------|
| Sodium dodecyl sulfate gel electrophoresis | 37,500    | 33,600     | 24,200      | 14,600     | 13,800    | 12,700     | 5,200       |
| gel composition a                 | 41,000    | 32,700     | 23,900      | 14,100     | 13,400    | 12,400     | 4,600       |
| gel composition б                | 39,800    | 32,800     | 23,100      | 14,300     | 12,400    | 12,400     | 4,600       |
| Gel filtration                    | 41,600    | 28,300     | 21,700      | 14,700     | 13,100    | 13,100     | 4,500       |
| Amino acid analysis (Minimal molecular weight) | 36,930    | 33,840     | n.d. а      | 16,440     | n.d. а    | 10,260     | n.d. а      |

а.n.d. = not determined

TABLE II
Isoelectric properties of cytochrome c oxidase subunits from yeast

Isoelectric points were determined by gel isoelectric focusing in the presence of urea (see "Methods"). All values given represent the average of at least four independent determinations.

| Subunit | Isoelectric Point |
|---------|------------------|
| I       | 6.7-6.8          |
| II      | 6.9-7.0          |
| III     | 5.1-5.3          |
| IV      | 4.7-4.8          |
| V       | 4.3-4.4          |
| VI      | 4.2-4.3          |
| VII     | 4.9-5.0          |

two classes of subunits which can be distinguished both by their site of synthesis and by their hydrophobicity.

DISCUSSION

The results of this study show that the apoprotein component of yeast cytochrome c oxidase is composed of seven polypeptides which differ in molecular weight, isoelectric point, and hydrophobicity. It has been shown earlier that the three largest polypeptides are synthesized on mitochondrial ribosomes whereas the four smallest polypeptides are synthesized on cytoplasmic ribosomes (1, 5, 9). We have now shown that this intriguing duality of origin is reflected in the chemical properties of these polypeptides. The mitochondrially made polypeptides are larger, less acidic, and more hydrophobic than the cytoplasmically made polypeptides.

Throughout this paper we have referred to these polypeptides as subunits of cytochrome c oxidase. In the absence of decisive biochemical data on the function of a polypeptide in any enzyme, it is difficult to state with certainty that a given polypeptide is a bona fide subunit and not merely a contaminant. This difficulty is amplified for membrane-bound enzymes for which one must also decide "where the enzyme ends and the membrane begins" and for which one can envision subunit functions other than catalysis and regulation. We have been aware of this difficulty since the beginning of our studies on yeast cytochrome c oxidase (2). However, on the basis of the following recent observations we are inclined to consider it likely that all seven polypeptides not only are physically associated but also are probably subunits of the enzyme (a) All seven polypeptides copurify through a series of preparative procedures including DEAE-cellulose chromatography, hydroxylapatite chromatography, and sucrose gradient centrifugation. (b) An antibody which cross-reacts with only one polypeptide precipitates all seven polypeptides (11). (c) Antibodies specific for either polypeptide II, VI, or V plus VII

| Subunit | I  | II  | III | IV  | V  | VI  |
|---------|----|-----|-----|-----|----|-----|
| Amino Acid | Cytochrome c Oxidase |
| α-lylase | 4.95 | 4.77 | 7.20 | 8.87 | 6.65 |
| histidine | 2.23 | 2.23 | 2.11 | 2.10 | 1.29 |
| arginine | 3.20 | 3.20 | 3.11 | 3.11 | 4.23 |
| aspartic acid | 6.80 | 7.76 | 8.15 | 11.67 | 12.04 |
| threonine | 5.47 | 5.12 | 4.89 | 5.62 | 1.81 |
| leucine | 6.07 | 7.28 | 8.08 | 5.39 | 5.40 |
| glutamic acid | 6.47 | 6.55 | 6.19 | 6.31 | 15.52 |
| proline | 4.86 | 4.32 | 4.32 | 4.32 | 4.32 |
| glycine | 7.44 | 13.90 | 14.82 | 8.41 | 4.64 |
| alanine | 8.86 | 7.96 | 7.01 | 6.77 | 9.46 |
| cysteine | 1.41 | 1.41 | 1.49 | 1.49 | 1.50 |
| valine | 7.93 | 6.26 | 5.38 | 7.43 | 7.03 |
| methionine | 2.38 | 3.20 | 2.74 | 2.96 | 0.81 |
| leucine | 6.07 | 7.43 | 6.21 | 4.72 | 3.33 |
| isoleucine | 10.31 | 11.47 | 8.51 | 6.69 | 9.29 |
| tyrosine | 4.20 | 3.46 | 3.18 | 2.54 | 3.51 |
| phenylalanine | 5.47 | 6.28 | 3.79 | 2.16 | 5.02 |
| tryptophan | 2.11 | 2.08 | 1.96 | 1.88 | 2.08 |

α The polarity (42) is defined as mole percent of the hydrophilic amino acids (Val, Ile, Leu, Phe, Asp, Glu, Ser, and Thr).

Minimum molecular weight

| Subunit | 128,000 | 126,930 | 33,840 | 16,440 | 10,260 |

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inhibit cytochrome c oxidase activity (11). (d) Nuclear petite mutants of yeast which specifically lack cytochrome c oxidase also lack Polypeptide III (8). (e) Polypeptide I is buried in the interior of the enzyme and is thus unlikely to be a contaminant (11a). This evidence strongly suggests that all seven polypeptides are tightly associated in a functional complex, but does not necessarily imply that each of the seven polypeptides is required for enzymatic activity. Indeed, it has been claimed that not all of the polypeptides present in purified preparations of cytochrome c oxidase are required for enzymatic activity (43, 44). However, the enzymatic activities of the preparations upon which this claim was based were very low.

Apart from catalysis, at least three functions can be envisioned for the subunits of an oligomeric membrane-bound enzyme such as cytochrome c oxidase. These include regulation, membrane binding, and transmembranous ion or electron transport. Polypeptide subunits that function in binding or transmembranous electron transport may be expected to be “integral” (45) or “internal” (11a) proteins which are at least partly buried in the membrane (cf. also Ref. 11a). Such proteins usually exhibit a high degree of hydrophobicity for one or more of the following reasons: (a) they contain a high percentage of apolar amino acid residues (46); (b) they have an asymmetrical distribution of polar and apolar amino acids along their polypeptide chain (47, 48); and (c) they are folded so as to expose a large number of apolar amino acid residues (45). On the other hand, polypeptide subunits that function in catalysis or regulation need not exhibit these properties. They may well be relatively hydrophilic proteins with the properties of “peripheral” membrane proteins (45) such as the subunits of F 
1
ATPase or cytochrome c of the inner mitochondrial membrane. Both types of polypeptide subunits appear to be present in yeast cytochrome c oxidase. At least two of the cytoplasmically made subunits are water-soluble in the absence of added detergent and have a low percentage of apolar amino acid residues. All three of the mitochondrially made subunits are insoluble in the absence of added detergents and at least two of these subunits have a high percentage of apolar amino acids. One of these hydrophobic mitochondrial products, Subunit I, would be large enough to span the lipid bilayer of the inner mitochondrial membrane, assuming a near globular symmetry of the protein.

Our finding that the mitochondrially made subunits are insoluble and exhibit a high degree of hydrophobicity confirms, at the level of a single enzyme, earlier reports (49, 50) that mitochondrial translation products are insoluble components of the inner mitochondrial membrane. Recent reports which appeared while this work was in progress have shown that mitochondrially made components of Neurospora cytochrome c oxidase (4) and yeast rutamycin-sensitive ATPase (51) also contain a high percentage of apolar amino acids.

There is tentative evidence indicating that the heme prosthetic groups of yeast cytochrome c oxidase are associated with the low molecular weight cytoplasmically made subunits (52). If these observations are correct, then our finding that the cytoplasmically made subunits are hydrophilic and water-soluble would support the view that the heme prosthetic groups are at or near the surface of the inner mitochondrial membrane. Because it has been suggested that cytochrome a is on the outside of the inner mitochondrial membrane whereas cytochrome a3 is on the inside of the inner mitochondrial membrane (53–55) one possible function for one or more of the hydrophobic mitochondrially made subunits could be the transport of electrons from cytochrome a to cytochrome a3 across the inner mitochondrial membrane as postulated by the chemiosmotic hypothesis (56). Subunit I, which is large enough and which may penetrate through the lipid bilayer of the inner mitochondrial membrane, is an obvious candidate for this function particularly in view of the recent finding that this subunit is inaccessible to chemical probes used for the covalent labeling of certain amino acids (11a). The precise mechanism by which this type of electron transport might be accomplished awaits further study.

In summary, the present results document chemical differences between the mitochondrially made and cytoplasmically made subunits of cytochrome c oxidase. With these differences in mind we can now begin to explore the functions and interactions of these subunits.

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