Characterization of a Seventh Different Subunit of Beef Heart Cytochrome *c* Oxidase. Similarities between the Beef Heart Enzyme and That from Other Species†

Nancy W. Downer, Neal C. Robinson,‡ and Roderick A. Capaldi*

ABSTRACT: Beef heart cytochrome *c* oxidase has been resolved into seven subunits by electrophoresis in highly cross-linked gels containing urea and sodium dodecyl sulfate. The molecular weights of the polypeptides are estimated to be I, 35 400; II, 24 100; III, 21 000; IV, 16 800; V, 12 400; VI, 8200; and VII, 4400. It has been shown that subunits II and III can coelectrophorese on standard sodium dodecyl sulfate–polyacrylamide gels and appear as a single component with an apparent molecular weight of 22 500. This accounts for previous reports that the beef heart enzyme contains only six subunits. Amino acid analysis of the isolated subunits I, II, and III revealed that they have polarities of 35.5, 44.7, and 39.9%, respectively. All three subunits have an extremely high leucine content and a low percentage of basic amino acids relative to subunits IV–VII. The size, number, and properties of subunits in the beef heart cytochrome *c* oxidase complex suggest that it has essentially the same subunit structure as the complexes isolated from *Saccharomyces cerevisiae* and *Neurospora crassa*.

Recent studies have found that beef heart cytochrome *c* oxidase is a multisubunit complex apparently consisting of six different subunits with molecular weights from 4000 to 40 000 (Briggs et al., 1975; Yamamoto and Orii, 1974; Kornblatt et al., 1973; Kuboyama et al., 1972; Rubin and Tzagoloff, 1973). The standard sodium dodecyl sulfate ($NaDodSO_4$[1])–polyacrylamide gel systems used in these studies do not provide a complete separation of the smaller subunits, nor are they adequate for the determination of molecular weights smaller than 10 000 (Williams and Gratzer, 1971). In order to be able to use various protein modifying reagents to study the arrangement of the subunits in membrane-bound cytochrome *c* oxidase, we were looking for methods which would separate the subunits sufficiently for us to determine the distribution of label among them. The method developed by Swank and Munkres (1971) for $NaDodSO_4$–urea gel electrophoresis in highly cross-linked gels was found to be ideally suited for this purpose.

Here we show that this method not only gives significantly better resolution of the low-molecular-weight polypeptides but also reveals a seventh component of beef heart cytochrome *c* oxidase. Studies are reported which confirm that this component is a polypeptide subunit of the enzymic complex and not an artifact of the conditions used to run $NaDodSO_4$–urea gels.

Experimental Procedure

Cytochrome c Oxidase from Beef Heart Mitochondria. Beef heart mitochondria were isolated as described by Crane et al. (1956). ETP and "green membrane" were prepared as described by Tzagoloff et al. (1968) in steps 1 and 2 of their method for preparing oligomycin-sensitive ATPase. The "green membrane" fraction was then resuspended to a protein concentration of 20 mg/ml in 0.01 M Tris–0.25 M sucrose buffer, pH 7.4. Cytochrome *c* oxidase was prepared as described by Capaldi and Hayashi (1972). This method exposes the enzyme to both deoxycholate and cholate in the course of purification by ammonium sulfate fractionation. Samples of cytochrome *c* oxidase were also prepared using Triton X-100 (Rohm and Haas) as the solubilizing detergent (Sun et al., 1968) and using cholate alone (Yonetani, 1961). Small aliquots of enzyme (20–50 mg/ml) were stored at −20 °C, either before or after being dialyzed against buffer to remove residual cholate and ammonium sulfate.

Gel Electrophoresis. System 1. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was run on pre-formed Bio-Phore gels containing 12.0% total acrylamide (acrylamide–bisacrylamide, 40:1) in a buffer system containing 0.205 M Tris, 0.205 M acetic acid, and 0.1% (w/v) sodium dodecyl sulfate at pH 6.4.

System 2. Polyacrylamide gel electrophoresis was run in the presence of sodium dodecyl sulfate and 8 M urea in gels containing 12.5% total acrylamide (acrylamide–bisacrylamide, 10:1). Acrylamide solutions were made by dissolving 11.4 g of acrylamide and 1.14 g of bisacrylamide in a total volume of 100 ml which contained 8 M urea, 0.1% sodium dodecyl sulfate, 0.75% (w/v) Temed, and 0.1 M H_3PO_4 adjusted to pH 6.8 with Tris base. The solution was deaerated under vacuum (5–15 min), and 0.07% (w/v) ammonium persulfate was added. The solution was immediately pipetted into glass tubes (0.5 × 15 cm) that had been treated with Siliclad (Clay Adams). The reservoir buffer contained 0.1% sodium dodecyl sulfate and 0.1 M H_3PO_4 adjusted to pH 6.8 with Tris base. Samples were run for 15–20 h at a constant current of 2.5 mA/gel.

Two-dimensional electrophoresis employed 12% cylindrical gels and buffer system 1 for the first dimension. The cylindrical gels were then attached at the top of a 1–3-mm thick polyac-

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‡ Present address: Department of Biochemistry and Biophysics, University of California, Davis, California 95616.

[1] Abbreviations used are: ETP, beef heart submitochondrial particles; $NaDodSO_4$, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; Temed, *N,N,N′,N′*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane.

rylamide slab using a 1% solution of agarose in reservoir buffer. A separate sample well was formed in the agarose in order to hold a sample of cytochrome *c* oxidase. The gel system used in the second dimension was the same as in system 2, except that the slab contained 15% total acrylamide with an acrylamide–bisacrylamide ratio of 50:1. Slab gels were run at a constant current of 0.1 mA/mm^2 for 15–20 h.

All samples for gel electrophoresis were prepared by adding sufficient dissociating buffer (8 M urea, 4% sodium dodecyl sulfate, 40 mM dithiothreitol, 0.01 M phosphate, pH 8.3) so that the final sodium dodecyl sulfate concentration was at least 2%. Samples were heated to 37 °C for 30 min or 50 °C for 1 min.

Before staining, gels were soaked for 5 h in 5:5:1 methanol–water–acetic acid to remove sodium dodecyl sulfate and urea and at the same time to fix proteins. This step assured reproducible staining of polypeptides. (Without fixation, subunits I, III, and VII were most susceptible to variation.) All gels were stained by two sequential incubations for 15 min at 70 °C in solutions of 0.2% Coomassie blue in 5:4:1 methanol–water–acetic acid. Gels were destained in a Bio-Rad diffusion destainer. Densitometric traces of the gels were made at 550 nm on a Beckman DU spectrometer equipped with a Gilford linear transport attachment. Gels were calibrated for molecular weight determination using the following standard proteins: bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, myoglobin, lysozyme, cytochrome *c*, bovine pancreatic trypsin inhibitor, and cyanogen bromide fragments of cytochrome *c* prepared as described by Gross (1967).

Gel Filtration in Sodium Dodecyl Sulfate–Urea. Cytochrome *c* oxidase (20 mg) was dissolved in 1 ml of buffer containing 5% sodium dodecyl sulfate, 8 M urea, and 0.05 M NaH_2PO_4 (pH 7.2) by heating to 37 °C for 30 min. Chromatography was carried out at room temperature in a column (1.5 × 84 cm) of Sephadex G-100 Superfine equilibrated with 5 mM sodium dodecyl sulfate, 8 M urea, 0.05 M NaH_2PO_4, pH 7.2. Blue dextran was used to determine the void volume and a drop of mercaptoethanol was included in samples as a measure of the included volume of the column.

Gel Filtration in Triton X-100. Cytochrome *c* oxidase (10 mg) was dissolved in 1 ml of Tris-HCl buffer, pH 8.0, $I = 0.10$, containing 20 mg of Triton X-100. Chromatography was carried out at 4 °C in a column (1.0 × 60 cm) of Sepharose 4B equilibrated with 1 mM Triton X-100, 0.09 M NaCl, 0.02 M Tris-HCl, pH 8.0. The void volume and included volume were determined as above.

Analytical Procedures. Protein concentration was determined by the method of Lowry et al. (1951) with some modifications to facilitate solubilization of membrane proteins. The alkaline copper reagent used was fivefold more concentrated than that described by Lowry et al., while the Folin-Ciocalteu phenol reagent was diluted 1:11 with distilled water before use. The assay was performed in the usual manner except that the volumes of sample, alkaline copper reagent, and diluted phenol reagent were in the ratio 1:1:3. The final concentrations were roughly equivalent to those in the standard Lowry. Color development could also be carried out at 50 °C for 10 min instead of at room temperature for 30 min. Bovine serum albumin was used as a standard.

Heme a concentration was estimated by the pyridine hemochromogen difference spectrum (Williams, 1964) with $\Delta\epsilon_{587-620} = 21.7$ mM^{-1} cm^{-1} or from the reduced spectrum of cytochrome *c* oxidase in either 1% Triton X-100 or 0.05% deoxycholate, 0.1 M phosphate, pH 7.0, using $\Delta\epsilon_{603-630} = 16.5$ mM^{-1} cm^{-1}. Both methods gave equivalent results.

Cytochrome *c* oxidase activity was measured spectrophotometrically at 25 °C by following the oxidation of ferrocytochrome *c* at 550 nm in 0.05 M phosphate buffer, pH 7.0, containing 0.5% Tween 80. Activities were expressed as MA_0, the initial molecular activity, in units of moles of cytochrome *c* oxidized per mole of heme a_3 per second. $MA_{0_{max}}$ and $K_{m_{app}}$ were determined as described by Vanneste et al. (1974).

Samples for amino acid analysis were hydrolyzed for 24 h with 6 N HCl in a sealed tube under vacuum in a 100 °C oven. HCl was removed by rotary evaporation at 30 °C and samples were analyzed in a Technicon automatic amino acid analyzer according to the method of Spackman et al. (1958).

Materials. Tween 80, deoxycholic acid, and cholic acid were all obtained from Sigma. Urea used for polyacrylamide gel electrophoresis and gel filtration chromatography was either Schwarz/Mann Ultra-Pure or Baker reagent grade that had been recrystallized from 95% ethanol. The *N,N*-methylenebisacrylamide and acrylamide used here were obtained from Eastman Kodak or Aldrich chemicals (Gold Label). Preformed polyacrylamide gels (Bio-Phore) were purchased from Bio-Rad, Inc. Protein standards for calibration of gels were the best grade available from Sigma. Bovine pancreatic trypsin inhibitor (Trasylol) was purchased from FBA Pharmaceuticals.

Results

Electrophoresis in Highly Cross-Linked Sodium Dodecyl Sulfate–Urea Gels. Electrophoresis of purified cytochrome *c* oxidase (9.6–11.3 nmol of heme a/mg of protein) in the presence of sodium dodecyl sulfate and Tris-acetate buffer, pH 6.4 (system 1), revealed six major polypeptide components as described by Briggs et al. (1975) and shown in Figure 1a. In order to improve the resolution of the smaller subunits and obtain better estimates of their molecular weights, electrophoresis was carried out in highly cross-linked polyacrylamide gels (12.5% total acrylamide in the ratio of acrylamide–bisacrylamide, 10:1) in the presence of 8 M urea and sodium dodecyl sulfate with Tris-phosphate buffer at pH 6.8 (Figure 1c). The increased resolving power under these conditions (system 2) derives from a combination of factors including the presence of 8 M urea in the gels, increases in both the acrylamide concentration and the percentage of cross-linker, and the use of Tris-phosphate for the reservoir buffer (Swank and Munkres, 1971).

The most striking difference observed was that gel electrophoresis in system 2 revealed two major components in the 20 000–30 000 molecular weight range. Also, better resolution of the doublet at 10 000–12 000 molecular weight (system 1) showed that at least two additional minor components were present. The major components resolved in system 2 are tentatively designated as subunits I–VII. Table I lists the apparent molecular weights determined for the major polypeptides of cytochrome *c* oxidase under the two different conditions of electrophoresis and compares them with corresponding values obtained by gel filtration in sodium dodecyl sulfate and/or Gdn·HCl. The apparent molecular weights determined by all methods for subunits I through V were in excellent agreement. Significant differences were found only for subunits VI and VII which have molecular weights below 10 000 and therefore fall in a region of the standard curve for sodium dodecyl sulfate gel electrophoresis where the shape of an $NaDodSO_4$–protein complex varies very little with the size of the protein (Williams and Gratzer, 1971; Reynolds and Tanford, 1970). Our results indicate that, for cytochrome *c* oxidase subunits, the method

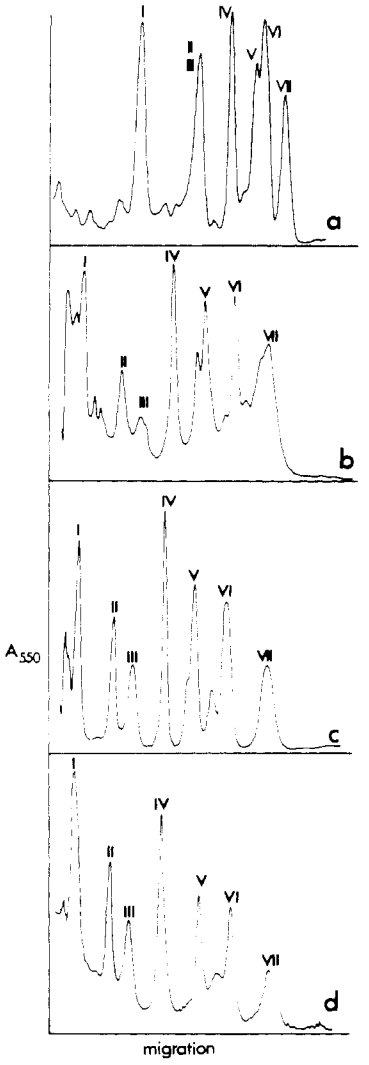

FIGURE 1: (a) Densitometric traces of sodium dodecyl sulfate–polyacrylamide gels of beef heart cytochrome *c* oxidase run on a 12% Biophore gel, acrylamide–bisacrylamide 40:1 and run as described for system 1. (b,c,d) Densitometric traces of sodium dodecyl sulfate–polyacrylamide gels, 12.5% gels, acrylamide–bisacrylamide 10:1, run in the presence of urea as described under system 2. The profiles show cytochrome *c* oxidase complex at different stages in its isolation and purification. (b) Green membrane; (c) complex isolated according to Capaldi and Hayashi (1972); and (d) purified cytochrome *c* oxidase after chromatography on Sepharose 4B in presence of Triton X-100. Subunits are labeled I–VII.

of Swank and Munkres does discriminate between differently sized complexes in this range. (These authors estimate that their system is capable of determining molecular weights of oligopeptides to within ±18%.) Also, the molecular weights determined by this method agree fairly well with values obtained by the more reliable method of gel filtration in Gdn·HCl. In obtaining average values of the molecular weights, we have used all of the data for subunits I–V and data from Gdn·HCl gel filtration and $NaDodSO_4$–urea gel electrophoresis for subunits VI and VII.

Two-Dimensional Gel Electrophoresis. While the appearance of additional low-molecular-weight components was an expected consequence of gel electrophoresis in system 2, there was no obvious reason for better separation of polypeptides in the higher molecular weight range where overall resolution is roughly equivalent between the two systems. Several explanations were considered. There is some precedent for changes in protein mobility as a function of ionic strength or buffer composition (see e.g., Campagnoni and Magno, 1975). Alternatively the new component might be derived from other polypeptides by aggregation, proteolysis, or some other modification, perhaps as a consequence of having urea in the gels.

As a first step in determining the origin of the additional component, a two-dimensional separation (Figure 2) was performed by first electrophoresing the cytochrome *c* oxidase in a cylindrical gel (system 1), then transferring this gel to the top of a polyacrylamide slab and electrophoresing in a second dimension using the reservoir buffer from system 2. The polyacrylamide concentration in the slab had to be altered (15% total, acrylamide–bisacrylamide, 50:1) to avoid having the edge of the slab shrink away from the side spacers. A second sample of enzyme, applied along with the cylindrical gel, indicates the migration of cytochrome *c* oxidase subunits in the second dimension. It can be seen that the pattern obtained with the 15% polyacrylamide is qualitatively similar to that with 12.5% gels except that an additional component near subunit VI, which is occasionally observed in 12.5% gels, is clearly resolved in this gel.

Subunits II and III (as designated in system 2) are seen to have coelectrophoresed in the first dimension where they appeared as a single component with apparent molecular weight of 22 500. In fact, subunit III ran at the trailing edge of the band indicating that there were two separable polypeptides even under the conditions used in system 1. Other experiments suggest that the shift in relative mobilities of these two polypeptides is at least in part a consequence of substituting Tris-phosphate for Tris-acetate buffer. A standard 12% gel (acrylamide–bisacrylamide, 40:1) run in Tris-phosphate without urea shows two incompletely separated polypeptides with relative mobilities and apparent molecular weights similar to those determined for subunits II and III in system 2.

The additional components around subunits V and VI all appeared to derive from the unresolved doublet seen in system 1. As in the case of subunits II and III, the fact that some polypeptides do not fall on a single diagonal of the two-dimensional slab shows that there are small changes in their relative

TABLE I: Molecular Weights of the Polypeptide Subunits of Beef Heart Cytochrome *c* Oxidase.

Analytical Method	I	II	III	IV	V	VI	VII
$NaDodSO_4$–Urea gel electrophoresis, 12.5% total acrylamide–bisacrylamide 1:10	35 300 ±2 000	25 200 ±400	21 000 ±800	16 200 ±800	12 100 ±800	6 700 ±900	3400 ±200
$NaDodSO_4$ gel electrophoresis, 12.0% Bio-Phore gels[a]	36 000	22 500[b]		17 200	12 500	11 200	8000
Column chromatography in 6 M Gdn·HCl[a]				17 000	12 500	9 700	5300
Column chromatography in $NaDodSO_4$[a]	35 000	23 000					
Average of determinations by various methods	35 400	24 100	21 000	16 800	12 400	8 200	4400

[a] Briggs et al., 1975. [b] Not a single component and therefore not included in average.

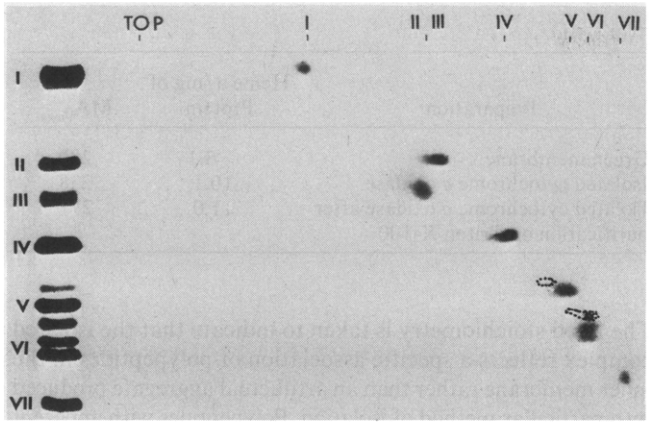

FIGURE 2: Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophorogram showing the relationship of bands observed in systems 1 and 2. The positions of six polypeptide bands after electrophoresis in the cylindrical gel (system 1) are indicated at the top of the slab. Migration of these components in the slab in the presence of 8 M urea (system 2 with modification described in Experimental Procedure) is compared with a sample of purified cytochrome *c* oxidase run at the left-hand side of the slab. The dotted lines indicate components that stained too weakly to show up in the photograph of the gel.

mobilities between the two buffer systems.

Gel Chromatography in Sodium Dodecyl Sulfate–8 M Urea. To establish that subunits II and III are actually distinct polypeptide components of the isolated cytochrome oxidase complex, they were isolated for further characterization. Separation of subunits II and III by gel filtration was accomplished on a Sephadex G-100 column using phosphate buffer in the presence of 5 mM sodium dodecyl sulfate and 8 M urea. The changes in size, shape, and/or sodium dodecyl sulfate binding that occur in the presence of urea in this buffer system and that facilitate the separation of subunits II and III by polyacrylamide gel electrophoresis are seen to similarly influence their behavior on a Sephadex column. The improved resolution of cytochrome *c* oxidase subunits by sodium dodecyl sulfate column chromatography in the presence of 8 M urea can be seen by comparing the elution profile obtained for the dissociated enzyme in this system (Figure 3a) with that obtained for the same amount of enzyme run under the same conditions but without 8 M urea (Figure 3b). The gels displayed at the top of the figure show the polypeptide composition of various column fractions as analyzed by electrophoresis in system 2. Whereas subunits I, II, and III all eluted in or close to the void volume of a Sephadex G-100 column in aqueous sodium dodecyl sulfate buffer (gels not shown), subunits II and III entered the beads in the presence of urea. Further, the penetration of the smaller subunits into the Sephadex beads is greater in the presence of urea and leads to some separation of these subunits. The fact that these polypeptides can penetrate G-100 Sephadex more readily when dissolved in $NaDodSO_4$ and 8 M urea than in $NaDodSO_4$ alone, probably indicates that the $NaDodSO_4$–protein complex is smaller in the presence of urea than in water. This could result from less binding of $NaDodSO_4$ and/or a different shape of the $NaDodSO_4$–protein aggregate. Alternatively, of course, urea might alter the effective pore size of the gel.

On close inspection of gels showing the polypeptides in different fractions from the column (Figure 3), it can be seen that polypeptide IV appears as a closely spaced doublet. We have also on occasion seen subunits I and VI appear as doublets. The fact that heme a is distributed among many of the dissociated and denatured subunits of cytochrome oxidase is indicated by

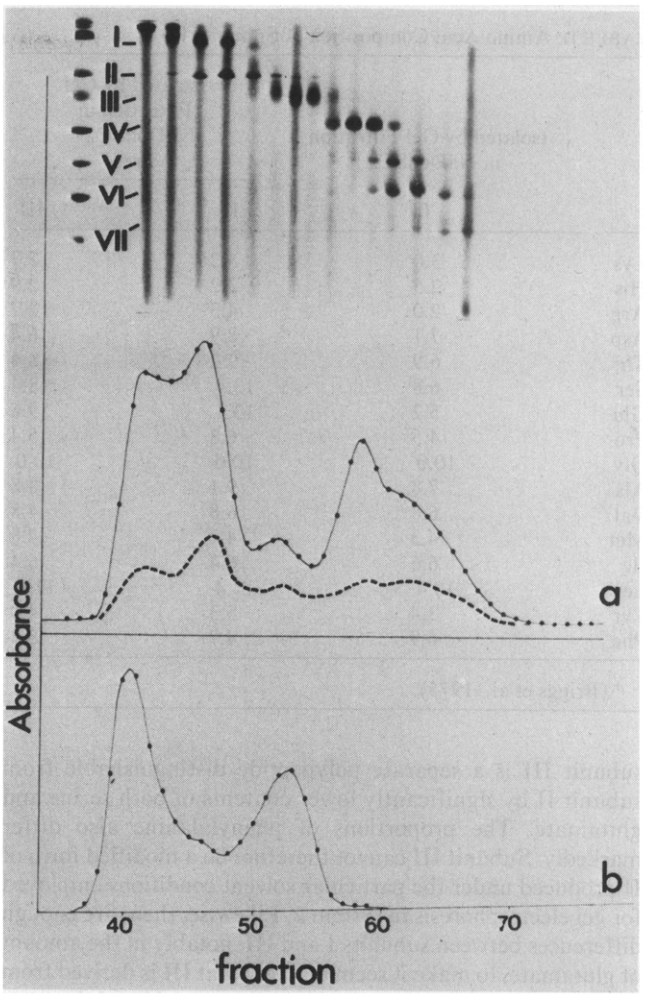

FIGURE 3: Elution profiles of reduced and dissociated cytochrome *c* oxidase chromatographed on Sephadex G-100 as described in Experimental Procedure (a) in presence of sodium dodecyl sulfate–8 M urea and (b) in sodium dodecyl sulfate alone. Absorbance profiles at 280 nm are indicated by solid lines. The polypeptide composition of sodium dodecyl sulfate–urea column fractions, as analyzed by electrophoresis in system 2, is indicated by gels shown above their respective fractions. Cytochrome *c* oxidase electrophoresed in the same system is shown at the left. The lower trace (dashed line) in a is the absorbance profile at 422 nm reflecting the distribution of heme a among different polypeptides.

the absorbance profile at 422 nm in Figure 3. It is possible that polypeptides with associated heme migrate slightly differently from heme-free polypeptide molecules and that this phenomenon could explain the occasional appearance of closely spaced doublets for certain subunits.

Some Properties of Subunits II and III. Subunit III could be isolated in relatively pure form directly from an $NaDodSO_4$–urea column. Crude subunit II was contaminated with subunit I which could be removed by re-chromatographing the pooled fractions that were enriched in II. After isolation by gel filtration, subunits II and III ran as single bands on $NaDodSO_4$–urea gel electrophoresis and displayed the same mobilities as in the dissociated enzyme. They therefore appear to represent stable, discrete species. As can be seen in Figure 3, bands corresponding to subunit III appear broader than bands for most polypeptides in this size range. This is a consistent finding for which we have as yet no explanation.

The amino acid compositions of subunits II and III are given in Table II together with the composition of subunit I as isolated previously (Briggs et al., 1975). These data confirm that

TABLE II: Amino Acid Composition of Subunits I–III.

	Isolated by Gel Filtration in $NaDodSO_4$[a]	Isolated by Gel Filtration in $NaDodSO_4$–Urea	
	I	II	III
Lys	3.0	3.2	2.7
His	2.5	3.2	2.9
Arg	2.0	4.3	2.7
Asp	7.1	8.9	6.4
Thr	6.9	9.2	8.4
Ser	6.8	13.4	8.4
Glu	5.2	13.1	9.6
Pro	4.5	6.8	5.4
Gly	10.0	10.0	10.0
Ala	7.8	9.1	8.8
Val	6.8	6.8	4.8
Met	4.3	4.7	2.8
Ile	6.6	6.4	6.4
Leu	10.4	14.4	11.4
Tyr	3.4	5.3	4.0
Phe	6.9	4.7	8.3

[a] (Briggs et al., 1975).

subunit III is a separate polypeptide distinguishable from subunit II by significantly lower contents of both serine and glutamate. The proportions of phenylalanine also differ markedly. Subunit III cannot therefore be a modified form of II produced under the particular solvent conditions employed for gel electrophoresis in system 2. Likewise, there are enough differences between subunits I and III, notably in the amount of glutamate, to make it seem unlikely that III is derived from I by proteolysis. Swank et al. (1971) have considered the same potential artifacts. They showed that the mitochondrially synthesized polypeptides of *Neurospora crassa*, which included polypeptides corresponding to subunits I, II, and III reported here, were stable in $NaDodSO_4$–urea and not subject to proteolysis under the conditions used for $NaDodSO_4$–urea gel electrophoresis.

The composition of subunit II purified here by gel filtration in $NaDodSO_4$–urea is very similar to that described by Briggs et al. (1975). Several factors can be suggested to explain why most of subunit III had been removed from subunit II as isolated by gel filtration in $NaDodSO_4$ alone. It has subsequently been shown that the conditions used to dissociate the cytochrome *c* oxidase complex for gel filtration (100 °C, 1 min) lead to some irreversible aggregation of subunits I and III. This result is not unexpected considering that they are the most apolar polypeptides in cytochrome oxidase. We suspect, therefore, that a large proportion of subunit III (as well as I) eluted in the void volume of the column used to purify II. In addition, our studies indicate that III probably runs slightly ahead of II in phosphate buffer. In the course of re-chromatography, fractions from the center of the peak for crude subunit II would have been only slightly contaminated with III.

Removal of Minor Polypeptide Components. The relative staining intensities of the seven subunits separated on $NaDodSO_4$–urea polyacrylamide gels are found to be roughly constant in three different preparations of cytochrome *c* oxidase. These include complexes isolated with deoxycholate and cholate (Capaldi and Hayashi, 1972), with cholate alone (Yonetani, 1961) and with Triton X-100 (Sun et al., 1968).

TABLE III.

Preparation	Heme a/mg of Protein	$MA_{0_{max}}$
Green membrane	3.1	200
Isolated cytochrome *c* oxidase	10.1	238
Isolated cytochrome *c* oxidase after purification on Triton X-100	11.0	271

The fixed stoichiometry is taken to indicate that the isolated complex reflects a specific association of polypeptides in the inner membrane rather than an artifactual aggregate produced by a particular method of isolation. Polypeptides with apparent molecular weights of 8500 and 13 100, and several with molecular weights in the range 50 000–80 000, also appear routinely in these preparations, although their proportions are variable.

We have been able to demonstrate that, through given purification procedures, the proportion of several of the so-called "minor components" can be significantly reduced without decreasing the enzymic activity of the complex. Figure 1 shows the composition of cytochrome *c* oxidase in several stages of purification. The profile (Figure 1b) shows "green membrane", an inner membrane derivative, still in membranous form, which contains roughly two to three times as much protein per heme a as the isolated cytochrome *c* oxidase. The profile in Figure 1c shows isolated cytochrome *c* oxidase and the lower profile shows the isolated enzyme after gel filtration on Sepharose 4B in the presence of Triton X-100 (Figure 1d). It can be seen that high-molecular-weight components (>40 000) are removed on going from "green membrane" to purified cytochrome *c* oxidase and are further removed by gel filtration in Triton X-100. A polypeptide of molecular weight 13 100 is also removed during purification of cytochrome *c* oxidase from "green membrane" and is further extracted by gel filtration of the detergent-dispersed enzyme. These purification procedures do not decrease the electron-transfer activity of cytochrome *c* oxidase (Table III), and this is taken as evidence that the aforementioned polypeptides are not integral to the enzyme complex. We have been able to purify the component of molecular weight 13 100 (M. Briggs, unpublished observation) and obtain its amino acid composition. The mole fractions of basic amino acids (with which the dye Coomassie blue associates) in this polypeptide are comparable to those in subunits IV–VII. There is no apparent reason therefore why the 13 100-dalton polypeptide should not stain normally, and the staining profile of purified enzyme can be taken to indicate that this polypeptide is present in less than stoichiometric amounts with the polypeptides we designate as subunits of the complex.

We have not been able to remove the polypeptide with an apparent molecular weight of 8500. The fact that it varies in amount from preparation to preparation is at present the only basis for excluding it as a subunit of the enzyme.

It remains to be determined whether each of the seven polypeptides we have isolated and designated as a subunit of beef heart cytochrome *c* oxidase is homogeneous. Clearly, the fact that each migrates as a single band on gels is not an adequate criterion. Poyton and Schatz (1975) have presented very convincing evidence that each of the seven subunits identified for yeast cytochrome *c* oxidase is indeed a single, homogeneous component. Technical difficulties have so far prevented similar experiments on the beef heart complex. N-Terminal analysis and/or sequencing of the N-terminal portion of the different

TABLE IV: Subunit Structure of Cytochrome *c* Oxidase from Different Sources.

	S. cerevisiae		*N. crassa*[c]	*L. migratoria*[d]	Beef Heart[e]
I	40 000[a]	40 000[b]	41 000	38 000	35 400
II	33 000	27 300	28 500	24 000	24 100
III	22 000	25 000	21 000	19 000	21 000
IV	14 500	13 800	16 000	14 500	16 800
V	12 700	13 000	14 000	12 500	12 400
VI	12 700	10 200	11 500	10 000	8 200
VII	4 600	9 500	10 000	8 000	4 400

[a] Reference for this column: Poyton and Schatz (1975). [b] Reference for this column: Rubin and Tzagoloff (1973). [c] Sebald et al. (1973). [d] Weiss et al. (1972). [e] This study.

TABLE V: Percent Polar Amino Acids in Cytochrome *c* Oxidase and Selected Subunits.

	Whole Complex	I	II	III
Beef Heart[a]	39.7	35.5	44.7	39.9
S. cerevisiae[b]	39.2	34.7	42.1	
N. crassa[c]	38.5	32.5	40.1	35.8

[a] This study and Briggs et al., 1975. [b] Poyton and Schatz (1975). [c] Sebald et al. (1973).

chains will be needed to establish beyond peradventure the homogeneity of the different fractions we have obtained.

Discussion

Cytochrome *c* oxidase is ultimately defined as the complex of polypeptides, coenzymes, and cofactors that catalyze the oxidation of ferrocytochrome *c* by molecular oxygen and couple the energy derived from this reaction to the synthesis of ATP. The issues of how many subunits actually constitute the enzyme, which subunits participate in catalysis or coupling, and which subunits, if any, play a purely structural role have been approached primarily through studies which use cytochrome *c* oxidase from beef heart. This is because the quantities of enzyme necessary for biochemical characterization and reconstitution are obtained most easily from this source. On the other hand, *Saccharomyces cerevisiae* and *Neurospora crassa* lend themselves to studies of the biogenesis of cytochrome *c* oxidase, and the subunit structures of the purified enzymes from these organisms have been characterized as a basis for these studies.

Methods for purifying cytochrome *c* oxidase from all sources generally yield a complex containing one copper atom per heme a and at least 10 nmol of heme a per mg of protein (Poyton and Schatz, 1975; Rubin and Tzagoloff, 1973; Kuboyama et al., 1972; Sebald et al., 1973). The enzymes from *Saccharomyces cerevisiae, Neurospora crassa,* and *Locusta migratoria* have been resolved into seven different polypeptides, all with molecular weights less than 40 000 (Poyton and Schatz, 1975; Sebald et al., 1973; Weiss et al., 1972). The beef heart enzyme, on the other hand, has been resolved so far into only six different polypeptides (Rubin and Tzagoloff, 1973; Kornblatt et al., 1973; Briggs et al., 1975) and it has been generally accepted that one of the large, mitochondrially synthesized polypeptides found in other species is missing from the beef heart enzyme. Our present results account for this reported difference by showing that subunits II and III of the beef heart enzyme comigrate under the conditions of gel electrophoresis normally used for subunit analysis and therefore appear as a single band on gels. These two polypeptides can be separated by using the system of $NaDodSO_4$–urea polyacrylamide gel electrophoresis first described by Swank and Munkres (1971).

Similarities in the subunit composition of cytochrome *c* oxidase from all eukaryotic organisms are now apparent. The molecular weights of the seven different subunits are reasonably constant among different species (Table IV), given the limitations outlined already in the methods used for their determination.

As in the other species studied, the subunits of beef heart cytochrome *c* oxidase seem to fall into two categories according to physical properties, the larger subunits (I–III) being considerably more hydrophobic than the smaller ones (IV–VII). Subunits I and III are particularly hydrophobic with polarities below 40% in all species (Table V). Subunit II has a polarity ranging between 40.1 and 44.7% in the different species. This is still significantly lower than the average polarity of the smaller subunits (48.2%) and lower than the average value of 47% observed for a variety of soluble proteins (Capaldi and Vanderkooi, 1972). The similarities in composition between analogous subunits from beef heart and *Neurospora crassa* are particularly striking. Subunits I–III all contain large amounts of leucine (mol % > 11.1) and relatively small amounts of the basic amino acids lysine, arginine, and histidine. Subunit II from both these species shows an unusually large complement of glutamate and serine.

The results outlined above indicate considerable conservation in the structural genes of cytochrome *c* oxidase in eukaryotic organisms. Studies on the biogenesis of this enzyme in *Saccharomyces cerevisiae* and *Neurospora crassa* have established that the three largest subunits in both these organisms are synthesized on mitochondrial ribosomes, while the four smallest subunits are cytoplasmic in origin (Schatz and Mason, 1974). Although the site of synthesis of the various subunits has not been determined for cytochrome *c* oxidases from mammalian mitochondria, studies of mitochondrial protein synthesis from several mammalian sources (Mockel and Beattie, 1975) have shown that the size and distribution among size classes of proteins synthesized in these mitochondria are the same as that observed in *Saccharomyces cerevisiae* and *Neurospora crassa* (Swank et al., 1971; Tzagoloff et al., 1968). Thus it seems quite likely that subunits I–III of beef heart cytochrome *c* oxidase are also products of mitochondrial biosynthesis. Given that the beef heart cytochrome *c* oxidase now appears to be quite comparable to the enzymes from other sources, in terms of both subunit structure and biosynthesis, information from studies of certain mutant strains which give rise to defects in the biogenesis of cytochrome *c* oxidase can be related more effectively to biochemical studies on beef heart enzyme directed at determining the roles of various polypep-

tides in electron transport and/or coupling function.

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