Origin of Mitochondrial Enzymes. V. The Polypeptide Character and the Biosynthesis of Rat Liver Cytochrome *c* Oxidase Polypeptides by Mitochondria

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Abstract

Isolated rat liver mitochondria were labeled in vitro with L-[^{14}C]leucine. Sixty percent of the incorporated radioactivity was found to reside in subunits 1, 2, and 3 of cytochrome *c* oxidase with apparent molecular weights of approximately 33,000, 25,000, and 20,000, respectively. The results indicate that these are the predominant products of protein synthesis under the conditions employed. The enzyme complex, as derived by immunoprecipitation, was found to contain four additional polypeptides with apparent molecular weights of 17,000, 12,500, 7000, and 3500. A comparison of electrophoretic profiles of the rat liver and beef heart enzyme reveals that the apparent molecular weights of all polypeptides are remarkably similar.

Introduction

A number of investigators have examined both the electrophoretic profile and function of polypeptides synthesized by mitochondria of yeast or

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Neurospora crassa, and have succeeded in establishing functional roles for specific mitochondrial translation products in inner membrane enzyme complexes involved in electron transport and phosphorylation in these organisms [1–6]. On the other hand, less emphasis has been applied to the study of the mitochondrial synthetic products in mammalian systems and, in the literature to date, there is much uncertainty as to the number, molecular weight, and functional roles of these proteins [7–11]. Costantino and Attardi [7] have noted the labeling of 10 distinct polypeptides ranging in apparent molecular weight from 11,000 to 42,000 by mitochondria of HeLa cells incubated in the presence of the inhibitor of cytoplasmic protein synthesis, emetine. In contrast, Jeffreys and Craig [8] reported the labeling of only four or five mitochondrial polypeptides in the HeLa cells under similar conditions and Burke and Beattie [10] observed the labeling of eight distinct polypeptides by rat liver mitochondria in vitro.

The functional roles of any of the specific proteins synthesized by mammalian mitochondria are even less clear. Beattie et al. [12] reported that polypeptides synthesized by rat liver mitochondria in vitro were not apparently associated with oligomycin-sensitive ATPase, cytochromes $b-c_1$ (complex III), or purified cytochrome c oxidase. However, Jeffreys and Craig [9] report that mitochondria of either human or mouse cells can synthesize a subunit of cytochrome c oxidase with an apparent molecular weight of 20,000, and Yatscoff et al. [11] have indicated that two subunits of cytochrome c oxidase (with apparent molecular weights of 45,000 and 29,000) are synthesized by mitochondria in cultures of bovine tracheal cells. In contrast to these findings, a number of studies with *Neurospora* and yeast demonstrate that the three largest polypeptide components of cytochrome c oxidase are major products of mitochondrial protein synthesis [1, 4, 13] either in vivo or in vitro [14, 15].

Because of these discrepancies as to the number and function of products of mammalian protein synthesis, we have sought to investigate these parameters using an electrophoretic gel system which we believe affords a much clearer resolution of inner membrane polypeptides. The gel system used is that of Swank and Munkres [16], containing 8 M urea; it has been shown to resolve completely the polypeptide constituents of cytochrome c oxidase [17, 18] and ubiquinone-cytochrome c reductase (complex III) [18]. In addition, we have employed a rabbit antiserum to cytochrome c oxidase as an analytical tool in characterizing the products of in vitro rat liver mitochondrial protein synthesis.

The labeling of three major products of rat liver mitochondrial protein synthesis in vivo or in vitro has been reported by Ibrahim and co-workers [19]. Our results are consistent with this finding but we establish herein

the identity of these polypeptides as being subunits I, II, and III of cytochrome c oxidase. In addition, we present the subunit profile of rat liver cytochrome c oxidase. Electrophoresis of the immunoprecipitate of rat liver cytochrome c oxidase indicates a subunit composition very similar to that of beef heart cytochrome c oxidase [20] as resolved in the same electrophoretic system.

Materials and Methods

Mitochondria

Male Sprague-Dawley rats, weighing 500–600 g, were obtained from Charles River Breeding Labs, Inc. As part of a chronic ethanol feeding experiment (to be reported elsewhere), the animals were fed a nutritionally well-balanced liquid diet in which carbohydrate, protein, and fat contributed 69.9, 23.3, and 4.6% of the total calories, respectively [21]. Animals were maintained on this liquid diet for 4 months during which they sustained a weight gain of 0.92 ± 0.13 g/day, similar to that of animals fed standard rat chow *ad libitum* (0.87 ± 0.02 g/day). Mitochondria were prepared according to Chappell and Hansford [22], using a medium containing 250 mM sucrose, 10 mM THAM [tris(hydroxymethyl)aminomethane], and 1 mM EDTA (ethylenediaminetetraacetic acid), pH 7.8 [23]. Sterile reagents and conditions were maintained throughout the isolation [24]. Final pellets were drained well and homogenized by hand in 2–3 ml of isolation medium. Protein concentrations, as determined by the method of Lowry et al. [25], were adjusted to approximately 10 mg/ml for use in incorporation experiments. Mitochondria were contaminated with 5% microsomal protein on a weight basis as calculated from determinations of glucose-6-phosphatase activity. Isolated mitochondria from animals fed the liquid diet were normal as judged by their respiratory characteristics as well as by their contents of cytochromes.

Amino Acid Incorporation into Total Protein

Amino acid incorporation by mitochondria was measured in the sterile incubation system described by Beattie and Ibrahim [23]. In all experiments 10 mg of mitochondrial protein was incubated in duplicate with uniformly labeled L-[^{14}C]leucine in a total volume of 10 ml at 30°C in a metabolic shaker. To determine rates of incorporation, aliquots of the system were delivered at intervals onto strips of Whatman No. 3 filter paper and processed by the procedure of Mans and Novelli [26], utilizing cold 10% TCA (trichloroacetic acid) containing 50 mM L-leucine in the initial washes.

Strips were counted in scintillation vials containing 10 ml of Scintiverse cocktail (Fisher Scientific Company) and 1 ml distilled water in a Beckman CPM-100 liquid scintillation counter with an efficiency for ^{14}C of 90%.

Fractionation of Incubated Mitochondria

When the number and/or character of the labeled components was to be determined, incorporation was stopped by mixing the flask contents with an equal volume of ice-cold 20 mM L-leucine and 250 mM sucrose, pH 7.8. Mitochondria were then reisolated and washed according to the method of Burke and Beattie [10] and mitochondrial membranes were prepared using dilute acetic acid and Lubrol [10]. The pellet was washed by suspension and recentrifugation with 5% TCA, washed three times with distilled water, and then dissolved in 0.5 ml 1% SDS (sodium dodecyl sulfate). Aliquots were used for the following: protein determination; determination of specific radioactivity as described earlier; or electrophoresis as described later. In experiments in which enzyme activities were measured, the derived Lubrol pellets were brought directly into homogeneous suspension in 2% Triton X-100, 0.2 M potassium phosphate, pH 7.

Immunoprecipitation

Rabbits were immunized with beef heart cytochrome c oxidase [27] and immune sera were harvested according to Campbell et al. [28]. Crude γ-G globulin was precipitated from sera by use of ammonium sulfate [29]; the precipitate was dissolved in 50 mM potassium phosphate, pH 7, and dialyzed for 48 hr at 4°C versus 200 volumes of the buffer. The preparation was rendered sterile by subsequent passage through an appropriate Millipore filter. Derived γ-G globulin preparations were titrated with a constant amount of mitochondria as indicated later.

Well-washed mitochondria were suspended in a system containing 2% (Vol/Vol) Triton X-100, 200 mM potassium phosphate, pH 7, and centrifuged for 10 min at 5000g. Cytochrome contents in the supernatant were determined from reduced minux oxidized difference spectra using a millimolar extinction coefficient of 12 for the 605–630 nm absorbance peak of cytochrome aa_3 and 19.1 for the 550–540 nm absorbance peak of cytochrome $c+c_1$ [30]. Equal aliquots of the supernatant were then mixed with varying amounts of immune γ-G globulin in a final volume of 7 ml containing 0.29% Triton X-100 and 50 mM potassium phosphate, pH 7. After incubation for 1 hr at 38°C and for 12 hr at 0°C, tubes were centrifuged for 20 min at 10,000g, and the pellets were resuspended once in 50 mM potassium phosphate, pH 7, and reisolated. In control experiments, identical proced-

ures were followed using γ-G globulin of equal protein concentration derived from sera of the same rabbits before immunization. No evidence of cytochrome c oxidase precipitation was observed with nonimmune γ-G globulin preparations.

Electrophoresis

Samples were dissolved in the dissociation medium of Swank and Munkres [16], heated 20 min at 70°C, and then electrophoresed in 8% (unless otherwise indicated) polyacrylamide gels (15 cm) containing 0.1% SDS and 8 M urea. Electrophoresis was conducted at 2.5 mA for about 12 hr as described by Swank and Munkres [16]. Horse liver alcohol dehydrogenase, lactic dehydrogenase, sperm whale myoglobin, lysozyme, and cortrosyn were used to calculate molecular weights as described by Weber and Osborn [31]. Immediately after electrophoresis gels were either sliced for radioactive counting (as indicated later) or stained in Coomassie Blue R-250 according to Weber and Osborn [31] and subjected to densitometry in an Isco gel scanner.

Counting of Gels

Gels were cut into 1.0-mm slices with a Hoefer Scientific SL 280 electronic gel slicer. Samples of two consecutive gel slices were digested in scintillation vials in 1 ml of 30% hydrogen peroxide for 12 hr at 55°C. Twenty milliliters of scintillation cocktail containing a mixture of 3 parts toluene solution (6 g PPO, 2,5-diphenyloxazole, and 0.1 g POPOP, 1,4-bis[2-(5-phenyloxazoyl)] benzene, per liter) and 1 part Triton X-100 was then added. Samples were counted as described previously.

Enzyme Assays

All assays were performed spectrophotometrically at 25°C in triplicate and used freeze-thawed mitochondria to obviate permeability problems [32]. Cytochrome c oxidase activity was determined according to Elliot et al. [33]. Succinic dehydrogenase activity was determined according to King [34] using PMS (phenazine methosulfate) and DCIP (2,6-dichlorophenolindophenol). NADH dehydrogenase activity (NADH:ferricyanide reductase) was determined using 1 mM potassium ferricyanide. Antimycin A_3 was added at a level of 0.6 μmol/g mitochondrial protein, observing conditions regarding its use recommended by previous workers [35, 36]. In both succinic and NADH dehydrogenase assays, reference cuvettes were constituted to compensate for any nonenzymatic reduction of acceptor. All

enzyme assays were linear during the period of measurement and saturated with respect to substrates.

Chemicals

Uniformly labeled L-[^{14}C]leucine (290 Ci/mole) was obtained from ICN Chemical and Radioisotope Division. All protein standards except cortrosyn (Organon), amino acids, Bicine (N,N-bis[2-hydroxyethyl] glycine), ATP, phosphoenol pyruvate, pyruvate kinase, DCIP, PMS, chloramphenicol, and cycloheximide were obtained from Sigma Chemical Company. Sodium dithionite and Triton X-100 were obtained from J. T. Baker Chemical Company. PPO and POPOP were obtained from New England Nuclear, antimycin A_3 from Upjohn Chemical Company, acrylamide from Eastman Chemical Company, and N,N'-methylenebisacrylamide from Aldrich Chemical Company. Lubrol WX was a gift from ICI United States, Inc.

Results

Throughout our experiments, the incorporation of leucine by isolated mitochondria was found to be first order with respect to the amount of mitochondrial protein in the incubation at either level of precursor leucine used (Fig. 1). As expected, the uptake of leucine was completely inhibited by chloramphenicol and insensitive to cycloheximide. However, it is important to note that the results of Fig. 1 demonstrate as well that mitochondria derived from animals fed the liquid diet are competent insofar as amino acid incorporation is concerned. Previous reports indicate that mitochondria from animals fed standard laboratory diets incorporate approximately 4000 dpm/mg of total mitochondrial protein when incubated under identical conditions of medium, protein concentration, time, and precursor specific activity [10, 19]. Our experiments exhibit good agreement with these findings.

In order to study further the products of mitochondrial protein synthesis, Lubrol pellets were prepared according to Burke and Beattie [10]. Table I presents the results of a representative experiment wherein the pellet contained approximately 13% of the total protein and 50% of the total radioactivity of the parent mitochondria. The purification resulted also in an approximate fourfold increase in both specific radioactivity and the specific activity of NADH dehydrogenase [NADH:K_3Fe(CN)$_6$ reductase]. A further characterization of the Lubrol pellet was attempted insofar as other inner mitochondrial membrane enzymes were concerned. We found that neither cytochrome c oxidase nor succinic dehydrogenase activities survived the
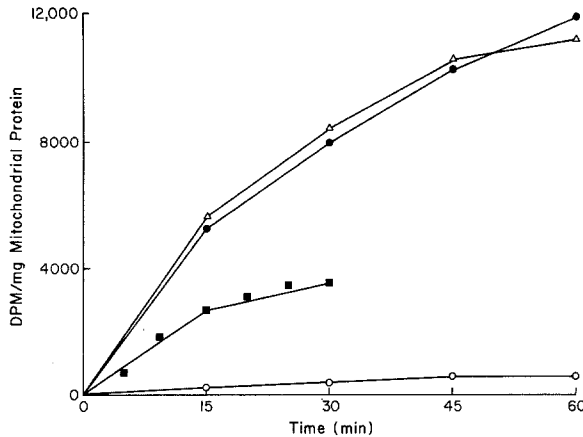
Figure 1. Characteristics of the incorporation of L-[^{14}C]leucine by isolated rat liver mitochondria. Mitochondria were incubated in the complete system as indicated in Materials and Methods at levels of either 0.25 (■) or 1.0 μCi/m (●) of L-[^{14}C]leucine. Incorporation rates were determined in a medium containing 50 mM Bicine, pH 7.6; 10 mM MgCl$_2$; 1 mM EDTA; 5 mM potassium phosphate, pH 7.6; 90 mM KCl; 2 mM succinate; 5 mM phosphoenolpyruvate; 2 mM ATP; 10 μg/ml pyruvic kinase, plus 22.5 μg of an amino acid mixture minus leucine (according to Roodyn et al. [40]). Effects of the inhibitors chloramphenicol (○) and cycloheximide (△) were assessed at concentrations of 100 μg/ml in the presence of 1 μCi/ml L-[^{14}C]leucine.

TABLE I. Derivation of Lubrol-Insoluble Components of Mitochondrial Membranes[a]

	Whole mitochondria	Lubrol insoluble	Specific activity ratio[b]
Protein (mg)	9.8	1.3	—
Dpm/mg	9514	34,593	3.6
	(7116±2077)	(33,016±2231)	(4.5±1.2)
NADH:K$_3$Fe(CN)$_6$ reductase	3.3	12.7	3.9

[a]Isolated mitochondria were labeled in vitro with 1 μCi/ml L-[^{14}C]leucine for 30 min, and treated with dilute acetic acid and Lubrol as described in Materials and Methods. Figures in parentheses are mean ± SD for three animals. Enzyme activities are expressed as micromoles of electron acceptor reduced per minute per milligram of mitochondrial protein.

[b]Specific activity of Lubrol pellet divided by that of whole mitochondria.

treatment, reflecting either solubilization of more hydrophilic components and/or inactivation by the acid extraction (not shown).

When scanned densitometrically, electrophoretic separations of the Lubrol pellet (Fig. 2A) revealed approximately 16 distinct polypeptides with apparent molecular weights between 10,000 and 60,000. When the distribution of radioactivity was determined following electrophoresis, it was found that three polypeptides with apparent molecular weights of 33,000, 25,000,
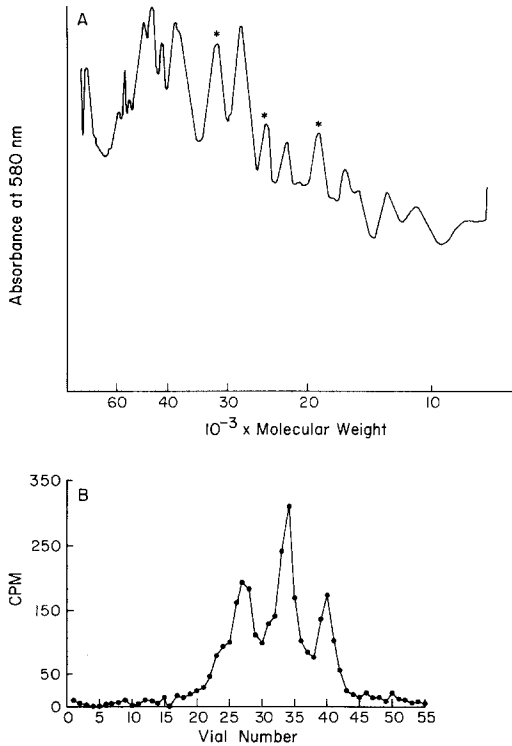

Figure 2. The electrophoretic resolution of the polypeptide components and of the principal products of in vitro mitochondrial protein synthesis in the Lubrol-insoluble pellet. Lubrol-insoluble pellets were derived from mitochondria following their incubation with 1 μCi/ml L-[^{14}C]leucine for 30 min in the complete system (Materials and Methods). (A) Derived from scans of electrophoretic separations containing 75 μg of Lubrol-insoluble protein. Asterisks indicate polypeptides which correspond in apparent molecular weight to three labeled components of (B). For determination of labeled products of protein synthesis, 150 μg of Lubrol-insoluble protein (31,438 dpm/mg protein) were subjected to electrophoresis (Fig. 2B).

and 20,000 are synthesized by mitochondria in vitro (Fig. 2B). These three components contained the bulk of the incorporated label in the Lubrol pellet and, on the basis of molecular weight, they correspond precisely to three of the components of the Lubrol pellet (asterisked in Fig. 2A). Burke and Beattie [10] have similarly detected the predominant labeling of three polypeptides in the Lubrol pellet from rat liver mitochondria. The apparent molecular weights of the polypeptides in their preparation were 40,000, 27,000, and 20,000. The differences in apparent molecular weights for the largest product of mitochondrial synthesis could well stem from the incorporation of urea into the Swank and Munkres electrophoretic system. In this regard, Capaldi et al. [18] found that subunit 1 of beef heart cytochrome c oxidase displays an apparent molecular weight of 35,300 in the presence of urea as opposed to an apparent molecular weight of 38,000 in the absence of urea. Thus, inasmuch as we submit evidence later that the largest of the three labeled products of the Lubrol pellet is most likely the largest subunit of rat liver cytochrome c oxidase, we believe that a similar effect of urea on electrophoretic mobility is responsible for the differences in apparent molecular weight observed by Burke and Beattie and ourselves.

When isolated mitochondria which had been labeled in vitro were treated with an optimal level of immune γ-G globulin, 60% of the total radioactivity was precipitated (Table II). Under these conditions, essentially

TABLE II. Immunoprecipitation of Cytochrome c Oxidase from Mitochondria Labeled in Vitro[a]

	DPM	Cytochrome c oxidase Activity (%)	Cytochrome $c+c_1$ content (%)
Mitochondrial lysate	10,425	100	100
Remaining in supernatant after immunoprecipitation	3,903	5	100
Removed by antibody	62.6%	95	0

[a]Mitochondria were incubated in vitro for 30 min with 1 μCi/ml L-[14C]leucine for 30 min under the conditions indicated; they were then washed, reisolated, and lysed in 2% Triton X-100, 0.2 M potassium phosphate, pH 7. An aliquot of the lysate containing 0.7 nmol cytochrome aa_3 was treated with an optimal level of γ-G globulin prepared from serum directed against beef heart cytochrome c oxidase as described in Materials and Methods. Cytochrome c oxidase activity and cytochrome $c+c_1$ content were also determined as described in Materials and Methods.
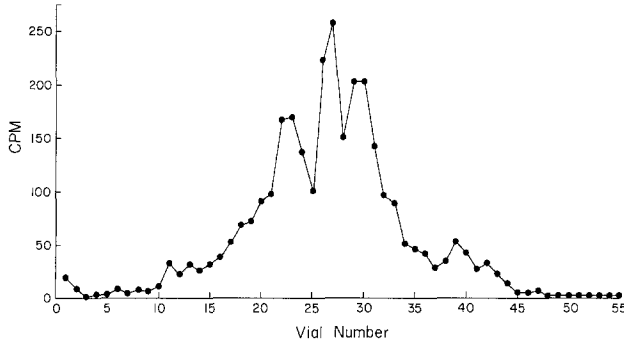
Figure 3. The electrophoretic resolution of the products of in vitro mitochondrial protein synthesis in an immunoprecipitate of rat liver cytochrome c oxidase. The immunoprecipitate derived in the experiment of Table II (approximately 6000 dpm) was subjected to electrophoresis, gel slicing, and liquid scintillation counting as described in Materials and Methods.

all of the cytochrome c oxidase activity and no cytochrome $c+c_1$ were removed from the supernatant. On the other hand, when mitochondria were treated with γ-G globulin derived from normal rabbit sera, neither cytochrome c oxidase activity nor radioactivity was removed from the supernatant (not shown).

Upon subjection of the immunoprecipitate from the experiment of Table II to electrophoresis, it was found that the radioactivity therein resided principally in three polypeptides (Fig. 3). A comparison of the radioactivity profile of the immunoprecipitate (Fig. 3) with that of the Lubrol pellet (Fig. 2B) shows that the same three polypeptides, with respect to both apparent molecular weight and apparent labeling stoichiometry, were precipitated by the antibody, establishing the role of these polypeptides as the mitochondrially synthesized subunits of cytochrome c oxidase. Similar results were obtained with all the animals employed in these studies.

When all the proteins of the immunoprecipitate of rat liver cytochrome c oxidase were visualized with Coomassie Blue (Fig. 4A) and the pattern compared to that of the beef heart enzyme which had been employed as the antigen (Fig. 4B), a marked similarity in both the number and relative mobilities of the polypeptide constituents was apparent. The numbering of the polypeptide components of rat liver oxidase was accomplished according to Downer et al. [20]. In the view of these authors the unnumbered components (Fig. 4B) are contaminants. One of these (migrating between subunits 4 and 5) has an apparent molecular weight of 13,100; its removal by gel
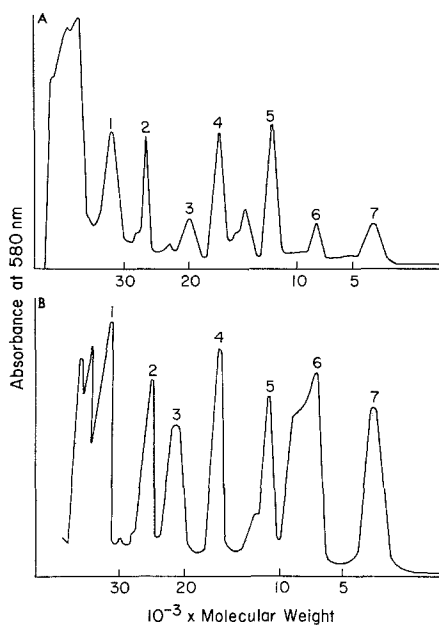
Figure 4. A comparison of the polypeptide compositions of beef heart and rat liver cytochrome c oxidase preparations. An immunoprecipitate of cytochrome c oxidase from isolated and lysed rat liver mitochondria (0.5 nmol cytochrome aa_3) (A) and a preparation of beef heart cytochrome c oxidase (~ 100 μg protein) (B) were subjected to electrophoresis in 12.5% polyacrylamide gels as described in Materials and Methods. In both (A) and (B) the presence of additional components of low mobility is obvious. In the case of rat liver mitochondria (A) this is due in part to the presence γ-G globulin components and in part to contaminant reductase activities [17]. The latter type of contamination can also be seen in (B). The sharper resolution of components in these separations (as opposed to those of Figs. 2 and 3) is due to the use of 12.5% gels rather than 8%.

filtration in Triton X-100 results in an enhanced V_{max} and heme-to-protein ratio. The other minor component is seen as a shoulder on peak 6 in the beef heart enzyme; it has an apparent molecular weight of 8500 and is considered a contaminant because it varies in relative content from preparation to preparation [20].

Table III presents a comparison of the apparent molecular weights of each subunit of beef heart cytochrome c oxidase as determined by Downer et al. [20] and by this laboratory, versus those of the immunoprecipitate of the rat liver enzyme. It is quite evident that there is a close correspondence

TABLE III.   Apparent molecular weights of pro-
teins of beef heart and rat liver cytochrome c
oxidase[a]

	Rat liver	Beef heart (1)	Beef heart [20] (2)
1	35,000	35,000	35,400
2	27,000	25,000	24,000
3	20,000	22,000	21,000
4	17,000	16,000	16,800
5	12,500	12,000	12,400
6	7,000	6,500	8,200
7	3,500	3,500	4,400

[a]An immunoprecipitate of rat liver cytochrome c
oxidase and a preparation of the beef heart enzyme
(1) (both as described in Fig. 4) were electro-
phoresed in 12.5% polyacrylamide gels as de-
scribed in Materials and Methods. For purposes of
comparison, the findings of Downer et al. [20] are
also listed (2).

between the two species insofar as apparent molecular weights are concerned.
For each known subunit of the beef heart enzyme, there is a polypeptide of
identical or closely agreeing apparent molecular weight in the rat liver
immunoprecipitate. Two apparent minor components of the rat liver
immunoprecipitate (between components 4 and 5—Fig. 4A) are at this time
considered to be contaminants though this point will have to be established
in further experimentation.

Discussion

Many investigators have reported that the three largest polypeptides in
cytochrome c oxidase of yeast or *Neurospora crassa* are synthesized by
mitochondria [1, 4, 13–15] and are the major products of synthesis both in
vivo and in vitro [14, 15]. Similarly, Ibrahim et al. [19] previously demon-
strated that the three prominent labeled components of the Lubrol pellet of
rat liver mitochondria are synthesized to similar extents in vivo and in vitro.
Our results confirm the results of Ibrahim et al. [19] and extend them with the
finding that the three predominant labeled products of in vitro mammalian

mitochondrial protein synthesis are subunits of cytochrome c oxidase. Proof of this is provided in part by findings presented herein and in part by recent results of other laboratories (see later). In our experiments, more than 60% of the total radioactivity incorporated into protein by isolated mitochondria was precipitated by a level of γ-G globulin that completely precipitated cytochrome c oxidase (Table II). In addition, the apparent molecular weights of the three labeled components of immunoprecipitates (Fig. 3) and the Lubrol pellets (Fig. 2B) were found to be identical. We believe also that the closely similar apparent stoichiometries of labeling of the three components in each type of preparation provide further support for our conclusion. The previously observed similarities in in vivo and in vitro synthesis of mitochondrial components insoluble in Lubrol [19] would suggest, therefore, that as much as 60% of the amino acid incorporation by isolated rat liver mitochondria is involved in the production of subunits 1, 2, and 3 of cytochrome c oxidase.

As indicated earlier recent work of other laboratories serves to confirm our results. Hundt and Kadenbach [37] have purified rat liver cytochrome c oxidase to a heme aa_3-to-protein ratio of 14 and, in such preparations, they find seven components displaying apparent molecular weights of 38,000, 24,500, 20,500, 14,400, 12,400, 10,300, and 8500, upon electrophoresis in the absence of urea. In addition, Höchli and Hackenbrock (unpublished observations) have found seven components in purified cytochrome c oxidase preparations (heme aa_3-to-protein ratio of 9.5) from rat liver. The polypeptides exhibit apparent molecular weights of 34,000, 26,800, 23,700, 17,000, 12,500, 9500, and 3600 upon electrophoresis in SDS+8 M urea. A close comparison of these values with those of the first column of Table III will establish that, but for two exceptions, there is excellent agreement in our respective findings. Although the discrepancies in apparent molecular weights assigned to subunits 3 and 6 by Höchli and Hackenbrock and by ourselves remain unexplained at this time, it should be added that the work of these investigators also establishes a large effect of urea on the apparent molecular weight of subunit 1 of rat liver cytochrome c oxidase. They determine that the apparent molecular weight of this entity is 43,500 upon electrophoresis in the absence of urea (as opposed to the preceding value of 34,000).

In sum, both of the foregoing sets of findings serve to support our contention that the three principal labeled products of in vitro rat liver mitochondrial protein synthesis found in Lubrol pellets and immunoprecipitates are cytochrome c oxidase components. In addition, they provide confirmation of our deduction, based on immunologic evidence, that rat liver and beef heart cytochrome c oxidase are very similar entities in both the number and apparent molecular weights of constituent subunits.

The incomplete recoveries of incorporated radioactivity of total mito-
chondrial proteins in either derived Lubrol pellets or immunoprecipitates
of cytochrome c oxidase are to be expected. Successive extractions of mito-
chondria with dilute acetic acid and a Lubrol solution could be expected to
remove not only the water-soluble and more readily solubilized mito-
chondrial components, simultaneously enriching the insoluble fraction in
hydrophobic inner membrane complexes, but such extractions would also
remove labeled products of synthesis which were not integrated into intact
enzyme complexes during the course of the incubation. The first type of
extractions would serve to explain the observed enrichments of specific
radioactivity and NADH:K_3Fe(CN)$_6$ reductase (Table I); the second type
could well underlie, at least in part, the incomplete recovery of radioactivity
in Lubrol pellets. On the other hand, the immune γ-G globulin selectively
removes cytochrome c oxidase (and presumably unintegrated subunits) from
mitochondrial lysates. The precipitations were not only quantitative with
respect to cytochrome c oxidase (Table II) but they were also free of gross
contamination (Fig. 4A). As a consequence, labeled products of synthesis
which were unrelated to cytochrome c oxidase, as well as subunits of the
enzyme for which the immune γ-G globulin preparation might have been
deficient in its content of specific antibodies, would have remained unprecip-
itated and would have thus contributed to the losses in radioactivity which
were observed (Table II).

It must be emphasized that we are aware that subunits 1–3 of cytochrome
c oxidase are not the only products of mammalian mitochondrial protein
synthesis. Recent work with cultured cells [38, 39] reaffirms the findings of
earlier investigators that mammalian mitochondria have a capacity to
manufacture a discrete, though limited number of polypeptides. However,
under the conditions employed, our work establishes that subunits 1–3 of
cytochrome c oxidase are the three principal labeled products formed in
vitro by rat liver mitochondria. It should be noted that the conditions of our
experimentation do not eliminate the possibility that contaminating poly-
peptides might contribute to the labeling patterns observed for both immuno-
precipitates and Lubrol pellets. At this time we consider this a remote
possibility at best inasmuch as the two intrinsically different techniques each
yield preparations containing three components which contain the pre-
ponderance of radioactivity and which display identical apparent molecular
weights upon electrophoresis. Future experiments will, however, be directed
to obtaining an unequivocal answer to this question.

In past experimentation we prepared rat liver cytochrome c oxidase with
heme-to-protein ratios of 10 by conventional procedures. However, such
preparations were invariably contaminated with at least two forms of NADH

dehydrogenase activity and further efforts in this direction were abandoned (Davidian and Penniall, unpublished experiments). As a consequence, we turned to the use of a rabbit antiserum to beef heart cytochrome c oxidase as an experimental tool inasmuch as it was possible to demonstrate the quantitative cross-reactivity of such preparations with the rat liver enzyme complex [33]. Our findings by this approach establish a fundamental consistency, with respect to the character of in vitro protein synthetic activity, of mitochondria of mammalian cells and those of yeast and *Neurospora*, and they suggest as well a remarkable conservatism of the mitochondrial genome, at least insofar as cytochrome c oxidase is concerned.

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