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Electrophoretic Behavior of Cytochrome *b* in a Partially Purified Preparation and Evidence for High Molecular Weight Associated Mitochondrial Translation Products†

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ABSTRACT: A partially purified preparation of cytochrome *b* lacking cytochromes *c*, c_1, and a–a_3 was isolated from yeast submitochondrial particles. The preparation contained 7 nmol of heme *b*/mg of protein and upon dodecyl sulfate gel electrophoresis separated into four major bands with apparent molecular weights of 31 000, 35 000, 48 000, and 50 000. The 31 000-dalton band, identified as cytochrome *b* by comparison with total mitochondrial translation products, showed normal migration behavior during dodecyl sulfate electrophoresis in different concentrations of acrylamide. Furthermore, this polypeptide migrated at a molecular weight of 31 000 when the preparation was heated in dissociation medium at 20, 37, 70, or 100 °C, when phenylmethanesulfonyl fluoride, the protease inhibitor, was present or absent, and in dodecyl sulfate–urea gels. By contrast, cytochrome *b* in the intact mitochondrial membrane displayed anomalous migration behavior in gels of different acrylamide concentrations. Two proteins of higher molecular weight are present in the immunoprecipitates of labeled mitochondria treated with the specific antiserum against cytochrome *b*. These polypeptides are products of mitochondrial protein synthesis as they are labeled in the presence of cycloheximide, not labeled in the presence of chloramphenicol, and absent in petite mutants. These mitochondrial translation products do not copurify with cytochrome *b* as they are not present in the partially purified cytochrome *b* preparation obtained from yeast cells labeled in the presence of cycloheximide. These proteins do not appear to be precursors of cytochrome *b* as the addition of a short or long chase of unlabeled amino acid did not alter the labeling of these high molecular weight proteins relative to cytochrome *b*. Furthermore, varying the time of pulse label in the presence of cycloheximide from 3 to 30 min also did not indicate any precursor–product relationship between these high molecular weight proteins and cytochrome *b*.

Our previous studies on the purification of cytochrome *b* from yeast mitochondria and its biogenesis on mitochondrial ribosomes raised two unanswered questions (Lin & Beattie, 1978; Lin et al., 1978). First, the actual molecular weight of cytochrome *b* was not conclusively demonstrated because proteolytic digestion to solubilize the proteins was a necessary step in the overall purification scheme. The purified cytochrome *b* polypeptide thus obtained had a molecular weight of 28 000 based on dodecyl sulfate gel electrophoresis and 28 800 based on sucrose gradient centrifugation (Lin & Beattie, 1978); however, when immunoprecipitates obtained from labeled yeast mitochondria treated with the specific antiserum against cytochrome *b* in the presence of phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$[1]), the protease inhibitor, were analyzed by gel electrophoresis, the major labeled band in the immunoprecipitate migrated with a molecular weight of 31 000 (Lin et al., 1978). Alternately, the differences in molecular weight of the purified protein and the major band of the immunoprecipitate may have occurred because of the various conditions used for electrophoresis. Recently, several groups have reported that the migration of cytochrome *b* in b–c_1 complexes isolated from either beef heart (Bell & Capaldi, 1976; Marres & Slater, 1977) or yeast (Groot et al., 1978) mitochondria varies depending on the gel conditions used. Similarly, the molecular weight of cytochrome *b* varies when total yeast mitochondrial translation products were examined on polyacrylamide gels of different concentrations (Groot et al., 1978; Cabral et al., 1978).

In the present study, we have reevaluated the molecular weight of cytochrome *b* under various conditions of gel electrophoresis and solubilization using both intact mito-

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[1] Abbreviation used: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

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chondria and a partially purified preparation of cytochrome *b*. The data indicate that the apparent molecular weight of cytochrome *b* varies during electrophoresis when this protein is present in the mitochondrial membrane or in an intact b–c_1 complex. By contrast, cytochrome *b* when partially purified has a molecular weight of 31 000 under all conditions used for the determination.

The second unanswered question raised by our previous study was the identity of the one to two polypeptides of higher molecular weight other than cytochrome *b* which are coprecipitated by the specific antiserum to cytochrome *b* (Lin et al., 1978). One possibility is that these proteins are very hydrophobic and merely adhere to the immunoprecipitate formed between cytochrome *b* and its antibody and, hence, have a completely nonspecific association with cytochrome *b*. Alternately, these polypeptides may be an integral part of the cytochrome b–c_1 complex or identical with the large subunits of the cytochrome *b* complex isolated from yeast mitochondria by Marjanen & Ryrie (1976) and, hence, coprecipitate with cytochrome *b*. In the present study, the synthesis of these proteins on mitochondrial ribosomes is demonstrated. Furthermore, these mitochondrially translated polypeptides are not apparently identical with the "core" proteins of the cytochrome b–c_1 complex and are not present in a partially purified preparation of cytochrome *b*.

Materials and Methods

Growth of Yeast. The strain of yeast used in this investigation was a diploid strain of *Saccharomyces cerevisiae* used in this laboratory for several years. Cells were grown aerobically at 30 °C with 2% galactose as carbon source, under the conditions described previously (Brown & Beattie, 1977).

Preparation of Mitochondria and Submitochondrial Particles. Yeast cells were harvested by centrifugation for 5 min at 5000*g*, washed with distilled water, and suspended with STE buffer (0.25 M sucrose, 0.02 M Tris-HCl, and 1 mM EDTA containing 1 mM $PhCH_2SO_2F$, pH 7.5). Cells were then broken by shaking with glass beads two times for 20 s in a Bronwill shaker, as described previously (Kim & Beattie, 1973). The suspension was centrifuged for 10 min at 1000*g* to remove unbroken cells and debris. After sedimenting at 18000*g* for 20 min, mitochondria were washed by suspension with a glass–glass homogenizer in STE buffer and centrifuged for 20 min at the same speed. The mitochondrial pellet was resuspended in sodium phosphate buffer (0.1 M Na_2HPO_4/NaH_2PO_4, 0.5 mM EDTA, and 1 mM $PhCH_2SO_2F$, pH 7.5) and sonicated for 2 min in 15-s bursts in a Branson sonifier (Branson Instruments, Inc., Danbury, CT) at a power setting of 4.5. The suspension was centrifuged for 10 min at 4300*g* in a Sorvall SS 34 rotor. The pellet was discarded and the supernatant was centrifuged for 30 min at 93000g_{av} in a Spinco no. 40 rotor. The pellet of submitochondrial particles (SMP) was resuspended in sodium phosphate buffer and the protein concentration adjusted to 10 mg/mL.

Purification of Spectrally Pure Cytochrome b. All manipulations for isolation of cytochrome *b* were performed at 0–4 °C. Centrifugations were performed in a Sorvall SS 34 rotor for 10 min at the speeds indicated. A 20% solution of sodium cholate, pH 8.0, was added to a SMP suspension at a protein concentration of 10 mg/mL to give a final concentration of cholate of 3.5%. After 75 mg of KCl/mL was slowly added, the suspension was stirred for 1 h at 0 °C and then centrifuged at 12000*g*. The supernatant was left overnight in the cold room (4 °C) and centrifuged again at 35000*g*. The pale gray pellet was discarded and solid ammonium sulfate was added slowly to the supernatant to a final concentration of 16%. The suspension was allowed to stand for 90 min at 0 °C with constant stirring before centrifugation at 35000*g*. The yellowish green supernatant was discarded and the reddish precipitate containing cytochrome *b* was used in further experiments.

For spectral analysis, the pellet containing cytochrome *b* was homogenized in sodium phosphate buffer and the protein concentration adjusted to approximately 3 mg/mL. Sodium cholate at a final concentration of 3.5% was added to dissolve the protein and to reduce the turbidity. Difference absorption spectra were obtained with a Cary Model 15 at room temperature with dithionite in the sample cuvette and potassium ferricyanide in the reference cuvette. The heme *b* content of the preparation was calculated as described previously (Lin & Beattie, 1978).

Labeling in Vivo. Cells were harvested in early stationary phase and washed twice with 100 mL of water. The cell pellet was weighed and resuspended in incubation medium containing 2% glucose and the inorganic salts described by Saltzgaber & Schatz (1978) to a concentration of 200 mg of wet cells/mL. The cell suspension was shaken for 5 min in a water bath at 30 °C, after which cycloheximide, to a final concentration of 1 mg/mL, was added and the incubation continued for 5 min. Labeled [^{35}S]methionine (75–100 μCi/100 mg of cells) was then added and the incubation was continued for the times indicated. Labeling was stopped 5 min before harvesting, by the addition of 10 mM unlabeled methionine.

The cells were then washed with 20 mL of water containing 0.5 mg of cycloheximide/mL and mitochondria prepared.

Preincubation with Chloramphenicol. Cells were grown at 30 °C in culture medium containing 5% glucose, for a period of 12 h necessary to reach an early stationary phase. To an aliquot of the cell culture, an alcoholic solution of chloramphenicol, 4 mg/mL of culture, was added. Another aliquot served as control. Incubation was continued for 2 h, after which cells were harvested and washed thoroughly three times with water. The washed cells were resuspended to the initial volume with fresh culture medium containing 0.1% glucose and 2.3% ethanol, incubated for another hour and then harvested. Both sample and control cells were washed twice with water, and equal weights of cells were resuspended in incubation medium and labeled with [^{35}S]methionine in the presence of cycloheximide, as described above.

Electrophoresis and Autoradiography. Disk electrophoresis in glass tubes of 12-cm length was performed in gels of different porosities (5–15% acrylamide) by using sodium dodecyl sulfate–phosphate buffer, according to Weber & Osborn (1969), and by using sodium dodecyl sulfate–urea buffer, according to Swank & Munkres (1971), for gels of 10% acrylamide.

Slab gels (10 cm long, 1.2 mm thick) were prepared according to Studier (1973), with minor modifications, and left overnight at room temperature for aging. The discontinuous sodium dodecyl sulfate–Tris buffer system, described by Laemmli (1970), was used for electrophoresis. The stacking gels were 5% acrylamide and the running gels were 5%, 7.5%, 10%, 12.5%, or 15% acrylamide, respectively. In some cases a 10–15% gradient gel was used.

The disk and slab gels were stained for 1 h with 1.25% Coomassie Blue in 45% methanol–9.2% acetic acid (v:v) and destained overnight in 5.5% methanol–7.5% acetic acid (v:v). In order to eliminate the variations due to gel elongation and shrinkage during the staining and destaining processes, the length of the gel was determined before and after staining and the position of the tracker dye was marked with a small needle.

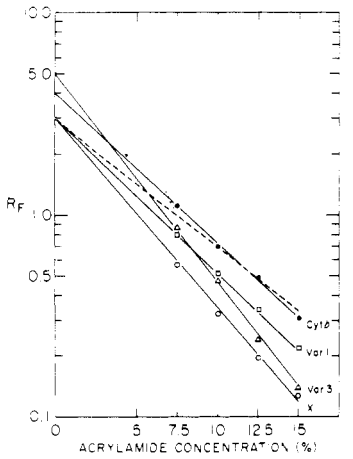

FIGURE 1: Ferguson plot of mitochondrial translation products. (□—□) VAR 1; (△—△) VAR 3; (●—●) cytochrome *b*; (○—○) χ, a mitochondrially synthesized polypeptide with a molecular weight of 67 000; (- - -) carbonic anhydrase, 30 000-dalton standard.

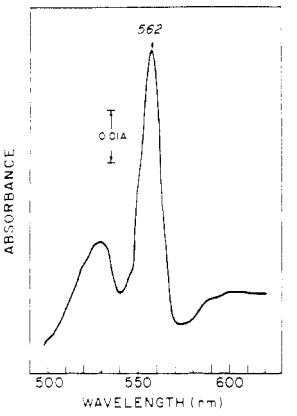

FIGURE 2: Difference spectrum of the cytochrome *b* preparation from yeast mitochondria. Dithionite-reduced vs. ferricyanide-oxidized spectrum was recorded at room temperature. Sample was suspended in sodium phosphate buffer, pH 7.5, containing 3.5% cholate at a protein concentration of 2.9 mg/mL.

The low molecular weight "Pharmacia" kit, used as a calibration standard, covered the molecular weight range between 14 400 and 94 000. Slab gels were dried for autoradiography on Whatman 3 MM filter paper by using a drying device similar to that described by Fairbanks et al. (1965) and exposed at −70 °C to Kodak NS-5T X-ray film for 1 week. Autoradiograms were scanned with a Canalco, Model J, microdensitometer.

Sample Preparations. The following dissociating systems were used to solubilize the proteins for electrophoresis: for disk gels prepared in sodium dodecyl sulfate–phosphate buffer, 0.01 M sodium phosphate, pH 7.2, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 10% glycerol; for the sodium dodecyl sulfate–urea system, 0.01 M Tris phosphate, pH 6.8, 1% sodium dodecyl sulfate, 8 M urea, 1% 2-mercaptoethanol; for the discontinuous buffer system (slab gels), 0.05 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 2 mM EDTA, 150 mM 2-mercaptoethanol, 10% glycerol. Samples treated with sodium dodecyl sulfate–urea buffer were heated for 10 min at 60 °C and stored overnight at room temperature. For electrophoresis in the other buffers, the samples were treated either overnight at 20 °C, for 2 h at 37 °C, for 20 min at 70 °C, or for 3 min at 100 °C. Bromophenol Blue (0.1%) in 25% glycerol was added as tracker dye to each sample, prior to electrophoresis.

Labeled mitochondria were adjusted to 2–6 mg of protein/mL and to 30 000–40 000 counts/10–20 μL of dissociating medium.

Ferguson Plots. The relative mobilities of cytochrome *b*, of other mitochondrial translational products, and of the standard proteins were plotted against the acrylamide concentration, according to Ferguson (1964). The "free mobilities" of the proteins were obtained by extrapolating the respective lines to "zero" concentration of acrylamide.

Materials. L-[4,5-^{3}H]Leucine (54 Ci mmol^{-1}) was obtained from Amersham, and L-[^{35}S]methionine (500 Ci mmol^{-1}) was obtained from New England Nuclear. Chloramphenicol, cycloheximide, phenylmethanesulfonyl fluoride, and 2-mercaptoethanol were from Sigma; acrylamide, bisacrylamide, and Temed (*N,N,N′,N′*-tetramethylenediamine) were from Eastman. Other chemicals used were of the highest purity.

Results

Molecular Weight Determination of Cytochrome b. Yeast cells were labeled with [^{35}S]methionine in the presence of cycloheximide under nongrowing conditions and the translation products present in intact mitochondria were examined by dodecyl sulfate gel electrophoresis at concentrations of acrylamide varying from 7.5% to 15% (Figure 1). The band corresponding to cytochrome *b* was identified by its identical migration in 10% acrylamide gels to that observed for the major band present in a specific immunoprecipitate. In addition, the identities of subunit II of cytochrome oxidase and cytochrome *b* were established by comparison with gel patterns of mutants lacking cytochrome *b* (Claisse et al., 1977). The anomalous migration behavior of cytochrome *b* at different concentrations of acrylamide as compared with standard proteins or to other mitochondrial products is clear. At low concentrations of acrylamide, the apparent molecular weight of cytochrome *b* by using carbonic anhydrase as a 30 000-dalton standard is less than 30 000, while, at higher acrylamide concentrations, its molecular weight is significantly greater than 30 000. In addition, Figure 1 shows that VAR 1 (Perlman et al., 1977) plus a minor product of mitochondrial protein synthesis with a molecular weight of 67 000 migrate the same as do standard proteins in different concentrations of acrylamide, while VAR 3, presumably a subunit of the oligomycin-sensitive ATPase (Perlman et al., 1977), also displays anomalous behavior. We have confirmed the results of Cabral et al. (1978) that subunit I of cytochrome oxidase behaves abnormally under these conditions, while subunits II and III behave normally (data not shown).

All the data indicating that cytochrome *b* behaved anomalously during dodecyl sulfate gel electrophoresis were obtained with either intact mitochondria or a purified b–c_1 complex (Groot et al., 1978). We felt that it would be of interest to study the migration of cytochrome *b* in a partially purified preparation in which cytochromes *a* and c_1 had been completely removed as determined by spectral analysis (Figure 2). The addition of ascorbate did not reduce this cytochrome, indicating a lack of cytochrome c_1 in the preparation. The specific heme content of this preparation obtained by ammonium sulfate fractionation of cholate-solubilized submitochondrial particles was 7.1 nmol/mg of protein, a value comparable to that observed in the b–c_1 complex obtained from yeast mitochondria (Siedow et al., 1978). When analyzed on a 10% acrylamide gel in the presence of sodium dodecyl sulfate, the presence of 4 major polypeptides with apparent molecular weights of 31 000, 35 000, 48 000, and 50 000 was revealed as well as 4-5 minor bands (Figure 3). The identification of the band of 31 000 daltons as cytochrome *b* was

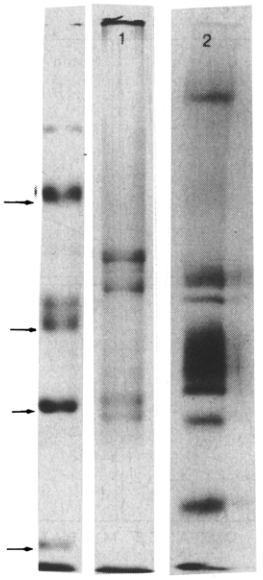

FIGURE 3: Identification of the 31 000-dalton polypeptide in the partially purified cytochrome *b* preparation as cytochrome *b*. Labeled mitochondrial translation products were separated on the same 10% polyacrylamide slab gel with a partially purified cytochrome *b* preparation. The gel was stained with Coomassie Blue and then dried for autoradiography. Lane 1 contains a stained cytochrome *b* preparation and lane 2 the labeled mitochondrial translation products. The arrows indicate the positions of the standard proteins bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), and myoglobin (17 200).

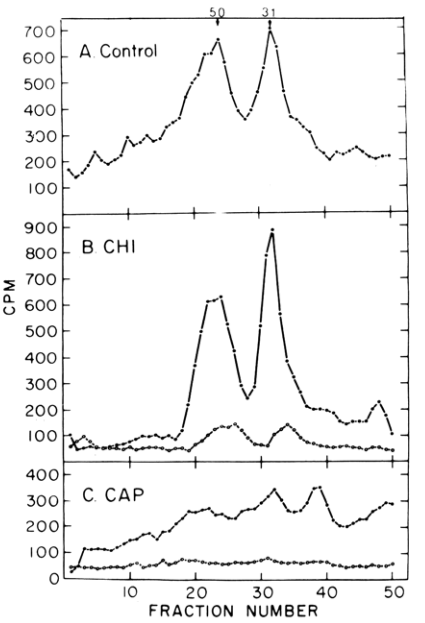

FIGURE 4: Immunoprecipitates formed with labeled mitochondria and cytochrome *b* antiserum. Washed, early stationary phase yeast cells were suspended at 250 mg/mL in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1% glucose and 2.3% ethanol (A) plus either (B) 100 μg of cycloheximide/mL or (C) 4 mg of chloramphenicol/mL. After 10-min incubation at 30 °C, 50 μCi of [^{3}H]leucine/mL was added and the incubation continued for 30 min. Unlabeled leucine (10 mM) was then added for another hour. Cells were harvested and mitochondrial extracts prepared, as described under Materials and Methods. Mitochondrial suspensions at 10 mg of protein/mL were treated according to Lin et al. (1978), with either preimmune serum (O—O) or with specific anticytochrome *b* serum (●—●), and the immunoprecipitate was allowed to form at 4 °C overnight. The mixture was centrifuged at 12000*g* for 10 min and then washed and depolymerized. The immunoprecipitates were analyzed by disk electrophoresis in 10% acrylamide. The arrows indicate calculated molecular weights of 50 000 and 31 000.

made possible by comparison with an autoradiograph of mitochondrial products subject to electrophoresis on the same slab gel (Figure 3), or with the labeled band present in the immunoprecipitate formed when labeled mitochondria were incubated with the specific antiserum against cytochrome *b*. The identity of the 35 000-dalton polypeptide is unknown; however, the higher molecular weight bands may be the so-called core proteins present in the b–c_1 complex as they have a similar molecular weight and comigrate with the core proteins of an isolated complex during gel electrophoresis (data not shown).

This preparation of cytochrome *b* was also subject to electrophoresis at various acrylamide concentrations ranging from 5% to 15%. The band corresponding to cytochrome *b* in this preparation migrated normally and the R_f values obtained when plotted against acrylamide concentration extrapolated back to the same point as do the standard proteins. The molecular weight of cytochrome *b* calculated as the mean of four determinations at each acrylamide concentration was 31 000.

The determinations were all performed on a cytochrome *b* preparation obtained when $PhCH_2SO_2F$ was added to all solutions used after the yeast cells were harvested. The molecular weight of cytochrome *b*, when the purification was performed in the absence of $PhCH_2SO_2F$, was unchanged. In addition, we varied the temperature used to solubilize the preparation prior to dodecyl sulfate gel electrophoresis: 100 °C for 3 min, 70 °C for 20 min, 37 °C for 2 h, or 20 °C overnight. In all cases a similar migration behavior of the bands in the preparation was observed. Similarly, the molecular weight of the band corresponding to cytochrome *b* was 31 000 after electrophoresis in sodium dodecyl sulfate–urea (Swank & Munkres, 1971). These results are in contrast to those of Capaldi et al. (1977), who reported that the polypeptides of complex III, especially cytochrome *b*, had different migrations depending on the conditions used to dissociate the proteins. One major difference observed was the increase in material remaining at the top of the gel in the samples which had been solubilized by heating to 100 °C. This may reflect aggregation of cytochrome *b* as a slight decrease in the staining intensity in the band corresponding to cytochrome *b* was observed.

Mitochondrial Translational Products Associated with Cytochrome b. In our previous studies on the biogenesis of cytochrome *b* in yeast mitochondria, we reported that one or two polypeptides of higher molecular weight in addition to cytochrome *b* were coprecipitated by the specific antiserum to cytochrome *b* (Lin et al., 1978). As seen in Figure 4, the labeling of these high molecular weight polypeptides is insensitive to cycloheximide and sensitive to chloramphenicol. Some nonspecific radioactivity is present in the immunoprecipitate formed by using mitochondria obtained from yeast labeled in the presence of chloramphenicol; however, no sharp peaks are observed corresponding in molecular weight to the labeled bands present in immunoprecipitates obtained from control cells or those labeled in the presence of cycloheximide.

The labeled mitochondrial products of higher molecular weight present in the immunoprecipitate migrate with a similar molecular weight (48 000–50 000) to the "core" proteins present in the isolated b–c_1 complex and the partially purified cytochrome *b* preparation (Figure 3). It was of interest to learn whether these possible "core" proteins in the isolated cytochrome *b* preparation were mitochondrial translation products and possibly those present in the immunoprecipitate. Hence, cytochrome *b* was partially purified from yeast cells

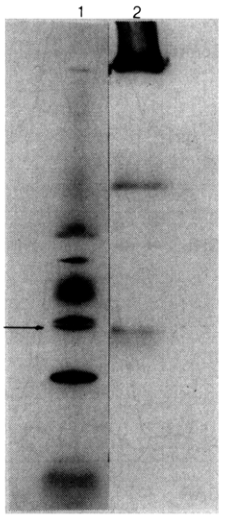

FIGURE 5: Comparison of total mitochondrial translation products and a labeled preparation of cytochrome *b*. Cells were labeled in vivo with [^{35}S]methionine in the presence of cycloheximide and fractionated as described under Materials and Methods. Electrophoresis was in 10–15% gradient polyacrylamide gels. Lane 1 contains an extract of labeled mitochondria and lane 2 a partially purified cytochrome *b* preparation. The arrow indicates cytochrome *b*.

labeled with [^{35}S]methionine in the presence of cycloheximide and the labeled proteins were studied by autoradiography. Considerable difficulties were encountered in achieving a pure preparation of cytochrome *b* when using small amounts of labeled mitochondria as starting material. Often contamination by other mitochondrial products, especially the subunits of cytochrome oxidase, was observed on the autoradiographs even when the proteins corresponding to those molecular weights were only barely detectable after staining with Coomassie blue. This problem was avoided by adding unlabeled mitochondria to the small amount of labeled mitochondria to yield sufficient starting material; however, the starting specific activity was considerably lowered. Figure 5 indicates that the purified cytochrome *b* preparation obtained in this manner contains a major labeled band with a molecular weight of 31 000, cytochrome *b*, plus a distinct band with a molecular weight of 67 000, a minor band when the total mitochondrial translation products are analyzed (cf. Figures 3 and 6). It should be noted that none of the labeled bands in the cytochrome *b* preparation correspond to any of the subunits of cytochrome oxidase. In addition, the two "core" proteins associated with cytochrome *b* during its separation from the b–c_1 complex are not labeled in the presence of cycloheximide. It thus appears that the mitochondrially synthesized proteins of 48 000–50 000 molecular weight present in the immunoprecipitate formed from the antiserum against cytochrome *b* are not the "core" proteins of the b–c_1 complex. Furthermore, these proteins are not associated with cytochrome *b* during its separation from the b–c_1 complex.

Another possibility is that the higher molecular weight proteins present in the immunoprecipitates are precursors of cytochrome *b* which are modified posttranslationally. To examine this possible precursor–product relationship, yeast cells were labeled with [^{3}H]leucine for 30 min, unlabeled leucine was then added, and the incubation was continued for 10 min or 2 h. After either the short or long chase, the high molecular weight material was still present in the immunoprecipitate. No decrease in radioactivity was observed in the polypeptides of higher molecular weight as compared with the labeling of the 31 000 molecular weight polypeptide corresponding to cytochrome *b* (data not shown).

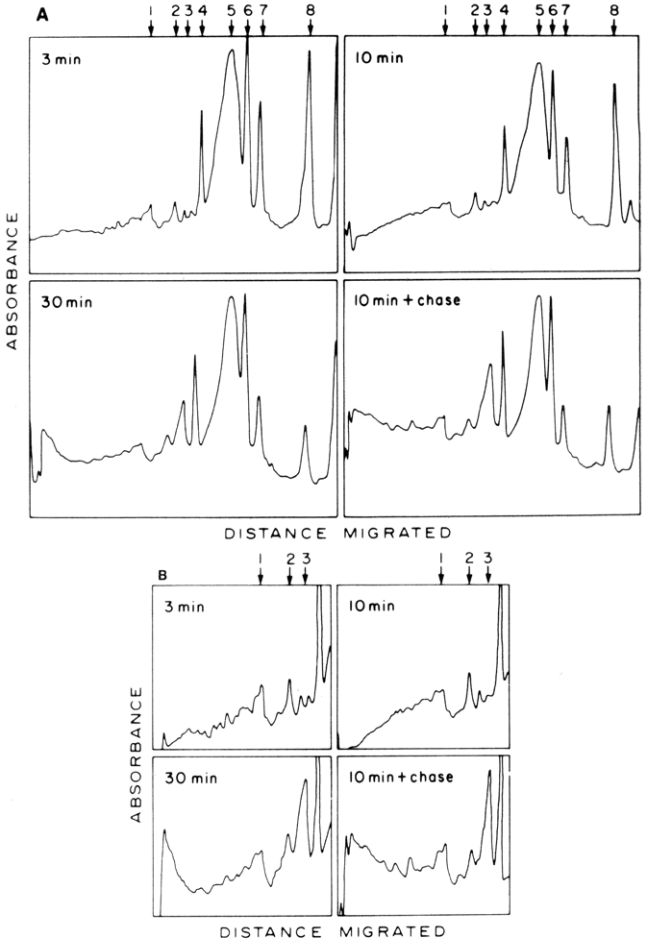

FIGURE 6: Time-course of labeling of mitochondrial translation products with [^{35}S]methionine. Cells were labeled in the presence of cycloheximide, as described under Materials and Methods for the periods of time indicated, with or without a 20-min chase. The labeled mitochondrial translation products were separated on 10% polyacrylamide slab gels and analyzed by scanning the autoradiograms with a Canalco microdensitometer. The numbers over the arrows identify the following: (1) an unknown polypeptide of 67 000 daltons; (2) an unknown polypeptide of 50 000 daltons; (3) VAR 3; (4) VAR 1; (5) cytochrome oxidase, subunit I; (6) cytochrome oxidase, subunit II; (7) cytochrome *b*; (8) cytochrome oxidase, subunit III. (A) The autoradiogram of mitochondrial labeled polypeptides was scanned at an arbitrary sensitivity scale. (B) The higher molecular weight mitochondrial polypeptides were analyzed at an expanded sensitivity scale.

In a further approach to learn whether the high molecular weight proteins were possible precursors of cytochrome *b*, the time course of labeling of mitochondrial products was examined by varying the time of the pulse with radioactive methionine from 3 to 30 min. Cytochrome *b* as well as the major subunits of cytochrome oxidase was labeled very rapidly, reaching a maximum after 3 min (Figure 6A). The amount of label present in subunits I (band 5) and II (band 6) of cytochrome oxidase as well as in VAR 1 (band 4) remained constant when the time of labeling was increased from 3 to 30 min. By contrast, the amount of label in the bands corresponding to both cytochrome *b* (band 7) and subunit III (band 8) of cytochrome oxidase decreased 50% or more with increasing time of incubation. The decrease in labeling in these two polypeptides was also observed during a 20-min chase with unlabeled methionine after an initial 10-min pulse labeled with [^{35}S]methionine.

The time course of labeling of the subunit of the oligomycin-sensitive ATPase, VAR 3 (band 3), differs completely from that of the other mitochondrial translation products.

Table I: Effect of Chloramphenicol Preincubation on Labeling of Mitochondria Products[a]

mitochondria	% total radioact.[b] control[c]	preincubn with CAP[d]	% change +121
VAR 3	24.4	41.2	+68
VAR 1	2.0	3.7	+85
cyt ox I	29.8	25.3	no change
cyt ox II	10	9.9	no change
cytochrome *b*	2.3	4.8	+109
cyt ox III	31.1	15.2	−49

[a] Cells were labeled in vivo with [^{35}S]methionine in the presence of cycloheximide after growth in the presence or absence of chloramphenicol, as outlined under Materials and Methods. The labeled mitochondrial translation products were electrophoresed on 10% polyacrylamide slab gels. The autoradiograms of the dried gels were scanned at 540 nm with a Gilford 240 spectrophotometer to which a Model 2410-s linear transporter was attached. [b] The percentage values were calculated by measuring the area under the respective labeled band. [c] 122 000 counts min^{-1} mg^{-1}. [d] 270 000 counts min^{-1} mg^{-1}.

After a pulse of 3 or 10 min, two very small bands are visible in the region of the gel corresponding to VAR 3; however, when the time of incubation was increased to 30 min, one large highly labeled band appeared in the gel. A similar increase in the labeling of VAR 3 was observed during the 20-min chase after the initial 10-min pulse. Changes in the labeling of subunit I of cytochrome oxidase were also apparent during increasing time of incubation. The labeled band present in the autoradiogram becomes more homogeneous and less diffuse over the gel, despite the maintenance of the same peak absorbance.

The time course of labeling of the polypeptides of molecular weight greater than that of VAR 1 was more closely examined by using an expanded scale on the gel scanner (Figure 6B). Two polypeptides (band 1) which migrate very close together with a molecular weight of approximately 67 000 were labeled rapidly with a 3-min pulse and maintained a constant amount of label throughout the incubation. By contrast, a less highly labeled polypeptide (band 2) with a molecular weight of approximately 50 000 was labeled to the same extent after a 3- or 10-min pulse, but continuing the incubation time to 30 min or addition of a 20-min chase with unlabeled methionine resulted in a significant decrease in labeling of this polypeptide.

Effect of Chloramphenicol on the Labeling of Cytochrome b. Previously, several groups (Tzagoloff, 1973; Ibrahim et al., 1973; Katan et al., 1976) reported that preincubation of growing yeast in chloramphenicol for 2–3 h prior to pulse labeling in the presence of cycloheximide resulted in increased radioactivity present in isolated mitochondria. Subsequently, the increased rates of mitochondrial protein synthesis observed under these conditions were shown to result from an increased rate of chain initiation on mitochondrial polysomes (Ibrahim & Beattie, 1976). The increased resolution of mitochondrial translation products afforded by slab gel electrophoresis and the positive identification of the various labeled polypeptides present in the gel has permitted us to analyze which mitochondrial products contain increased label after the preincubation with chloramphenicol. The specific radioactivity of the isolated mitochondria obtained after this treatment was increased 50% as compared with control mitochondria (Table I). The amount of label in each band was determined by scanning the autoradiogram and measuring the area under each peak. As seen in Table I, labeling into three mitochondrial products VAR 3, VAR 1, and cytochrome *b* is nearly doubled after the preincubation in chloramphenicol. The label in subunits I and II of cytochrome oxidase remains constant after this treatment, while that in subunit II of cytochrome oxidase decreased.

Discussion

Several different values for the molecular weight of cytochrome *b* from the respiratory chain of yeast as well as mammalian mitochondria have been reported (Weiss, 1976). These varying estimates may have occurred because of species differences in the molecular weight, while others may have resulted because of the anomalous behavior of cytochrome *b* during gel electrophoresis (Marres & Slater, 1977; Groot et al., 1978). From their studies, Groot et al., (1978) have concluded that cytochrome *b* has a true molecular weight of 55 000. By contrast, Weiss & Ziganke (1974) have suggested that cytochrome *b* from *Neurospora* exists as a dimer with a molecular weight of 56 000. In all of these studies, where an anomalous migration of cytochrome *b* was observed, this protein was present either in the intact mitochondrial membrane or associated with other proteins in the cytochrome b–c_1 complex.

Hence, we decided to reinvestigate the behavior of cytochrome *b* during sodium dodecyl sulfate gel electrophoresis using a partially purified preparation. For these studies, it was impossible to use our previously purified cytochrome *b* since proteolytic digestion was a necessary step during the purification (Lin & Beattie, 1978). By solubilizing submitochondrial particles in low concentrations of cholate followed by ammonium sulfate precipitation, we have been able to obtain a spectrally pure cytochrome *b* preparation which contains four major polypeptides. The band in this preparation corresponding to cytochrome *b*, identified by comparison with total mitochondrial translation products, displayed normal migration behavior during sodium dodecyl sulfate electrophoresis in different concentrations of acrylamide with an apparent molecular weight of 31 000. It thus appears that considerable changes occur when cytochrome *b* is separated from cytochrome c_1 and other associated proteins in the b–c_1 complex. Perhaps cytochrome *b* is buried in the complex in such a way that it incompletely binds sodium dodecyl sulfate. Alternately, the conformation of cytochrome *b* may change upon cleavage of the cytochrome b–c_1 complex. This suggestion is supported by the observations that two specifically and kinetically different species of cytochrome *b* are present in mitochondria and an isolated cytochrome b–c_1 complex but cannot be observed when the complex has been split (Weiss, 1976). We have concluded from these studies that the actual molecular weight of cytochrome *b* is 31 000. In addition, this polypeptide migrates with an identical molecular weight in the sodium dodecyl sulfate–urea gels of Swank & Munkres (1971).

The second major objective of the current study was to elucidate the nature of the proteins which coprecipitate with cytochrome *b*, when mitochondria are treated with the specific antiserum against cytochrome *b* (Lin et al., 1978). This antiserum was shown to be specific for cytochrome *b* by immunodiffusion and counterimmunoelectrophoresis. The data obtained in this study indicate that these polypeptides are indeed products of protein synthesis on mitochondrial ribosomes, as they are labeled in intact yeast cells in the presence of cycloheximide but not labeled in the presence of chloramphenicol. Furthermore, these labeled proteins are not present in the immunoprecipitates formed in a petite mutant of yeast lacking mitochondrial protein synthesis (Lin et al., 1978). These proteins in the immunoprecipitate have a molecular weight of approximately 50 000 but do not appear

to be the so-called "core" proteins of the b–c_1 complex. The partially purified preparation of cytochrome b described above contains two proteins of 48 000–50 000 molecular weight which copurify with the core proteins of a purified b–c_1 complex (C. A. Battie, R. A. Weiss, and D. S. Beattie, unpublished results). However, when this cytochrome b preparation was isolated from cells labeled in the presence of cycloheximide in yeast cells, these proteins did not contain any label. This result was anticipated since Katan et al. (1976) have previously reported that cytochrome b is the only polypeptide of the cytochrome b–c_1 complex of yeast synthesized within the mitochondria. Furthermore, these results suggest that none of the proteins which are associated with cytochrome b during purification are products of mitochondrial protein synthesis with the exception of the 67 000-dalton polypeptide.

These higher molecular weight polypeptides also do not appear to be precursors of cytochrome b. Addition of a short or long chase with unlabeled amino acid did not alter the amount of radioactivity in the higher molecular weight polypeptides of the immunoprecipitate relative to that in cytochrome b. Varying the time of pulse labeling in the presence of cycloheximide from 3 to 30 min also did not indicate any precursor–product relationship between the high and low molecular weight products of mitochondrial protein synthesis.

These studies have indicated, however, that mitochondrial translation products are labeled in vivo with very different kinetics. One explanation for the loss of label in cytochrome b and other proteins is that of Costantino & Attardi (1977) who suggested that cytoplasmically synthesized proteins may be necessary to stabilize mitochondrial products in HeLa cells.

The identity of these higher molecular weight polypeptides which coprecipitate with cytochrome b remains unclear, but several possible explanations are suggested by the experimental results. These polypeptides may be migrating in sodium dodecyl sulfate–acrylamide gels at or near the true molecular weight of cytochrome b, as determined from the Ferguson plots. Alternately, they may represent cytochrome b present in the dimeric form. Perhaps, when cytochrome b has been precipitated as an immune complex with its antibody, the further solubilization with detergent is affected. Alternately, these associated polypeptides may be nonspecifically trapped in the antigen–antibody complex and, hence, be unrelated to cytochrome b.

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