Cytochrome *c* Oxidase from Rat Liver Mitochondria: Purification and Characterization†

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ABSTRACT: A method is described for the rapid purification of soluble, highly active cytochrome *c* oxidase from rat liver mitochondria. Triton X-114 is used to solubilize mitochondria resulting in a membranous fraction enriched in cytochrome *c* oxidase. This fraction is solubilized in 4% sodium cholate in the presence of ammonium sulfate. The purified oxidase is then obtained by ammonium sulfate fractionation initially in the presence of cholate and subsequently in the presence of Tween 20. The entire procedure requires 48 h. The purified oxidase exhibits spectral properties characteristic for cytochrome *a*, while no other heme is evident. The purified oxidase contains a 44-fold increase in heme *a* compared with whole mitochondria and averages 9.5 nmol of heme *a*/mg of protein. The iron to heme *a* ratio is 1 and the copper to iron ratio is 1.2–1.5. The phosphorus content is 1.47 μg/mg of protein. The electron transfer activity given as a first-order rate constant is 56.3 s per mg of protein per mL. The turnover number per molecule of heme *a* in the purified oxidase is 80% of the turnover number found for solubilized mitochondria. The pH optimum for the oxidase is 6.0–6.3. The K_m for reduced cytochrome *c* is 10–15 μM. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis resolves the purified cytochrome *c* oxidase into 6 subunits with apparent molecular weights of 43 500, 24 500, 15 500, 12 000, 10 500, and 9000. Sodium dodecyl sulfate–polyacrylamide gels containing urea resolve the oxidase into 7 subunits with apparent molecular weights of 34 000, 26 800, 23 700, 17 000, 12 500, 9500, and 3600. Analysis of the amino acid composition reveals a high methionine content, compared with beef heart cytochrome *c* oxidase, and a low polarity of 38.53% indicative of a transmembrane protein. The minimum molecular weight of the purified oxidase is 105 000 based on the heme *a* content, 115 000 based on the six subunit composition, 119 500 based on the amino acid composition, and 127 000 based on the seven subunit composition. Ouchterlony double immunodiffusion reveals that the rat liver oxidase contains immunodeterminants common with, but not immunologically identical to, beef heart cytochrome *c* oxidase.

Cytochrome *c* oxidase is the terminal enzyme of the respiratory chain of mitochondria. It functions as an electron carrier between cytochrome *c* and oxygen and participates in the conservation of metabolic energy during the synthesis of ATP. Cytochrome *c* oxidase has been isolated and purified from various sources including *Saccharomyces cerevisiae* (Mason et al., 1973; Poyton & Schatz, 1975), *Neurospora crassa* (Sebald et al., 1973), and beef heart (Yonetani, 1961; Griffiths & Wharton, 1961; Kuboyama et al., 1972; Volpe & Caughey, 1974). The composition of cytochrome *c* oxidase from different sources is similar. All cytochrome *c* oxidases contain heme *a* and copper with the heme *a* content of purified preparations ranging from 9 to 12 nmol per mg of protein. The heme *a* to iron ratio is 1.0 and non-heme iron has not been demonstrated. Copper to iron ratios range from 1.0 to 1.3. Cytochrome *c* oxidase is a lipoprotein with the lipid content of the solubilized, purified enzyme varying from 1 to 2% (Mason et al., 1973; Awashi et al., 1971) to 20% (Kuboyama et al., 1972; Volpe & Caughey, 1974). The amount of bound lipid appears to be dependent on the nature of the detergents used for solubilization and purification of the enzyme. $NaDodSO_4$[1]–polyacrylamide gel electrophoresis of cytochrome *c* oxidase purified from *Saccharomyces cerevisiae* and *Neurospora crassa* reveals 7 subunits with molecular weights ranging from below 10 000 to 40 000 (Poyton & Schatz, 1975; Sebald et al., 1973). Isolated beef heart cytochrome *c* oxidase is reported to contain 6 (Briggs et al., 1975; Phan & Mahler, 1976), 7 (Downer et al., 1976), or 8 (Bucher & Penniall, 1975) subunits.

Only one report has appeared describing a method for the isolation and characterization of cytochrome *c* oxidase from rat liver mitochondria. Jacobs et al. (1966a,b) used a combination of Triton X-114 and X-100 for solubilization and reported a purified oxidase of 15 000–20 000 molecular weight. Using their method, we were unable to obtain a spectrophotometrically stable, active cytochrome *c* oxidase and we have therefore developed a purification method which can be carried out within 48 h and which results in a highly purified and active enzyme. Since a reliable method for the isolation of liver mitochondrial cytochrome *c* oxidase was not available, the enzyme was not heretofore adequately characterized. This communication describes our procedure and some of the characteristic properties of purified cytochrome *c* oxidase from rat liver.

Methods and Materials

Experimental Procedures. Cytochrome *c* oxidase was purified from rat liver mitochondria which were prepared in 0.25 M sucrose according to Schneider (1948). All steps of the purification procedure were carried out at 0–4 °C. Centrifugation was done in a Beckman ultracentrifuge (Model L3-50) for 15 min at 105 000*g*. Ammonium sulfate concentrations were calculated according to Yu et al. (1972).

Analytical Procedures. Protein concentrations were determined according to Lowry et al. (1951). Iron was measured according to Doeg & Ziegler (1962). Copper was estimated according to Wharton & Rader (1970). Total phosphorus was determined by the method of Ames & Dubin (1960) after extensive dialysis of the enzyme against distilled water for 48 h with two changes of the dialysate.

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride; Temed, *N,N,N′,N′*-tetramethylethylenediamine; IgG, immunoglobulin G.

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Absolute absorption spectra were measured in a Cary spectrophotometer (Model 14) at room temperature. Heme *a* concentration was determined in a Beckman spectrophotometer (Model 25). The millimolar extinction coefficient used for the quantitation of heme *a* was 12 for the reduced minus oxidized form at 604 nm (van Gelder, 1966).

The electron transfer activity of cytochrome *c* oxidase was determined polarographically with a Clark oxygen electrode (Yellow Springs, Ohio) at 25 °C. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.5% Tween 20, 2 mg of asolectin, 30 mM sodium ascorbate, 22.5 μM cytochrome *c*, and 0.1 mM TMPD in a total volume of 2 mL. The oxygen content of the reaction mixture was calculated by the method described by Estabrook (1967). The reaction was started by addition of oxidase. To determine the cytochrome *c* oxidase activity of mitochrondria, 20 mg of mitochondrial protein was first solubilized immediately before use in 1 mL of 0.25 M sucrose containing 0.5% sodium deoxycholate.

Micelles of asolectin, a crude mixture of soybean phospholipids, were obtained by sonicating 20 mg of asolectin suspended in 1 mL of 50 mM potassium phosphate (pH 7.0), containing 1 mM EDTA, for 10 min in an ice bath at 40–60 W with a Branson sonifier (Model W185). After sonication, the mixture was centrifuged for 10 min at 20 000*g* and the sediment was discarded.

Electron transfer activity of cytochrome *c* oxidase was expressed as μatoms oxygen consumed per min per mg of protein. Turnover numbers and first-order rate constants were calculated assuming that two electrons are transferred and two cytochrome *c* hemes are oxidized per atom of oxygen reduced.

Amino acid analysis was performed by AAA laboratory, Seattle, with a Durrum Analyzer (Model D-500). The samples were hydrolyzed in 6 N HCl at 110 °C for 24 h prior to analysis.

NaDodSO$_4$-Polyacrylamide Gel Electrophoresis. The procedure of Fairbanks et al. (1971) was used with the following modifications: samples of purified cytochrome *c* oxidase (2 mg/mL) were digested for 2 h at 75 °C. The gels were 12 cm long and contained 12% acrylamide (acrylamide:bis(acrylamide) = 40:1.5). Ammonium persulfate (0.05%) and 0.025% Temed were used to catalyze polymerization of the acrylamide. The gel buffer and the reservoir buffer contained 0.1% NaDodSO$_4$ in a Tris-acetate buffer, pH 7.4. Electrophoresis was performed at a constant current of 5 mA per gel for 3 h. After completion of electrophoresis, gels were fixed in 25% 2-propanol, 10% acetic acid for 6 h, stained with 0.05% Coomassie Brilliant Blue in 25% 2-propanol, 10% acetic acid for 8 h, and destained in 10% 2-propanol, 10% acetic acid. Densitometric traces of the gels were recorded at 580 nm with a gel scanning system (Instrumentation Specialties Co., Lincoln, Neb.).

Preparation of Antibodies. Immunization of rabbits with cytochrome *c* oxidase, collection of antiserum, purification of the IgG fraction, and Ouchterlony double immunodiffusion assays were carried out as described by Hackenbrock & Miller-Hammon (1975).

Materials. The animals used in these studies were 200-g male Sprague-Dawley rats. Triton X-114, sodium cholate and cytochrome *c* (horse, type VI) were purchased from Sigma Chemical Corp. (St. Louis, Mo.) and were used without further purification. Ammonium sulfate, enzyme grade, was obtained from Schwarz/Mann Research Laboratory (Orangeburg, N.Y.). Asolectin was from Associated Concentrates (Woodside, L.I., N.Y.). All other chemicals used were reagent grade. Beef heart cytochrome *c* oxidase was generously provided by Professor Tsoo E. King, Department of Chemistry, State University of New York, Albany, N.Y.

Results

Purification Procedure. Rat liver mitochondria were incubated at a protein concentration of 25 mg/mL in 1.5% Triton X-114 in 0.2 M potassium phosphate pH 7.0 for 2 h which yielded a membranous fraction enriched in cytochrome *c* oxidase. When the membranous cytochrome *c* oxidase was subjected to NaDodSO$_4$-polyacrylamide gel electrophoresis, 7 major bands were resolved, 5 of which corresponded to 5 of the bands characteristic of purified beef heart cytochrome *c* oxidase (Figure 3). Two major and a few minor bands were visible which had no equivalent bands in the purified beef heart oxidase. In most of our preparations of membranous cytochrome *c* oxidase, no other heme proteins could be detected by spectral analysis.

In order to solubilize the cytochrome *c* oxidase, the membranous fraction was resuspended in 0.2 M potassium phosphate, pH 7.0, to a protein concentration of 10 mg/mL and an equal volume of 8% sodium cholate in water was added. The mixture was brought to 30% ammonium sulfate saturation by slowly adding freshly ground ammonium sulfate. The pH was adjusted to 7.6–7.8 with 1 M potassium hydroxide and the mixture was stirred slowly for 14–16 h. The turbid suspension was centrifuged and the brownish sediment was discarded. Approximately 60–70% of the cytochrome *c* oxidase was recovered in the supernatant.

The supernatant was diluted 1:2 with 0.1 M potassium phosphate (pH 7.4), to decrease the cholate concentration. Powdered ammonium sulfate was added to 45% saturation. A green flocculation formed at this concentration of ammonium sulfate. The mixture was stirred slowly for 5–10 min in a small glass vial which resulted in a jelly-like precipitate adhering to the stirring rod and the walls of the vial. This jelly-like precipitate was similar to that described by Yu et al. (1975) during the preparation of their phospholipid-rich cytochrome *c* oxidase from beef heart. The remaining turbid supernatant was decanted and centrifuged in order to recover as much of the enzyme as possible. The green sediment which formed during centrifugation was combined with the jelly-like precipitate recovered from the small vial in the previous step.

The combined jelly-like precipitate was dissolved in 0.1 M potassium phosphate (pH 7.4), containing 1.5% sodium cholate to give a protein concentration of 5–10 mg/mL. Saturated ammonium sulfate solution was added until the protein solution became slightly turbid, usually at about 25% ammonium sulfate saturation. Stirring was continued for 10 min after which the solution was left on ice for 30 min. The mixture was then centrifuged and the sediment discarded.

Saturated ammonium sulfate was added to the supernatant until a green precipitate became visible, which occurred at about 35–37% ammonium sulfate saturation. The mixture was centrifuged and the green sediment was dissolved in 0.1 M potassium phosphate containing 0.25% Tween 20 at a concentration of approximately 5 mg protein per mL. The precipitation with saturated ammonium sulfate and the subsequent centrifugation were repeated 2–3 times or until a heme *a* concentration of at least 8 nmol/mg of protein was obtained. The ammonium sulfate concentration necessary to precipitate the cytochrome *c* oxidase was found to decrease in each successive step to approximately 32% for the final precipitation. The final cytochrome *c* oxidase precipitate had an oily consistency. This precipitate was dissolved in 0.1 M potassium phosphate (pH 7.4), containing 0.5% Tween 20, and dialyzed

TABLE I: Cytochrome *c* Oxidase from Rat Liver Mitochondria.

step	heme *a* (nmol/mg of prot.)	purification factor	electron transfer act. atoms O per mg prot per (min)	purification factor	turnover number electrons per heme *a* per s
1. mitochondria	0.230 ± 0.011 (3)	1.0	1.09 ± 0.117 (3)	1.0	156.7
2. membranous cytochrome *c* oxidase	3.91 ± 1.21 (5)	17.0	5.69 ± 2.13 (5)	5.2	48.7
3. sodium cholate extract	5.61 ± 1.12 (5)	24.4	16.05 ± 4.98 (5)	14.7	95.3
4. ammonium sulfate fraction I	6.08 ± 1.42 (3)	26.4	15.36 ± 4.95 (3)	14.1	84.3
5. purified enzyme	9.49 ± 0.91 (5)	41.3	37.99 ± 7.68 (5)	34.9	133.3

[a] Heme *a* content, specific activity and turnover number at various steps of purification were determined as described in Methods. Numbers in parentheses indicate number of experiments.

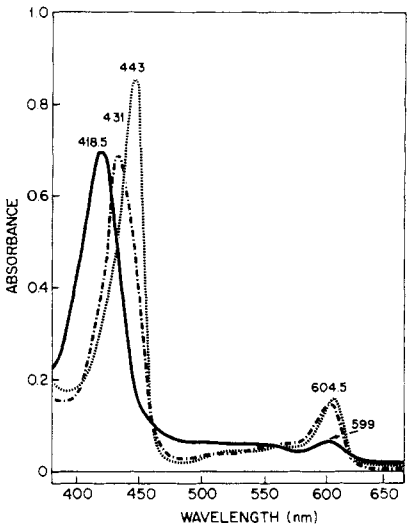

FIGURE 1: Absorption spectra of purified rat liver cytochrome *c* oxidase. The sample contained 0.76 mg of protein/mL of 0.1 M potassium phosphate (pH 7.4), containing 0.5% Tween 20; (—) oxidized; (- - -) reduced with sodium dithionite; (— - — - —) reduced plus CO.

overnight against the phosphate–Tween buffer to remove the ammonium sulfate. It was observed that the oxidase aggregated when stored in 0.25% Tween 20, whereas it remained in solution in 0.5% Tween 20.

The specific heme *a* content increased 41-fold relative to whole mitochondria (Table I), and the specific activity increased by a factor of 35. The yield was generally 25–30% of the total mitochrondrial heme *a* content. If more than three ammonium sulfate precipitations in Tween 20 were necessary, the yield decreased to as little as 10–15%.

Heme a, Iron, and Copper Content. Heme *a* content of the purified enzyme averaged 9.5 nmol/mg of protein (Table I), while the iron content was 9–9.5 nmol/mg of protein. These figures are similar to cytochrome *c* oxidase preparations from beef heart (Griffiths & Wharton, 1961; Kuboyama et al., 1972; Volpe & Caughey, 1974; Phan & Mahler, 1976; Capaldi & Hayashi, 1972; Fowler et al., 1962). The equality of iron and heme *a* content indicates the absence of non-heme iron. The ratio of copper to iron in the purified enzyme was 1.2–1.5, in agreement with the values found by Yonetani (1961) and Griffiths & Wharton (1961) for the oxidase from beef heart. However, more recent reports give copper to iron ratios of 1–1.2 (Kuboyama, 1972; Volpe & Caughey, 1974).

Lipid Content. Phospholipid was estimated as inorganic phosphorus. The total phosphorus content of the purified enzyme was determined to be 1.47 μg/mg of protein, equivalent to 37 μg of phospholipid/mg of protein, based on the general assumption that 1 μg of phosphorus corresponds to 25 μg of phospholipid.

Absorption Spectra. The absolute absorption spectra of the purified enzyme (Figure 1) exhibited absorption maxima characteristic for cytochrome *a*. These are essentially the same as reported for beef heart cytochrome *c* oxidase (Yonetani, 1961; Kuboyama et al., 1972; Volpe & Caughey, 1974). The oxidized form showed a characteristic α band at 599–600 nm and a Soret band at 418–419 nm. Reduction with dithionite typically shifted the α band to 604 nm and the Soret band to 443 nm. A shoulder at 420 nm remained. Addition of CO to the reduced form of the enzyme caused a slight decrease of the absorption maximum at 604 nm, the appearance of a shoulder at 590 nm, and a shift in the Soret band from 443 to 431 nm.

Electron Transfer Activity. The average electron transfer activity of the purified enzyme determined polarographically with cytochrome *c* as electron donor was 38 μatoms of oxygen per min per mg of protein (Table I) and the apparent first-order rate constant k' was 56.3 s per mg of protein per mL. By comparison, k' values obtained for beef heart cytochrome *c* oxidase have been reported as 13.5 (Yonetani, 1961), 42.6 (Fowler et al., 1962), 46.2 (Volpe & Caughey, 1974), and 48 per mg of protein per mL (Kuboyama et al., 1972).

The turnover number of the purified enzyme was 80% of the value obtained with solubilized mitochondria (Table I). At intermediate purification steps, the turnover number was considerably decreased which may have been due to an increase in aggregation of the cytochrome *c* oxidase molecules rendering a portion of the enzyme inaccessible to substrate. Alternatively, it is known that the microenvironment, i.e., the lipids or detergents bound to the oxidase molecule, affects the electron transfer activity (Awashi et al., 1971; Chuang & Crane, 1973; Robinson & Capaldi, 1977). The Tween detergents are most effective compared with other detergents in restoring electron transfer activity in lipid depleted cytochrome *c* oxidase from beef heart (Yu et al., 1975; Robinson & Capaldi, 1977). It is therefore possible that the turnover number of rat liver cytochrome *c* oxidase is decreased at intermediate purification steps because the presence of detergents bound to the enzyme such as cholate or Triton X-114 does not provide a microenvironment optimal for electron transfer. Replacement of these detergents with Tween 20 in the latter steps of purification, however, may reconstitute the microenvironment to one more nearly approaching that of the enzyme in the intact mitochondrion.

Rat liver cytochrome *c* oxidase showed optimal activity between pH 6.0 and 6.3, when assayed in 0.05 M potassium phosphate containing 0.5% Tween 20 (Figure 2). The K_M for reduced cytochrome *c* was estimated as 10–15 μM, which is well within the range found for beef heart cytochrome *c* oxidase

TABLE II: Comparison of Amino Acid Compositions of Cytochrome *c* Oxidases Purified from Rat Liver and from Beef Heart Mitochondria.

amino acid	rat liver (residues/nmol of heme *a*)	%	beef heart (residues/nmol of heme *a*)	%
Lys	38	4.0	28	3.91
His	23	3.48	20	2.79
Arg	31	3.27	21	2.93
Asp	71	7.48	52	7.26
Thr	61	6.43	51	7.12
Ser	66	6.95	53	7.40
Glu	66	6.95	52	7.26
Pro	54	5.69	48	6.70
Gly	73	7.69	53	7.40
Ala	76	8.01	55	7.68
Cys	10	1.05	7	0.98
Val	57	6.01	45	6.28
Met	39	4.11	13	1.82
Ile	50	5.27	40	5.59
Leu	104	10.96	79	11.03
Tyr	35	3.69	39	4.05
Phe	55	5.80	43	6.0
Trp	30	3.16	27	3.77
total residues	949	100.0	716	100.0
mol wt	119 492		80 054	
polarity	38.53%		38.69%	

[a] Values for the beef heart enzyme are taken from Kuboyama et al. (1972). The amino acid analysis of the rat liver enzyme was performed as described in Methods. The polarity is defined as the sum of the residue mole percentages of the polar amino acids Lys, His, Arg, Asp, Thr, Ser, & Glu (Capaldi & Vanderkooi, 1972).

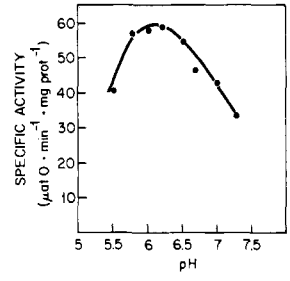

FIGURE 2: Effect of pH on the activity of cytochrome *c* oxidase. Oxygen consumption was determined polarographically. The system contained 0.05 M potassium phosphate buffer, 1 mM EDTA, 30 mM sodium ascorbate, 22.5 μM cytochrome *c*, 0.1 mM TMPD, 1–2 μg of purified rat liver cytochrome *c* oxidase in a final volume of 2.0 mL.

(Volpe & Caughey, 1974; Nicholls, 1974). Lineweaver–Burk plots were linear for cytochrome *c* concentrations tested (2–22.5 μM). At all concentrations, cytochrome *c* was kept fully reduced by ascorbate and TMPD.

Asolectin was routinely included in the reaction medium and was generally necessary for maximal oxidase activity. Without addition of asolectin, most purified enzyme preparations showed 70–80% of the maximal activity. Occasional preparations showed no loss of activity when asolectin was omitted from the medium.

Amino Acid Composition. The amino acid composition of purified rat liver cytochrome *c* oxidase containing 9.95 nmol of heme *a*/mg of protein was similar to that reported for beef heart enzyme (Kuboyama et al., 1972; Table II). Differences between the mole percentages of amino acids in the rat liver and beef heart enzyme were less than 10%. A significant difference was that twice as much methionine was present in the rat liver enzyme. The total amount of amino acids per heme *a* was higher in rat liver cytochrome *c* oxidase which is consistent with the lower heme *a* content per mg of protein. The polarity of rat liver cytochrome *c* oxidase, defined as the residue percentage of the polar amino acids, Glu, Asp, Lys, Arg, His, Ser, Thr (Capaldi & Vanderkooi, 1972), was calculated to be 38.53%. A value of 38.69% was calculated for beef heart cytochrome *c* oxidase using the amino acid composition published by Kuboyama et al. (1972).

Stability. Storage of purified cytochrome *c* oxidase in 0.1 M potassium phosphate, pH 7.4, containing 0.5% Tween 20 on ice for 6 days led to a 40–50% decrease of specific activity while the absolute absorption spectra showed only a minor change. In the Soret region the ratio of the reduced to oxidized form decreased from 1.2–1.25 to 1.1. This decrease was accompanied by an increase in the shoulder of the reduced form at 420 nm. In addition, the difference between the reduced and oxidized form at 604 nm decreased by 20%. These observations indicate a decrease in the reducibility of heme *a* in the enzyme as suggested previously by Griffiths & Wharton (1961) in their studies with beef heart cytochrome *c* oxidase. Storage in 0.1 M potassium phosphate, pH 7.4, containing 0.5% Tween 20 liquid nitrogen and subsequent thawing led to a 20% decrease in the difference between the reduced and oxidized form at 604 nm and a decrease of 60–70% in the specific activity. The electron transfer activity of the purified oxidase was much more sensitive to aging and to freezing and thawing than were the spectral characteristics of the protein. Our observations suggest that storage of the oxidase up to 7 days is preferably carried out in ice to avoid adverse effects of freezing and thawing on the electron transfer activity. However, storage in liquid nitrogen is preferred to preserve the enzyme over periods of several weeks or months.

Subunit Composition. Electrophoresis of the purified oxidase in $NaDodSO_4$–polyacrylamide gels resolved six subunits with apparent molecular weights of 43 500, 24 500, 15 500, 12 000, 10 500, and 9000 (Table III). The mobilities of these subunits corresponded closely to the mobilities of six of the seven subunits of the beef heart enzyme (Figure 3). $NaDodSO_4$–polyacrylamide gel electrophoresis of rat liver cytochrome *c* oxidase in the presence of urea further resolved the

TABLE III: Molecular Weight Estimations of the Subunits of Cytochrome *c* Oxidase from Rat Liver and Beef Heart Mitochondria Determined by $NaDodSO_4$–Polyacrylamide Gel Electrophoresis.[a]

subunit no.	rat liver cytochrome *c* oxidase		beef heart cytochrome *c* oxidase	
	$NaDodSO_4$	$NaDodSO_4$ & urea	$NaDodSO_4$	$NaDodSO_4$ & urea
I	43 500	34 000	41 500	35 300
II	24 500	26 800	22 600	25 200
		23 700		21 000
III	15 500	17 000	15 000	16 200
			13 200	
IV	12 000	12 500	11 300	12 100
V	10 500	9 500	10 200	6 700
VI	9 000	3 600	7 900	3 400

[a] Results obtained by two different procedures are compared; $NaDodSO_4$ procedure, 12% acrylamide as described in Methods; $NaDodSO_4$ and urea procedure, 12.5% acrylamide plus 8 M urea according to Downer et al. (1976). Calculations of apparent molecular weights in the urea-free system were based on the mobilities of the following standard proteins: catalase (60 000), ovalbumin (43 000), glyceraldehyde-3-phosphate dehydrogenase (37 000), myoglobin (17 200), cytochrome *c* (12 400). Standard proteins for the urea containing gel system were: bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (29 000), soybean trypsin inhibitor (21 700), myoglobin (17 200), lysozyme (14 300), cytochrome *c* (12 400), bovine pancreatic trypsin inhibitor (6200), cyanogen bromide fragments of cytochrome *c* (8300 and 4100).

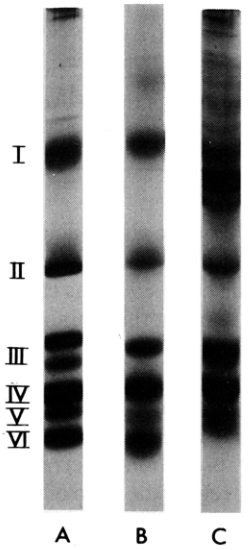

FIGURE 3: Comparison of purified rat liver cytochrome *c* oxidase with purified beef heart cytochrome *c* oxidase by $NaDodSO_4$–polyacrylamide gel electrophoresis. Electrophoresis was performed in 12% acrylamide gels as described in Methods. (A) beef heart cytochrome *c* oxidase; (B) rat liver cytochrome *c* oxidase; (C) membranous rat liver cytochrome *c* oxidase.

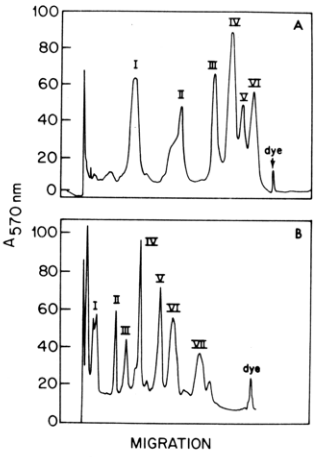

FIGURE 4: Densitometric traces of polyacrylamide gels of purified rat liver cytochrome *c* oxidase: (A) 12% acrylamide, electrophoresis performed in the presence of $NaDodSO_4$ as described in Methods; (B) 12.5% acrylamide, electrophoresis performed in the presence of $NaDodSO_4$ and 8 M urea according to Downer et al. (1976).

enzyme into 7 major subunits (Figure 4). Preliminary results indicate that subunit II in the $NaDodSO_4$–polyacrylamide gels could be resolved into two main components in the presence of urea. The apparent molecular weights of the subunits of rat liver cytochrome *c* oxidase differed somewhat depending on the presence or absence of urea in the gel system (Table III). However, in any one gel system the molecular weights of subunits of rat liver oxidase always corresponded closely to those of the subunits of beef heart oxidase.

To more accurately estimate the apparent molecular weights of rat liver cytochrome *c* oxidase subunits, $NaDodSO_4$ gel electrophoresis was carried out at five different acrylamide concentrations and the mobilities of the subunits were plotted against the total acrylamide concentration according to Ferguson (1964). Such plots revealed that the free mobilities extrapolated to zero acrylamide concentration were very similar for the subunits II through VI (Figure 5). The free mobilities of these subunits were also very close to those of the standard proteins. Subunit I showed an anomalous mobility; thus a calculation of the molecular weight of subunit I based on the mobilities of the standard proteins may not be reliable. An anomalous mobility of subunit I has also been observed for the cytochrome *c* oxidases isolated from beef heart (Phan & Mahler, 1976) and *Saccharomyces cerevisiae* (Poyton & Schatz, 1975; Phan & Mahler, 1976). Such behavior may be related to the finding that subunit I is a relatively hydrophobic protein (Capaldi & Vanderkooi, 1972).

Molecular Weight of Rat Liver Cytochrome c Oxidase. The minimum molecular weight of rat liver oxidase based on the heme *a* or on the iron content was estimated as 105 000. Assuming that the monomeric form of the protein contains one of each of the different subunits, the sum of the apparent molecular weights of the six subunits has a minimum molecular weight of 115 000. If the apparent molecular weights of the seven subunits found in the presence of urea are used, the minimum molecular weight of the monomeric protein is estimated as 127 100. A molecular weight of 119 500 is estimated from the amino acid composition.

Immunologic Characteristics. Rat liver cytochrome *c* oxidase was compared immunologically with beef heart cytochrome *c* oxidase. Ouchterlony double immunodiffusion

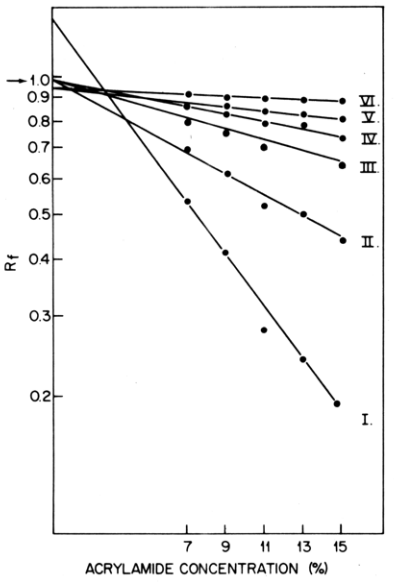

FIGURE 5: Ferguson plot of mobility of the subunits of rat liver cytochrome *c* oxidase in $NaDodSO_4$–polyacrylamide gels. The ratio of acrylamide:bis(acrylamide) was 40:1.5 for all concentrations used. The arrow indicates the intercept on the ordinate for the standard proteins (catalase, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, myoglobin, cytochrome *c*).

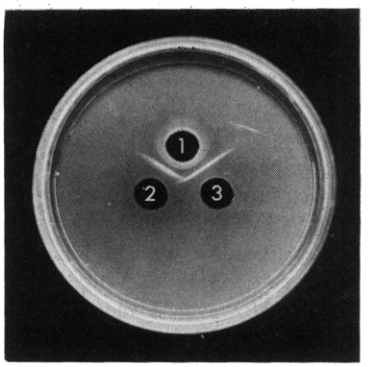

FIGURE 6: Immunological comparison of beef heart and rat liver cytochrome *c* oxidase. Double immunodiffusion in 0.65% agarose plates containing 1% Triton X-100. (Well 1) contains rabbit antibody prepared against beef heart cytochrome *c* oxidase; (well 2) purified rat liver cytochrome *c* oxidase; (well 3) purified beef heart cytochrome *c* oxidase. Photographed after 72 h of diffusion at 23 °C.

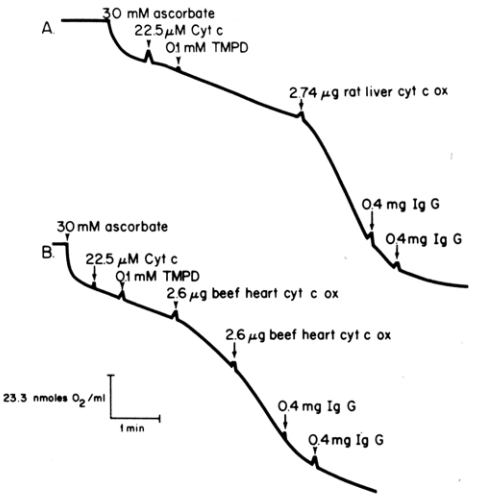

FIGURE 7: Immunotitration of cytochrome *c* oxidase from rat liver and beef heart. Polarographic traces of the inhibition of cytochrome *c* oxidase from rat liver (a) and beef heart (b) by rabbit antibody prepared against beef heart cytochrome *c* oxidase. The conditions for the assay were as described in Methods.

showed that cytochrome *c* oxidase from rat liver cross-reacted with antibody produced in rabbits against beef heart cytochrome *c* oxidase (Figure 6). When the liver and beef heart enzymes were present in adjacent wells, there was spurring at the junction of the two immunoprecipitates (Figure 6) suggesting that the two oxidases are not immunologically identical but that they have common immunodeterminants. The electron transfer activity of both rat liver and beef heart cytochrome *c* oxidases was completely inhibited by the IgG fraction of rabbit antiserum produced against the beef heart enzyme (Figure 7).

Discussion

The method described here for the isolation of rat liver cytochrome *c* oxidase yields a highly purified, active, and stable enzyme. Jacobs et al. (1966a,b) showed that Triton X-114 in the presence of 0.2 M potassium phosphate (pH 7.0) partially solubilizes mitochondria resulting in a membranous residue that does not contain heme proteins other than cytochrome *c* oxidase. The elimination of other heme proteins by Triton X-114 is a major advantage over most other methods which usually start with the solubilization of whole mitochondria and require several fractionation steps with ammonium sulfate before cytochrome *c* oxidase is free of other heme proteins, mainly of cytochrome *b* (Yonetani, 1961; Griffiths & Wharton, 1961; Fowler et al., 1962). Only Kuboyama et al. (1972) succeeded in eliminating the cytochrome b–c_1 complex from the respiratory chain before the solubilization of cytochrome *c* oxidase by sequential extraction of beef heart Keilin–Hartree particles with 1% and 2% sodium cholate. We preferred to use Triton X-114 to prepare membranous cytochrome *c* oxidase since we could obtain a 17-fold increase in the heme *a*/mg of protein content in this way.

We were not able to satisfactorily solubilize the membranous cytochrome *c* oxidase in Triton X-100 and chromatograph the enzyme on DEAE-cellulose (Jacobs et al., 1966a,b). We found the enzyme to be altered significantly by Triton X-100 in that it could no longer be reduced with dithionite. Therefore, we further purified the cytochrome *c* oxidase by ammonium sulfate precipitation after solubilization of the membranous oxidase in 4% cholate. This high cholate concentration was necessary in order to extract an appreciable amount of enzyme from the membranous cytochrome *c* oxidase fraction. It should be cautioned here that excessive use of cholate could be a disadvantage since cholate is known to extract phospholipids from cytochrome *c* oxidase, especially in the presence of ammonium sulfate (Kuboyama et al., 1972; Awashi et al., 1971; Yu et al., 1975). We found that, following solubilization, several consecutive ammonium sulfate precipitation steps in the presence of 1.5% cholate were deleterious to the cytochrome *c* oxidase, whereas, after transferral of the oxidase into buffer containing Tween 20, ammonium sulfate precipitation could be repeated several times without impairing the electron transfer activity. The percentage of ammonium sulfate necessary for effective purification varied from one preparation to another sometimes by differences of 1–2%. Kuboyama et al. attributed such variations to differences in the lipid content of the enzyme in different purification steps.

The purification procedure for cytochrome *c* oxidase described in this communication is rapid and can be completed in 48 h. The purity of the enzyme at 9–10 nmol of heme *a*/mg of protein is comparable to the purity of most preparations of cytochrome *c* oxidase from beef heart (Yonetani, 1961; Grif-

fiths & Wharton, 1961; Volpe & Caughey, 1974; Fowler et al., 1962) and yeast (Mason et al., 1973).

The purified rat liver cytochrome *c* oxidase described in this report has a high electron transfer activity compared with most enzyme preparations of beef heart (Yonetani, 1961; Kuboyama et al., 1972; Volpe & Caughey, 1974; Fowler et al., 1962). This was not anticipated, since the purified rat liver enzyme was found to have a low lipid content compared with preparations of beef heart oxidase with high electron transfer activities (Kuboyama et al., 1972; Volpe & Caughey, 1974; Fowler et al., 1962). However, Tween 20, which was present in the assay medium, has been shown to substitute for lipids and to activate lipid depleted beef heart enzyme to 50–60% of the maximum rate of the lipid rich enzyme (Yu et al., 1975). The activity of the lipid depleted beef heart enzyme has been restored 90% in the presence of Tween 80 (Robinson & Capaldi, 1977). Since rat liver cytochrome *c* oxidase aggregated in the absence of Tween 20, we did not attempt to measure oxidase activity in the absence of this detergent.

The subunit composition of cytochrome *c* oxidase from four different organisms, *Saccharomyces cerevisiae* (Poyton & Schatz, 1975), *Neurospora crassa* (Sebald et al., 1973), *Locusta migratioria* (Weiss et al., 1972), and beef heart (Downer et al., 1976), has been found to be very similar. All these oxidases consist of seven subunits, three that have apparent molecular weights between 20 000 and 40 000 and four that have apparent molecular weights below 20 000. In our laboratory only six subunits were found for both rat liver and beef heart cytochrome *c* oxidase electrophoresed in $NaDodSO_4$–polyacrylamide gels without urea. However, in the presence of urea, seven bands were resolved, indicating that both beef heart and rat liver cytochrome *c* oxidase consist of at least seven subunits, and therefore are similar in this respect to the oxidases from other sources.

The minimum molecular weight of rat liver cytochrome *c* oxidase calculated from the sum of the apparent molecular weights of the subunits is 115 000–127 000 compared with 105 000 obtained by calculation from the heme *a* content. Based on the assumption that the functional oxidase isolated from beef heart and yeast contains one heme *a* and one heme a_3, it has been concluded that the functional oxidase is a dimer of approximately 200 000 daltons (Kuboyama et al., 1972; Phan & Mahler, 1976; Robinson & Capaldi, 1977). However, cytochrome *c* oxidase of *Neurospora crassa* has a heme *a* content of 14 nmol/mg of protein (Sebald et al., 1973); thus it contains one heme *a* per 70 000 molecular weight. The minimum molecular weight based on the sum of the seven subunits is 150 000, indicating that the monomeric protein, consisting of one of each subunit, contains two heme *a* groups. For beef heart and also for the rat liver oxidase such agreement has not been demonstrated. Since beef heart cytochrome *c* oxidase preparations with as high as 10–11 nmol of heme *a*/mg of protein have been shown to contain some contaminants (Briggs et al., 1975; Penniall et al., 1976), the stoichiometry of heme *a*/oxidase protein remains unclear. However, it is likely that a monomeric, functional oxidase consisting of one of each subunit and containing 2 heme *a* groups exists also for the beef heart enzyme as well as the rat liver enzyme.

The amino acid composition of rat liver cytochrome *c* oxidase does not differ significantly from that of the beef heart enzyme. Consequently, the polarities of the two enzymes are similar, suggesting they have a similar degree of hydrophobicity. In general, hydrophilic proteins have polarities greater than 45% (Capaldi & Vanderkooi, 1972). Previous studies have shown that the polarity of cytochrome *c* oxidase from different sources is below 40% (Capaldi & Vanderkooi, 1972). Polarities below 40% have been found to be typical for proteins strongly associated with membranes such as carotenoid glycoprotein, chlorophyll proteins, and rhodopsin which require detergents and organic solvents for extraction from their corresponding membranes (Capaldi & Vanderkooi, 1972). The low polarity of 38.53% for the purified rat liver cytochrome *c* is consistent with the findings that cytochrome *c* oxidase is a *completely* transmembranous integral protein in the energy transducing membrane of the rat liver mitochondrion (Hackenbrock & Miller-Hammon, 1975).

That rat liver and beef heart cytochrome *c* oxidases are immunologically similar but not identical was demonstrated by the cross-reaction of rat liver cytochrome *c* oxidase with antibodies elicited against the beef heart enzyme utilizing double immunodiffusion. This antibody also inhibited completely the electron transfer activity of rat liver oxidase. Although Elliott et al. (1971) showed earlier that antibody developed against beef heart cytochrome *c* oxidase precipitated a partially purified rat liver oxidase from solution, they did not obtain total inhibition of the electron transfer activity of cytochrome *c* oxidase from either beef heart or rat liver with their antiserum.

The rat liver cytochrome *c* oxidase prepared in our laboratory is 8–10 times more active than the enzyme prepared by Jacobs et al. (1966a,b). Unfortunately the authors did not give any data regarding the heme *a* content of their preparation. They reported a molecular weight of 15 000–20 000 for their oxidase as estimated by ultracentrifugation, which differs from ours considerably. The fact that the minimum molecular weight of our enzyme determined by $NaDodSO_4$–polyacrylamide gel electrophoresis is 127 000, and by gel filtration on agarose is 300 000–400 000, suggests that the purified enyzme exists as a trimer or a tetramer.

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References

Ames, B. N., & Dubin D. T. (1960) *J. Biol. Chem. 235,* 769–775.

Awashi, Y. C., Chuang, T. F., Keenan, T. W., & Crane, F. L. (1971) *Biochim. Biophys. Acta 226,* 42–52.

Briggs, M., Kamp, P. F., Robinson, N. C., & Capaldi, R. A. (1975) *Biochemistry 14,* 5123–5128.

Bucher, J. R., & Penniall, R. (1975) *FEBS Lett. 60,* 180–184.

Capaldi, R. A., & Hayashi, H. (1972) *FEBS Lett. 26,* 261–263.

Capaldi, R. A., & Vanderkooi, G. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 930–932.

Chuang, T. F., & Crane, F. L. (1973) *J. Bioenerg.* 4, 563–578.

Doeg, K. A., & Ziegler, D. M. (1962) *Arch. Biochem. Biophys. 97,* 37–40.

Downer, N. W., Robinson, N. C., & Capaldi, R. A. (1976) *Biochemistry 15,* 2930–2936.

Elliott, W. B., Holbrook, J. P., & Penniall, R. (1971) *Biochim. Biophys. Acta 251,* 277–280.
Estabrook, R. W. (1967) *Methods Enzymol. 10,* 41–47.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10,* 2606–2617.
Ferguson, K. A. (1964) *Metabolism 13,* 985–1002.
Fowler, L. R., Richardson, S. H., & Hatefi, Y. (1962) *Biochim. Biophys. Acta 64,* 170–173.
Griffiths, D. E., & Wharton, D. C. (1961) *J. Biol. Chem. 236,* 1850–1856.
Hackenbrock, C. R., & Miller-Hammon, K. (1975) *J. Biol. Chem. 250,* 9185–9197.
Jacobs, E. E., Andrews, E. C., Cunningham, W., & Crane, F. L. (1966a) *Biochem. Biophys. Res. Commun. 25,* 87–95.
Jacobs, E. E., Kirkpatrick, F. H., Jr., Andrews, E. C., Cunningham, W., & Crane, F. L. (1966b) *Biochem. Biophys. Res. Commun. 25,* 96–104.
Kuboyama, M., Yong, F. C., & King, T. E. (1972) *J. Biol. Chem. 247,* 6375–6383.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265–275.
Mason, T. L., Poyton, R. O., Wharton, D. C., & Schatz, G. (1973) *J. Biol. Chem. 248,* 1346–1354.
Nicholls, P. (1974) *Biochim. Biophys. Acta 346,* 261–310.
Penniall, R., Elliott, W. B., van Buren, K. J. H., & van Gelder, B. F. (1976) *J. Bioenerg. Biomembr. 8,* 271–292.
Phan, S. H., & Mahler, H. R. (1976) *J. Biol. Chem. 251,* 257–276.
Poyton, R. O., & Schatz, G. (1975) *J. Biol. Chem. 250,* 752–761.
Robinson, N. C., & Capaldi, R. A. (1977) *Biochemistry 16,* 375–380.
Schneider, W. C. (1948) *J. Biol. Chem. 176,* 259–266.
Sebald, W., Machleidt, W., & Otto, J. (1973) *Eur. J. Biochem. 38,* 311–324.
van Gelder, B. F. (1966) *Biochim. Biophys. Acta 118,* 36–46.
Volpe, J. A., & Caughey, W. S. (1974) *Biochem. Biophys. Res. Commun. 61,* 452–459.
Weiss, H., Lorenz, B., & Kleinow, W. (1972) *FEBS Lett. 25,* 49–51.
Wharton, D. C., & Rader, M. (1970) *Anal. Biochem. 33,* 226–229.
Yonetani, T. (1961) *J. Biol. Chem. 236,* 1680–1688.
Yu, C. A., Yu, L., & King, T. E. (1972) *J. Biol. Chem. 247,* 1012–1019.
Yu, C. A., Yu, L., & King, T. E. (1975) *J. Biol. Chem. 250,* 1383–1392.

Cross-linking of Ubiquinone Cytochrome *c* Reductase (Complex III) with Periodate-Cleavable Bifunctional Reagents†

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ABSTRACT: Two novel cross-linkers, disuccinimidyl tartarate (DST) and *N,N'*-bis(3-succinimidyloxycarbonylpropyl)tartaramide (SPT), have been synthesized. These reagents span 6 and 18 Å, respectively, between functional groups and contain a *vic*-glycol bond which can be cleaved with periodate under mild reaction conditions. Both DST and SPT have been used to examine the near-neighbor relationships of polypeptides in ubiquinone cytochrome *c* reductase (complex III) from beef heart mitochondria. Among the cross-linked products resolved were pairs containing I + II, II + VI, I + V, and VI + VII. Polypeptides III and IV, a cytochrome *b* aproprotein, and the cytochrome c_1 hemoprotein, respectively, were also resolved in several cross-linked products.

Ubiquinone cytochrome *c* reductase or complex III is an oligomeric protein which spans the mitochondrial inner membrane and is involved in electron transport and in the coupling of this reaction to ATP synthesis or ion transport. The complex contains b heme, c_1 heme, and non-heme-iron centers in the molar ratio 2:1:1 (for review, see Rieske, 1976). The polypeptide composition of complex III has been studied in several laboratories (Das Gupta and Rieske, 1973; Yu et al., 1974; Hare and Crane, 1974; Gellerfors and Nelson, 1975; Bell and Capaldi, 1976). By using highly resolving $NaDodSO_4$–polyacrylamide gel systems, we have been able to identify nine different polypeptides in complex III with molecular weights ranging from 50 000 to 4400 (Bell and Capaldi, 1976; Capaldi et al., 1977). The smallest component, polypeptide IX, represents 1–2% by weight of the protein and is only clearly seen when large amounts of protein are electrophoresed.

As one approach to studying the arrangement of polypeptides in complex III, we have used DSP and DTBP, two cleavable bifunctional reagents to cross-link neighboring polypeptides through their available lysine residues (Smith and Capaldi, 1977). With low levels of either reagent, several pairs of polypeptides were covalently linked together, including I + II, II + VI, I + V, and VI + VII. With higher levels of DSP, an aggregate containing all of the polypeptides was obtained and this is, presumably, the complex III monomer with an apparent molecular weight of 310 000.

Unfortunately, the amount of information that could be obtained using either DSP or DTBP was limited by technical problems. Both reagents are bridged by a disulfide bond and thus separation of cross-linked products for analysis must be done in the absence of reducing agents. Several components of complex III behave anomalously when β-mercaptoethanol

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