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Characterization of Cytochrome Oxidase Purified from Rat Liver

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Abstract

The purpose of this study was to characterize the physical properties of cytochrome *c* oxidase from rat liver. The enzyme was extracted from isolated mitochondria with nonionic detergents and further purified by ion-exchange chromatography on DEAE Bio-Gel A. The purified enzyme contained 9.64 nmol heme a/mg protein and one iron atom plus one copper atom for each heme a. The specific activity of the final preparation was 146 μmol of ferrocytochrome *c* oxidized/min · mg protein, measured at pH 5.7. The spectral properties of the enzyme were characteristic of purified cytochrome oxidase and indicated that the preparation was free of cytochromes *b*, *c*, and c_1. In analytical ultracentrifugation studies, the enzyme sedimented as a single component with an $S^{20,w}$ of 5.35*S*. The Stokes radius of the enzyme was determined by gel filtration chromatography and was equal to 75 Å. The molecular weight of the oxidase calculated from its sedimentation coefficient and Stokes' radius was 180,000, indicating that the active enzyme contained two heme a groups. The purified cytochrome oxidase was also subjected to dodecyl sulfate–polyacrylamide gel electrophoresis in order to determine its components. The enzyme was resolved into five polypeptides with the molecular weights of I, 27,100; II, 15,000; III, 11,900; IV 9800; and V, 9000.

Introduction

The purpose of this study was to isolate and characterize rat liver cytochrome *c* oxidase (E.C. 1.9.3.1).

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Cytochrome oxidase is a constituent of the inner mitochrondrial membrane. It is the terminal member of the electron transfer chain and consists of two heme a groups, two coppers, and several polypeptide components [1, 2]. The enzyme has been purified from beef heart, *Saccharomyces cerevisiae, Neurospora crassa,* and *Locusta migratoria* in order to characterize its physical properties and subunits [1, 2]. The mechanisms regulating the synthesis of this enzyme have been investigated extensively in *S. cerevisiae* and *N. crassa*. In these cells the enzyme has been reported to consist of seven different polypeptide components [3–7], three of which are synthesized on mitochondrial ribosomes and the other four synthesized on cytoplasmic ribosomes and imported into the organelles [3, 4, 8, 9]. Furthermore, the availability of the cytoplasmically synthesized components influences the rate of formation of the complementary components synthesized in the mitochondria [4, 10]. It has also been shown that the synthesis of the enzyme's constituents in *S. cerevisiae* is regulated by the availability of oxygen and glucose to those cells [8, 10, 11]. Therefore, the investigations on the biosynthesis of cytochrome oxidase have provided important insights into the interactions of the mitochondrial and cytoplasmic protein synthesizing machineries in eukaryotic cells.

The aim of this study was to purify and characterize cytochrome oxidase from a mammalian organism which could serve as a suitable model for further investigations on the regulation of biosynthesis of the enzyme in different physiological states. In this investigation, the enzyme was purified from rat liver mitochondria and its composition and physical properties were defined.

Materials and Methods

Preparation of Cytochrome Oxidase

Cytochrome oxidase was purified from rat liver using a modification of the procedures of Jacobs et al. [12–14]. The enzyme was prepared from male Sprague-Dawley rats (350–500 g). Ten to fifteen animals were used for each preparation. The animals were killed by decapitation and the livers were removed immediately and placed in ice-cold saline (0.9% w/v NaCl). All subsequent operations were carried out at 0–4°C. The livers were sliced, washed with saline, and homogenized in sucrose buffer (0.25 M sucrose, 20 mM Tris, 1 mM EDTA, pH 7.4) using a Potter–Elvehjem glass homogenizer fitted with a motor-driven Teflon pestle. A 12.5% (w/v) tissue homogenate was prepared. The homogenate was centrifuged at 500*g* for 10 min and the supernatant was collected. The supernatant was centrifuged at 15,000*g* for 15 min and the mitochondrial pellets were saved. The pellets

were resuspended in 50 mM potassium phosphate buffer, pH 7.2, and centrifuged once more at 15,000*g* for 15 min. The washed mitochondrial pellets were suspended in 50 mM potassium phosphate buffer, pH 7.2, to a concentration of approximately 15 mg protein/ml and 16.5 ml of 20% (v/v) Triton X-114 was added for each 100 ml of the mitochondrial suspension. The mitochondria–Triton suspension was placed in a cold room for 4 h and stirred vigorously. Subsequently, the suspension was centrifuged at 20,000 rpm for 1 h in a Sorvall SS34 rotor. The pellets from this last centrifugation contained most of the cytochrome oxidase originally present in the suspension. The pellets were suspended in 50 mM potassium phosphate buffer, pH 7.2 (to approximately half the volume of the mitochondria–Triton suspension) and the suspension was centrifuged at 30,000 rpm for 60 min in a Spinco type 30 rotor. This step was carried out to remove the Triton X-114 and any cytochrome *c* and c_1 contaminating the oxidase preparation.

The pellets from the last centrifugation were resuspended in minimal volume of 100 mM potassium phosphate buffer, 5% (v/v) Triton X-100, pH 7.2, and the suspension was placed in the cold for 1 h with gentle stirring to allow for the complete solubilization of the enzyme. The suspension was then centrifuged at 20,000 rpm for 40 min in a Sorvall SS34 rotor. The supernatant from this last centrifugation was a highly active and spectrally pure preparation of cytochrome *c* oxidase.

The preparation obtained in the last step was diluted with 3 volumes of glass-distilled water and loaded onto a DEAE Bio-Gel A column (0.9 cm × 10 cm) equilibrated with 25 mM potassium phosphate buffer, 1% (v/v) Triton X-100, pH 7.2. The enzyme formed a narrow dark brown band at the top of the column. Up to 100 mg of protein was loaded onto the column. The column was washed with 35 ml of 25 mM potassium phosphate buffer, 1% (v/v) Triton X-100, pH 7.2, followed by 35 ml of 50 mM potassium phosphate buffer, 1% (v/v) Triton X-100, pH 7.2. The enzyme was eluted off the column with 150 mM potassium phosphate buffer, 1% (v/v) Triton X-100, pH 7.2, and the fractions (1 ml) containing the higher activities of cytochrome oxidase were pooled. The eluted enzyme constituted the fully purified cytochrome oxidase preparation to be described in the Results section. The modifications introduced in the procedures of Jacobs et al. [13, 14] removed minor contamination by cytochrome *b* in the intermediate steps of the isolation and increased the overall yield.

Analytical Methods

Protein was determined by the method of Lowry et al. [15] with bovine serum albumin serving as standard.

Iron was determined by the method of Doeg and Ziegler [16].

Copper was determined by method C of Brumby and Massey [17].

Lipids were extracted by the procedure of Folch et al. [18], and lipid phosphorus was determined by the method of Chen et al. [19].

The concentration of cytochrome $a+a_3$ in isolated mitochondria was determined by difference spectra at 23°C according to the method of Estabrook and Holowinsky [20] using a Cary 15 recording spectrophotometer.

Heme a was determined from the absorbance at 604 nm minus that at 630 nm in dithionite-reduced preparations of cytochrome oxidase; the extinction coefficient used ($\Delta E_{604\,nm-630\,nm}$) was 16.5 (mM cm)$^{-1}$ [21].

Cytochrome oxidase activity was determined by the spectrophotometric method of Smith [22]. The activity measurements were carried out with a Cary 15 recording spectrophotometer at 23°C. The enzyme preparation was diluted with 50 mM potassium phosphate buffer, 0.5% (v/v) Tween 80, pH 7.0, to an appropriate concentration and incubated for 30 min prior to the assay. A sample (25 µl) of the diluted preparation was added to a cuvette containing 2 ml of 30 µM cytochrome *c* (96–98% reduced) in fourfold diluted McIlvaine's standard buffer (25 mM citric acid, 50 mM Na_2HPO_4) at pH 5.7 [23]. The reaction was allowed to proceed for 2 min while the absorbance at 550 nm was continuously recorded. Fifteen microliters of 0.5 M potassium ferricyanide were then added to the cuvette and the optical density was again measured. Water served as a blank. The first-order velocity constant for the aerobic oxidation of cytochrome *c* was determined from the rate of decrease in absorbance at 550 nm as described by Smith [22]. Cytochrome *c* (Sigma, type III, from horse heart) was reduced with soidum dithionite and the excess dithionite was removed by aeration. The pH of the assay buffer wass varied in the pH optimum studies to the values indicated in the Results section.

Velocity Sedimentation and Gel Filtration

Sedimentation velocity studies were conducted at 20°C with a Beckman Model E analytical ultracentrifuge equipped with an AN-D rotor. The sample to be analyzed was concentrated by dialysis against solid polyethylene glycol and then dialyzed for 24 h at 4°C against 150 mM potassium phosphate buffer, 1% (v/v) Triton X-100, pH 7.2. The sample was adjusted to a protein concentration of 5 mg/ml with dialysis buffer and then subjected to velocity sedimentation. The experiments were carried out with a double sector cell and a 12-mm centerpiece at 44,770 rpm.

Gel filtration chromatography studies were carried out with Sephadex G-200 and Bio-Gel A-5m columns (0.8 cm × 75 cm). The gel materials

were equilibrated with 0.1 M potassium phosphate buffer, 1% (v/v) Triton X-100, pH 7.2, and the elutions were carried out with the same buffer at 5°C. The various molecular markers used in the chromatography experiments and their Stokes' radii were horse heart myoglobin, 18.8 Å [24]; chicken heart lactic dehydrogenase (E.C. 1.1.1.27). 47.0 Å [25]; and bovine liver L-glutamic dehydrogenase (E.C. 1.4.1.3), 83.9 Å [26]. The elution of myoglobin was determined from its absorbance at 502 nm. The elutions of the other markers were determined from their activities. Lactic dehydrogenase activity was assayed by the method of Bergmeyer et al [27]. L-glutamic dehydrogenase activity was assayed by the method of Schmidt [28]. Dextran Blue 2000 was used to determine the void volume of the Sephadex columns, and its elution was determined from its absorbance at 635 nm.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis studies were carried out according to the method of Weber and Osborn [29]. The gels were prepared in tubes 12 cm long and 0.7 cm in diameter. The gel system consisted of 1 cm of 3% acrylamide, 2% methylenebisacrylamide, followed by 8.5 cm of 15% acrylamide, 2% methylenebisacrylamide prepared in the running buffer. The running buffer was 60 mM sodium phosphate, 0.1% (w/v) SDS, pH 7.0. The 3% acrylamide portion was included to minimize the time required for entry of the sample proteins into the gel at the initiation of electrophoresis. The protein in a sample to be analyzed was precipitated with 7 volumes of cold acetone [6] and pelleted by centrifugation at 1500 *g* for 15 min at 4°C. The resulting pellet was solubilized in 3.5% (w/v) SDS, 2% (v/v) β-mercaptoethanol to a protein concentration of 1 mg/ml, and the solution was placed at 85°C for 2 h. The sample was then dialyzed at room temperature for 20 h against 1 liter of 0.1% (w/v) SDS, 0.1% (v/v) β-mercaptoethanol in 10 mM sodium phosphate buffer, pH 7.2. Just prior to electrophoresis 300 µl of the sample was mixed with 100 µl of a solution of bromphenol blue and glycerol (0.14 mg/ml bromphenol blue, 50% v/v glycerol). From this final mixture, 25- to 50-µl aliquots were applied to individual gels. Standards prepared exactly as the unknown samples were run on parallel gels. Electrophoresis was carried out at a current of 3 mA/gel for 13 h. At the termination of electrophoresis, the gels were removed from the tubes and fixed for 12 h in 10% (w/v) trichloroacetic acid, 50% (v/v) methanol. The gels were stained in 0.25% (w/v) Coomassie brilliant blue, 10% (v/v) acetic acid, 50% (v/v) methanol for 2.5 h at 30°C. The gels were destained by diffusion in 10% (v/v) acetic acid. The stained

gels were scanned at 550 nm with a Gilford Model 240 spectrophotometer fitted with a linear transport attachment.

The standards used and their molecular weights were bovine serum albumin, 66,000; ovalbumin, 46,000; bovine erythrocyte carbonic anhydrase (E.C. 4.2.1.1), 30,000; horse heart myoglobin, 16,900; and horse heart cytochrome *c*, 12,400.

Results

The recovery of cytochrome oxidase at the various steps of purification is summarized in Table I. Approximately 11% of the starting amount of the enzyme was recovered in the final step of purification as determined by heme a recovery. As indicated in Table II, the heme a/protein ratio in the final preparation was 9.64 nmol/mg. The enzyme preparation had a high capacity to oxidize ferrocytochrome *c* and contained one atom of iron and one atom of copper for each heme a. These results are in agreement with the findings reported for the enzymes purified from heart and

TABLE I. Typical recovery of cytochrome oxidase from 100 g of rat liver at the various steps of purification

	Protein	Recovered heme a (nmol)
Initial	21 g	1000[a]
Mitochondrial pellet	1.3 g	270
Triton X-100-soluble extract	44 mg	170
Fully purified	11 mg	110

[a] Rat liver content of heme a was obtained from Ades [30].

TABLE II. Composition of purified cytochrome oxidase[a]

Parameter	Amount
Heme a/protein[b]	9.64 ± 0.50 (7)
Total iron/heme a	1.18 ± 0.23 (4)
Total copper/heme a	1.09 ± 0.05 (4)
Specific activity[c]	146 ± 9 (3)

[a] The determinations were carried out as described in the Materials and Methods section. The values are expressed as mean ± SEM and the number of different enzyme preparations used in each of the determinations is given in parentheses.

[b] nmol/mg.

[c] μmol ferrocytochrome *c* oxidized/min · mg protein.

yeast [1, 5, 6, 31, 32]. No lipid phosphorus was detected in chloroform–methanol (2:1) extracts of enzyme preparations which had been dialyzed against water, suggesting that the oxidase preparations were depleted of phospholipids. It is possible, however, that the enzyme preparations may have contained some tightly bound phospholipids which could not be extracted with neutral chloroform–methanol. Such findings have been reported for beef heart cytochrome oxidase [33, 34].

The activity expressed by cytochrome oxidase was dependent on the detergent in which the enzyme was solubilized prior to the assay. As shown in Fig. 1, the Triton X-100-solubilized enzyme has some capacity to oxidize reduced cytochrome *c*, and the reaction had a pH optimum at 5.7. However, when the Triton-solubilized enzyme was preincubated with Tween 80 prior to the assay, the specific activity of the preparation is

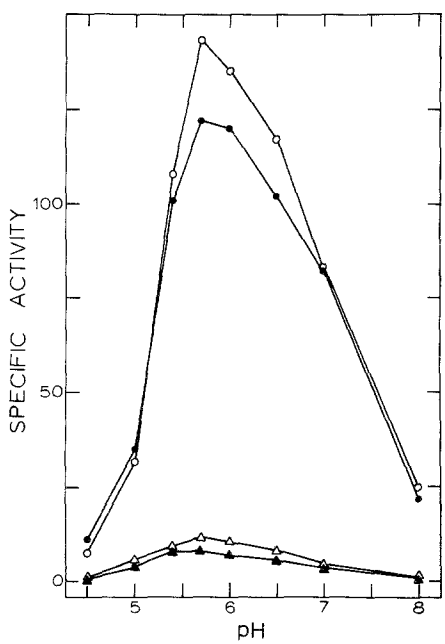

Figure 1. The pH optimum of the cytochrome oxidase reaction and the effects of preincubation with Tween 80 on the activity of the Triton X-100-solubilized enzyme. The enzyme preparations were diluted to the proper concentrations either with 50 mM potassium phosphate buffer plus 0.5% (v/v) Triton X-100, pH 7.2 (△, ▲), or with 50 mM potassium phosphate buffer plus 0.5% (v/v) Tween 80, pH 7.2 (○, ●), and preincubated for 30 min prior to the assay. Activity measurements were carried out as described in the Materials and Methods section. Two different preparations were used in these determinations. Specific activity is expressed in micromoles of ferrocytochrome *c* oxidized per minute per milligram of protein.

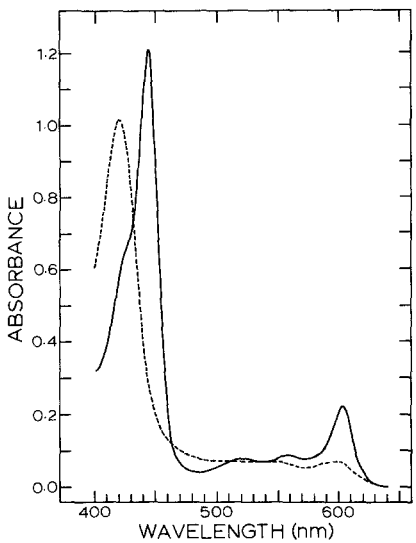

Figure 2. Absolute absorption spectra of the cytochrome oxidase preparation. The spectra were recorded on a Cary 15 spectrophotometer at 23°C with water serving as the blank. Reduction was achieved by adding a few sodium dithionite crystals directly to the cuvette containing the enzyme preparation and immediately covering the cuvette. The solid line represents the reduced preparation.

increased 13-fold at the optimum pH. The mean specific activity reported in Table II was determined with Tween 80 preincubated preparations. The dependence of lipid-depleted cytochrome oxidase preparations on preincubation with either phospholipids or various nonionic detergents especially of the Tween and Emasol type for the restoration of their activities has been documented [1, 35].

The spectral properties of the cytochrome oxidase preparation are shown in Fig. 2. The oxidized enzyme had a γ absorption band at 420 nm which shifted to 443 nm upon reduction of the enzyme with sodium dithionite. The reduced enzyme had α and β absorption bands at 604 and 520 nm, respectively. Additional spectral properties of the enzyme are summarized in Table III together with values suggested by Lemberg [35] as criteria for purity based on the properties of beef heart cytochrome oxidase. The ratio of the absorbance at 604 nm to that at 550 nm of the reduced enzyme suggests that the preparation was practically free of cytochromes *b*, c_1, and *c*. The reduction of the enzyme was complete within 5 min after the addition of sodium dithionite, unlike the heart preparations which require 30 min for complete reduction [35]. The ratio of the absorbance at 443 nm to that at 420 nm of the reduced enzyme,

TABLE III. Spectral properties of purified cytochrome oxidase [a]

Absorbance ratios	Liver	Lemberg [35]
$\frac{\gamma Fe^{2+}}{\alpha Fe^{2+}}$	5.32 ± 0.10	5.0–5.1
$\frac{A_{604}}{A_{550}}$	2.83 ± 0.03	Greater than 2, almost 3
$\frac{A_{420}}{A_{444}}$	0.486 ± 0.010	< 0.5
$\frac{\gamma Fe^{3+}}{\alpha Fe^{3+}}$	12.4 ± 0.9	7.5–9.5
$\frac{\alpha Fe^{2+}}{\alpha Fe^{3+}}$	2.85 ± 0.13	2.0–2.2
$\frac{\gamma Fe^{2+}}{\gamma Fe^{3+}}$	1.23 ± 0.01	1.25–1.3

[a] The absorbance characteristics of the liver preparation are presented together with the values suggested by Lemberg [35] as criteria for purified cytochrome oxidase. The values for the liver enzyme are expressed as mean ± SEM and were obtained from the absorption spectra of three different enzyme preparations.

which has been proposed as an indicator for the presence of modified or irreducible cytochrome oxidase [35], was within the suggested range. The results in Fig. 2 and in Table III indicate that the liver preparation had the spectral characteristics of purified cytochrome oxidase.

The properties of the isolated enzyme were investigated further by analytical ultracentrifugation. The sedimentation profiles observed by Schlieren optics indicated that the preparation consisted of a single sedimenting component. A typical sedimentation profile is presented in Fig. 3. The average sedimentation coefficient ($S^{20,w}$) of the enzyme obtained from these studies was 5.35*S* (two determinations), assuming a value of 0.740 ml/g for the partial specific volume [34, 36].

In order to estimate the molecular weight of cytochrome oxidase, the Stokes radius of the enzyme was determined by gel filtration chromatography. Cytochrome oxidase eluted with the void volume when the preparation was filtered through a column of Sephadex G-200, indicating that the enzyme was excluded from this type of gel. Therefore, the chromatography studies were carried out with Bio-Gel A-5m which resolves in the molecular weight range of 10,000 to 5 million. Myoglobin, lactic dehydrogenase, and glutamic dehydrogenase served as markers. A typical elution profile is shown in Fig. 4. The elution volume of a given molecule subjected to gel filtration chromatography has been shown to depend on its Stokes radius [37, 38]. The column was calibrated with the three markers which cochromatographed with cytochrome oxidase, and

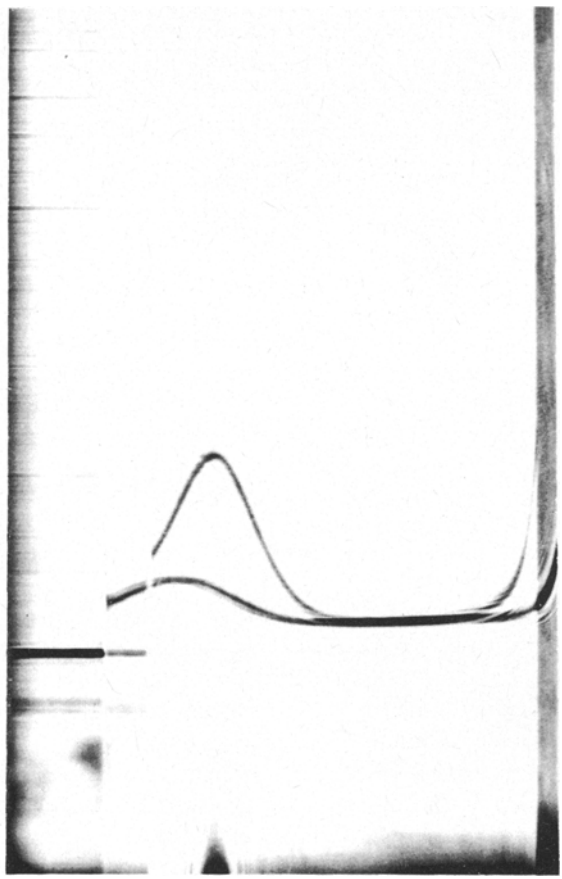

Figure 3. Sedimentation profile of cytochrome oxidase. The enzyme was solubilized in 150 mM potassium phosphate buffer, 1% (v/v) Triton X-100, pH 7.2, to a protein concentration of 5 mg/ml. The experiment was carried out in a Beckman Model E analytical ultracentrifuge under the conditions described in the Materials and Methods section. The picture was taken 105 min after the rotor had reached a speed of 44,770 rpm. Sedimentation is from left to right.

the elution volumes of the markers were indeed related to their respective Stokes radii as seen in Fig. 5. The Stokes radius of the enzyme as determined from its elution volume was 75 Å (two determinations). The molecular weight of the enzyme was estimated from the values of its sedimentation coefficient and Stokes radius using the classic hydrodynamic equations [38] and by assuming again a value of 0.740 ml/g for the partial specific volume. The calculated molecular weight was 180,000, which

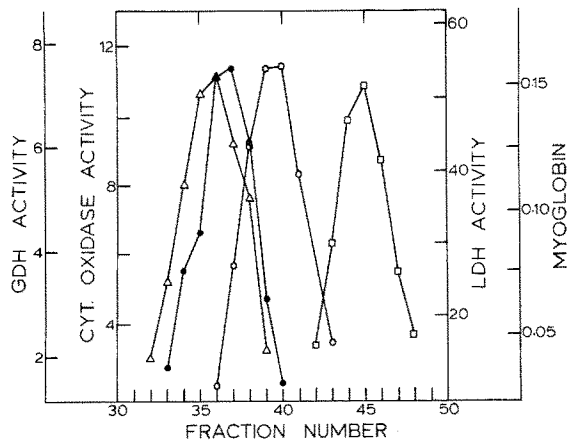

Figure 4. *Elution of cytochrome oxidase from Bio-Gel A-5m.* The column characteristics and the elution procedures are described in the Materials and Methods section. Fractions of 50 drops were collected. The elutions of the markers are expressed as myoglobin (Mb, □), absorbance at 502 nm; lactic dehydrogenase (LDH, ○), nmol NADH oxidized/min · 25 µl; cytochrome oxidase (●), nmol ferrocytochrome *c* oxidized/min · 25 µl; and L-glutamic dehydrogenase (GDH, △), nmol NADH oxidized/min · 25 µl.

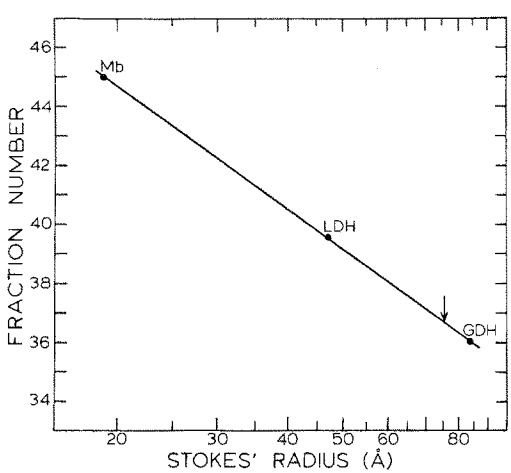

Figure 5. Relationship between elution volume and Stokes radius. The data were obtained from the results in Fig. 4. The arrow indicates the elution of cytochrome oxidase.

together with the heme a/protein values in Table II suggested that the enzyme contained two heme a groups per active molecule.

In order to determine the polypeptide composition of the liver cytochrome oxidase, the purified enzyme was subjected to polyacrylamide

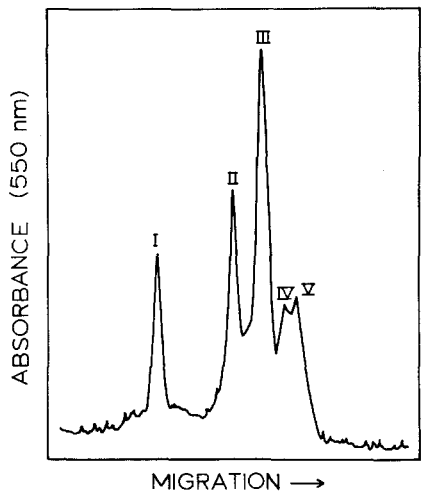

Figure 6. The polypeptide constituents of cytochrome oxidase determined by dodecyl sulfate–polyacrylamide gel electrophoresis. The electrophoretic analyses were carried out as described in the Materials and Methods section.

gel electrophoresis in the presence of sodium dodecyl sulfate. Five polypeptide constituents were distinguished in the liver enzyme as seen in Fig. 6. Gels containing concentrations of acrylamide lower than 15% failed to resolve polypeptides III–V into distinct components. The molecular weights of the components were determined from the mobilities of marker proteins run on parallel gels and are given in Table IV. It should be noted

TABLE IV. The molecular weights of the polypeptide constituents of cytochrome oxidase [a]

Subunit	Molecular weight	Suggested stoichiometry
I	27,100 ± 600	1
II	15,000 ± 500	1
III	11,900 ± 400	2
IV	9,800 ± 300	1
V	9,000 ± 400	1

[a] The molecular weights were determined from dodecyl sulfate–polyacrylamide gel electrophoretic analyses of the enzyme under the conditions described in the Materials and Methods section. The values are expressed as mean ± SEM. Five different cytochrome oxidase preparations were used in these determinations. The stoichiometries of the components were determined from the relative areas of the various peaks in densitometric tracings of 10 gels as described in the text.

that the molecular weight of the smallest standard used in the electrophoresis studies was 12,400, and thus the sizes of the smaller components reported in Table IV had to be estimated by extending the curve relating the mobilities of the markers to their molecular weights. This procedure obviously introduced some error into these determinations. The electrophoretic patterns obtained with the enzyme were identical to those obtained with preparations of the enzyme which had been subjected to gel filtration on Sephadex G-200 prior to electrophoresis as an additional check on the purity. Finally, there was no evidence of protein aggregation seen either on top of the 3% acrylamide or on top of the 15% acrylamide portions of the gels. This was an important consideration in view of the report that cold acetone precipitation of beef heart cytochrome oxidase led to aggregation of some of its subunits [6]. The aggregates could not be dissociated with 1% SDS–1% mercaptoethanol but have been dissociated with 2% SDS–2% mercaptoethanol [6]. Although the liver enzyme was precipitated with cold acetone, the precipitates were solubilized in 3.5% SDS–2% mercaptoethanol and heated at 85°C for 2 h prior to electrophoresis to minimize the possibility of such artifacts.

Quantitations of the relative areas (area under peak/molecular weight of protein) of polypeptides I, II, III, and IV+V in densitometric tracings of 10 gels suggested the molar stoichiometries indicated in Table IV. However, it is not possible to determine if peak III represents one protein or two different proteins with very similar mobilities. It should be pointed out that the determination of the stoichiometries cannot be considered rigorous because the binding of Coomassie blue to a protein depends on the protein's amino acid composition [4, 36]. Furthermore, the individual peaks in the densitometric tracings were not separated enough for very accurate determinations of their areas. The sum of the molecular weights of the five components multiplied by their respective stoichiometries equals $84{,}700 \pm 1000$, which is about half the value obtained for the molecular weight of the enzyme. If cytochrome oxidase consists of two identical subunits each with a heme a, not necessarily positioned in the same manner in each subunit [1], a molecular weight of $169{,}400 \pm 1400$ would be obtained for the enzyme from the electrophoretic studies. This value, based on the assumptions just stated, is close to the molecular weight of the enzyme obtained from the sedimentation and gel filtration studies.

Discussion

In this study cytochrome *c* oxidase was isolated from rat liver mitochondria. The enzyme preparation had a high capacity to oxidize ferro-

cytochrome *c*, contained one iron atom and one copper atom per heme a, and possessed the spectral characteristics of purified cytochrome *c* oxidase. The purified enzyme sedimented as a single component with an $S^{20,w}$ of 5.35*S* and had a Stokes radius of 75 Å as determined by gel filtration chromatography. The molecular weight of cytochrome oxidase calculated from its sedimentation coefficient and Stokes radius was 180,000, which suggested that the enzyme contained two heme a groups. Finally, Polyacrylamide gel electrophoresis studies indicated that the oxidase consisted of five polypeptide constituents ranging in molecular weight from 9000 to 27,100.

Cytochrome oxidase was originally discovered by MacMunn [39] and rediscovered by Keilin [40] who named it cytochrome because of its wide distribution and its role in cell respiration. The active enzyme appears to contain two heme a groups whose functions are different [1], thus the enzyme is also referred to as cytochrome $a+a_3$. One heme group (of cytochrome *a*) interacts with cytochrome *c*, and the other heme group (of cytochrome a_3) interacts with oxygen [1]. The two hemes have been distinguished by the capacity of cytochrome a_3 only to bind cyanide and carbon monoxide and by the changes in the spectral properties of the enzyme upon binding of these ligands [1, 41, 42].

The enzyme was purified for the first time independently by Yakushiji and Okunuki [43] and by Straub [44] from beef heart, and the procedures introduced by these investigators have become standard methods for isolating cytochrome oxidase. The enzyme is extracted from isolated mitochondria with ionic detergents such as cholate or deoxycholate, and the extraction is followed by ammonium sulfate fractionation to separate cytochromes *b*, *c*, and c_1 from cytochrome $a+a_3$ [1, 45]. The preparations obtained by this procedure have also been subjected to adsorption chromatography on alumina gels or to ion-exchange chromatography on DEAE-cellulose to ensure purity [5, 6, 45]. The ammonium sulfate fractionation procedure was applied to rat liver without much success during the initial phase of this study.

The procedure which Jacobs et al. [12–14] introduced for purifying rat liver cytochrome oxidase was used in this investigation. In this method the enzyme is extracted from mitochondria with nonionic detergents, and the extracted enzyme is purified further by ion-exchange chromatography. The studies of Jacobs et al. [13, 14] on the liver oxidase were mainly concerned with the reaction characteristics of this enzyme in reconstitued membranes.

The aim of this study was to characterize the physical properties of liver cytochrome oxidase in the rat, an animal which would be convenient to use as a model for investigating the regulation of synthesis of this enzyme

TABLE V. Subunit compositions of various cytochrome oxidase preparations

S. cerevisiae [6]	*S. cerevisiae* [7]	*N. crassa* [4]	*L. migratoria* [46]	*Beef heart* [47]
40,000	40,000	41,000	38,000	35,400
27,300	33,000	28,500	24,000	24,100
25,000	22,000	21,000	19,000	21,000
13,800	14,500	16,000	14,500	16,800
13,000	12,700	14,000	12,500	12,400
10,200	12,700	11,500	10,000	8,200
9,500	4,600	10,000	8,000	4,400

in higher cells. The determination of the polypeptide constituents of the liver enzyme was fundamental to this purpose. In Table V the protein components of cytochrome oxidase preparations obtained from various cell types are presented. The enzymes consist of seven polypeptides, and the molecular weights of the individual components correlate remarkably well in the various oxidases. The results from this study indicate that the rat liver enzyme may lack the largest component.

It is difficult to establish whether or not all the polypeptides constituting cytochrome oxidase are necessary for the expression of enzyme function. The problem lies in the definition of cytochrome oxidase; that is, whether the capacity to oxidize ferrocytochrome *c* should be the only criterion for the definition of the enzyme or whether other characteristics such as hydrophobicity and the capacity to bind to membranes should also be considered. Several reports have indicated that active cytochrome oxidase prepared from beef heart and from yeast could be obtained with fewer polypeptide constituents than reported in Table V [36, 48, 49]. Phan and Mahler [49] were able to obtain active cytochrome oxidase preparations consisting of four polypeptide components from both yeast and beef heart. These oxidase preparations lacked the larger polypeptide components and appeared to have lost some of their hydrophobic properties since they remained soluble in phosphate buffer in the absence of any added detergent. Therefore, the possibility suggested by Phan and Mahler [49] that cytochrome oxidase may consist of a catalytic component and a membrane binding component is quite attractive.

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