Biosynthesis of Cytochrome c Oxidase by Isolated Rat Liver Mitochondria*

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When isolated mitochondria which have been labeled with [3H]leucine are solubilized and treated with antiserum specific for cytochrome c oxidase, labeled polypeptides which correspond to the three largest polypeptides of this enzyme are immunoprecipitated. This indicates that the three largest polypeptides of cytochrome c oxidase which have $M_r$ of 66,000, 39,000, and 23,000 are synthesized by isolated mitochondria whereas the three smallest ones which have $M_r$ of 14,000, 12,500, and 10,000 are not. The smallest polypeptides are probably synthesized on cytoplasmic ribosomes as has been demonstrated in other systems by in vitro studies. These results are the first demonstration that isolated mammalian mitochondria are capable of synthesizing some of their own polypeptide components.

The antiserum used in this study was prepared to highly purified cytochrome c oxidase (12.4 nmol of heme $a + a_3$/mg of protein) from rat liver mitochondria. This antiserum gives a single precipitin line when tested by the Ouchterlony double diffusion technique. Its specificity has been demonstrated by the fact that: 1) only precipitates heme $a + a_3$ not hemes $b$, $c$, or $c_{1}$, when added to solubilized mitochondria, 2) inhibits cytochrome c oxidase activity at least 85%, and 3) precipitates only those polypeptides found in purified cytochrome c oxidase when added to solubilized mitochondria labeled in vivo.

It has been demonstrated by in vivo experiments that mitochondria from several sources are capable of synthesizing some of their peptide components (1-9). At least three mitochondrial enzyme complexes, cytochrome c oxidase, coenzyme $Q_{10}$-cytochrome $c$ reductase, and oligomycin-sensitive ATPase have been shown to contain translation products of both mitochondrial and cytoplasmic ribosomes (2-6, 9). Thus, these enzymes are a result of coordinate function between two separate cellular protein synthesis systems. Such in vivo studies are dependent on the use of cycloheximide to inhibit protein synthesis on cytoplasmic ribosomes, and chloramphenicol to inhibit protein synthesis on mitochondrial ribosomes, so that polypeptide translation products of these two systems can be studied independently of each other. This approach has provided considerable information, but the possibility of nonspecific side effects of these drugs (10, 11) has prompted us to develop a protein synthesis system using isolated rat liver mitochondria to study the products of mitochondrial translation. This circumvents possible problems inherent in using inhibitors, ensures that the products are indeed synthesized in mitochondria and provides information as to whether this organelle can synthesize completed peptides in the absence of the intact cell. In addition, the results from these in vitro studies can be used to complement those obtained from in vivo studies. The rat liver system also offers the opportunity to compare mitochondrial protein synthesis in a mammalian system to that in lower organisms which have been used to obtain most of the data available to date.

The approach has been to: 1) incubate isolated mitochondria with radioactive leucine, 2) solubilize the labeled mitochondria with detergent, 3) add antibodies specific for cytochrome c oxidase, and 4) analyze the immunoprecipitates by SDS-polyacrylamide gel electrophoresis to determine whether any labeled peptides present correspond in molecular weight to completed cytochrome c oxidase peptide components. A similar approach has been used by Poyton and Groot (12) for the yeast system.

The purification and characterization of cytochrome c oxidase from rat liver mitochondria was reported in the previous paper (13). The present communication describes the biosynthesis of polypeptides by isolated mitochondria, the preparation and characterization of antiserum specific for cytochrome c oxidase, and the use of this antiserum to study the synthesis of this enzyme by isolated mitochondria. The results obtained indicate that the three largest polypeptides of rat liver cytochrome c oxidase are synthesized by isolated mitochondria whereas the three smallest ones are not. These results are analogous to those obtained using the yeast system (12) and are the first demonstration that isolated mammalian mitochondria are capable of synthesizing some of their own polypeptide components. Some of this work has been reported previously in preliminary form (14).

**EXPERIMENTAL PROCEDURES**

Materials

White Sprague-Dawley rats of both sexes weighing 100 to 160 g were obtained from Holtzman Laboratories, Madison, WI. Lactoperoxidase, N,N,N',N'-tetramethylmethylenediamine, glycine, sodium dodecyl sulfate, and Triton X-100 were obtained from Sigma. Methylenbisacrylamide and ammonium persulfate were obtained from Bio-Rad. Di-cyclodecyl isovalerate and Triton-X-100 were obtained from Dupont. Acrylamide was obtained from ICN. BBOT (2,5-bis[2-(5-tert-butylbenzoxa-...
Method

Preparation of Antiserum—Antiserum was prepared essentially as described by Mason et al. (15). Control serum was collected prior to injection of the cytochrome c oxidase antigen. Purified cytochrome c oxidase was sonicated for 1 min at maximum power in the Branson Sonifier method of Smith and Conrad (16). S-175 and the submitochondrial particles were collected by centrifugal weighing about 100 g. After 4 h, the animal was killed, and enzyme was assayed at zero time and after 30 min according to the method of Smith and Conrad (16). 1.0 ml and incubated overnight at 0°C. After centrifugation of the supernatant fraction of solubilized mitochondria after immunoprecipitation revealed that the addition of control serum did not precipitate any heme (Fig. 2A) whereas the addition of antiserum precipitated at least 90% of the heme. As shown in Fig. 2B, Specificity was also demonstrated by the ability of antiserum to inhibit cytochrome c oxidase activity. As shown in Table 1, addition of control serum caused no significant inhibition of enzyme activity while antiserum resulted in at least 85% inhibition after incubating under conditions which prevent precipitation. Finally, antiserum specificity was demonstrated by its ability to precipitate only cytochrome c oxidase from solubilized mitochondria which had been labeled in vivo with [14C]leucine (Fig. 3).

Preparation of Specific Antiserum against Cytochrome c Oxidase—Antiserum prepared against the most highly purified fraction of cytochrome c oxidase (13) was tested for purity by the Ouchterlony double diffusion test. Only those samples which exhibited a single precipitin line when tested against solubilized mitochondria were used subsequently. When antiserum was titrated for its ability to precipitate heme a + a3, it was found (Fig. 1) that 0.2 ml of antiserum/nmol of heme a + a3 was required to obtain 100% precipitation. All subsequent immunoprecipitations were performed using this ratio of antiserum to heme a + a3.

In Vivo Labeling of Mitochondrial Proteins—Three milliliter of [3H]leucine (50 Ci/mmol) was injected intraperitoneally into an animal weighing about 100 g. After 4 h, the animal was killed, and mitochondria were prepared as described (13). The mitochondria were sonicated for 1 min at maximum power in the Branson Sonifier. The submitochondrial particles were collected by centrifugation for 1 h at 100,000 g. The particles were resuspended in 0.25 M sucrose at 20 mg/ml and solubilized with sodium cholate and KCl as described above. Purified cytochrome c oxidase (13) which had been labeled with [14C]leucine was added to the solubilized particles in order to provide an internal reference marker upon subsequent polyacrylamide gel electrophoretic analysis. The mixture was incubated overnight with control serum, centrifuged to remove any precipitated material, and subsequently treated in the same manner with specific antiserum. The resulting immunoprecipitate was washed five times with 200 mM sodium phosphate, pH 7.0, containing 1% Triton X-100 and treated with 5% SDS, 0.1% 2-mercaptoethanol at room temperature until precipitates were completely dissolved. Glycerol, bromophenol blue, and sodium phosphate, pH 7.0 (final concentration, 0.05 M) were added to the samples; they were heated at 100°C for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis.

In Vitro Labeling—Freshly isolated mitochondria (13) were incubated at a concentration of 0.5 to 1.0 mg/ml in a mixture modified from Coote and Work (17) which contained: 30 mM Bicine, 15 mM potassium phosphate, 10 mM magnesium acetate, 90 mM potassium chloride, 5 mM ATP, 0.5 mM NAD, 20 mM nicotinamide, 35 mM ammonium sulfate, 50 μM each amino acid (except leucine), and 100 μCi/ml of [3H]leucine (5 mCi/μmol). pH 7.6. When included, cycloheximide or chloramphenicol were present at concentrations of 200 μg/ml or 1.0 mg/ml, respectively. Labeling was initiated by the addition of mitochondrion and carried out at 37°C. After labeling for either 5 or 20 min, the incubation was "chased" for 5 or 10 min, respectively, with a 500-fold excess of unlabeled leucine. After pelleting the labeled mitochondria, they were washed twice with 0.25 M sucrose, 10 mM unlabeled leucine, and sonified as described above. After sequential treatment with control serum followed by antiserum, the resulting immunoprecipitate was washed and prepared for SDS-gel electrophoresis as described above.

Polyacrylamide Gel Electrophoresis—Gel electrophoresis was carried out as previously described (13). Gels containing radiolabeled polypeptides were divided into 1-mm slices. Each slice was incubated overnight at 37°C in a capped glass scintillation vial containing 10 ml of scintillation fluid, consisting of 0.5% 2,5-diphenyloxazole, 0.005% 1,4-bis(2-(5-phenyloxazoly)benzene, and 3% Protosol in toluene. The incubated samples were monitored for 14C and 125I content under conditions yielding 20% efficiency for 14C with no detectable spillover into the 125I channel and 40% efficiency for 125I with equal counting rates in both channels.

RESULTS

Preparation of Specific Antiserum against Cytochrome c Oxidase—Antiserum prepared against the most highly purified fraction of cytochrome c oxidase (13) was tested for purity by the Ouchterlony double diffusion test. Only those samples which exhibited a single precipitin line when tested against solubilized mitochondria were used subsequently. When antiserum was titrated for its ability to precipitate heme a + a3, it was found (Fig. 1) that 0.2 ml of antiserum/nmol of heme a + a3 was required to obtain 100% precipitation. All subsequent immunoprecipitations were performed using this ratio of antiserum to heme a + a3.

Specificity was also demonstrated by the ability of antiserum to inhibit cytochrome c oxidase activity. As shown in Table 1, addition of control serum caused no significant inhibition of enzyme activity while antiserum resulted in at least 85% inhibition after incubating under conditions which prevent precipitation. Finally, antiserum specificity was demonstrated by its ability to precipitate only cytochrome c oxidase from solubilized mitochondria which had been labeled in vivo with [3H]leucine (Fig. 3).

FIG. 1. Titration of antiserum against cytochrome c oxidase. The titration was carried out as described under "Methods."
Studies on Cytochrome c Oxidase from Rat Liver Mitochondria

Protein Synthesis by Isolated Mitochondria—Several different incubation conditions were tested for their ability to support protein synthesis in isolated mitochondria. The best results were obtained with a modification of the system described by Coote and Work (17) and are shown in Fig. 4. Synthesis displayed linear kinetics for about 1 h and resulted in the incorporation of 72 pmol of leucine/mg/h. Synthesis was inhibited 10 to 15% by cycloheximide, which may indicate a small amount of contamination by cytoplasmic ribosomes. However, chloramphenicol inhibited the reaction 80 to 85%, indicating that the protein is synthesized primarily on mitochondrial ribosomes. Analysis of the polypeptide products

Table I

**Inhibition of cytochrome c oxidase activity by antiserum prepared to highly purified cytochrome c oxidase**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Specific activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>210.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Solubilized mitochondria + Control serum</td>
<td>200.0</td>
<td>85.0</td>
</tr>
<tr>
<td>+ Antiserum</td>
<td>31.5</td>
<td>85.0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>169.0</td>
<td>0</td>
</tr>
<tr>
<td>Solubilized mitochondria + Control serum</td>
<td>169.0</td>
<td>0</td>
</tr>
<tr>
<td>+ Antiserum</td>
<td>23.7</td>
<td>86.0</td>
</tr>
</tbody>
</table>

Activity is expressed as the first order rate constant = \( \ln(A_f/A_i) \) / protein (mg/ml).

Figure 2. Selective precipitation of heme a + a₃ by antiserum against cytochrome c oxidase. The experiment was carried out as for Fig. 1. A, 0.2 ml of control serum added to solubilized mitochondria containing 1 nmol of heme a + a₃ in a reaction volume of 1 ml. B, 0.2 ml of antiserum added to solubilized mitochondria containing 1 nmol of heme a + a₃, in a reaction volume of 1 ml. Absorbance in the 601 nm region is due to heme a + a₃. Absorbances in the 550 nm and 520 nm regions are due to hemes b, c, and c₃.

Figure 3. In vivo labeling of cytochrome c oxidase. Mitochondria, labeled in an intact animal with \(^{3}H\)leucine, were solubilized, mixed with \(^{125}I\) labeled purified cytochrome c oxidase, and treated with specific antiserum to cytochrome c oxidase as described under "Methods." The immunoprecipitates were then analyzed by SDS-polyacrylamide gel electrophoresis. **O**, \(^{125}I\)-labeled cytochrome c oxidase added as an internal standard; **.-.-.**, \(^{3}H\)leucine.

Figure 4. Effect of cycloheximide and chloramphenicol on the kinetics of \(^{3}H\)leucine incorporation into isolated mitochondria. The experiment is described under "Methods." **-** - control; **-** - plus cycloheximide; **-** - plus chloramphenicol; **-** - plus cycloheximide and chloramphenicol.
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FIG. 5. A, in vitro labeling of total mitochondrial protein with [3H]leucine. Isolated mitochondria were labeled with [3H]leucine for 20 min, “chased” with unlabeled leucine for 10 min, solubilized, and analyzed by SDS-polyacrylamide gel electrophoresis as described under "Methods." B, isolated mitochondria were labeled with [3H]leucine, solubilized, and treated with specific antiserum to cytochrome c oxidase as described under "Methods." The immunoprecipitates were then analyzed by SDS-polyacrylamide gel electrophoresis. C, purified cytochrome c oxidase (13) was labeled with [125I] as described under "Methods" and analyzed by SDS-polyacrylamide gel electrophoresis. This preparation has not been immunoprecipitated and the gel profile shows disproportionately high amounts of label in the region of the three smallest polypeptides. This reflects the presence of Triton X-100 which becomes labeled during the iodination of the enzyme and migrates in this region.

TABLE II
Distribution of [3H]leucine incorporated into polypeptides by isolated mitochondria

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incorporation</th>
<th>Protein Specific activity</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>mg</td>
<td>cpm/mg</td>
</tr>
<tr>
<td>Intact mitochondria</td>
<td>185,000</td>
<td>185</td>
<td>1,000</td>
</tr>
<tr>
<td>Membrane (SMP)</td>
<td>167,000</td>
<td>102</td>
<td>1,637</td>
</tr>
<tr>
<td>Soluble proteins</td>
<td>15,000</td>
<td>77</td>
<td>195</td>
</tr>
</tbody>
</table>

a Fractions were prepared as previously described (13).
b Labeling was for 15 min followed by a 5-min "chase" with 500-fold excess of unlabeled leucine.
SMP, submitochondrial particle.

TABLE III
Immunoprecipitation of [3H]leucine-labeled polypeptides from isolated mitochondria by antiserum against cytochrome c oxidase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Labeling conditions</th>
<th>Incorporation</th>
<th>Protein Specific activity</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Chloramphenicol</td>
<td>cpm</td>
<td>% total</td>
<td>cpm/mg</td>
</tr>
<tr>
<td>Solubilized mitochondria</td>
<td>609,000</td>
<td>100</td>
<td>70,000</td>
<td>100</td>
</tr>
<tr>
<td>Control serum precipitate</td>
<td>472</td>
<td>0.07</td>
<td>383</td>
<td>0.55</td>
</tr>
<tr>
<td>Antiserum precipitate</td>
<td>17,292</td>
<td>2.8</td>
<td>278</td>
<td>0.39</td>
</tr>
</tbody>
</table>

a Labeling was carried out as described under "Methods."
b 0.2 ml of control serum added to solubilized mitochondria containing 1 nmol of heme a + a3 in a reaction volume of 0.52 ml.
c 0.2 ml of antiserum added to solubilized mitochondria containing 1 nmol of heme a + a3 in a reaction volume of 0.52 ml.

from such incubations revealed that over 90% of the label is found in the membrane fraction, while only 8% of the label enters soluble polypeptides and these are of low specific activity (Table II). The results of electrophoresis of solubilized labeled mitochondria on SDS-polyacrylamide gels reveal that virtually all of the labeled polypeptides range in M, between 20,000 and 70,000. Little or no synthesis of peptides smaller than 20,000 occurred under the conditions used (Fig. 5A). This suggests that if isolated mitochondria do synthesize polypeptides of cytochrome c oxidase, they must all have M, higher than 20,000.

To determine whether this spectrum of labeled peptides included cytochrome c oxidase peptides, the labeled mitochondria were solubilized and treated sequentially with control serum followed by antiserum to cytochrome c oxidase. During labeling, a "chase" with unlabeled leucine was used in an attempt to ensure that all labeled peptides were completed peptide chains. As shown in Table III, significant amounts of radiolabeled polypeptides were precipitated only with antiserum. In addition, the synthesis of these precipitated polypeptides was more than 98% sensitive to chloramphenicol, indicating that they were synthesized on mitochondrial ribosomes. Electrophoretic analysis of the immunoprecipitates reveals three polypeptides with M, of 66,000, 42,000, and 24,000 (Fig. 5C). These correspond well within experimental limits to the three largest polypeptide components of highly purified cytochrome c oxidase (Fig. 5C). Therefore, isolated mitochondria synthesize polypeptides which are identical in size with the three largest components of cytochrome c oxidase and which are precipitated by specific antiserum prepared against that enzyme. The small amount of protein synthesized having a M, range of 12,000 to 14,000 represents less than 4% of the total protein synthesized and does not seem to indicate that the small polypeptides of this enzyme are synthesized on mitochondrial ribosomes.

DISCUSSION

Ever since the discovery of DNA in mitochondria (18),
there has been a continuing interest in the extent of the autonomy of these organelles. The mitochondrial DNA in lower organisms is much larger than that of higher organisms and thus potentially contains more genetic information (19). This large difference in mitochondrial genome size raises the question of whether investigation will reveal substantial differences in the gene content of these DNAs. It may be, for example, that much of the mitochondrial DNA in lower organisms is "spacer DNA" with no genetic function as suggested by Bernardi et al (20). Hybridization studies have established that mitochondrial ribosomal and transfer RNAs are coded by the mitochondrial genome in both higher and lower organisms (18, 21). Many studies have been done to gain insight into the possibility that this genome also codes for mitochondrial proteins. In lower eukaryotes, it has been demonstrated by in vivo experiments that polypeptide components of at least three mitochondrial membrane-bound enzyme complexes, cytochrome c oxidase, coenzyme QH2-cytochrome c reductase, and oligomyelin-sensitive ATPase are synthesized on mitochondrial ribosomes (2-6, 9). Recently, Poyton and Groot have demonstrated that isolated mitochondria from yeast are also capable of the synthesis of polypeptide components of the cytochrome oxidase complex (12). With regard to mammalian cells, several workers (17, 22, 23) have demonstrated that isolated mitochondria can synthesize polypeptides of similar size to ones obtained in vivo, but it has not been clear from these studies whether the polypeptides from the two systems are identical.

In the present study, antiserum to highly purified cytochrome c oxidase selectively precipitated six polypeptides from total solubilized mitochondria which had been labeled in vivo. These six polypeptides co-migrated with highly purified cytochrome c oxidase on SDS-polyacrylamide gels. When polypeptides synthesized by isolated mitochondria were treated with this same antiserum, selective precipitation of three polypeptides occurred. These also co-migrated in SDS-polyacrylamide gels with the three largest components of highly purified oxidase. Thus, this is the first evidence that polypeptides synthesized by isolated mammalian mitochondria are apparently identical with those synthesized in vivo. In addition, while there have been conflicting results in the literature as to whether isolated mitochondria from higher systems are capable of the synthesis of completed polypeptide chains (24), our results seem to indicate within the limits of gel analysis that they are able to do so.

In addition to demonstrating that isolated mitochondria are capable of the biosynthesis of completed polypeptides, these experiments indicate that of the six polypeptides present in cytochrome c oxidase, only the three largest ones having molecular weights greater than 20,000 are made in this organelle. This agrees well with the results of Poyton and Groot (12) who found that only the three largest components of the seven polypeptides in the corresponding yeast enzyme are synthesized by isolated mitochondria. Presumably the polypeptides having molecular weights less than 20,000 are synthesized on cytoplasmic ribosomes. This is consistent with the notion of coordinate synthesis between the cytoplasmic and mitochondrial protein synthesis systems and agrees with results obtained in vivo using metabolic inhibitors (2-6, 9). While it had been suggested that the side effects of such inhibitors might obscure the true results (10, 11), the agreement found between in vivo studies and those using isolated mitochondria indicates that this need not be a problem.

While these experiments using isolated mitochondria emphasize that these organelles have a certain degree of autonomy with respect to protein synthesis, the fact that they can synthesize bona fide mitochondrial polypeptides cannot be taken as evidence that these polypeptides are coded in the mitochondrial genome. Such evidence does seem to be forthcoming, nonetheless. The possibility that the polypeptides synthesized on mitochondrial ribosomes are the products of nuclear genes has been considered unlikely because of a lack of evidence for transport of nuclear mRNAs into mitochondria (25). Moreover, several laboratories have demonstrated the presence of poly(A)-containing RNA in mitochondria which hybridizes with mitochondrial DNA (26-28). These are presumably mitochondrial mRNAs to be used for translation on mitochondrial ribosomes. The most definitive evidence has come from cell-free synthesis studies directed by mitochondrial mRNA. Padmanaban et al. (29) have isolated an mRNA fraction from yeast mitochondria capable of directing the synthesis of the three largest polypeptide components of cytochrome c oxidase. This mRNA fraction hybridizes preferentially to mitochondrial DNA, suggesting these polypeptides are indeed products of the mitochondrial genome. Similar experiments using rat liver mitochondria are in progress in our laboratory to obtain information on the extent to which polypeptides are coded by the mitochondrial genome of higher organisms.

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