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Studies on the Biogenesis of an Enzymatically Active Complex III of the Respiratory Chain From Yeast Mitochondria

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Complex III isolated from yeast mitochondria catalyzed an antimycin A and Diuron-sensitive coenzyme QH_2-cytochrome *c* reductase activity with a turnover number of 15.7 sec^{-1} and contained 10 nmoles of cytochrome *b* and 4.6 nmoles of cytochrome c_1 per mg of protein. Electrophoresis in sodium dodecyl sulfate acrylamide gels resolved Complex III into 10 bands with apparent molecular weights of 50,000, 40,000, 30,000, 29,000, 24,000, 17,000, 16,000, 12,000, 8,400, and 5,800. Yeast cells were labeled under nongrowing conditions with (^{35}S)-methionine in the absence or presence of inhibitors of cytoplasmic or mitochondrial protein synthesis. Labeled Complex III was isolated by immunoprecipitation from detergent-solubilized mitochondria using antiserum raised against the purified complex. Analysis of the immunoprecipitates by polyacrylamide gel electrophoresis revealed that a 30,000-dalton protein, cytochrome *b*, as well as 16,000-dalton protein were labeled in the presence of cycloheximide, indicating that they are products of mitochondrial protein synthesis. Immunoprecipitates from mitochondria obtained from cells labeled in the presence of chloramphenicol contained a new radioactive peak with a molecular weight of 100,000. In addition, significant decreases in the labeling of the proteins with molecular weights of 50,000, 40,000, 30,000, and 16,000 were observed. When Complex III was isolated by immunoprecipitation from intact spheroplasts after a 5-minute pulse with (^{35}S)-methionine, the 100,000-dalton protein was labeled in the immunoprecipitate whether or not chloramphenicol was present; however, after a 1-hour chase with unlabeled methionine, decreased labeling of the 100,000-dalton protein was observed concomitant with an increased labeling of the 50,000- and 40,000-dalton proteins. These results suggest that a protein with a molecular weight of 100,000 may either be a precursor or a partially assembled form of other proteins of Complex III, most probably the two largest polypeptides.

Key words: mitochondria, protein synthesis, cytochrome *b*-c_1, biogenesis, repiratory chain

Early studies in this laboratory had indicated that the formation of Complex III of the yeast mitochondrial respiratory chain, as measured by the development of coenzyme QH_2-cytochrome *c* reductase activity, required both mitochondrial and cytoplasmic pro-

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tein synthesis [1–3]. One product of mitochondrial protein synthesis in Complex III was later shown to be cytochrome *b* in both Neurospora crassa [4] and in Saccharomyces cerevisiae [5, 6]. Even more recent studies have demonstrated that mitochondrial DNA of S. cerevisiae codes for cytochrome *b* [7–9]. By contrast, two other components of Complex III, cytochrome c_1 [10] and a 25,000-dalton protein that may be the iron-sulfur protein [11], are synthesized in the cytoplasm. The possibility that other proteins of Complex III may be synthesized by mitochondria has not been ruled out, since most studies on the biogenesis of this complex were performed with enzymatically inactive complexes that may have lost key proteins [5, 12].

Recently, a method was published for the isolation of an enzymatically active, antimycin A-sensitive Complex III that contains levels of cytochrome *b* and c_1 exceeding those reported for highly purified complexes from beef heart [13]. In the present study, Complex III was isolated by this method in order to establish its protein composition and to study the biogenesis of the component proteins by immunotechniques. The results obtained indicated that Complex III of yeast contains 10 proteins ranging in molecular weight from 5,800 to 50,000 and that at least one protein, cytochrome *b*, and possibly a second with a molecular weight of 16,000 are synthesized by mitochondria. Evidence is also presented for the possible existence of a high molecular weight precursor of the two largest proteins of the complex.

EXPERIMENTAL PROCEDURES

Preparation of Complex III

Submitochondrial particles were prepared by sonicating mitochondria isolated from commercially grown Baker's yeast (Red Star) as previously described [14]. Complex III was isolated from the submitochondrial particles according to the method of Siedow et al [13] with the following modifications. Desalting by Biogel column chromatography was omitted and instead the purified complex, precipitated at 60% ammonium sulfate, was resuspended in 0.1 M sodium phosphate buffer, pH 7.4, twice, and centrifuged at 100,000g for 3 hours. The final precipitate was resuspended in either 0.1 M sodium phosphate buffer, pH 7.4, or was prepared for electrophoresis as described below.

Assay Methods

Protein determinations, performed on isolated Complex III, were made by the Bio-Rad Protein Assay Kit. All other protein determinations were by the method of Lowry et al [15]. Succinate dehydrogenase was assayed at 25°C by the method of King [16] and succinate- and coenzyme QH_2-cytochrome *c* reductase activities were assayed according to Hatefi and Rieske [17], using either sodium succinate (10 mM) or the coenzyme Q analog, 6-decyl,2,3-dimethoxy,5-methyl,1,4-benzoquinone (25 μg/ml) in 25 mM sodium phosphate buffer, pH 7.6. The coenzyme Q analog was reduced and added as a solution in ethanol. Antimycin A, dissolved in ethanol, was added to a final concentration of 5 μg/ml; Diuron, also dissolved in ethanol, was added to a final concentration of 50 μM. Enzyme activity was calculated using a millimolar absorption coefficient of 18.5 for cytochrome *c*.

Spectral analysis was performed with the isolated Complex III at a concentration of 0.5–1.0 mg of protein per ml in 0.1 M sodium phosphate buffer, pH 7.4, at 25°C in a Perkin-Elmer Dual Wavelength Spectrophotometer. The cytochrome content was calculated from the dithionite versus ferricyanide spectrum according to the method of Vanneste [18].

Gel Electrophoresis

Complex III was prepared for gel electrophoresis by resuspending the 100,000g pellet in dissociation solution consisting of 0.1 M Tris-acetate buffer, pH 8, 5% sodium dodecyl sulfate, 5% mercaptoethanol, and 10% glycerol at a protein concentration of 3–5 mg/ml and allowing it to stand overnight at room temperature.

Sodium dodecyl sulfate electrophoresis in tubes was performed according to Weber and Osborn [19], using the Tris/glycine buffer system. The acrylamide concentration of the lower acrylamide gel was varied from 7.5% to 15% while a 5% acrylamide stacking gel was used. Slab gels were prepared in the same manner with either a 10% or a 10–15% gradient acrylamide lower gel and a 5% upper gel [20]. Tube gels were prepared for counting by slicing into 1-mm pieces, dissolving the pieces in 400 μl of 30% hydrogen peroxide at 60°C for 18 hours and counting in 10-ml Liquiscint. Slab gels were dried for autoradiography on Whatman 3MM filter paper using a Bio-Rad slab gel drier. Autoradiography of slab gels was performed using Kodak NS-5T x-ray film.

Preparation of Antibodies to Complex III

Purified Complex III solubilized in 0.1 M sodium phosphate buffer, pH 7.6, and mixed with an equal volume of Freund's complete adjuvant was injected into rabbits subcutaneously in three equal doses of 1 mg protein each over a period of 2 months. Immunodiffusion analysis of the antibody was performed for 5 days in plates prepared with 1% agarose containing 38 mM Tris-glycine, pH 8.6, 150 mM NaCl, 1% Triton X-100 (w/v) and sodium azide (1 g/l). Wells contained 50–100 μl antiserum, 0.2–0.3 mg of purified Complex III dissolved in 38 mM Tris-glycine, pH 8.6, with 5% Triton X-100 or 0.6 mg of submitochondrial particles and other ammonium sulfate pellets resulting during isolation dissolved in the same medium as described for Complex III with the addition of 0.5 mg sodium dodecyl sulfate per mg protein [21].

Labeling of Yeast Cells and Immunoprecipitation of Complex III

Labeling of wild-type yeast strain D273-10B in vivo was performed essentially as described previously [20] using (^{35}S)-methionine. Preincubations of 5 minutes in 1 ml of incubation media were performed in the presence of 4 mg per ml of chloramphenicol, 1 mg per ml of cycloheximide, or no inhibitor prior to isotope addition. The cells were incubated for 20 minutes with the isotope, washed, and mitochondria-prepared as described previously [22]. For the immunoprecipitation of Complex III, mitochondria were suspended at a concentration of 10 mg/ml in 10 mM sodium phosphate, pH 7.2, containing 150 mM NaCl, 1% Lubrol and 0.5% deoxycholate for 2 hours at 4°C [6]. Solubilized mitochondria were centrifuged at 15,600g for 7 minutes and the resulting supernatants were combined with antiserum in a ratio of 1:3 and incubated for 18 hours at 4°C. After washing the precipitates four times with solubilizing buffer, the precipitates were dissolved in the electrophoresis dissociation buffer at a concentration of 10–20 $\times 10^3$ counts per minute per μl and incubated overnight at room temperature prior to electrophoresis.

Pulse-Chase Experiments

Spheroplasts were obtained and labeled with (^{35}S)-methionine according to Ibrahim and Beattie [22] with 4 mg per ml of chloramphenicol or no inhibitor included in the incubation medium. After 5 minutes, aliquots were removed and mixed with 1 mM phenylmethylsulfonyl fluoride and lysed immediately in two volumes of boiling 4.5% sodium dodecyl sulfate solution containing 2.25 mM EDTA and 2.25 mM EGTA, pH 7.4. After

5 minutes, the lysates were diluted 10-fold with 3% Triton X-100 solution containing 150 mM NaCl, 100 mM sodium phosphate, pH 7.4, and 1 mM EDTA, allowed to stand for 30 minutes at room temperature, and then centrifuged at 1,650g for 30 minutes at 22°C. The resulting supernatants were combined with antiserum in a ratio of 1:1 or 1:2 and incubated overnight at room temperature. The precipitates were washed with the 3% Triton solubilizing buffer and prepared for electrophoresis as described above. The remaining spheroplasts were washed two times with 1 M sorbitol and then incubated for 1 hour in medium containing 10 mM nonradioactive methionine in the absence of chloramphenicol before processing as described above.

RESULTS

Properties of Isolated Complex III

The purification of Complex III from baker's yeast by the method of Siedow et al [13] in our laboratory yielded a multiprotein complex which catalyzed an antimycin and Diuron-sensitive coenzyme QH_2-cytochrome *c* reductase activity (Table I). The maximum activity obtained was 4.34 μmoles min^{-1} mg^{-1} of protein, which represents a 38.5-fold purification of the enzyme activity present in submitochondrial particles. The isolated Complex III contained very low succinate dehydrogenase activity and undetectable succinate-cytochrome *c* reductase activity. The difference spectrum of the isolated Complex III is typical of those reported previously for yeast [23] and indicates the absence of contamination with cytochromes a-a_3 (Fig. 1). The content of cytochromes *b* and c_1 was 10.7 and 4.6 nmoles per mg of protein respectively, a value comparable to that reported by Siedow et al [13].

Sodium dodecyl sulfate electrophoresis of purified Complex III was performed in gels of various acrylamide concentrations as well as in 10–15% gradient gels. As seen in Figure 2, Complex III separated into ten bands numbered in order of increasing mobility with approximate molecular weights of 50,000, 40,000, 30,000, 29,000, 24,000, 17,000, 16,000, 12,000, 8,400, and 5,800. These molecular weights were calculated by comparing the mobility of the bands of Complex III with standard molecular weight proteins in either 10% acrylamide gels or in a 10–20% acrylamide step gel [24].

Biogenesis of Complex III

To study the biosynthesis of Complex III, immunoprecipitation techniques were employed to isolate the complex from mitochondria using antiserum raised in rabbits. Double diffusion analysis in agar showed a sharp, single precipitin line when the antiserum was tested against purified Complex III and a fainter line when tested against Triton X-100 sodium dodecyl sulfate-solubilized submitochondrial particles. Control serum from nonimmunized rabbits failed to produce any precipitin lines under similar test conditions.

To establish whether the antiserum could be used to isolate Complex III, gel electrophoresis of immunoprecipitates was formed using solubilized mitochondria obtained from yeast cells pulse-labeled with (^{35}S)-methionine. Analysis of immunoprecipitates in 10% acrylamide tube gels revealed radioactive peaks corresponding in molecular weight to the major bands of isolated Complex III, ie, 50,000, 40,000, 30,000, 24,000, and 17,000 (Fig. 3).

Several points should be noted. First, in the 10% acrylamide gel, proteins with a molecular weight less than 12,000 run with the dye front so that only the proteins of higher molecular weight can be studied in this system. Secondly, bands 3 and 4 are not resolved and run as one labeled peak. The minor peak with a molecular weight of 70,000

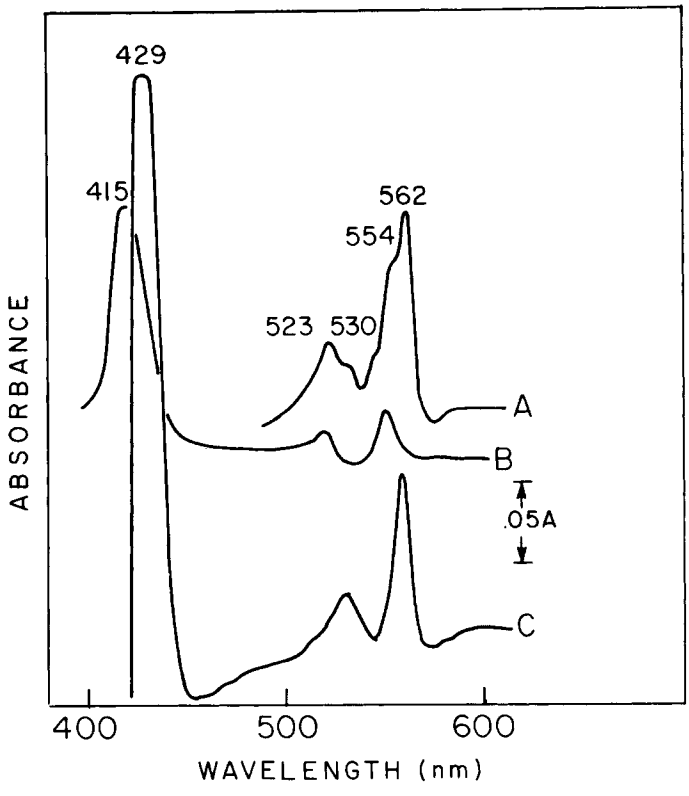

Fig. 1. Difference spectra at 25°C of purified Complex III obtained from yeast mitochondria. Curve A, sample reduced by dithionite and reference oxidized by ferricyanide. Curve B, sample reduced by ascorbate and reference oxidized by air. Curve C, sample reduced by dithionite and reference reduced by ascorbate. Concentration of Complex III was 0.8 mg protein per ml.

TABLE I. Properties of Complex III Purified From Baker's Yeast

Activity	Submitochondrial particles	Complex III
Succinate dehydrogenase (μmol/min/mg)	0.296	0.017
Coenzyme Q-cytochrome *c* reductase (μmol/min/mg)	0.112	4.34
+ Antimycin A	–	0.29
+ Diuron	–	0.41
Cytochrome *b* (nmol/mg protein)	–	10.7
Cytochrome c_1 (nmol/mg protein)	–	4.6

Enzymes were assayed and cytochrome content determined as described under "Experimental Procedure."

observed in the gel may resent contamination with succinate dehydrogenase, either because of incomplete solubilization of the two complexes or from slight cross-reactivity of the antiserum with Complex II. Preimmune serum also precipitated some radioactive material, but this precipitation was not concentration dependent and the gel patterns obtained did not indicate the presence of radioactive peaks.

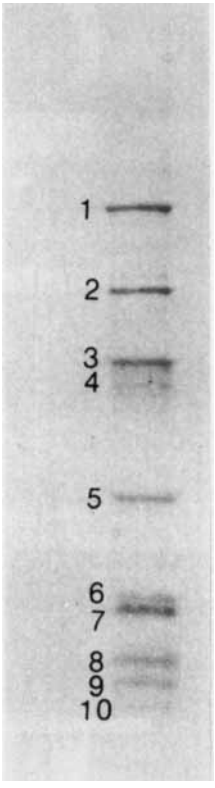

Fig. 2. Peptide composition of isolated Complex III after electrophoresis in a 10–15% polyacrylamide gradient gel. The numbers indicate the proteins of Complex III in order of decreasing molecular weight.

To identify proteins of Complex III synthesized on mitochondrial ribosomes, yeast cells were labeled in vivo in the presence of cycloheximide, a specific inhibitor of cytoplasmic protein synthesis. Electrophoresis of the immunoprecipitate formed with the antiserum against Complex III revealed two radioactive proteins with molecular weights of 30,000 and 16,000 (Fig. 3, CHI). The peak at 30,000 daltons most probably represents cytochrome *b*, a known mitochondrial product which is present in immunoprecipitates formed with specific antiserum to cytochrome *b* from yeast [6]. The labeled protein of 16,000 daltons is, at present, of unknown origin. Labeling of this peak, however, did not decrease after repeated washing of the immunoprecipitate or if the mitochondria were prepared for immunoprecipitation by solubilization in 3% Triton in 154 mM NaCl and 100 mM sodium phosphate, pH 7.4. Although the 16,000-dalton peak appears broad and split in Figure 3, in most experiments a single peak was obtained.

Yeast cells were also labeled in the presence of concentration of chloramphenicol sufficient to block mitochondrial protein synthesis. The content of labeled Complex III present in the mitochondrial membrane was drastically decreased in cells labeled in chloramphenicol, as evidenced by the 50% decrease in radioactivity present in immunoprecipitates formed using the optimal antigen-antibody ratio. The gel pattern of these immunoprecipitates revealed a new prominently labeled band with an apparent molecular weight

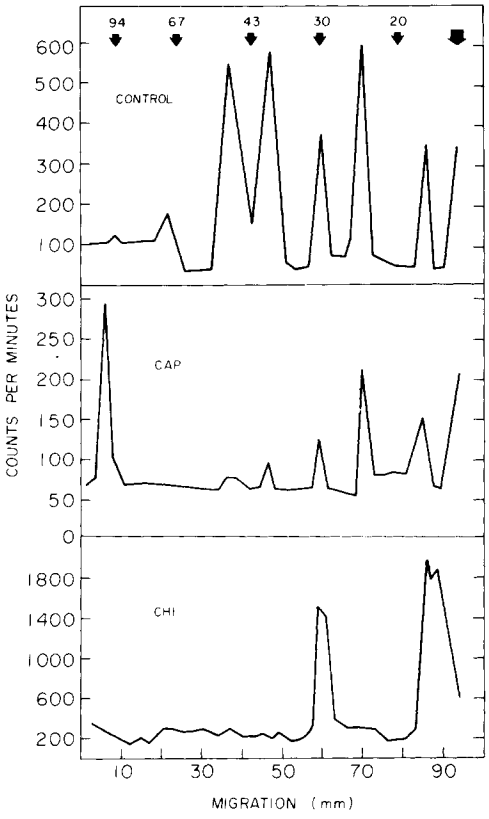

Fig. 3. Separation by SDS gel electrophoresis of Complex III immunoprecipitated from detergent-solubilized mitochondria. Immunoprecipitates were prepared for electrophoresis in 10% acrylamide gels as described in Methods. Control refers to cells pulse-labeled with (^{35}S)-methionine without inhibitors; CAP to cells labeled with (^{35}S)-methionine in the presence of 4 mg/ml chloramphenicol; CHI to cells labeled with (^{35}S)-methionine in the presence of 1 mg/ml cycloheximide. The numbers refer to the migration of standard proteins of given molecular weight.

of 100,000 concomitant with a large decrease in labeling of the two bands of 50,000 and 40,000 daltons (Fig. 3). The decrease in labeling of the 30,000-dalton peak was expected, since this peak was labeled in the presence of cycloheximide and hence is a protein synthesized by chloramphenicol-sensitive mitochondrial ribosomes. The decreased labeling of bands 1 and 2 was unexpected, however, as these latter two proteins are not labeled in the presence of cycloheximide and thus are not products of mitochondrial protein synthesis.

One possible explanation for these results is that the 100,000-dalton protein is a precursor of the two largest subunits of Complex III. When mitochondrial protein synthesis is blocked by chloramphenicol, this putative precursor may not be processed and may thus accumulate in the membrane. To look for possible precursors, spheroplasts were pulse-labeled for 5 minutes in the presence or absence of chloramphenicol prior to solubilization and immunoprecipitation of Complex III. In the control cells, radioactive methionine was incorporated into a band of 100,000 daltons, as well as into those of

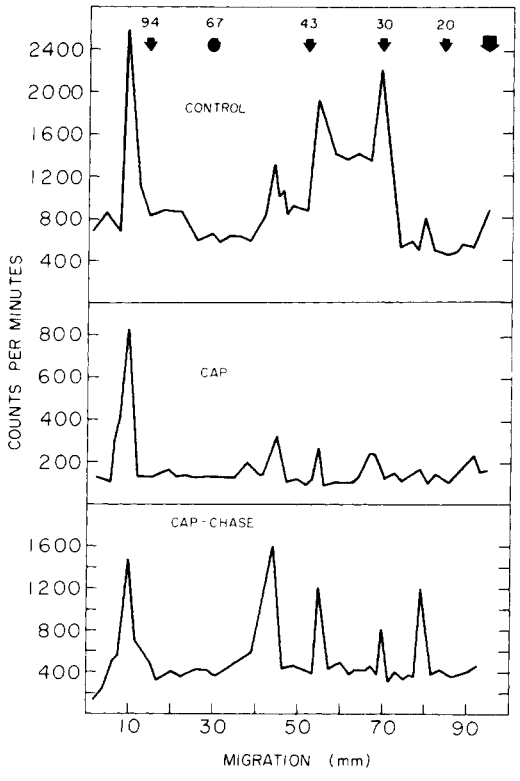

Fig. 4. Gel electrophoresis of Complex III immunoprecipitated from spheroplasts pulse-labeled for 5 minutes with (^{35}S)-methionine. The spheroplasts were lysed in SDS and immunoprecipitated as described in Methods prior to electrophoresis in 7.5% acrylamide gels. CAP refers to cells labeled in the presence of 4 mg/ml chloramphenicol, and CAP-Chase refers to cells pulse-labeled for 5 minutes with (^{35}S)-methionine in the presence of 4 mg/ml chloramphenicol and then washed and chased for 1 hour with unlabeled methionine in the absence of chloramphenicol.

50,000, 40,000, and 30,000 (Fig. 4). When chloramphenicol was present during the pulse, a greater than 50% decrease in labeling in the immunoprecipitate was observed. Most of the label was incorporated into a band with a molecular weight of 100,000 with minor incorporation into the other bands. After a 2-hour chase with unlabeled methionine, the relative labeling of the 100,000-dalton decreased, whereas the relative labeling of the other four proteins increased. The increases in labeling of all peaks during the chase may reflect the continued synthesis of membrane proteins during the time necessary for the cold methionine to equilibrate with the radioactive methionine in the yeast cells.

DISCUSSION

Purification of Complex III from yeast mitochondria in our laboratory by the method of Siedow et al [13] yielded an enzymatically active Complex III which contained 10 distinct polypeptides with molecular weights ranging from 50,000 to 5,800. Cytochrome *b*-c_1-enriched complexes have been previously isolated from S. cerevisiae [13, 23], N. crassa [4, 25], and beef heart [26, 27]. The reported subunit compositions, both in terms of

the number of polypeptides and their molecular weights, vary in the different preparations. Some of the reported differences might have resulted from factors such as species variation, loss of protein components during isolation, the anomalous migration behavior in polyacrylamide of certain membrane-bound proteins [20, 26, 28, 29], and difficulties inherent in assigning accurate molecular weights after gel electrophoresis. Our conclusion that the enzymatically active cytochrome b-c_1 complex of yeast contains ten polypeptides was based on electrophoretic patterns obtained by three types of electrophoresis. In particular, the step gel electrophoresis [24] permitted the resolution of the low weight proteins which may migrate with the dye front in other systems. Three other groups [25, 26, 27] have reported that Complex III from beef heart also contains nine or ten proteins.

The results of the present study confirm previous reports [4–6] that a 30,000-dalton protein of Complex III, presumably cytochrome *b*, is translated on mitochondrial ribosomes; however, the immunoprecipitates obtained from cells labeled with (^{35}S)-methionine in the presence of cycloheximide using the antiserum against the complex contained a second labeled protein with a molecular weight of 16,000. This band does not appear to be a proteolytic breakdown product of cytochrome *b*, as immunoreplica studies with antisera against cytochrome *b* [6] only revealed a labeled band at 30,000 daltons, not at 16,000 [Clejan and Beattie, unpublished results]. The possibility exists that this lower molecular weight polypeptide is analogous to the cytochrome *b*-associated peptide [29] and hence coimmunoprecipitates with cytochrome *b*. In previous studies, a radioactive peak with a 16,000–17,000 molecular weight was seen in the immunoprecipitates obtained with antisera against cytochrome *b* [6, 20] or against the b-c_1 complex [5]. In studies with N. crassa [4], a protein of this size in an isolated complex was not synthesized by mitochondria. One explanation may be that this protein was lost during the isolation of enzymatically inactive preparations of Complex III and was not present, or that Neurospora differ from yeast. Alternatively, this protein may be a contaminant in the preparation we used to obtain antibodies against the complex.

In the present study, we have also obtained evidence that suggests that, under certain experimental conditions, a high molecular weight protein may be associated with Complex III. A highly labeled peak with a molecular weight of 100,000 was observed in the immunoprecipitates obtained from mitochondria isolated from whole cells labeled when mitochondrial protein synthesis was blocked by chloramphenicol. The labeled peak at 100,000 daltons does not represent nonspecific immunoprecipitation, as it is never observed in the immunoprecipitates from mitochondria labeled without inhibitors. Double-label studies have indicated that this protein is only labeled in the presence of chloramphenicol [Battie and Beattie, unpublished observations]. Decreases in labeling of the two largest molecular weight proteins, the "core" proteins, were observed concomitant with the appearance of the 100,000-dalton peak. The decreased labeling of the two core proteins in chloramphenicol was unexpected as these proteins are not labeled in the presence of cycloheximide, indicating that they are not mitochondrial translation products. Our preliminary studies with spheroplasts have suggested that the 100,000-dalton may either be a precursor of these two proteins or a partially assembled complex of them. The 100,000-dalton protein was labeled in spheroplasts in the absence of chloramphenicol and showed decreased labeling after an hour's chase in unlabeled methionine.

Evidence for precursor forms of mitochondrial proteins synthesized in the cytoplasm has already been established for the F_1-ATPase [30], subunit V of the b-c_1 complex [11], and for the small subunits of cytochrome oxidase [31, 32]. All of these proteins are synthesized as large precursors which are processed posttranslationally, presumably in the mitochondria. The results of the present study also suggest that mitochondrial protein

synthesis may be necessary for either the processing of the large precursor or its assembly into Complex III. Katan et al [6] had also reported a decreased labeling of the 40,000- and 17,000-dalton proteins in Complex III isolated by ammonium sulfate fractionation from yeast that had been labeled in chloramphenicol. By contrast, mitochondrial protein synthesis does not appear necessary for processing the precursor subunits of F_1-ATPase or subunit V of Complex III [33]. Further studies to establish a precursor-product relationship between these proteins are currently in progress.

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