Purification and Characterization of Cytochrome c Oxidase from Rat Liver Mitochondria

Ralph J. Rascati and Peter Parsons

The Journal of Biological Chemistry
Vol. 254, No. 5, Issue of March 10, pp. 1586-1593, 1979

Cytochrome c oxidase has been purified from rat liver mitochondria using affinity chromatography. The preparation contains 10.5 to 13.4 nmol of heme a + a3 per mg of protein and migrates as a single band during polyacrylamide gel electrophoresis under nondissociating conditions. It has a heme a/a3 ratio of 1.12 and is free of cytochromes b, c, and c1 as well as the enzymes, NADH dehydrogenase, succinic dehydrogenase, cytochrome c oxidase, oligomycin-sensitive ATPase, and ATPase. The enzyme preparation consists of six polypeptides having apparent Mr of 60,000, 59,000, 23,000, 14,000, 12,500, and 10,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The peptide composition is similar to those found for cytochrome c oxidase from other systems. The enzymatic activity of the purified enzyme is completely inhibited by carbon monoxide or cyanide, partially inhibited by Triton X-100 and dramatically enhanced by Tween 80 or phospholipids.

Our studies have been prompted by an interest in the cellular site of synthesis of mitochondrial proteins, particularly in mammalian systems. Studies based on specific inhibition of either cytoplasmic protein synthesis or mitochondrial protein synthesis using whole cells of lower systems have now indicated that at least three mitochondrial proteins, cytochrome c oxidase, oligomycin-sensitive ATPase, and cytochrome b are synthesized in part on cytoplasmic ribosomes and in part on mitochondrial ribosomes (1). While these experiments have been done very carefully under well controlled conditions, there remains the possibility that erroneous results might occur due to nonspecific or indirect effects of such inhibitors (1, 2). We, therefore, carried out our studies using isolated mitochondria to avoid this problem and to determine whether the isolated organelle could carry out the synthesis of completed mitochondrial peptides. Cytochrome c oxidase was studied because preliminary findings in our laboratory indicated that isolated rat liver mitochondria could synthesize polypeptides associated with this enzyme.

Our approach to the study of the biosynthesis of cytochrome c oxidase has been to: (a) purify and characterize the enzyme, (b) prepare specific antibodies to the enzyme, (c) incubate isolated mitochondria with radioactive leucine, (d) treat sulfonated mitochondrial proteins labeled in this manner with antibodies to cytochrome c oxidase, and (e) analyze the immunoprecipitates by SDS-polyacrylamide gel electrophoresis to determine whether any newly synthesized, radioactively labeled cytochrome c oxidase peptides are present.

This paper describes the purification and characterization of the cytochrome c oxidase. Purification procedures for this enzyme have been reported for bovine heart (3-9), yeast (3, 10), Neurospora crassa (11), Locusta migratoria (12), and rat liver (13). However, in our hands, the procedures available when we initiated this work yielded preparations which were low in heme a + a3 content and exhibited a large number of bands on SDS-polyacrylamide gel electrophoresis. We have developed a procedure including affinity chromatography on cytochrome c-Sepharose which results in an active enzyme of very high heme a + a3 content. Ozawa et al. (14) have used a similar affinity column to obtain cytochrome c oxidase with high heme a content from beef heart mitochondria. Our enzyme has been characterized with respect to subunit composition and various other physical, chemical, and enzymatic properties. Experiments detailing the preparation of specific antisera against cytochrome c oxidase and the mitochondrial biosynthesis of some of the peptides of this enzyme are presented in the following paper (15). Some of this work has been reported previously in preliminary form (16).

EXPERIMENTAL PROCEDURES

Materials

White Sprague-Dawley rats of both sexes used for all experiments were obtained from Holtzmann Laboratories, Madison, WI. Horse heart cytochrome c type III, polyethylleuline (30,000 daltons), N,N,N',N'-tetramethylethylenediamine, glycine, sodium dodecyl sulfate, Tween 80, Triton X-100, and twice crystallized bovine serum albumin were obtained from Sigma. Sephadex G-15 and Sepharose 4B were obtained from Pharmacia. Methylenebisacrylamide and ammonium persulfate were obtained from Bio-Rad. Cyanogen bromide and acrylamide were purchased from Eastman Organic. Whatman DE52 DEAE-cellulose was purchased from Reeve Angel. Oligomycin Q10 was a generous gift from Merck, Sharp and Dohme Laboratories.

Preparation of Chromatographic Resins

Cytochrome c-Sepharose—Sepharose 4B was activated by reaction with cyanogen bromide (CNBr) according to the procedure of Cucrcecasas (17). Twenty milliliters of packed resin was diluted with 20 ml of distilled water and 2 g of CNBr were added. The reaction was maintained at pH 11.0 by addition of 2 N NaOH, dropwise at 30°C until proton release stopped. The activated resin was washed rapidly

The abbreviations used are: SDS, sodium dodecyl sulfate; SMP, submitochondrial particles.

1586
by suction filtration with 20 volumes of 0.2 M sodium citrate, pH 6.5. Twenty milliliters of cytochrome c (4 mg/ml) in 0.2 M sodium citrate, pH 6.5, was added to the activated washed resin and the mixture was gently stirred at 0-4°C for 16 to 24 h to allow covalent linkage of the cytochrome c to the Sepharose.

**DEAE-cellulose**—Whatman DE52 microgranular, preswollen resin was equilibrated in 20 mM sodium phosphate, pH 7.0, containing 1% Triton X-100.

Poly(lysine)-Sepharose—Sepharose 4B was prepared as outlined above for purification of cytochrome c-Sepharose with the following exceptions: 0.1 M sodium carbonate, pH 8.5 was used to wash the CNBr-activated resin and the poly(lysine) (10 mg/ml) was dissolved in this same buffer.

**Sephadex G-15**—Sephadex G-15 was swollen and equilibrated in 20 mM sodium phosphate, pH 7.0, containing 1% Tween 80.

**Purification of Cytochrome c Oxidase**

The following procedure is for 1 g of intact mitochondrial protein. All steps were performed at 0-4°C.

**Preparation of Mitochondria**—Mitochondria were prepared by the method of Parsons and Simpson (18) using a 4% homogoneate to improve the yield of organelles. Mitochondria were washed four times with 0.37 ml of saturated ammonium sulfate (0°C) per ml of S,. This mixture was centrifuged for 1 min at maximum power in the Branson Sonifier-S-175. The broken mitochondrial particles were centrifuged for 1 h at 100,060 x g in a Sorvall SS-34 rotor. The supernatant which contained no cytochrome oxidase was discarded and the pellet (SMP) was resuspended in 0.25 M sucrose.

**Solution of Cytochrome Oxidase**—Twenty per cent sodium cholate was added to a final concentration of 3 mg/mg of SMP protein. Ammonium sulfate (saturated at 0°C) was then added dropwise until the ratio of 0.34 ml/mg of SMP solution which brought the final concentration of ammonium sulfate in the mixture to 95% saturation. Gentle stirring for 2 h followed by centrifugation for 15 min at 40,000 x g in a Sorvall SS-34 rotor yielded a supernatant (S1) which contained all the cytochrome oxidase.

**Precipitation of Cytochrome Oxidase**—The S1 fraction was brought to 45% saturation with ammonium sulfate by mixing directly with 0.37 ml of saturated ammonium sulfate (0°C) per ml of S,. This mixture was centrifuged for 15 min at 40,000 x g in a Sorvall SS-34 rotor. The supernatant (S2) was discarded and the oily, dark green pellet (P2) was dissolved in a minimum volume of 20 mM sodium phosphate (pH 7.0), 1% Tween 80.

**Desalting**—The P2 fraction was desalted on a Sephadex G-15 column (1.5 x 25 cm) equilibrated in the same buffer used to dissolve P2. All colored eluate fractions were combined and diluted 10-fold with elution buffer (G-15 eluate).

**Cytochrome c-Sepharose Chromatography**—The G-15 eluate was applied to a cytochrome c-Sepharose affinity column (1.5 x 25 cm) equilibrated with 20 mM sodium phosphate (pH 7.0), 1% Tween 80. After washing successively with 3 column volumes each of 20 mM sodium phosphate (pH 7.0), 1% Tween 80, and 200 mM sodium phosphate (pH 7.0), 1% Tween 80, the cytochrome oxidase eluted with 100 mM sodium phosphate (pH 7.0), 1% Triton X-100. The green-colored fractions were combined and diluted 5-fold with 1% Triton X-100.

**DEAE-cellulose Chromatography**—The diluted eluate from the previous column was applied to a DEAE-cellulose column (1.5 x 7 cm) equilibrated with 20 mM sodium phosphate (pH 7.0), 1% Triton X-100. The column was washed successively with 2 column volumes of 20 mM sodium phosphate (pH 7.0), 1% Triton X-100, and 50 mM sodium phosphate (pH 7.0), 1% Triton X-100. The latter buffer usually causes spreading of the green cytochrome c oxidase on the resin. Occasionally, some oxidase is eluted by this buffer but this is of low purity and is discarded. The enzyme is eluted with 100 mM sodium phosphate (pH 7.0), 1% Triton X-100. The eluted colored fractions are combined and diluted 5-fold with distilled water.

**Poly(lysine)-Sepharose Chromatography**—The eluted eluate from the previous column was applied to a poly(lysine)-Sepharose column (0.9 x 4 cm) equilibrated with 20 mM sodium phosphate (pH 7.0), 0.1% Triton X-100. The column was then washed successively with 3 column volumes each of 20 mM sodium phosphate (pH 7.0), 0.1% Triton X-100, and 40 mM sodium phosphate (pH 7.0), 0.1% Triton X-100. Further washing was carried out with at least 1 column volume of 80 mM sodium phosphate (pH 7.0), 0.1% Triton X-100, and until the oxidase spread to approximately the middle of the column. The enzyme was eluted with 160 mM sodium phosphate (pH 7.0), 0.1% Triton X-100.

The purification scheme is outlined in Fig. 1.

**Enzyme Assays**

**Cytochrome c Oxidase Activity**—Cytochrome c oxidase activity was assayed at 23°C by a modification of the procedure of Smith and Conrad (19). A 1-ml assay contained 40 mM potassium phosphate (except where otherwise noted), pH 7.0, 0.5% Tween 80, 30 µM ferrocyanochrome c, and enzyme. Ferrocyanochrome c was prepared by adding a few grains of sodium dithionite to 1% solution of cytochrome c in assay buffer followed by aeration to remove excess dithionite. The activity of the enzyme is expressed as the first order rate constant. An extinction coefficient, \( \Delta E_{600} \), of 18.5 Mm\(^{-1}\) cm\(^{-1}\) was assumed (19).

**Assays of Enzymatic Impurities**—

1. NADH dehydrogenase was measured by the procedure of DeBernard (20). Reduction of ferricyanide was determined in a 1-ml assay containing 40 mM potassium phosphate, pH 7.5, 0.5 mM ferricyanide, and 0.5 mM NADH. An extinction coefficient, \( \Delta E_{492} \), of 1.0 Mm\(^{-1}\) cm\(^{-1}\) was used (20).

2. Cytochrome Q-cytochrome c reductase was measured by a modification of the procedure of Riske (21). Reduction of cytochrome c was monitored in a 1-ml assay containing 40 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 20 mM sodium azide, 0.08% bovine serum albumin, 50 µg of reduced cytochrome Q, and 210 µg cytochrome c. An extinction coefficient, \( \Delta E_{550} \), of 18.5 Mm\(^{-1}\) cm\(^{-1}\) was assumed (19).

3. Succinic acid dehydrogenase was assayed by the method of King (22). Dichloroindophenol reduction was measured in a 1-ml assay containing 50 mM potassium phosphate, pH 7.8, 1.5 mM sodium cyanide, 20 mM sodium succinate, 0.5 mM dichloroindophenol, 1 mg of bovine serum albumin, and 1 mM phenazinemethosulfate. An extinction coefficient, \( \Delta E_{550} \), of 2.0 Mm\(^{-1}\) cm\(^{-1}\) was used (22).

4. ATPase activity was measured indirectly by the ability of antibodies to the most highly purified fraction of cytochrome c oxidase (see Ref. 15) to inhibit the conversion of \([\text{H}]\text{ATP}\) to \([\text{H}]\text{ADP}\) by

![Fig. 1. Purification scheme for cytochrome c oxidase. Abbreviations used are: HSS, high speed supernatant; SMP, submitochondrial particles; S, supernatant; P, pellet; TW, Tween 80; TX, Triton X-100; NaP, sodium phosphate.](http://www.jbc.org/Downloadedfrom/2011.10.31/fig1.png)
### Purification of cytochrome c oxidase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Heme a + a₃</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Heme recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>nmol</td>
<td>nmol/mg protein</td>
<td>K (min⁻¹) × 10⁻⁵</td>
<td>K (min⁻¹) mg⁻¹</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1000</td>
<td>140</td>
<td>0.14</td>
<td>44.0</td>
<td>44</td>
</tr>
<tr>
<td>SMP</td>
<td>550</td>
<td>123</td>
<td>0.23</td>
<td>40.2</td>
<td>13</td>
</tr>
<tr>
<td>S₁</td>
<td>292</td>
<td>152</td>
<td>0.56</td>
<td>49.3</td>
<td>169</td>
</tr>
<tr>
<td>P₂</td>
<td>285</td>
<td>160</td>
<td>0.58</td>
<td>37.3</td>
<td>131</td>
</tr>
<tr>
<td>G-15</td>
<td>200</td>
<td>124</td>
<td>0.62</td>
<td>38.0</td>
<td>190</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>15.8</td>
<td>76</td>
<td>5.00</td>
<td>11.4</td>
<td>729</td>
</tr>
<tr>
<td>DEAE</td>
<td>6.0</td>
<td>30</td>
<td>5.50</td>
<td>4.1</td>
<td>679</td>
</tr>
<tr>
<td>Polylysine</td>
<td>2.0</td>
<td>23</td>
<td>11.50</td>
<td>1.8</td>
<td>915</td>
</tr>
</tbody>
</table>

*a Both methods for heme determination cited under "Experimental Procedures" gave similar results.

*b Activity is expressed as the first order rate constant - ln(A₀/Aₜ) + protein (mg/ml).

### RESULTS

The results of the purification of cytochrome c oxidase according to the scheme given in Fig. 1 are presented in Table I. Purification of the enzyme will be discussed on the basis of its heme a + a₃/protein ratio since heme is an obvious, relatively stable marker of cytochrome oxidase. Preparation of SMP from intact mitochondria as well as subsequent solubilization and ammonium sulfate fractionation of these particles each results in a small purification of the enzyme; no heme a + a₃ is lost up to this point. The use of cytochrome c-Sepharose as an affinity column resulted in a further 8-fold purification and loss of about 50% of the heme a + a₃. Elution from this resin required the presence of both detergent and salt, indicating both ionic and hydrophobic interactions involved in the binding of enzyme. Oxidase could not be eluted by 20 mM sodium phosphate, 1% Triton X-100, but was slowly eluted by 50 mM sodium phosphate, 1% Triton X-100 and readily eluted by 100 mM sodium phosphate, 1% Triton X-100. However, it is curious that as much as 200 mM sodium phosphate could be used in the presence of Tween 80 without eluting the enzyme from the column. Chromatography on DEAE-cellulose results in only a small purification. This step appears to be an important one, however, since attempts to eliminate it result in preparations whose purity has never been higher than 8.6 nmol of heme a + a₃/mg of protein. Occasionally, some heme a + a₃ with very low heme/protein ratios elutes with the low salt wash buffers. This material may be enzyme which is somewhat altered in structure and must be removed in order to obtain the levels of purity normally observed upon subsequent poly(L-lysine) chromatography. Poly(L-lysine)-Sepharose was chosen as a resin for the purification of cytochrome oxidase because poly(L-lysine) is a known competitive inhibitor of the activity of this enzyme and therefore has binding affinity for the enzyme. The inter-
action with this column appears to be primarily ionic since the concentration of enzyme eluted was accomplished by a stepwise salt gradient. The final purity of the enzyme obtained from several preparations is 10.5 to 13.4 nmol of heme a + a₃/mg of protein.

The complete procedure results in an 82-fold purification of the enzyme using intact mitochondria as the starting material. The recovery of enzyme as judged by its heme content is approximately 16%. The concentration of enzyme obtained in this final purification step is approximately 2 to 3 mg/ml.

Sonication during the preparation of SMP particles sometimes results in the loss of as much as 50% of the oxidase activity. Further activity is lost upon introduction of Triton X-100. While the specific activity of the enzyme based on activity per mg of protein does increase at each purification step, the apparent enzyme activity per nmol of heme (turnover rate) decreases during purification so that the enzyme in the final fraction has an apparent activity which is 23 to 30% that of intact mitochondria.

When this preparation was analyzed by polyacrylamide gel electrophoresis under nondissociating conditions, a single protein band was observed (Fig. 2). As little as 1 µg of protein can be detected by the staining method used. Therefore, since 200 µg of protein were applied to these gels, the preparation is judged to be greater than 99% pure. This, however, does not exclude the possibility that there are contaminants which co-migrate with the oxidase.

Subsequent analysis of this enzyme preparation by polyacrylamide gel electrophoresis in the presence of SDS consistently revealed the presence of six polypeptide components having apparent Mₐ of 66,000, 39,000, 23,500, 14,000, 12,500, and 10,000 (Fig. 3). In some preparations, there was a small amount of protein on the leading side of the 39,000 component which, when present, constituted less than 2% of the total protein. The three low molecular weight components are variable in the extent of their resolution. Enzymatic iodination of the enzyme preparation prior to gel electrophoresis significantly enhances resolution of these components and results in the appearance of three distinct components in this molecular weight range (see Ref. 15, Figs. 3 and 5c).

In summary, we have developed a procedure which results in an 82-fold purification of cytochrome c oxidase. The enzyme preparation has a heme a + a₃/protein ratio of 10.5 to 13.4 nmol/mg, migrates as a single band during electrophoresis under nondissociating conditions and consists of six polypeptide components.

**Properties of Cytochrome c Oxidase**

**Enzymatic Properties**—The enzymatic activity was determined as described under "Experimental Procedures." The effects of various parameters on this activity are discussed below.

**pH**—The enzyme was tested for its ability to oxidize cytochrome c at various pH values. The pH of the assay was varied from 3.0 to 12.0 by 0.5-pH unit increments. The results (Fig. 4) demonstrate that there is a very sharp optimum around pH 6.0.

**Ionic Strength**—The activity of the enzyme was tested with regard to the ionic strength of the assay buffer. The concentration of potassium phosphate, pH 7.0, was varied from 10 to 200 mM. It can be seen in Fig. 5 that the activity shows a broad optimum from 10 to 70 mM. At higher concentrations of phosphate, however, enzymatic activity is severely impaired. This effect is not limited to potassium phosphate alone; equivalent concentrations of potassium chloride produce the same effect.

**Inhibitors**—Table II shows the effect of cyanide and carbon monoxide on enzyme activity. The presence of 0.01 mM KCN completely inhibits enzyme activity as does bubbling CO through the enzyme solution for 10 min prior to assay.

**Detergents**—Cytochrome c oxidase is a membrane-bound
Studies on Cytochrome c Oxidase from Rat Liver Mitochondria

Fig. 5. Effect of potassium phosphate concentration on cytochrome c oxidase activity. Activity was measured as described under “Experimental Procedures.”

TABLE II
Inhibition of cytochrome c oxidase by KCN and CO

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Specific activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>680.9</td>
<td>100</td>
</tr>
<tr>
<td>+ 0.01 mM KCN</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>+ CO*</td>
<td>0.0</td>
<td>100</td>
</tr>
</tbody>
</table>

*The enzyme fraction was from poly(L-lysine)-Sepharose; heme/protein = 12.4 nmol/mg.
Carbon monoxide was bubbled through the enzyme fraction for 10 min prior to assay. Twenty microliters of enzyme was used in a 1.0-ml assay volume.

enzyme. Presumably in situ, this binding involves membrane phospholipids which may be very important for enzyme activity. As shown in Table III, each enzyme purification procedure which results in a decrease in the turnover rate of the enzyme is accompanied by the removal of significant amounts of phospholipid. Further experiments indicate that addition of phospholipid or Tween 80 to the enzyme results in significant increases in enzyme activity (Table IV). Unlike Tween 80 or phospholipids, Triton X-100 inhibits enzyme activity 46%. This is approximately equal to the 44% loss of enzyme activity (units per nmol of heme) which occurs during cytochrome c-Sepharose chromatography (when Triton X-100 is first introduced into the procedure) as shown in Table I.

Spectral Properties—The spectral properties of the purified enzyme are given in Figs. 6 and 7. Fig. 6 shows the spectra of the most highly purified cytochrome c oxidase fraction in the oxidized and reduced forms. The oxidized form shows a broad peak in the region of 595 nm and a sharp peak at 424 nm. The reduced form shows sharp peaks at 601 nm and 441 nm. No traces of contamination by any other cytochromes are evident as shown by the lack of absorption between 540 and 570 nm. Determination of the heme a + a3 content of enzyme from the difference in absorbance at 601 nm and 630 nm after dithionite reduction gave results which were in agreement with those obtained by the pyridine hemochromogen method (see “Experimental Procedures”). Fig. 7 shows the difference spectrum obtained between a sample of enzyme reduced with sodium dithionite and a sample through which carbon monoxide has been bubbled and then reduced with sodium dithionite. The difference in absorbance between 445 nm and 430 nm in this spectrum was used in determining the heme a3 content as described under “Experimental Procedures.” The heme a/a3 ratio calculated from this data for the purified enzyme is 1.12, in good agreement with active cytochrome c oxidase (25).

Contaminating Enzymes—Coenzyme Q-cytochrome c reductase, NADH dehydrogenase, succinic acid dehydrogenase,
and ATPase are mitochondrial inner membrane enzymes and are associated with cytochrome c oxidase as part of the electron transport chain. Because of this association and the fact that each of these enzymes also has some hydrophobic properties, the most highly purified fraction of cytochrome c oxidase was examined for possible contamination by these enzymes. Coenzyme-Q-cytochrome c reductase, NADH dehydrogenase, and succinic acid dehydrogenase were measured directly and in each case the enzyme was present in intact mitochondria but was completely absent from the most highly purified fraction of cytochrome oxidase (Table V). In the case of NADH dehydrogenase, which has been reported by Pen- niall (30, 31) to be present in his preparation of cytochrome c oxidase from rat liver mitochondria, we have calculated that if it is present at all there is less than 1% contamination by this enzyme. Ninety-five per cent of the NADH dehydrogenase activity present in intact mitochondria fractionates into the 20 mM sodium phosphate, pH 7.0, 1% Tween 80 wash during cytochrome c-Sepharose chromatography. Thus, essentially all of this enzyme is separated from cytochrome c oxidase at this step. In the case of both coenzyme-Q-cytochrome c reductase and succinic acid dehydrogenase, we have calculated that any contamination of the preparation by either of these enzymes would be less than 0.1%. The locations of these two enzyme activities during fractionation were not determined directly, but the majority of cytochromes b and c₁, which are integral components of coenzyme-Q-cytochrome c reductase were found in the 20 and 200 mM sodium phosphate, pH 7.0, 1% Tween 80 washes during cytochrome c-Sepharose chromatography. We have not determined the location of succinic acid dehydrogenase. ATPase activity was determined indirectly by measuring the ability of antibodies to the most highly purified fraction of cytochrome c oxidase to inhibit the ATPase activity of sonicated or solubilized mitochondria. There was no inhibition of ATPase activity (Table VI) whereas cytochrome c oxidase activity was inhibited by more than 85% (data not shown, see Ref. 15). This indicates that there are no antibodies to ATPase in the antisem to cytochrome c oxidase and suggests, therefore, that the most highly purified fraction of this enzyme is free of contamination by ATPase.

Non-heme Iron—NADH dehydrogenase, coenzyme-Q-cytochrome c reductase, and succinic acid dehydrogenase each contain measurable quantities of non-heme iron (21, 32, 33). Since these enzymes are not detectable in our enzyme preparation and since cytochrome c oxidase in other systems does not contain non-heme iron (6), one would not expect our most highly purified fraction of cytochrome c oxidase to contain non-heme iron. Analysis indicates that the total iron concentration approximates that of the heme iron. Therefore, the enzyme is not heavily contaminated with non-heme iron.

### DISCUSSION

Purification of cytochrome c oxidase from rat liver mitochondria has yielded an enzyme which is suitable for the study of its biosynthesis by isolated mitochondria. The procedure includes two resins, cytochrome c-Sepharose and polyl-(L-lysine)-Sepharose, which we have attempted to use as affinity resins. While the precise interactions between the resins and the enzyme during purification are not known, some observations are worth noting. First, ionic interactions seem to be involved during purification of the enzyme using cytochrome c-Sepharose. The p1 of the cytochrome c is 10.65 while that of the rat liver oxidase was found to be 5.6. Thus, upon chromatography at pH 7.0, resin-linked substrate and enzyme have opposite net charges and, as expected, increases in salt concentration are needed to release bound enzyme. Also, the ability of this resin to bind cytochrome c oxidase in preference to cytochromes b and c₁ depends on the pH at which the Sepharose is activated and linked to cytochrome c. When

---

### TABLE V

| Table V
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay for contaminating enzymes in cytochrome c oxidase</strong></td>
</tr>
<tr>
<td>Enzyme activities were determined as described under &quot;Experimental Procedures.&quot; Abbreviations used are: Mt, mitochondria; Polylsine, poly(L-lysine)-Sepharose eluate.</td>
</tr>
<tr>
<td>Contaminating enzyme</td>
</tr>
<tr>
<td>CoQH₂-cytochrome c reductase</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
</tr>
<tr>
<td>Succinic acid dehydrogenase</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>---</td>
</tr>
</tbody>
</table>

1. unit = 1 μmol of substrate converted to product per min.
2. Polylsine = poly(L-lysine)-Sepharose eluate: this fraction had a heme a + a₃/protein = 12.4 nmol/mg.

---

### FIG. 7

The effect of carbon monoxide on the spectral properties of cytochrome c oxidase. Carbon monoxide was bubbled through the oxidase preparation for 10 min. The solution was then reduced with sodium dithionite and analyzed using reduced cytochrome oxidase as reference solution.

### TABLE VI

<table>
<thead>
<tr>
<th>Experiment 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (μmol)</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>-Antiserum</td>
</tr>
<tr>
<td>+Antiserum, no preincubation</td>
</tr>
<tr>
<td>+Antiserum, 30-min preincubation</td>
</tr>
</tbody>
</table>

**Experiment 2**

<table>
<thead>
<tr>
<th>Solubilized mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (μmol)</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>-Antiserum</td>
</tr>
<tr>
<td>+Antiserum, no preincubation</td>
</tr>
<tr>
<td>+Antiserum, 30-min preincubation</td>
</tr>
</tbody>
</table>

* Activity is expressed as picomoles of ATP converted to ADP in 15 min, see "Experimental Procedures."
prepared at pH 8.5 to 9.5, the cytochrome c-Sepharose binds all three hemoproteins in the presence of 100 mM phosphate, 1% Tween 80. However, when this resin is prepared at pH 6.5 to 7.5, it binds cytochrome c oxidase, but not cytochromes b or c₁ in the presence of this same buffer. The preferential binding of enzyme under these conditions seems to involve a hydrophobic interaction since replacement of the Tween 80 by Triton X-100 results in ready elution of the cytochrome c oxidase from this resin. The significant difference in structure between these two detergents is that Triton X-100 contains an aromatic phenol moiety while Tween 80 does not. Thus, it may be that there is a significant aromatic interaction between the respective hemes of cytochrome c oxidase and the cytochrome c-Sepharose during binding to the resin and Triton X-100 may effect elution because its aromatic moiety interferes with this interaction.

The complete purification method provides an enzyme preparation of high purity based on several criteria. Its heme a + a₈ content (10.5 to 13.4 nmol/mg of protein) is one of the highest reported for cytochrome c oxidase of comparable peptide composition obtained from any biological system (3, 10-12, 14, 34-38). The preparation contains no detectable cytochrome b, c, or c₁, as judged by spectral examination. It contains no measurable succinic acid dehydrogenase or coenzyme Q-cytochrome c reductase activity, appears to be free of ATPase and in contrast to the preparation of Penniall (30, 31) is also free of NADH dehydrogenase activity. Finally, the enzyme preparation migrates as a single band when analyzed by polyacrylamide gel electrophoresis under nondissociating conditions.

When the enzyme preparation is analyzed by SDS-polyacrylamide gel electrophoresis, it is resolved into six polypeptides having apparent Mᵦ of 66,000, 39,000, 23,500, 14,000, 12,500, and 10,000. Table VII shows that preparations of this enzyme from other sources are similar in that they all consist of six to seven polypeptides of comparable molecular weights. Their molecular weight profiles also agree well with that of the rat liver enzyme except that the latter contains a high molecular weight species of 66,000. Because of this molecular weight discrepancy, we have considered the possibility that this peptide is a contaminant which is not physically associated with the oxidase, but, rather, merely co-purifies with it. However, analysis indicates that the known membrane enzymes are absent and the enzyme preparation does migrate as a single protein band when analyzed by polyacrylamide gel electrophoresis under nondissociating conditions.

Studies on Cytochrome c Oxidase from Rat Liver Mitochondria

While this procedure yields an enzyme preparation of high purity, the turnover rate of the enzyme decreases during purification to 23 to 30% of its value in intact mitochondria. Pertinent to this, Triton X-100 inhibits enzyme activity some 46% (Table IV). The extent of inhibition is very similar to the apparent 44% loss of enzyme activity obtained upon cytochrome c-Sepharose chromatography which is the step at which Triton X-100 is introduced into the purification procedure. This suggests that the enzyme activity at this step and all subsequent steps is inhibited by the presence of Triton X-100. Another factor which appears to be important for enzyme activity is the enzyme-associated lipids. Purification procedures which result in decreased enzyme turnover rate also result in removal of significant amounts of phospholipid (Table III). These phospholipids may provide a somewhat hydrophobic or quasi-membranous environment required for optimal activity. This suggestion is supported somewhat by the increased enzyme activity obtained when preliminary attempts to restore activity by adding phospholipids (or Tween 80) to the purified enzyme were carried out (Table IV). Similar results have been obtained with the yeast and beef heart enzymes (36, 41). Thus, while it is impossible at this point to quantify the various parameters responsible for the apparent decrease in turnover rate of the enzyme during purification (Table I), it seems likely that inactivation due to the presence of Triton X-100 is very important and the removal of phospholipids may also contribute significantly.

An important question, as yet unanswered, is whether all of the polypeptides observed in the various oxidase preparations (Table VII) have a role in enzyme activity. This is a difficult question to answer at the moment, in part, because of our inability to precipitate by antibodies to individual polypeptides of the "true" enzyme complex. Thus, it is possible to detect such contaminants by comparing the polypeptide composition of the enzyme preparation with that of immuno precipitates obtained from treating the enzyme preparation with antibodies to individual polypeptides of the enzyme. Such an approach has been very useful in studying the composition of yeast cytochrome c oxidase (39) as well as gaining insight into its topography (40).

### Table VII

<table>
<thead>
<tr>
<th>Rat liver</th>
<th>Saccharomyces cerevisiae</th>
<th>Neurospora crassa</th>
<th>Beef heart</th>
<th>Locusta migratoria</th>
<th>Xenopus laevis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>daltons (× 10⁻²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>9.5</td>
<td>9.5</td>
<td>10.0</td>
<td>7.3</td>
<td>4.4</td>
</tr>
<tr>
<td>12.5</td>
<td>10.2</td>
<td>12.5</td>
<td>10.6</td>
<td>11.5</td>
<td>8.2</td>
</tr>
<tr>
<td>14.0</td>
<td>13.0</td>
<td>14.0</td>
<td>12.3</td>
<td>14.0</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>13.8</td>
<td>14.6</td>
<td>16.0</td>
<td>15.0</td>
<td>8.0</td>
</tr>
<tr>
<td>23.0</td>
<td>25.0</td>
<td>23.0</td>
<td>24.7</td>
<td>21.0</td>
<td>17.0</td>
</tr>
<tr>
<td>39.0</td>
<td>40.0</td>
<td>42.0</td>
<td>42.4</td>
<td>41.0</td>
<td>21.0</td>
</tr>
<tr>
<td>66.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19.0</td>
</tr>
</tbody>
</table>

* This report.
* Ref. 3.
* Ref. 10.
* Ref. 36.
* Ref. 37.
* Ref. 12.
* Ref. 38.
relative inexperience with multicomponent membrane-associated enzymes. In a single polypeptide enzyme, only a portion of its structure actually constitutes the catalytic site. However, the remainder of the molecule is clearly needed for the formation of that site and also can affect its activity. It is more difficult to delineate what constitutes the “complete enzyme” in a multicomponent enzyme because of the lack of defined limits such as is given by the covalent bonding of a single polypeptide enzyme. In the case of cytochrome oxidase, most experimental evidence suggests that the heme prosthetic groups are bound to the smaller (<20,000 daltons) polypeptides (5, 36, 42, 43), although Tzagoloff et al. (44) reach a different conclusion. Poyton and Schatz (39) and Kytan and Schatz (40) have demonstrated that antibodies to two of the larger polypeptides of the yeast enzyme (>20,000 daltons) inhibit catalytic activity. These authors interpret this to mean that these polypeptides are involved with the catalytic site. Phan and Mahler (36, 43) have been able to remove at least two of the large polypeptide components of the enzyme and retain 73% of its activity. These authors emphasize, therefore, that these large polypeptides are not part of the catalytic site and suggest that they may be involved either in assembly of the catalytic polypeptides or as “regulatory-integrative” components of the enzyme. At present, then, the data do not rule out participation of the large subunits in catalytic activity, nor does it rule it in. Rather, the role of the various polypeptides of this enzyme with respect to its activity remains to be defined and probably will not be resolved until reconstitution of the enzyme can be accomplished from individual polypeptides.

Acknowledgments—We wish to thank Mr. Alex Bonica for assistance in the preparation and characterization of the cytochrome c-Sepharose affinity column as well as very helpful discussion during the course of this investigation, and Drs. Lucille Smith and Diana Beattie for critical evaluation of the manuscript.

REFERENCES

Purification and characterization of cytochrome c oxidase from rat liver mitochondria.
R J Rascati and P Parsons


Access the most updated version of this article at http://www.jbc.org/content/254/5/1586

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/5/1586.full.html#ref-list-1