Two Forms of Cytochrome *b* in Yeast Mitochondria: Purification, Characterization, and Localization in the Inner Mitochondrial Membrane†

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ABSTRACT: Two forms of cytochrome *b* with molecular weights of 31 000 and 29 000 have been purified from yeast mitochondria by cholate extraction, ammonium sulfate precipitation, chromatography on Ultrogel AcA 44, and preparative gel electrophoresis in sodium dodecyl sulfate. Both purified proteins cross-reacted with the antiserum against cytochrome *b* purified earlier in our laboratory [Lin, L.-F. H., Clejan, L., & Beattie, D. S. (1978) *Eur. J. Biochem. 87*, 171–179] and revealed an identical fingerprint pattern after limited proteolysis with *Staphyloccus aureus* V8 protease. Antisera against the two forms of cytochrome *b* were raised and shown to cross-react with each other, suggesting that these two forms of cytochrome *b* share a common amino acid sequence with the exception of a 2000-dalton residue. Immunoprecipitates obtained with the antisera against both forms of cytochrome *b* and mitochondria isolated from yeast cells labeled with [^{3}H]leucine in vivo in the presence of cycloheximide contained a single labeled band with an apparent molecular weight of 31 000, suggesting that the primary translation product on mitochondrial ribosomes is the 31 000-dalton cytochrome *b* which is posttranslationally cleaved to the 29 000-dalton form. The antisera against both forms of cytochrome *b* partially inhibited coenzyme QH_2–cytochrome *c* reductase activity in mitoplasts and submitochondrial particles, suggesting that cytochrome *b* spans the inner mitochondrial membrane. Mitochondria and submitochondrial particles were treated with the nonpermeant reagent [^{125}I]diazobenzenesulfonate [DABS] and immunoprecipitated with the specific antisera against both forms of cytochrome *b*. In mitochondria, two major bands with molecular weights of 31 000 and 29 000 were labeled with [^{125}I]DABS, suggesting that both cytochromes *b* extend from the cytoplasmic surface of the inner membrane. By contrast, a single band with a molecular weight of 29 000 is labeled with [^{125}I]DABS in submitochondrial particles, suggesting that only this form of cytochrome *b* extends from the matrix side of the membrane.

The existence of two functionally different forms of cytochrome *b* in the coenzyme Q–cytochrome *c* reductase segment of the mitochondrial respiratory chain has been proposed. The two cytochromes *b* differ in the wavelength of their maximum light absorption, the kinetics of their oxidation and reduction, and their oxidation–reduction potentials (Chance 1958; Wainio, 1977; Von Jagow & Sebald, 1980). The functional properties which differentiate the two forms of cytochrome *b* can be readily distinguished in the intact membrane of either mitochondria or submitochondrial particles or in an isolated complex III; however, these differences disappear when cytochrome *b* has been removed from the other proteins with which it is associated in complex III.

Cytochrome *b* is a hydrophobic integral membrane protein with an apparent molecular weight of 30 000–31 000 when analyzed by sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis (Beattie et al., 1979; Weiss & Ziganke, 1974; Von Jagow et al., 1978). A dimeric form of cytochrome *b* with an apparent molecular weight of 58 000–60 000 has been observed when mild detergents such as deoxycholate or Triton X-100 are used for solubilization (Von Jagow et al., 1978; Weiss & Ziganke, 1976). The existence of a dimeric form of cytochrome *b* was not surprising, since complex III isolated from several types of mitochondria contains 2 mol of cytochrome *b* per mol of cytochrome c_1 (Rieske, 1976). The dimer of cytochrome *b* has been considered to be a homodimer (Von Jagow & Engel, 1980), since genetic evidence indicates the presence of a single gene for cytochrome *b* in yeast mitochondria (Nobrega & Tzagoloff, 1980). By contrast, Weiss & Ziganke (1976) previously reported the separation of two subunits of cytochrome *b* from *Neurospora crassa* mitochondria by means of hydroxylapatite chromatography in the presence of $NaDodSO_4$. These two forms of cytochrome *b* had similar amino acid composition, carboxy terminals, and fingerprint pattern after cleavage with cyanogen bromide. No further characterization of these two forms of cytochrome *b* has been reported.

In the present study, the isolation of two forms of cytochrome *b* with apparent molecular weights of 31 000 and 29 000 is reported. When an immunological approach with antisera directed against both proteins is used, it is concluded that the primary translation product of the cytochrome *b* gene is the 31 000-dalton form which is posttranslationally cleaved to the 29 000-dalton form. Furthermore, labeling studies of mitochondria and submitochondria with the membrane nonpermeant reagent diazobenzenesulfonate (DABS)[1] coupled with the immunoinhibition of coenzyme QH_2–cytochrome *c* reductase activity with antisera against both proteins suggest that cytochrome *b* spans the inner mitochondrial membrane.

Materials and Methods

Growth of Yeast and Preparation of Mitochondria. The strain of yeast used in these studies is a haploid strain, KL14-4A. Cells were grown aerobically at 30 °C with 3% galactose as carbon source, under the conditions described previously (Brown & Beattie, 1977). Growth medium con-

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DABS, diazobenzenesulfonate; DBH_2, decyl analogue of coenzyme Q [2,3-dimethoxy-5-methyl-6-(*n*-decyl)-1,4-benzoquinone]; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosyl-2-phenylethyl chloromethyl ketone.

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tained 0.3% yeast extract, 0.04% $CaCl_2$, 0.05% NaCl, 0.07% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, 0.12% $(NH_4)_2SO_4$, 0.5 mg % $FeCl_3$, and 3% galactose.

Yeast cells were harvested by centrifugation for 5 min at 5000*g*, washed with distilled water, and suspended in buffer containing 0.25 M sucrose, 0.02 M Tris-HCl, 1 mM EDTA, and 1 mM PMSF, pH 7.5 (STE). Cells were broken by shaking with glass beads 2 times for 20 s in a Bronwill shaker, and mitochondria were isolated as described by Kim & Beattie (1973). Mitochondria were also isolated from spheroplasts by a modification of the procedure described by Schatz & Kovacs (1974). Cells were harvested in late log phase, washed with distilled water, and centrifuged at 1200*g* for 5 min before an incubation in 20 mM Tris-HCl, 20 mM EDTA, and 50 mM β-mercaptoethanol, pH 9.0, for 15 min at 30 °C to soften the cell wall. After the cells were washed 3 times with distilled water and recentrifuged at 1200*g* for 5 min, they were suspended in 1 M sorbitol containing 20 mM KH_2PO_4, pH 7.4, at a concentration of 250 mg of cells/mL and lysed with 2 mg of Zymolyase 5000 per g of cells at 30 °C for 1 h in a metabolic shaker. After centrifugation at 1200*g* for 5 min, the sedimented spheroplasts were washed and resuspended in 0.6 M mannitol, 1 mM EDTA, 20 mM Tris-HCl, 1 mM PMSF, and 0.1% bovine serum albumin, pH 6.8, at a ratio of 3 g of cells/10 mL of buffer. The spheroplast suspension was shaken in 10–20-mL aliquots in a small Waring blender for 20–25 s at high speed. The broken suspension was centrifuged 5 min at 1200*g* to remove unbroken spheroplasts and mitochondria isolated (Schatz & Kovacs, 1974).

Mitoplasts were prepared by the digitonin fractionation procedure of Velours et al. (1977), with some modifications. Mitochondria, isolated from spheroplasts as described above, were suspended in 0.6 M mannitol and 10 mM sodium phosphate, pH 7.0, at a protein concentration of 10 mg/mL. A stock solution of 35 mg of digitonin/mL of water, prepared freshly by gently heating in a hot water bath, was added to the mitochondrial suspension to a final concentration of 3 mg of digitonin/10 mg of mitochondrial protein. After a 5-min incubation at 4 °C, the mitoplasts were sedimented by a 10-min centrifugation at 12000*g*.

Submitochondrial particles were prepared from yeast cells broken with the Bronwill shaker. The mitochondrial pellet was resuspended in sodium phosphate buffer (0.1 M Na_2HPO_4 and NaH_2PO_4, 0.5 mM EDTA, and 1 mM PMSF, pH 7.5) and sonicated for 2 min in 15-s bursts in a Branson sonifier at a power setting of 4.5. The suspension was centrifuged for 10 min at 8000*g*. The pellet was discarded, and the supernatant was centrifuged for 30 min at 100000*g*. The pellet was resuspended in sodium phosphate buffer containing PMSF and the protein concentration adjusted to 10 mg/mL.

Purification of Cytochrome b. A 20% solution of sodium cholate, pH 8.0, was added to the suspension of submitochondrial particles to a final concentration of 3.5%. After 75 mg of KCl/mL was added, the suspension was stirred for 1–3 h at 4 °C and then centrifuged 10 min at 12000*g*. The supernatant was kept at 4 °C overnight and centrifuged again at 35000*g* for 10 min.

Solid ammonium sulfate was added to the supernatant to a final concentration of 16% saturation. The suspension was maintained in an ice bath for 90 min with constant stirring and centrifuged 10 min at 35000*g*. The yellowish green supernatant was discarded, the red pellet was homogenized in sodium phosphate buffer containing PMSF, and the protein concentration was adjusted to approximately 3 mg/mL. Sodium cholate at a final concentration of 3.5% was added to dissolve the protein. This preparation contained spectrally pure cytochrome *b* (Beattie et al., 1979).

For further purification, the ammonium sulfate precipitate containing cytochrome *b* was solubilized in 0.1 M Tris–acetate, 5% sodium dodecyl sulfate, and 5% 2-mercaptoethanol at room temperature for 2 h and subjected to gel filtration through a 1-m Ultrogel ACA 44 column, as described by Weiss & Juchs (1978). The column was equilibrated with a buffer containing 0.1% sodium dodecyl sulfate, 2 mM dithiothreitol, 50 mM Tris–acetate, pH 7.0, and 0.02% sodium azide. The sample was eluted with the same buffer into approximately 300 fractions containing 1.6 mL. Several fractions containing the heme of cytochrome *b*, as determined by the optical density at 418 nm, were pooled and concentrated 10–20 times in a minicon B15 concentrator.

After analysis by dodecyl sulfate–polyacrylamide gel electrophoresis, the fraction obtained after Ultrogel chromatography containing proteins with molecular weights in the 30000 range was subjected to further electrophoresis under denaturing conditions on a super 10% acrylamide gel (25 × 12 × 0.4 cm thick). Super gels were stained with 0.25% Coomassie blue in 45% methanol and 9.2% acetic acid and destained by shaking in 5% methanol and 7.5% acetic acid. Two major bands with molecular weights of 31000 (protein I) and 29000 (protein II) were excised from the super gels and soaked in 0.1% dodecyl sulfate, 1 mM EDTA, 0.125 M Tris-HCl, pH 6.8, and 1 mM dithiothreitol for 1 h. These gel slices were further subjected to a preparative gel electrophoresis consisting of a modified funnel-shaped 10% acrylamide gel with its bottom attached by a close-ended dialyzing Spectrapor 1 membrane tubing with a molecular weight cutoff of 6000–8000. The preparative gel electrophoresis was performed in the cold room at 4 °C for 24 h until the dye front had entered the dialysis tubing. For removal of the dye, protein samples were extracted once with acidic acetone and twice with iced acetone (Chua & Blomberg, 1979).

Proteolytic Digestion. Limited proteolytic digestion of the purified proteins and labeled cytochrome *b* was performed by the method of Cleveland et al. (1977). So that samples of the protein for digestion could be obtained, the spectrally pure cytochrome *b* preparation was separated on a super gel (25 × 12 × 0.4 cm). Two major Coomassie blue stained bands, one corresponding to protein I of 31000 daltons and the other corresponding to protein II of 29000 daltons, were excised, trimmed to slices of 10 × 4 × 1 mm, and soaked in buffer containing 0.1% dodecyl sulfate, 1 mM EDTA, 0.125 M Tris-HCl, pH 6.8 and 1 mM dithiothreitol for 30 min prior to transfer to the sample well for digestion. Digestion was performed in a tall slab gel (14 cm long, 1 or 2 mm thick) containing 15% acrylamide with a stacking gel 4 cm long. *S. aureus* V8 protease (25 or 50 μg) was loaded into the sample well with the gel slice to be analyzed. Sample proteins were digested in the stacking gel for 40 min at 37 °C. The remaining electrophoresis was performed at 4 °C to avoid further digestion. The gel was fixed and stained with Coomassie blue.

Immunological Studies. Purified proteins I and II were each suspended in 0.2 mL of 10 mM sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate, and the final volume was adjusted to 1.0 mL with physiological saline. Two rabbits were injected with a total of 225 μg of protein of each protein in two equal doses intravenously in the ear. The second injection was 40 days after the first injection. Samples of blood were collected on the 40th day, the 47th day, and the 64th day after the first injection. Sera were obtained by centrifugation at 1500*g* for 20 min. In some experiments, an IgG was further

purified from the antisera against proteins I and II as described by Chan & Schatz (1979).

Control serum was obtained from nonimmunized rabbits and an IgG fraction obtained by the same procedure.

Counterimmunoelectrophoresis and immunodiffusion were performed as described previously (Lin et al., 1978). Agarose slices were made of 1% agarose, 150 mM NaCl, 1% Triton X-100, and 0.1% NaN_3 in 38 mM Tris–glycine, pH 8.6, also used as the running buffer. An aliquot of 10–20 μL of either purified proteins I or II (1.2 mg/mL) was placed in the cathodic well while an aliquot of 10 μL of antisera was placed in the anionic well. Electrophoresis was performed for 3 h at 4 mA/slice. After the electrophoresis, the agarose slices were pressed for 30 min to remove the buffer, rinsed, stained, and destained as previously described (Lin et al., 1978).

For immunoprecipitation, labeled mitochondria, mitoplasts, or submitochondrial particles were solubilized by the following techniques: (1) 1% Lubrol WX and 1% sodium deoxycholate at 4 °C for 1 h (Lin et al., 1978); (2) 1 M KCl and 1% Triton X-100 by shaking at 37 °C for 2 h (Bell et al., 1979); (3) 5% Triton X-100 and 0.1% sodium dodecyl sulfate by shaking at 37 °C for 2 h (Nelson & Mendel-Hartvig, 1977). The solubilized extracts were then centrifuged at 12000*g* for 10 min to remove any unsolubilized material.

An immunotitration curve was obtained by incubating a fixed amount of solubilized membrane extract (10 μL, 10 mg/mL) with varying amounts of antiserum. The mixtures of extract and serum were incubated at 4 °C for 14 h and then centrifuged for 2 min in an Eppendorf centrifuge. The immunoprecipitates were washed 3 times prior to addition of dissociation buffer (5% sodium dodecyl sulfate, 2 mM EDTA, 5% 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, and 10% glycerol). The samples were incubated at 37 °C for 2 h or at room temperature overnight. The dissociated precipitates were either counted in Liquiscint (National Diagnostics) or further analyzed by polyacrylamide gel electrophoresis.

Labeling of Cells and Electrophoresis. Cells were grown in growth medium containing 0.67% yeast nitrogen base, 0.2% yeast extract, 1% glucose, and 5 mCi/L [^{3}H]leucine for 10 generations prior to harvest. Cells were also labeled with [^{35}S]methionine or [^{3}H]leucine under nongrowing condition in the presence of cycloheximide as described previously (Beattie et al., 1979).

Slab gels (10 cm long, 1.2 mm thick) were prepared as described by Studier (1973) with minor modifications. The discontinuous sodium dodecyl sulfate–Tris buffer system (Laemmli, 1970) was used for electrophoresis. The stacking gels were 5% acrylamide, and the running gels were 10% acrylamide except where indicated. In some cases, longitudinal strips from a slab gel were also sliced manually by using a slicer made of a row of 100 razors. It was necessary to freeze the gel strips on a piece of flat dry ice prior to slicing. The gel slices were digested with a few drops of hydrogen peroxide at 70 °C for a minimum of 3 h and dissolved in Liquiscint prior to counting.

Enzymatic Assays. Cytochrome *c* reductase was assayed according to Brown & Beattie (1977) with the reduced form of 2,3-dimethoxy-5-methyl-6-(*n*-decyl)-1,4-benzoquinone (DBH_2) as substrate. The reduction of DB was carried out by the method of Rieske (1976).

For the inhibition experiments, 10, 20, or 40 μL of purified IgG fractions containing 8.5 mg of protein/mL were incubated with 10 μL (15 μg of protein) of 2% cholate-solubilized submitochondrial particle preparation or a mitoplast suspension for 45 min at room temperature before each assay. The same amounts of IgG fraction purified from control serum were used in parallel as a control.

Labeling of Mitochondria and Submitochondrial Particles with [^{125}I]DABS. ^{125}I-Labeled diazotized iodosulfanilic acid was prepared according to New England Nuclear product information. After addition of 45 nmol of sulfanilic acid as a carrier, 1 mCi of [^{125}I]iodosulfanilic acid (2200 Ci/mmol) in 10 μL of water was diazotized by adding 5 μL of 50 mM sodium nitrite and 5 μL of 0.1 N hydrochloric acid at 4 °C, followed by a neutralization with 170 μL of 100 mM phosphate buffer (pH 8.3).

An aliquot of 100 μL of mitochondria was suspended at 2 mg of protein/mL in 0.25 M sucrose and 10 mM sodium phosphate, pH 7.5, and a 100-μL aliquot of submitochondrial particles was suspended in 0.1 M sodium phosphate and 0.5 mM EDTA, pH 7.5. Both aliquots were incubated with 400 μCi of [^{125}I]DABS at 4 °C for 30 min with frequent vortexing. Labeling was terminated by addition of 5 mM histidine and immediate centrifugation. Iodinated mitochondria and submitochondrial particles were washed twice with 4 mL of 100 mM Tris-HCl and 5 mM histidine, pH 7.5, and twice with 100 mM Tris-HCl, and 2.5% bovine serum albumin and then solubilized either with 1% Lubrol–1% sodium deoxycholate at 4 °C for 1 h or with 1% Triton X-100–1 M potassium chloride at 37 °C for 2 h. After centrifugation at 12000*g* for 10 min to remove unsolubilized membranes, labeled mitochondria and submitochondrial particles were immunoincubated with the optimum amount of IgG fractions I and II and the control IgG fraction. Iodinated immunoprecipitates were analyzed on a 10% acrylamide gel electrophoresis and autoradiographed as described above.

Materials. L-[^{35}S]Methionine (1000 Ci mmol^{-1}) and L-[4,5-^{3}H]leucine (55 Ci mmol^{-1}) were obtained from Amersham. [^{125}I]Iodosulfanilic acid (2200 Ci mmol^{-1}) was purchased from New England Nuclear. Zymolyase 5000 was from Kirin Brewery, Japan. Sodium cholate and sodium deoxycholate were from Calbiochem. Digitonin, cycloheximide, Coomassie brilliant blue, phenylmethanesulfonyl fluoride, and β-mercaptoethanol were obtained from Sigma. Ultrogel AcA 44 was from LKB, Bromma; Minicon B15 concentrator was from Amicon. Acrylamide, bis(acrylamide), and TEMED (*N,N,N',N'*-tetramethylenediamine) were from Eastman. Protein A–Sepharose was purchased from Pharmacia, whereas *Staphylococcus aureus* V8 protease was from Miles Laboratories. Other chemicals used were of the highest purity.

Results

Purification of Cytochrome b from Growing Yeast. For further investigations of the properties of native cytochrome *b*, it was necessary to develop a new purification scheme, since proteolytic digestion was a necessary step in our previous preparation of purified cytochrome *b* (Lin & Beattie, 1978). Submitochondrial particles were solubilized with cholate followed by fractionation with 16% saturated ammonium sulfate to yield a red pellet containing spectrally pure cytochrome *b* separated from cytochrome c_1 (Beattie et al., 1979). The red pellet which consisted of four major polypeptides was solubilized as described under Materials and Methods prior to gel filtration on an Ultrogel column as described by Weiss & Juchs (1978). Four peaks containing heme, indicated by the absorbance at 418 nm, were concentrated and analyzed by $NaDodSO_4$–gel electrophoresis to indicate the polypeptide composition of each peak. The majority of cytochrome *b* heme as determined by the absorbance at 418 and 280 nm was present in the first peak from the column. The cytochrome

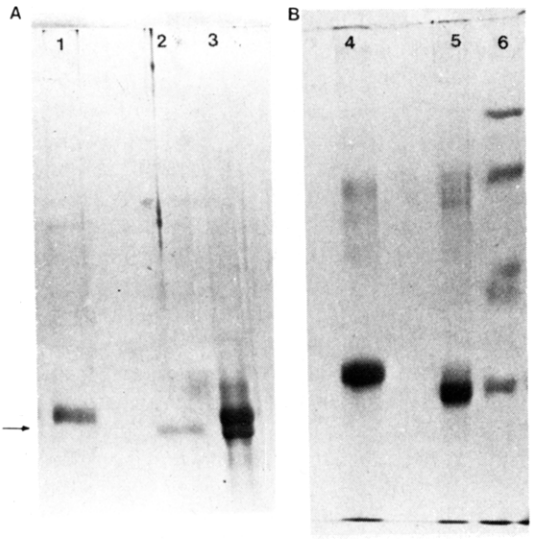

FIGURE 1: (A) $NaDodSO_4$–polyacrylamide gel electrophoresis of the two partially purified proteins excised from a prepartive gel as described under Materials and Methods. (Lane 1) Purified upper band (10 μg) with apparent molecular weight of 31,000; (lane 2) 10 μg of purified lower band with apparent molecular weight of 29 000; (lane 3) third peak obtained after Ultrogel chromatography. Arrow on the left indicates the migration of the M_r 30 000 standard. (B) (Lane 4) Upper band (30 μg); (lane 5) 30 μg of lower band; (lane 6) molecular weight standards corresponding to M_r 94 000, 67 000, 43 000, and 30 000, respectively, from the top of the gel.

b present in this fraction appeared to have aggregated and migrated slowly with an apparent molecular weight of 55 000 or greater, although a slight band migrating with a molecular weight of 31 000 was observed. The second peak from the column contained a prominent polypeptide with a molecular weight of 55 000 (which may possibly be a dimeric or aggregated form of cytochrome *b*) in addition to two bands with molecular weights of 31 000 and 29 000. The third peak from the column contained two major polypeptides with molecular weights of 31 000 and 29 000 corresponding to the reported molecular weight of cytochrome *b* (Beattie et al., 1979). The fourth peak from the column did not contain protein stainable with Coomassie blue and thus may contain free heme dissociated from cytochrome *b* leading to the observed absorption at 418 nm. The two major polypeptides of 31 000 and 29 000 daltons present in the third peak from the Ultrogel column were resolved and purified by electrophoresis on super slab gels, as described under Materials and Methods. Two single polypeptides, protein I of 31 000 daltons and protein II of 29 000 daltons, respectively, were obtained by this purification procedure (Figure 1A). No contaminating proteins were observed after electrophoresis of these two proteins in gels containing 10%, 12%, or 15% acrylamide. It should also be noted that these two proteins migrated with the apparent molecular weights of 31 000 and 29 000 at all acrylamide concentrations and did not exhibit anomalous migration behavior, as is true for cytochrome *b* in the mitochondrial membrane (Beattie et al., 1979). When larger amounts of protein were applied to the gels, occasionally a faint band of stained material with an approximate molecular weight of 67 000 was observed (Figure 1B). This material may represent aggregates of cytochrome *b* formed during the experimental manipulations, as was observed when the labeled band corresponding to cytochrome *b* was excised from a gel and subjected to further electrophoresis (Beattie & Clejan, 1980).

Identification of Protein I and Protein II as Apocytochrome b. For determination of which of these two purified proteins was antigenically related to cytochrome *b*, counterimmunoelectrophoresis of protein I and protein II was performed. As seen in Figure 2, both polypeptides formed obvious precipitin lines with the specific antiserum raised against the purified cytochrome *b* obtained by Lin et al. (1978). No precipitin line was observed when the same samples were electrophoresed against control serum. Similarly, immunodiffusion studies of the two proteins with the antiserum against purified cytochrome *b* revealed a line of identity. These results indicate that both protein I and protein II are antigenically similar to cytochrome *b*.

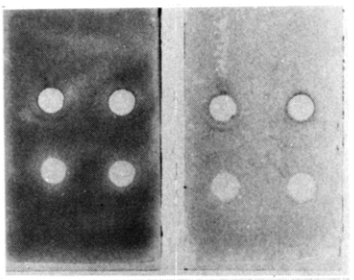

FIGURE 2: Counterimmunoelectrophoresis of protein I and protein II with antiserum to cytochrome *b*. The antiserum (10 μL) to cytochrome *b* was placed in the anodic wells (1 and 3) on the top, while 10 μL of preimmune serum were placed in wells 2 and 4 from left to right. Samples of purified proteins were placed in the cathodic wells on the bottom. Wells 1 and 2 contain 25 μg of protein I; wells 3 and 4 contain 24 μg of protein II.

As an additional approach to establish the relationship of protein I and protein II to apocytochrome *b*, a limited proteolysis of both proteins excised from a super gel was performed according to the method of Cleveland et al. (1977). A similar fingerprint pattern was obtained after digestion of protein I (Figure 3, lanes 1 and 2) and protein II (lane 3) with *S. aureus* V8 protease. The observed pattern differs completely from that obtained with protease alone (lane 4). Lanes 5 and 6 contain undigested protein II and protein I, respectively. The digestion pattern of protein II in lanes 1 and 2 includes a broad undigested protein band with the same mobility as the undigested band in lane 5 plus several bands migrating above it. Those polypeptides which migrate as if of greater molecular weight than the original protein may result from the interaction of hydrophobic peptide fragments obtained during digestion, either with the native apocytochrome *b* or with each other. These bound peptides may not dissociate during the subsequent electrophoresis. Limited proteolysis of protein I (lane 3) produced one peptide with the same mobility as undigested protein II, suggesting that protein II does indeed represent a part of protein I. These results plus the immunological response to the antiserum against cytochrome *b* suggest that proteins I and II are two forms of cytochrome *b* sharing a major peptide sequence with the exception of a 2000-dalton residue missing from protein II.

Biogenesis of Apocytochrome b-I and Apocytochrome b-II. For investigation of the relationship between apocytochrome *b* protein I and protein II and for studies on the biosynthesis of cytochrome *b*, antisera against both proteins were raised as described under Materials and Methods. An Ouchterlony double-diffusion test revealed that both antisera reacted with the third peak obtained after chromatography on Ultrogel AcA 44 (Figure 1), leading to the formation of significant precipitin lines. Counterimmunoelectrophoresis of the purified protein I against antiserum to protein I and of the purified protein II against antiserum to protein II revealed obvious precipitin lines. No precipitin lines were formed when either purified protein I or purified protein II was tested against control serum. Similarly, a precipitin line was observed between

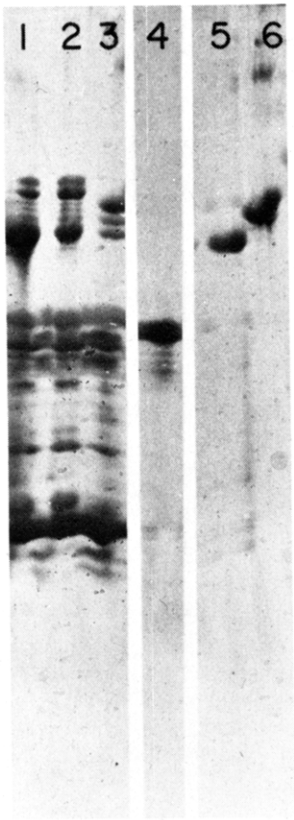

FIGURE 3: Peptide map after limited proteolytic digestion of the two forms of cytochrome *b*. (Lanes 1 and 2) Apocytochrome *b*-II (29 000 daltons) digested with 50 μg of *S. aureus* V8 protease; (lane 3) apocytochrome *b*-I (31 000 daltons) digested with 50 μg of *S. aureus* protease alone. (Lane 4) 50 μg of *S. aureus* V8 protease; (lane 5) undigested apocytochrome *b*-II; (lane 6) undigested apocytochrome *b*-I. Digestion was performed for 40 min at 37 °C in the stacking gel without current. The remaining electrophoresis was performed at 4 °C to prevent further digestion.

purified protein II and antiserum to protein I; however, only a partial reaction between purified protein I and antiserum against protein II was observed.

Mitochondria isolated from yeast cells grown in [^{3}H]leucine were immunotitrated with both antisera to learn whether these antisera could be used to immunoprecipitate cytochrome *b*. At the optimum ratio of antiserum to mitochondrial extract, both antisera precipitated between 3.5% and 5% of the radioactive material from labeled mitochondria. Gel electrophoresis of the immunoprecipitate obtained with optimal amounts of antiserum against protein I contained a prominent double band with a molecular weight of approximately 30 000 (Figure 4). Similarly, the labeling pattern of the immunoprecipitate formed with antiserum against protein II consisted of a major double band with a molecular weight of 30 000 (Figure 4). Several other labeled peaks of higher molecular weight were observed in both immunoprecipitates. These other labeled bands might have resulted from either nonspecific immunoprecipitation or from aggregation of cytochrome *b*.

An IgG fraction from both the antisera against proteins I and II and the control serum was prepared to avoid the presence of proteins other than cytochrome *b* in the immunoprecipitates obtained with crude antisera. An immunotitration curve with the IgG fraction was performed by using mitochondria prepared from yeast cells pulse labeled with [^{35}S]methionine in the presence of cycloheximide. As seen in Figure 5, increasing amounts of the IgG fractions obtained from antisera I and II precipitated 11–12% of the total radioactive material from the labeled mitochondria, while the

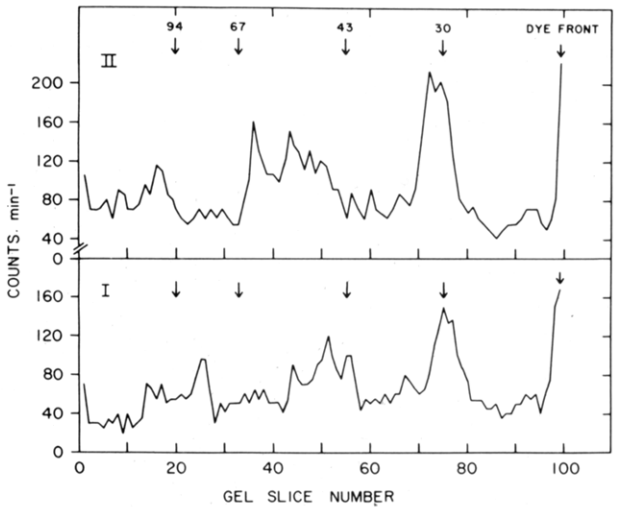

FIGURE 4: Electrophoretic analysis of cytochrome *b* immunoprecipitated from labeled yeast mitochondria. Labeled mitochondria were prepared from yeast cells grown in 0.67% yeast nitrogen base, 0.2% yeast extract, 1% glucose, and 5 mCi/L [^{3}H]lecuine for 10 generations and then solubilized in 1% Lubrol and 1% deoxycholate. After immunoincubation with both antisera to cytochrome *b*, the washed immunoprecipitates were analyzed by $NaDodSO_4$ gel electrophoresis and sliced every 2 mm with a Gilson gel slicer. Numbers above the arrows refer to molecular weights of the standards (I, immunoprecipitate obtained with antiserum to apocytochrome *b*-I; II, immunoprecipitate obtained with antiserum to apocytochrome *b*-II).

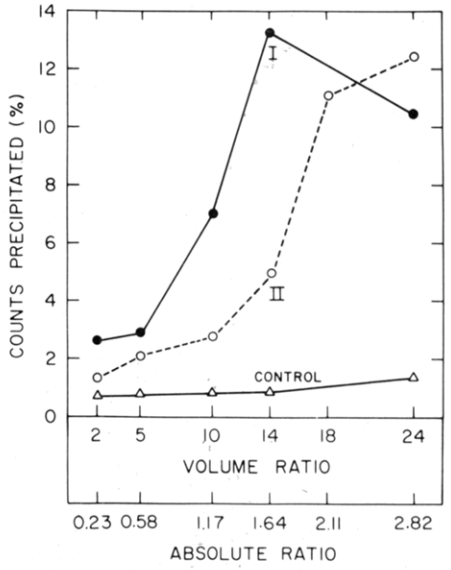

FIGURE 5: Immunotitration curve of mitochondria with IgG fractions against protein I and protein II. Labeled mitochondria were isolated from yeast cells pulse labeled with [^{35}S]methionine in the presence of cycloheximide and extracted with Lubrol–DOC as described under Materials and Methods. Increasing amounts of IgG fraction against apocytochrome *b*-I (●), apocytochrome *b*-II (○), and from preimmune serum (△) were incubated with 10 μL of mitochondrial extracts containing 8.5 mg/mL and 4.8×10^6 cpm/mL. Absolute ratio of IgG fraction to extract was calculated by dividing the volume of IgG by the protein concentration of the mitochondrial fraction.

IgG fraction from control serum precipitated less than 1% of the total radioactivity.

The IgG fractions prepared from antisera against proteins I and II were used in subsequent experiments to obtain immunoprecipitates from mitochondria isolated from yeast cells pulse labeled with [^{35}S]methionine in the presence of cycloheximide. The presence of proteins precipitated nonspecifically was avoided when mitoplasts were used instead of mitochondria as starting material for the immunoprecipitation. In this experiment, yeast cells were pulse labeled with [^{3}H]leucine

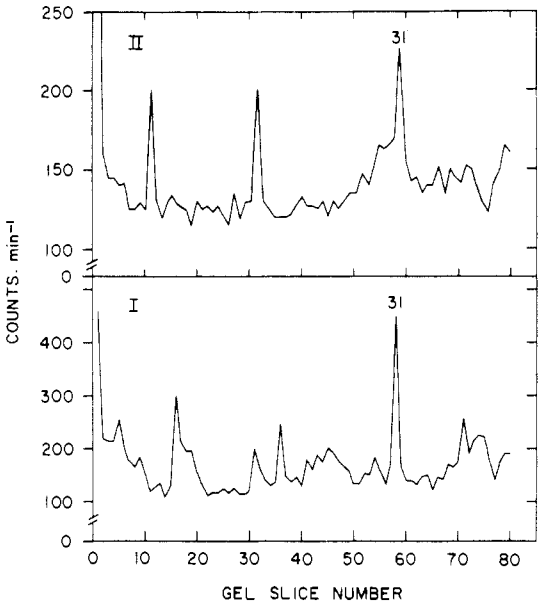

FIGURE 6: Electrophoretic analysis of proteins immunoprecipitated from labeled mitoplasts with IgG fractions against apocytochrome *b*-I and apocytochrome *b*-II. Yeast cells were pulse labeled with [^{3}H]leucine in the presence of cycloheximide. Spheroplasts were prepared prior to isolation of mitochondria and mitoplasts as described under Materials and Methods. The mitoplasts were extracted with 1% Lubrol and 1% deoxycholate prior to immunoprecipitation with the IgG fractions against the two apocytochromes *b*. After electrophoresis of the immunoprecipitates on slab gels, the slab gel strips (10 cm) were sliced manually and counted. Panel I is the immunoprecipitate formed with the IgG fraction against apocytochrome *b*-I (31 000 dalton). Panel II is the immunoprecipitate formed with antiserum against apocytochrome *b*-II (29 000 daltons).

in the presence of cycloheximide. Mitoplasts were solubilized with Lubrol and deoxycholate prior to immunoincubation with an optimum amount of IgG against protein I or II. The washed and dissociated immunoprecipitates were analyzed by slab gel electrophoresis, together with the labeled mitoplast as a control. The IgG fraction from the antiserum against protein I immunoprecipitated from labeled mitoplasts one prominent protein with a molecular weight of 31 000 (Figure 6). Several other peaks contained minor amounts of radioactivity. A parallel gel profile of the labeled mitoplasts also contained a pronounced peak of M_r 31 000 in addition to the other known mitochondrial translation products. Figure 6 also indicates that the IgG fraction against protein II immunoprecipitated one prominent labeled peak with an apparent molecular weight of 31 000. A second labeled band with a molecular weight of 58 000 was also present. This labeled band may represent an aggregate of cytochrome *b* present in the immunoprecipitate, since a peak of that molecular weight does not appear in the gel profile of mitoplasts. The minor peak with a molecular weight higher than 100 000 is probably a minor contaminant or an aggregate of cytochrome *b*. The observation that the IgG fractions against both proteins I and II precipitate one protein with the same apparent molecular weight suggests that only one form of cytochrome *b*, a polypeptide of 31 000 daltons, is synthesized on mitochondrial ribosomes and that this protein may be posttranslationally modified to the 29 000-dalton form.

Intramitochondrial Localization of the Two Forms of Cytochrome b. Our first approach was to study the inhibition of coenzyme QH_2–cytochrome *c* reductase activity by the IgG fractions prepared from the antisera obtained against proteins I and II. Initially, the activity of this enzyme complex was compared in intact mitochondria, in mitoplasts obtained with digitonin, and in submitochondrial particles obtained by sonication, using the reduced form of the decyl analogue of coenzyme Q as the substrate. The rate of cytochrome *c* reduction was slightly lower in the mitoplasts and submitochondrial particles, as compared to intact mitochondria (Table I). Upon addition of cholate to a final concentration of 1%, no change in the enzymatic activity of intact mitochondria was observed; however, a 10% increase in the activity in mitoplasts was observed and a 55% increase in the activity in submitochondrial particles. An aliquot of cholate-solubilized mitoplasts or submitochondrial particles was incubated, for 45 min at room temperature, with increasing amounts of the IgG fractions against apocytochrome *b*-I and *b*-II as well as the IgG fraction prepared from control serum prior to enzyme assay. In a parallel experiment, it was shown that a 45-min incubation of all three fractions at room temperature did not affect the activity of coenzyme QH_2–cytochrome *c* reductase. As seen in Figure 7, the IgG fractions obtained against both proteins I and II significantly inhibited DBH_2:cytochrome *c* reductase activity of mitoplasts and of submitochondrial particles. When the enzymatic activity obtained in the presence of control serum was used as the 100% value, the net inhibition by the IgG fractions against proteins I and II was 30% and 25%,

Table I: Effect of Cholate Treatment on DBH_2:Cytochrome *c* Reductase Activity in Mitochondria, Mitoplasts, and Submitochondrial Particles[a]

	DBH_2:cytochrome *c* reductase activity[b]		
	control	1% cholate	increase (%)
mitochondria	613.0	594.0	
mitoplasts	537.0	595.0	10.7
submitochondrial particles	571.0	888.0	55.5

[a] Mitochondria, mitoplasts, and submitochondrial particles were isolated and treated with cholate as described under Materials and Methods. [b] Enzyme activity is expressed as nanomoles of cytochrome *c* reduced per minute per milligram.

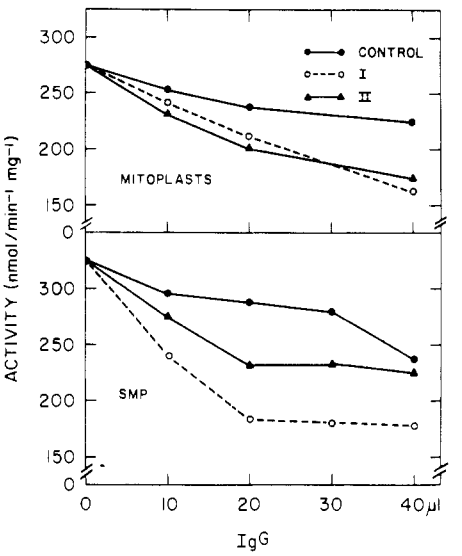

FIGURE 7: Immunoinhibition of coenzyme QH_2–cytochrome *c* reductase activity in mitoplasts and submitochondrial particles with IgG fractions against apocytochrome *b*-I and apocytochrome *b*-II. Mitoplasts and submitochondrial particles (SMP) were treated with cholate and incubated with increasing amounts of the IgG fractions for 45 min prior to assay as described under Materials and Methods. (●) IgG fraction from preimmune serum (control); (○) IgG fraction from antiserum against apocytochrome *b*-I (31 000 daltons); (△) IgG fraction from antiserum against apocytochrome *b*-II (29 000 daltons).

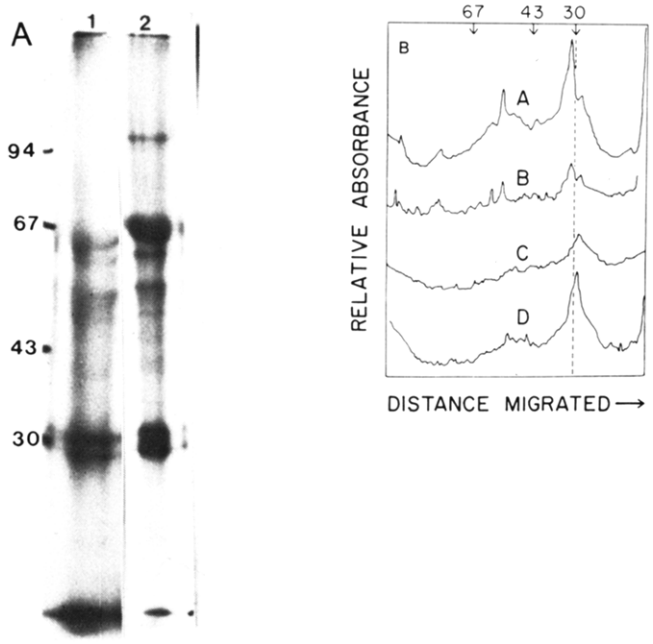

FIGURE 8: Gel patterns of autoradiograms of [^{125}I]DABS-treated mitochondria, submitochondria, and the immunoprecipitates formed with the antisera against apocytochromes *b*-I and *b*-II. Mitochondria and submitochondrial particles were treated with [^{125}I]DABS, solubilized with detergents, and treated with the respective IgG fractions as described under Materials and Methods prior to $NaDodSO_4$ gel electrophoresis. (A) Mitochondria prior to immunoprecipitation, 40 μg containing 110 000 cpm (lane 1); submitochondrial particles, 40 μg containing 120 000 cpm (lane 2). (B) Scanning trace of the autoradiograms of the immunoprecipitates. (Lane A) Mitochondria treated with IgG fraction against apocytochrome *b*-I (31 000 daltons); (lane B) mitochondria treated with IgG fraction against apocytochrome *b*-II (29 000 daltons); (lane C) submitochondrial particles treated with IgG fraction against apocytochrome *b*-I; (lane D) submitochondrial particles treated with antiserum against apocytochrome *b*-II.

respectively, in the mitoplasts. In the submitochondrial particles, the IgG fraction against protein I inhibited 35%, while that against protein II inhibited less than 20%. These results suggest that the antibodies raised against the two forms of apocytochrome *b* are capable of inhibiting cytochrome *c* reductase activity from both the matrix and cytoplasmic side of the inner membrane.

For confirmation of the localization of cytochrome *b* on both sides of the mitochondrial inner membrane, the membrane nonpenetrating reagent [^{125}I]DABS was used. Previous studies (Sears et al., 1971; Tinberg et al., 1974; Prochaska et al., 1980) had indicated that low concentrations of DABS do not permeate cellular or organelle membranes and hence react only with amino acid residues extending from or exposed on the surface of the membrane. Mitochondria and submitochondrial particles were incubated with 1 μM [^{125}I]DABS at 4 °C for 30 min. After extensive washing, the labeled mitochondria and submitochondrial particles were solubilized and immunoincubated with optimum amounts of the IgG fractions against proteins I and II and the IgG fraction obtained from control serum. The labeled mitochondria and submitochondrial particles as well as the immunoprecipitates from each of these were analyzed by 10% acrylamide gel electrophoresis and radioautographed. As seen in Figure 8, four proteins with molecular weights of approximately 65 000, 52 000, 31 000, and 29 000 in the mitochondria were labeled with [^{125}I]DABS (lane 1), while in submitochondrial particles (lane 2) seven labeled bands with molecular weights of 96 000, 68 000, 62 000, 55 000, 50 000, 31 000, and 29 000 were observed. The different surface labeling pattern of the mitochondria and the submitochondrial particles indicates the asymmetrical arrangement of the protein components in the mitochondrial inner membrane. A similar labeling pattern with [^{125}I]DABS was obtained when mitoplasts were used instead of mitochondria, suggesting that the outer membrane does not block labeling with DABS.

Immunoprecipitates obtained by the IgG fractions against proteins I and II and the control IgG fraction incubated with mitochondria are shown in Figure 8. A prominent labeled band with an apparent molecular weight of 31 000 with a lower molecular weight shoulder (M_r 29 000) were observed in the immunoprecipitate formed with the IgG fraction against protein I as well as a labeled band with a molecular weight of 56 000 (trace A). Four small peaks with molecular weights of 60 000, 55 000, 31 000, and 29 000 were in the immunoprecipitate obtained with the IgG fraction against protein II (trace B). No labeled protein was precipitated by the IgG fraction from control serum. The 31 000- and 29 000-dalton proteins in the immunoprecipitates obtained with the IgG against both proteins represents the two forms of cytochrome *b* with their antigenic active site exposed to the outside of the mitochondrial membrane. The two labeled bands of molecular weight between 55 000 and 60 000 in the immunoprecipitates may either by aggregates of cytochrome *b* or be proteins which nonspecifically precipitate with the IgG fractions (Beattie & Clejan, 1980).

The immunoprecipitates obtained from DABS-labeled submitochondrial particles reveal that one protein with a molecular weight of 29 000 is lightly labeled by using the IgG fraction against protein I (Figure 8, trace C) while a protein with a molecular weight of 29 000 is obtained with the IgG fraction against protein II (Figure 8, trace D). No proteins of higher molecular weight material contain label in these immunoprecipitates. The major [^{125}I]DABS-labeled protein in the immunoprecipitates from the submitochondrial particles and IgG against protein II contains less label than those in the immunoprecipitate from mitoplasts with IgG against protein I, suggesting that the higher molecular weight form of cytochrome *b* may be more exposed on the cytoplasmic side of the inner membrane than on the matrix side.

Discussion

In the present study, we report the separation of two identical forms of cytochrome *b* differing in their apparent molecular weight by M_r 2000. The evidence that protein I (31 000 daltons) and protein II (29 000 daltons) are two nearly identical forms of cytochrome *b* is as follows. Both proteins were present in the heme *b* containing fractions eluted from Ultrogel chromatography of a partially purified preparation of cytochrome *b*. Both purified proteins react immunologically with the antiserum against cytochrome *b* previously obtained in this laboratory (Lin et al., 1978). After limited proteolytic digestion with *S. aureus* V8 protease, both proteins have a similar fingerprint pattern. Antiserum against protein I cross-reacts with protein II, while the antiserum against both protein I and II immunoprecipitate a double peak with an approximate molecular weight of 30 000 from mitochondria isolated from yeast cells grown in [^{3}H]leucine. A 31 000-dalton protein is present in the immunoprecipitates obtained with both antisera from mitoplasts isolated from cells labeled in the presence of cycloheximide, indicating that this protein is a mitochondrial translation product. Finally, the antiserum against both proteins inhibits partially the activity of complex III measured as DBH_2:cytochrome *c* reductase in both mitoplasts and submitochondrial particles. While not one of the observations listed above provides conclusive proof that the

two purified proteins are indeed two different molecular weight forms of cytochrome *b*, all of the results taken together can best be interpreted as such. These results are in agreement with those previously reported by Weiss & Ziganke (1976). Two forms of cytochrome *b* with similar amino acid composition, peptide fragments, and carboxy terminals were isolated from *N. crassa* mitochondria by hydroxylapatite chromatography. The data of the current study have been interpreted to suggest that the original translation product of the cytochrome *b* gene is the 31 000-dalton form of this protein which is posttranslationally cleaved by proteolysis to the 29 000-dalton form. Labeling of both forms of cytochrome *b* after growth of yeast cells in [^{3}H]leucine is suggested by the presence of a double peak in the immunoprecipitate formed with antisera against either form of the protein. In addition, two labeled bands with slightly different molecular weights corresponding to cytochrome *b* are clearly evident in autoradiograms of mitochondrial translation products synthesized either in vivo at low temperatures (L. Clejan and D. S. Beattie, unpublished results) or in vitro (Finzi & Beattie, 1980; Finzi et al., 1981).

The mechanism of the proposed posttranslational processing is unclear at the present time; however, we are currently trying to eliminate the possibility that the lower molecular weight form of cytochrome *b* is generated by proteolysis during cell breakage or mitochondrial fractionation. It should be stressed that the 29 000-dalton protein is present in the same relative amounts when inhibitors of protein degradation, such as PMSF, TLCK, and TPCK, are added to all solutions during breakage and preparations. Varying the time of ammonium sulfate fractionation or cholate extraction does not affect the relative amount of the two proteins isolated; however, when mitochondria are suspended in phosphate buffer for several days at 4 °C, considerably greater amounts of the lower molecular cytochrome *b* are present, possibly due to endogenous mitochondrial proteases (T. J. Domenico and D. S. Beattie, unpublished observations). Studies are currently under way in our laboratory to determine the N- and C-terminal amino acids as well as the sequence of amino acids at the N terminus so that the relationship of these two forms of cytochrome *b* can be elucidated.

The conclusion that two forms of cytochrome *b* are present in the yeast mitochondrial membrane explains our previous observations on the biogenesis of this protein (Lin et al., 1978; Beattie et al., 1979). A purified cytochrome *b* peptide appeared to have a molecular weight of 28 000 based on both NnaDodSO_4–gel electrophoresis and sucrose gradient centrifugation (Lin & Beattie, 1978); however, the major band present in immunoprecipitates formed with the specific antiserum against this cytochrome *b* had an apparent molecular weight of 31 000 (Lin et al., 1978). The use of mild Pronase digestion during the purification of cytochrome *b*, a necessary step for solubilization of this protein, may have cleaved the same peptide bond involved in conversion of the 31 000-dalton form to the stable 29 000-dalton form. In addition, the well-documented anomalous migration of cytochrome *b* in the mitochondrial membrane (Beattie et al., 1979; Groot et al., 1978; Marres & Slater, 1977) may have prevented the clear observation of two polypeptide chains differing by only 2000 daltons. For example, the two polypeptides may migrate as one band in gel systems which cannot resolve such a difference in molecular weight.

The results of the present study also suggest that cytochrome *b* spans the inner mitochondrial membrane. Both antisera raised against the two different molecular weight forms of cytochrome *b* inhibited slightly the coenzyme QH_2–cytochrome *c* reductase activities to almost the same extent in mitoplasts and submitochondrial particles. Since each antiserum is cross-reactive with both purified proteins, these results do not permit the more precise localization of these two cytochromes *b* in the membrane. The labeling data of cytochrome *b* with the membrane nonpermeant reagent [^{125}I]DABS, however, suggest a possible arrangement of the two forms of cytochrome *b* in the membrane. Two DABS-labeled bands with molecular weights corresponding to those of the two forms of cytochrome *b* were labeled in mitochondria, suggesting that both cytochromes *b* extend from the cytoplasmic surface of the inner membrane. By contrast, a single protein with a molecular weight of 29 000 is labeled with [^{125}I]DABS in submitochondrial particles, suggesting that only the lower molecular weight form of cytochrome *b* extends from the matrix side of the membrane. These results also reveal a certain specificity of the antisera against the two proteins. For example, more radioactivity is present in the 31 000-dalton protein in the immunoprecipitate formed with the antiserum against that protein in mitoplasts, while more radioactivity is present in the 29 000-dalton protein in the immunoprecipitate formed with the antiserum against that protein in submitochondrial particles. The relative intensities of labeling in these two forms of cytochrome *b* from the two different sides of the membrane may be an accurate reflection of the relative positions of these proteins with cytochrome *b* more exposed on the cytoplasmic than on the matrix side of the membrane, since both mitoplasts and submitochondrial particles contain equal radioactive label after treatment with [^{125}I]DABS.

Previous studies with [^{35}S]DABS in beef heart mitochondria were also interpreted to suggest that cytochrome *b* spans the mitochondrial membrane (Bell et al., 1979). Weiss & Ziganke (1976) concluded, however, that the bulk of cytochrome *b* was buried in the interior of the membrane where it was inaccessible to labeling with iodine catalyzed by lactoperoxidase. In other studies in which specific antibodies to cytochrome *b* were used to inhibit electron transport, diJeso et al. (1978) concluded that cytochrome *b* is located near the outside of the inner membrane. Similarly, electron spin resonance studies in pigeon heart mitochondria indicated that the hemes of both cytochromes *b* are localized on the cytoplasmic side of the membrane (Case & Leigh, 1976). Further studies with lipophilic probes will establish more precisely the localization of cytochrome *b* in the interior of the membrane (Girdlestone et al., 1981).

The suggestion that two different forms of cytochrome *b* span the membranes is consistent with either of the two mechanisms recently proposed to explain the electrogenic movement of protons during electron transport in the respiratory chain. The protonmotive Q cycle proposes that the two functional forms of cytochrome *b* are localized on different sides of the membrane (Mitchell, 1975; Trumpower, 1981). Another recent proposed mechanism suggests that the cytochrome *b* dimer may be the proton translocator in the cytochrome b–c_1 region of the electron transport chain (Von Jagow & Engel, 1980). This model, also, would be consistent with the localization of both cytochromes *b* spanning the membrane. Our present data are consistent with but cannot distinguish between these two proposals for the topography of cytochrome *b*.

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