Biosynthesis of Polypeptides of Cytochrome c Oxidase by Isolated Mitochondria

(mitochondrial biogenesis/protein synthesis in vitro/cytochrome c oxidase subunits)

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ABSTRACT Yeast mitochondria, incubated with radioactive amino acids in a "protein-synthesizing mixture" containing an oxidizable substrate and an ATP-regenerating system, have been shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to incorporate label into polypeptides equivalent in molecular weight and relative amount to those made in vitro in the presence of cycloheximide. The ability of these isolated mitochondria to synthesize "native" polypeptides was assessed by examining the incorporation of label into subunits of cytochrome c oxidase (EC 1.9.3.1). An analysis of immunoprecipitates formed by incubating cholate extracts of labeled mitochondria with an antiserum against holocytochrome c oxidase revealed that label was incorporated into three polypeptides of sizes equivalent to those of cytochrome c oxidase subunits I, II, and III, shown from earlier studies in vitro to be translated on mitochondrial ribosomes. Further evidence that these polypeptides made in vitro are "native" and identical to subunits I, II, and III was provided by the observation that labeled polypeptides equivalent in size to subunits I–III are also immunoprecipitated by antiserum against subunits V+VII, an antiserum that can precipitate subunits I, II, and III only when they are complexed to the cytoplasmically synthesized subunits, V and VII, of the enzyme. These results suggest that isolated mitochondria are capable of synthesizing three subunits of cytochrome c oxidase and assembling them into a holoenzyme.

The biogenesis and assembly of a respiratory competent mitochondrial results from the joint expression of two distinct genetic systems, mitochondrial and cytoplasmic, composed of two discrete genomes and two physically separated protein synthetic systems. Although the means by which the expression of these two genetic systems is mediated is, as yet, uncertain, recent studies have revealed the importance of the coordinated functioning of mitochondrial and cytoplasmic protein synthesis in mitochondrial biogenesis. This coordination is particularly obvious for three complexes of the inner mitochondrial membrane—cytochrome c oxidase, cytochrome b, and oligomycin-sensitive ATPase—whose synthesis and assembly requires translation products from both mitochondrial and cytoplasmic protein synthetic systems (1–5).

Recently, it has been shown by studies in vitro with whole cells that cytochrome c oxidase (EC 1.9.3.1; ferrocytochrome c:oxygen oxidoreductase) from Saccharomyces cerevisiae (3, 4) and Neurospora crassa (6) is composed of three polypeptides translated on mitochondrial ribosomes and four polypeptides translated on cytoplasmic ribosomes. In both S. cerevisiae and N. crassa, this duality of origin is reflected in the chemical properties of the polypeptides themselves: the mitochondrially made polypeptides are hydrophobic and are, presumably at least, partially buried in the membrane lipid bilayer, whereas the cytoplasmically made polypeptides are relatively hydrophilic (6, 7). Although the functions of these polypeptides remain obscure, genetic and immunological studies with the yeast enzyme strongly suggest that all seven polypeptides are bona fide subunits of cytochrome c oxidase (8).

In order to study mitochondrial protein synthesis in more detail and its coordination with cytoplasmic protein synthesis, we have sought to establish a mitochondrial protein synthetic system in vitro from yeast that is capable of synthesizing complete and "native polypeptides." After analyzing, by sodium dodecyl sulfate (NaDodSO4)–polyacrylamide gel electrophoresis, the polypeptides made in several previously described mitochondrial systems in vitro, we have found that a system modified from Grivell’s (9) faithfully mimics yeast mitochondrial protein synthesis in vitro. We have been able to show that the three large subunits, I–III, of cytochrome c oxidase are translated in vitro in this system and that the polypeptides synthesized in vitro are integrated with the cytoplasmically made subunits, IV–VII, into a holoenzyme.

MATERIALS AND METHODS

Strains and Growth Conditions. The S. cerevisiae strains used in this study were D273-10B, α[p+], obtained from Dr. F. Sherman (University of Rochester), and DT-XII [α−], diploid, kindly provided by Dr. L. Kovalč (Bratislava, Czechoslovakia). Cells were grown on the lactate growth medium described by Omishi et al. (10).

Isolation of Mitochondria. Cultures in early to mid-exponential growth phase were harvested by centrifugation and washed once with distilled water. After the cells were washed, they were suspended in 0.1 M Tris·HCl (pH 9.3)–2.5 mM dithiothreitol and incubated at 28° for 20 min. Subsequently, the cells were harvested, suspended at a concentration of 1 g wet weight of cells per ml in 1.35 M sorbitol–0.05 M citrate-phosphate buffer (pH 5.8), and converted to spheroplasts (11) by either Helicase (Industrie Biologique Française, Gennevilliers, France) or Glusulase (Endo Laboratories, Garden City, N.Y.). The spheroplasts were harvested by centrifugation, washed once with 1.35 M sorbitol, and carefully resuspended at three times their original culture density in growth medium supplemented with 1 M sorbitol. They were then incubated for 90 min at 28° with gentle agitation on a rotary
Labeling In Vitro. Prior to labeling, mitochondria, suspended at a concentration of 1–2 mg of protein per ml, were preincubated for 5 min at 2° in a “protein-synthesizing mixture” containing: 20 mM Tris·HCl, 150 mM KCl, 10 mM KH₂PO₄, 10 mM MgCl₂, 5 mM α-ketoglutarate, 5 mM ATP, 5 mM phosphoenol-pyruvate, 60 mM mannitol, 30 µg/ml of pyruvate kinase, and 3 mg/ml of bovine serum albumin, pH 6.7. Labeling was initiated by the addition of L-[4,5-³H]-leucine (3 Ci/mmole) to 0.1 mCi/ml. After 30 min, the incorporation of label was terminated by a 10-min incubation, in the presence of nonradioactive leucine (5 mM), followed by a 5-min chase with 50 µg of puromycin per ml. Labeled mitochondria were harvested and washed three times with 0.6 M mannitol–1 mM EDTA–5 mM leucine (pH 6.7), suspended in 100 mM sodium phosphate–5 mM leucine (pH 7.2), and converted to submitochondrial particles by sonication (12). The submitochondrial particles were processed immediately for either NaDodSO₄-polyacrylamide gel electrophoresis or immunoprecipitation.

Labeling In Vivo. Published procedures were used for labeling cells in the presence of cycloheximide and for growing cells in the presence of L-[U-¹⁴C]leucine (0.33 Ci/mmole) (12, 13).

Immunoprecipitation of Cytochrome c Oxidase. The isolation of cytochrome c oxidase by immunoprecipitation was performed as described (13). Antisera used for this study were prepared and characterized as described by Poyton and Schats (8). The antiserum against the holoenzyme crossreacted with cytochrome c oxidase subunits I, II, IV, V, VI, and VII while antiserum against subunits V+VII crossreacted with only subunits V and VII.

Miscellaneous Procedures. Published procedures were followed for measurement of mitochondrial protein synthesis (9) and protein (14), and for determination of the radioactivity of polyacrylamide gels (13). Polyacrylamide gel electrophoresis in NaDodSO₄ was carried out in the discontinuous buffer system described (7).

RESULTS

Products of Mitochondrial Protein Synthesis In Vitro. The incubation of a mitochondrial fraction from S. carlsbergensis in the presence of ATP, an oxidizable substrate, and an ATP-regenerating system results in the incorporation of radioactive amino acids into a protein fraction that has been characterized as mitochondrial by its sensitivity to chloramphenicol and erythromycin and by its insensitivity to cycloheximide and ribonuclease (9). When mitochondria that have been isolated from S. cerevisiae are incubated in this in vitro system, modified to contain α-ketoglutarate in place of succinate, essentially the same results are obtained. Incorporation of [¹⁴C]leucine into protein is sensitive to chloramphenicol, insensitive to cycloheximide (Table 1), and linear with time for about 30 min. Since virtually all of the radioactivity incorporated into protein in this in vitro system remains with the membrane fraction on conversion of mitochondria to submitochondrial particles and is only removed from the membrane fraction by detergent treatment, we conclude that the polypeptides made in vitro, like those made by mitochondria in vivo, are most likely hydrophobic proteins of the inner mitochondrial membrane.

An analysis of the products of mitochondrial protein synthesis in vitro by NaDodSO₄-polyacrylamide gel electrophoresis (Fig. 1) reveals the presence of four major peaks of radioactivity corresponding to molecular weights of approximately 42,000, 32,500, 23,500, and 12,000. The migration of in vitro translation products with in vivo translation products, obtained from cells labeled in the presence of cycloheximide, on the same gel demonstrates that the size of the polypeptides

<table>
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<th>Additions</th>
<th>Radioactivity incorporated (cpm/mg of protein per 20 min)</th>
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<tr>
<td></td>
<td>Exp. 1</td>
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<tr>
<td>None</td>
<td>3500</td>
</tr>
<tr>
<td>Chloramphenicol, 10 µg/ml</td>
<td>314</td>
</tr>
<tr>
<td>Cycloheximide, 20 µg/ml</td>
<td>3550</td>
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L-[¹⁴C]Leucine was incorporated into protein in the “protein-synthesizing mixture” described in Materials and Methods. Mitochondria were isolated from strain D273-10B (Exp. 1) or DT-XII (Exp. 2) and suspended at 1.0 mg of protein per ml.
Fig. 2. Titration of mitochondrial membrane extracts (strain DT-XII) labeled in vitro and in vivo with rabbit antiserum to yeast cytochrome c oxidase. Membranes labeled in vivo were obtained from cells labeled with [14C]leucine in the presence of cycloheximide, and membranes labeled in vitro, from mitochondria labeled with [3H]leucine. Aliquots of the isolated mitochondria (in vivo, specific activity = 4.3 \times 10^4 \text{ cpm/mg of protein}; in vitro, specific activity = 4.2 \times 10^4 \text{ cpm/mg of protein}) containing equal amounts of protein were mixed and extracted with cholate and KCl (13). Small aliquots (0.025 ml) of the clarified extracts were mixed with the amounts of antiserum indicated on the abscissa and incubated for 10 hr at +4°. The immunoprecipitates were isolated by centrifugation, washed three times with 0.2 M sodium phosphate (pH 7.0)-1% Triton X-100, dissolved in 0.7 ml of NCS solubilizer (Amersham-Searle Corp.), and counted. The values are corrected for unspecified precipitation obtained with control serum (less than 7%). ○, In vitro; ×, in vivo.

synthesized by mitochondria both in vivo and in vitro are virtually identical.

*Synthesis of Cytochrome c Oxidase.* As shown earlier (13) cytochrome c oxidase can be isolated by immunoprecipitation from crude clarified extracts of mitochondria prepared by treatment with cholate in the presence of 1.1 M KCl and removal of insoluble material by centrifugation. The clarified extracts used in our experiments routinely contained 80-90% of the mitochondrial cytochrome aa3, 50-70% of the mitochondrial protein, and 55-60% of the radioactivity incorporated either in vitro by isolated mitochondria or in vivo by cells incubated in the presence of cycloheximide. Immuno-titration (Fig. 2) of clarified extracts from mitochondria labeled in vitro indicates that approximately 12% of the extracted radioactive material is precipitable by cytochrome c oxidase antiserum. This represents about half of the radioactivity precipitable from mitochondrial extracts labeled in vivo in the presence of cycloheximide. The explanation of this lower plateau value for mitochondria labeled in vitro remains to be elucidated but may reflect a lower rate of synthesis or turnover of cytochrome c oxidase in vitro.

Analysis of the radioactive protein immunoprecipitated from mitochondria labeled in vitro by NaDodSO4-polyacrylamide gel electrophoresis (Fig. 3, top) reveals that virtually all of the radioactivity migrates with subunits I, II, and III of cytochrome c oxidase that have been isolated by immunoprecipitation from cells labeled in vivo. This finding, together with the similarity in stoichiometries of these three polypeptides for mitochondria labeled in vivo and in vitro, strongly suggests that isolated mitochondria in our in vitro system are capable of synthesizing complete polypeptide subunits of cytochrome c oxidase.

While the electrophoretic pattern shown in Fig. 3 (top) is quite reproducible, occasionally a fourth peak of radioactivity is observed. Since this peak generally represents only a small percent of the total immunoprecipitable radioactivity and migrates with an apparent molecular weight of 6,000-10,000, it most likely consists of either incomplete polypeptide chains or proteolytic degradation products of subunits I, II, or III. The presence of this low molecular weight material in immunoprecipitates always coincides with the presence of similar low molecular weight material in NaDodSO4 gel electrophoretic patterns of total membrane proteins.

*Integration of Polypeptides Labeled In Vitro into Holocytochrome c Oxidase.* The use of antiserum to the holoenzyme in the above studies could not distinguish between the synthesis of subunits I, II, and III and their integration into the holoenzyme since this antiserum is capable of precipitating both integrated and unintegrated subunits. In order to determine the state (i.e., integrated compared with un-integrated) of the subunits synthesized in vitro and to use integration as a further criterion for establishing the “nativeness” of polypeptides synthesized in vitro, we have used an antiserum against subunits V+VII, which is specific for two cytoplasmically made...
subunits that can precipitate subunits I, II, and III only when they are integrated into a complex with either or both of these cytoplasmically made subunits (8). An analysis of the immunoprecipitates formed by incubating this antiserum with crude extracts of mitochondria labeled in vitro (Fig. 3, bottom) shows a distribution of radioactivity similar to that obtained with an antiserum to the holoenzyme. Thus, it appears that subunits I, II, and III labeled in vitro are "native" enough to combine with at least one, and possibly all four, of the cytoplasmically made subunits of the enzyme.

Our attempts to quantitate the distribution of subunits labeled in vitro between integrated and unintegrated states have been complicated by the finding that antiserum against subunits V+VII is less effective in immunoprecipitation than is antiserum to the holoenzyme (Table 2; see ref. 8). However, a rough determination of the degree of integration is afforded by comparing the immunotitration plateau values of both sera for mitochondria labeled in vitro and in vivo. Since it appears that subunits I, II, and III, which are present in immunoprecipitates from mitochondria labeled in vitro, are fully integrated (15), and that the decrease in the percentage of radioactivity precipitated by antiserum against subunits V+VII relative to antiserum against holoenzyme merely reflects a lesser number of antibody–antigen components in the system (8), we have used the ratio of radioactivity precipitated by anti-V+VII:anti-holoenzyme in vitro normalized to the ratio of radioactivity precipitated by anti-V+VII:anti-holoenzyme in vivo as a measure of the degree of integration of subunits I, II, and III labeled in vitro. As can be seen from Table 2, the ratio of radioactivity precipitated by anti-V+VII:anti-holoenzyme is 0.35 for mitochondria labeled in vitro and 0.46 for mitochondria labeled in vivo. Therefore, we conclude that the majority (i.e., 0.35/0.46 = 76%) of subunit I, II, and III molecules labeled in vitro are in the integrated state.

**DISCUSSION**

Although protein synthesis by isolated mitochondria was reported as early as 1958 (16), there has been a continuing controversy regarding the nature of polypeptides synthesized in vitro, particularly those isolated from higher eukaryotic cells (17). It has even been suggested by Hochberg et al. (18) that mitochondrial protein synthesis in vitro may be an artifact resulting from the binding of radioactive amino acids to mitochondrial membranes. This claim, however, now appears to have been incorrect (19), as recent studies have shown that the mitochondrial translation products in vitro and in vivo in HeLa cells (20), BHK-1 cells (21), and rat liver (19) give similar patterns on NaDodSO4-polyacrylamide gels. In this study we have observed that the polypeptides synthesized in vitro by yeast mitochondria during a 30-min incubation migrate on NaDodSO4 gels with mitochondrial proteins labeled in vitro during a 60-min incubation of cells in the presence of cycloheximide. These findings agree with results recently reported by Ibrahim et al. (22), although from a comparison of their electrophoretic gel patterns for mitochondrial polypeptides labeled in vitro and in vivo with those reported here, it would appear that the in vitro "protein-synthesizing mixture" chosen for this study is better able to reproduce the in vivo system. While these results demonstrate that isolated mitochondria are capable of synthesizing polypeptides similar in size to those made in vivo and suggest that isolated mitochondria are capable of making complete polypeptide chains (see ref. 17), they do not establish the identity of these polypeptides nor do they unequivocally demonstrate identity of mitochondrial translation products in vitro and in vivo.

In this study we have sought to establish by more rigorous criteria the ability of isolated yeast mitochondria to reproduce mitochondrial protein synthesis in vivo. To achieve this we have studied the incorporation of [3H]leucine in vitro into the polypeptides of a specific mitochondrial protein, cytochrome c oxidase, which requires both mitochondrial and cytoplasmic protein synthesis for its formation. We have found that isolated mitochondria are capable of incorporating label into three polypeptides which have been identified as subunits I, II, and III of cytochrome c oxidase by the following observations: (i) they migrate in NaDodSO4-polyacrylamide gels with subunits I, II, and III; (ii) they are precipitated by an antiserum to cytochrome c oxidase; and (iii) they are capable of complexing with at least one and possibly all four of the cytoplasmically made subunits (IV–VII) of the enzyme. In view of the virtually identical sizes of in vitro and in vivo labeled subunits and the apparent "nativeness" of subunits I, II, and III that have been labeled in vitro, we can conclude that polypeptide elongation and termination are most likely normal in our in vitro mitochondrial system. However, the ability of isolated mitochondria to initiate new polypeptides in this system remains to be determined.

**Table 2. Radioactivity immunoprecipitated from crude mitochondrial extracts by antisera to holocytochrome c oxidase and to subunits V and VII**

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<th>Antiserum</th>
<th>Percent of total mitochondrial radioactivity</th>
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<td>Immune precipitates from labeling in vitro</td>
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<tr>
<td>Anti-holocytochrome c oxidase</td>
<td>7.1</td>
</tr>
<tr>
<td>Anti-subunits V+VII</td>
<td>2.5</td>
</tr>
<tr>
<td>Ratio anti-V+VII/anti-holoenzyme</td>
<td>0.35</td>
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Immunoprecipitates were prepared from clarified extracts of solubilized mitochondrial membranes as described in Materials and Methods. These values represent plateau values from immunoprecipitation curves and have been normalized to reflect total percent of the radioactivity incorporated by correcting for the percentage of total counts present in the clarified extracts. These values were 57% for membranes labeled in vitro and 61% for membranes labeled in vivo.
side effects (12, 23), our independent confirmation demonstrates that the conclusions derived from these studies in vivo regarding the mitochondrial origin of subunits I, II, and III of cytochrome c oxidase are essentially correct.

Perhaps the most intriguing observation of the above study is that the major portion of subunit I, II, and III molecules that are labeled in vitro is integrated with the cytoplasmically made subunits of the enzyme. This suggests the existence of either a pool of unintegrated cytoplasmically made subunits inside of the mitochondrion or an equilibrium of cytoplasmically made subunits between the holoenzyme and a pool of unintegrated subunits. In either case, it seems important to examine further the role, if any, of such a pool in the synthesis of mitochondrially made subunits of cytochrome c oxidase, particularly in view of the increasing body of evidence from studies in vivo that suggests that the accumulation of cytoplasmically made proteins stimulates the synthesis of specific mitochondrially made proteins (19, 24, 25).

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