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Orientation of Complex III in the Yeast Mitochondrial Membrane: Labeling with [^{125}I] Diazobenzenesulfonate and Functional Studies with the Decyl Analogue of Coenzyme Q as Substrate[1]

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Abstract

Mitochondria (or mitoplasts) and submitochondrial particles from yeast were treated with [^{125}I] diazobenzenesulfonate to label selectively proteins exposed on the outer or inner surface of the inner mitochondrial membrane. Polyacrylamide gel analysis of the immunoprecipitates formed with antibodies against Complex III or cytochrome *b* revealed that the two core proteins and cytochrome *b* were labeled in both mitochondria and submitochondrial particles, suggesting that these proteins span the membrane. Cytochrome c_1 and the iron sulfur protein were labeled in mitochondria but not in submitochondrial particles, suggesting that these proteins are exposed on the cytosolic side of the inner membrane. The steady-state reduction of cytochromes *b* and c_1 was determined with succinate and the decyl analogue of coenzyme Q as substrates. Addition of the coenzyme Q analogue to mitochondria caused reduction of 15–30% of the total dithionite-reducible *b* and 100% of the cytochrome c_1: Addition of the coenzyme Q analogue to submitochondrial particles led to the reduction of 70% of the total dithionite-reducible cytochrome *b* but insignificant amounts of cytochrome c_1. A model to explain the topography of Complex III in the inner membrane is proposed based on these results.

Key Words: Mitochondria; cytochrome; Complex III; membrane; mitoplasts; submitochondrial particles; membrane orientation.

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[1]Abbreviations used: DABS, diazobenzene sulfonate; DBH_2, reduced form of decyl analogue of coenzyme Q (2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone); PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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Introduction

Complex III of the mitochondrial respiratory chain is a multiprotein enzyme complex which catalyzes the transfer of electrons from reduced coenzyme Q to cytochrome *c* (Rieske, 1976). The purified complex from yeast consists of 8–10 different polypeptides (Beattie *et al.*, 1980; Siedow *et al.*, 1978) including four oxidoreduction centers, cytochrome b_{562}, cytochrome b_{566}, cytochrome c_1, and an iron sulfur protein (ISP) (Trumpower *et al.*, 1980). This segment of the respiratory chain is also an energy-conserving site and during electron transport causes an electrogenic translocation of protons across the inner mitochondrial membrane (Leung and Hinkle, 1975; Alexandre *et al.*, 1980).

Considerable interest in the localization of the various protein components of Complex III in the inner mitrochondrial membrane has been generated to explain the unidirectional proton translocation at this site of the respiratory chain. Labeling studies with the membrane-nonpermeant reagent diazobenzene sulfanilic acid (DABS) in beef heart mitochondria have indicated that cytochrome c_1 and the ISP are on the cytoplasmic side of the inner membrane, while cytochrome *b* appears to span the membrane (Bell *et al.*, 1979). Using a similar approach with lactoperoxidase-catalyzed iodination of mitochondria from *Neurospora crassa,* Weiss and Ziganke (1976) concluded that the bulk of cytochrome *b* was localized in the interior of the membrane and hence almost inaccessible to the probe. In other studies in which specific antibodies to cytochrome *b* were used to inhibit electron transport, di Jeso *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 and Leigh, 1976).

In the present study, the arrangement of Complex III in the yeast mitochondrial membrane has been investigated by several different approaches. Proteins localized on the cytoplasmic and matrix surfaces of the inner membrane have been selectively labeled by treatment of mitochondria (or mitoplasts) and submitochondrial particles with [^{125}I]DABS. Complex III and cytochrome *b* were isolated by immunoprecipitation using specific antibodies (Beattie *et al.*, 1980; Chen and Beattie, 1981) and the labeled polypeptides examined by gel eletrophoresis. In addition, cytochrome *c* reductase activities, the steady-state reduction of cytochromes *b* and c_1 and the extra reduction induced by antimycin (Rieske, 1976) were studied in mitochondria and submitochondrial particles using both succinate and the decyl analogue of coenzyme Q (DBH_2) as substrate. A model for the organization of Complex III in the membrane is proposed on the basis of the data obtained in these experiments and those in the literature.

Experimental

Growth of Cells and Preparation of Mitochondria, Mitoplasts, and Submitochondrial Particles

The yeast strain, KL-14-4A, was grown aerobically to early stationary phase in a growth medium containing 2% galactose (Beattie, *et al.*, 1979). The cells were broken with glass beads and mitochondria prepared in a medium containing 0.25 M sucrose, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM phenylmethylsulfonylfluoride (PMSF) for enzymatic assays and for [^{125}I]DABS labeling. For spectral analysis, the cells were broken in a medium containing 0.65 M sorbitol, 10 mM KH_2PO_4, pH 6.3, 2 mM EDTA, 0.1 mM $MgCl_2$, 20 mM KCl, and 0.1% bovine serum albumin (Meunier-Lemesle *et al.*, 1980).

To obtain spheroplasts the cells were preincubated for 20 min at 30°C in a medium containing 20 mM Tris HCl, 20 mM EDTA, pH 9.0, and β-mercaptoethanol (35 μl per 100 ml buffer). Zymolyase 5000, suspended in the same buffer, was added to the cell suspension at a concentration of 3 mg/g cells. An initial reading at 800 nm of a 1:100 water dilution was recorded (A_0) and incubation at 30°C was carried out with gentle shaking until 10% of A_0 is reached (usually 1 hr). The spheroplasts were washed twice at 2000 g for 10 min with the above buffer from which β-mercaptoethanol was omitted. The spheroplasts were suspended in a medium containing 0.6 M mannitol, 1 mM EDTA, 1 mg/ml bovine serum albumin, and 10 mM KH_2PO_4, pH 6.8, and shaken in 10– to 20–ml aliquots in a small Waring blender for 20 to 25 sec at high speed. Mitochondria were then isolated as described by Schatz and 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 digitonin/ml, prepared freshly by gently heating in a hot water bath, was added to the mitochondrial suspension to a final concentration of 3 mg digitonin add 10 mg mitochondrial protein. After a 1-min incubation at 4°C, the mitoplasts were diluted 1:5 with the same buffer and sedimented by a 10-min centrifugation at 12000 *g*.

Submitochondrial particles were prepared by resuspending the mitochondrial pellet in sodium phosphate buffer (0.1 M Na_2HPO_4, NaH_2PO_4, 0.5 mM EDTA, pH 7.5) and sonicating in three 20-sec 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 100,000 *g*. The pellet was resuspended in sodium phosphate buffer and the protein concentration adjusted to 10 mg/ml.

Proteolytic Digestion

Mitochondria, mitoplasts, and submitochondrial particles were subjected to proteolytic digestion at room temperature for 90 min in a buffer containing 0.67 M sucrose, 50 mM Tris HCl, pH 7.8, and 1 mM histidine (Ball *et al.*, 1977). Trypsin was used at a concentration of 120 μg/mg of protein, and the digestion was stopped by addition of 480 μg/mg of trypsin inhibitor. Chymotrypsin and *Staphylococcus aureus* V-8 protease were used at a concentration of 200 μg/mg protein.

Enzymatic Assays

Succinate and coenzyme QH_2-cytochrome *c* reductase activities were assayed as described by Brown and Beattie (1977). The reduced analogue of coenzyme Q (DBH_2—2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone) was prepared as a 15 mM ethanolic solution. Values of V_{max} and K_m were calculated from Eadie–Hofstee plots (Fersht, 1977). Succinate dehydrogenase was assayed according to Kim and Beattie (1973).

Spectral Studies

The steady-state reduction of cytochromes *b* and c–c_1 by both succinate and DBH_2 were performed on mitochondria, mitoplasts, and submitochondrial particles obtained from the same yeast culture. Difference spectra of 0.6-ml samples were obtained at room temperature in a Perkin-Elmer Model 557 spectrophotometer by scanning the reduced versus the oxygen- or ferricyanide-oxidized sample. A 2-nm slit width and a scanning speed of 120 nm/min were used. After a base-line correction, either 250 μM of DBH_2 or 30 mM of succinate was added to the sample cuvette and the spectra recorded until a steady state of reduction was obtained. Antimycin (11.2 nmol) was then added and 10 ml of air was bubbled through the cuvette with a syringe. After 2 min the spectrum was recorded. In all cases an equal volume of either ethanol or water was added to the reference cuvette. Subsequently, a few grains of dithionite were added to the sample cuvette and the total cytochrome *b* content determined. For this purpose, the absorption pair 562–575 nm with a molar extinction coefficient of 25.6 mM^{-1} cm^{-1} was used. To determine the content of cytochrome c_1, the 553–539 nm wavelength pair with a molar extinction coefficient of 20.9 mM^{-1} cm^{-1} was used (Van Gelder, 1978).

Labeling of Mitochondria and Submitochondrial Particles with [^{125}]DABS

[^{125}I]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 5μl of 50 mM sodium nitrite and 5μl of 0.1 N hydrochloric acid at 4°C, followed by neutralization with 170 μl of 100 mM phosphate buffer, pH 8.3. An aliquot of 100 μl of mitochondria was suspended at 2 mg 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 with 1% Lubrol–1% sodium deoxycholate at 4°C for 1 hr. After centrifugation at 12,000 *g* for 10 min to remove unsolubilized membranes, 20 μl of labeled mitochondria and submitochondrial particles were immunoincubated with 30 μl of an IgG fraction prepared from antiserum against cytochrome *b* (Chen and Beattie, 1981) and from antiserum against Complex III. The iodinated immunoprecipitates were solubilized in 5% sodium dodecyl sulfate (SDS) and glycerol overnight at room temperature. The entire immunoprecipitated pellet was analyzed by SDS polyacrylamide gel eletrophoresis. After electrophoresis, the gels were dried and autoradiographed using Kodak film (Beattie *et al.,* 1979). The autoradiograms were scanned in a Canalco microdensitometer.

Materials

[^{125}]iodosulfanilic acid (2200 Ci nmol^{-1}) was purchased from New England Nuclear. Sodium cholate and sodium deoxycholate were from Calbiochem. Antimycin A, trypsin, digitonin, cycloheximide, Coomassie brilliant blue, phenylmethanesulfonyl fluoride, and β-mercaptoethanol were obtained from Sigma. Acrylamide, bisacrylamide, and TEMED (*N,N,N′,N′*-tetramethylenediamine) were from Eastman. Zymolyase 5000 and *S. aureus* V8 protease were from Miles Lab., Inc. α-Chymotrypsin was a Worthington product. Trypsin inhibitor was purchased from Boehringer, Mannheim. DBH_2 was a gift from Dr. B. Trumpower.

Results

Labeling of Proteins in the Mitochondrial Membrane with [^{125}I]DABS

Our initial approach to determining the localization of the different polypeptides of Complex III in the mitochondrial inner membrane involved

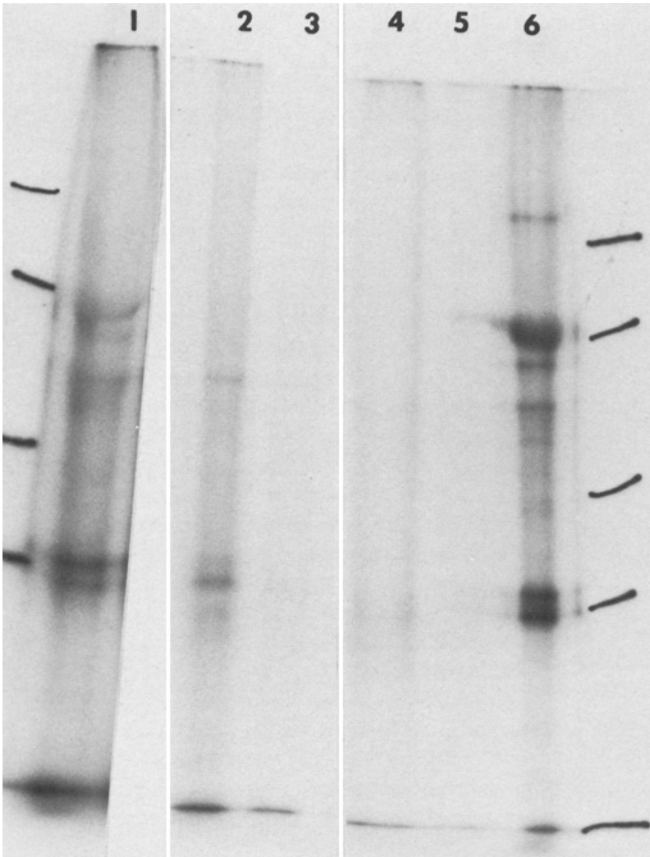

Fig. 1. Autoradiogram of SDS-polyacrylamide gel pattern of immunoprecipitates formed with antibodies against cytochrome *b* and mitochondria or submitochondrial particles labeled with [^{125}I]DABS. Mitochondria were reacted with [^{125}I]DABS for 30 min at 4°C and solubilized with 1% deoxycholate and 1% Lubrol prior to immunoincubation. Submitochondrial particles labeled with [^{125}I]DABS were solubilized with 1% Triton X-100 and 1 M KCl. Immunoprecipitation and gel electrophoresis were performed as described in the Experimental Section. Lane 1: mitochondria (103,000 cpm, 40 μg); lane 2: immunoprecipitate of labeled mitochondria; lane 3: labeled mitochondria treated with an IgG fraction from preimmune serum; lane 4: immunoprecipitate of labeled submitochondrial particles; lane 5: labeled submitochondrial particles treated with the IgG fractions from preimmune serum; lane 6: submitochondrial particles (140,000 cpm 40 μg). The lines on either side indicate the migration of standard-molecular-weight proteins from the top, 94,000, 67,000, 43,000, and 30,000.

labeling with the membrane-nonpermeant reagent [^{125}I]DABS. Previous studies (Sears *et al.*, 1971; Tinberg *et al.*, 1974; Prochaska *et al.*, 1980) had indicated that low concentrations of DABS and other sulfanilic acid derivatives do not permeate cellular or organelle membranes and hence only react with amino acid residues of proteins extending from or exposed on the surface of the membrane. Both cytochrome *b* and Complex III were immunoprecipitated from mitochondria and submitochondrial particles labeled with [^{125}I]DABS prior to SDS polyacrylamide gel electrophoresis and autoradiography as described in the Experimental section. In addition, the [^{125}I]DABS-labeled mitochondria and submitochondrial particles were also analyzed on the same gels for comparison. It should be noted that both mitochondria and submitochondrial particles were labeled to approximately the same extent under these conditions, such that the same number of counts was applied to the gel (104,000 and 140,000 total counts/min, respectively). As indicated in Fig. 1, a completely different labeling pattern with [^{125}I]DABS was observed in the mitochondria and submitochondrial particles. Four major polypeptide bands were labeled in mitochondria, while approximately 10 labeled bands can be identified in the submitochondrial particles. Furthermore, the intensity of the labeling of the bands in the submitochondrial particles was greater than that in the mitochondria with the exception of a heavily labeled band with an apparent molecular weight of 30,000. These results indicate that the profile of proteins labeled in submitochondrial particles differs completely from that of mitochondria, suggesting that an inversion of the inner mitochondrial membrane occurred during sonication. A similar labeling pattern was observed when mitoplasts instead of mitochondria were labeled with [^{125}I]DABS, suggesting that the outer membrane does not prevent the reaction of [^{125}I]DABS on the outer surface of the inner membrane.

The labeling pattern of the immunoprecipitates obtained with an IgG fraction of an antiserum against cytochrome *b* (Chen and Beattie, 1981) and an IgG fraction from preimmune serum with [^{125}I]DABS-labeled mitochondria and submitochondrial particles is shown in Fig. 1 (lanes 2 and 4). A prominent labeled band with a molecular weight of 31,000 is present in mitochondria, plus a second labeled band with an apparent molecular weight of 56,000. By contrast, a single very faint labeled band with an apparent molecular weight of 29,000 was observed in the immunoprecipitates obtained from submitochondrial particles. No labeled bands were observed using an IgG fraction from preimmune serum (Fig. 1, lanes 3 and 5).

The immunoprecipitates obtained from the same labeled preparation of mitochondria and submitochondrial particles using the antiserum against Complex III (Beattie *et al.*, 1980) are shown in Fig. 2. A comparison of the [^{125}I]DABS-labeled bands in these immunoprecipitates again indicates that

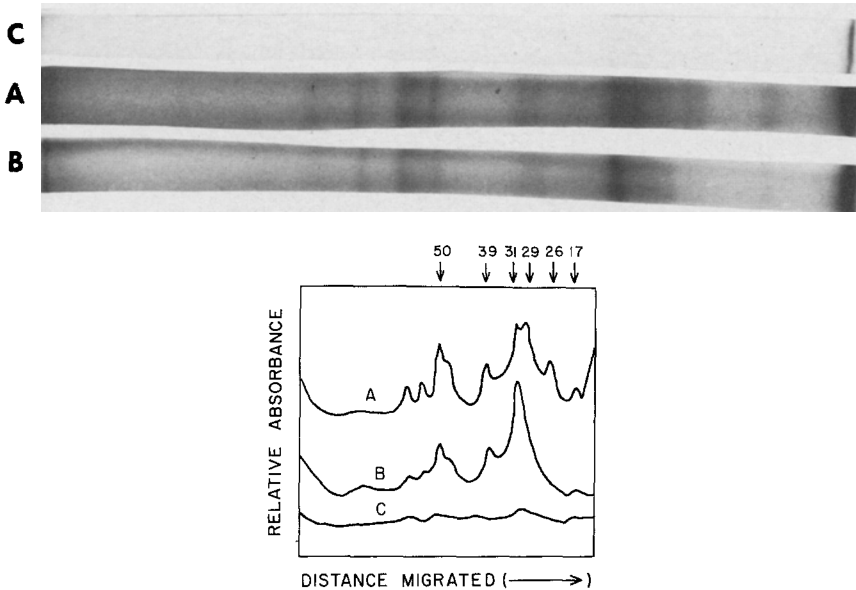

Fig. 2. Autoradiogram and scan of SDS-polyacrylamide gel pattern of immunoprecipitates formed with antibodies against Complex III and mitochondria or submitochondrial particles labeled with [^{125}I]DABS. Labeling and preparations for immunoprecipitation are as described in the legend to Fig. 1. Lane A: immunoprecipitate of labeled mitochondria; lane B: Immunoprecipitate of labeled submitochondrial particles; lane C: labeled mitochondria treated with preimmune serum.

different proteins are exposed on the surface of the inner mitochondrial membrane. Three bands with molecular weights of 29,000, 25,000, and 17,000 are labeled with [^{125}I]DABS in mitochondria (Fig. 2A) but not in submitochondrial particles (Fig. 2B), indicating that these proteins are localized on the outer surface of the inner membrane. By contrast, two proteins with molecular weights of 49,000 and 31,000 are heavily labeled with [^{125}I]DABS while two other proteins with molecular weights of 39,000 are lightly labeled with [^{125}I]DABS in both mitochondria and submitochondrial particles, suggesting that all of these proteins span the inner mitochondrial membrane. These proteins in the immunoprecipitate of Complex III have been tentatively identified as core protein I (49,000), core protein II (39,000), cytochrome *b* (31,000), cytochrome c_1 (29,000), the iron sulfur protein of Complex III (25,000), and subunit VI (17,000). The [^{125}I]DABS labeled band with an apparent molecular weight of 56,000 present in the immunoprecipitates obtained with antibodies against either Complex III or cytochrome *b* may represent a dimer of cytochrome *b* present in the membrane (Von Jagow

and Engel, 1980). Alternatively, this band may represent a nonspecific aggregate of cytochrome *b* or other proteins (Beattie and Clejan, 1980). In a separate experiment, the labeling pattern of immunoprecipitates obtained from [^{125}I]DABS-labeled mitoplasts was compared to mitochondria. A similar pattern of labeled polypeptides was observed, although quantitative differences in labeling of the peaks were observed. Both core protein I (39,000 daltons) and the iron sulfur protein (25,000 daltons) were more heavily labeled relative to cytochromes *b* and c_1 (31,000 and 29,000 daltons) in the immunoprecipitates from mitoplasts than in those from mitochondria, while that of core protein I (49,000 daltons) was less heavily labeled.

Cytochrome c *Reductase Activity in Mitochondria and Submitochondrial Particles*

Our second approach to the localization of Complex III in the inner mitochondrial membrane involved studies of its catalytic activity, cytochrome *c* reductase, using either succinate or the decyl analogue of coenzyme Q (DBH_2) as substrate. Previous studies (Brown and Beattie, 1977) in this laboratory have demonstrated that DBH_2 is an effective substrate to study coenzyme QH_2-cytochrome *c* reductase activity in mitochondria, whole cell extracts, or in a purified Complex III. In the present study the rates of cytochrome *c* reductase were compared in mitochondria and submitochondrial particles (Table I). The rates observed using the yeast strain KL-14-4A were similar to those previously reported for strain D273-10B (Brown and Beattie, 1977). The K_m for DBH_2 was determined to be 43 μM in mitochondria and 60 μM in submitochondrial particles (Table I). Determination of

Table I. Cytochrome *c* Reductase Activity of Mitochondria and Submitochondrial Particles[a]

	Mitochondria	Submitochondrial particles
DBH_2: Cyt *c* reductase	224.7 ± 27.0	446.1 ± 44.9
Plus 1% cholate	362.1 ± 27.3	674.8 ± 64.2
% increase	61%	51%
Succinate: Cyt *c* reductase	79.7 ± 4.6	156.4 ± 31.9
Plus 1% cholate	136.3 ± 6.4	192.6 ± 21.4
% increase	71%	23%
K_m (DBH_2)	43 μM	60 μM
V_{max} (DBH_2)	180	405

[a] Mitochondria and submitochondrial particles of KL-14-4A were prepared and the assay performed as described in the Experimental section and with 10–20 μg of protein in each assay. Specific activities are expressed as nanomoles of cytochrome *c* reduced per minute per milligram of protein. Values of K_m and V_{max} were determined using DBH_2 as substrate on one preparation of mitochondria and submitochondrial particles in the absence of cholate. Each value is the mean activity of six preparations ± the standard error of the mean.

V_{max} from Eadie–Hofstee plots (Fersht, 1977) indicated that under the usual assay conditions with saturating concentrations of DBH_2 the maximum rate of cytochrome *c* reduction was obtained. Addition of 1% cholate to the suspension of mitochondria or submitochondrial particles resulted in a 40–60% increase in activity which was completely inhibited by antimycin. These results suggest that some physical constraint which blocks the activity of the enzyme may be relieved by mild solubilization. By contrast, addition of cholate did not stimulate significantly the rate of succinate-cytochrome *c* reductase in submitochondrial particles, but caused an 80% increase in this activity in intact mitochondria. The latter increase may result from the greater accessibility of the substrate succinate to the dehydrogenase which is exposed in the matrix (Girdlestone *et al.*, 1981).

Steady-State Reduction of Cytochrome b

The addition of succinate to mitochondria or submitochondrial particles resulted in the reduction of cytochrome b_{562} to 72% of the total cytochrome *b* present (Fig. 4 and 5). Addition of antimycin and oxygen led to the classical crossover and the extra-reduction of cytochrome *b* with a concomitant 1-nm shift in the maximum absorption, the red shift (Rieske, 1976). A completely different pattern of cytochrome *b* was observed when DBH_2 was used as a reductant. Less than 30% of the total cytochrome *b* was reduced by DBH_2 in mitochondria (Fig. 4). Addition of antimycin caused an increase in the cytochrome *b* reduced to 90% of the total, a value comparable to the cytochrome *b* reduced by succinate in the presence of antimycin. The addition of DBH_2 to submitochondrial particles reduced 60% of the total cytochrome *b*; however, almost no cytochrome c–c_1 was reduced even after complete anaerobiosis was achieved (Fig. 5). Addition of antimycin resulted in the extra-reduction of cytochrome *b* with the red shift.

To ensure that a steady-state reduction was achieved with either succinate or DBH_2 as substrate, the time course of reduction in mitochondria was measured with both substrates (Fig. 6). With succinate as substrate, the

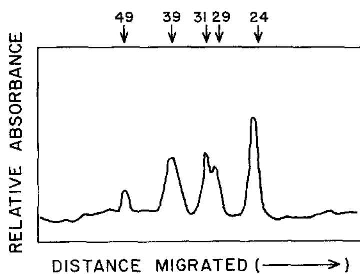

Fig. 3. Scan of autoradiogram of SDS-polyacrylamide gel pattern of immunoprecipitates formed with antibodies against Complex III and mitoplasts labeled with [^{125}I]DABS. Labeling and preparations for immunoprecipitation are as described in the legend to Fig. 1. Numbers above the trace indicate the molecular weights of the labeled proteins calculated from the migration of standard-molecular-weight proteins on the same gel.

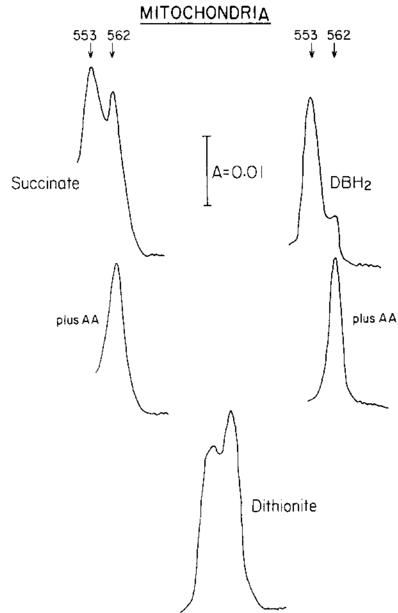

Fig. 4. Spectra of cytochrome *b* and cytochrome c–c_1 reduced with succinate or DBH_2 in mitochondria. Mitochondria were suspended in the medium described by Meunier-Lemesle *et al.* (1980) at pH 6.3 at a protein concentration of 2.5 mg/ml. Aliquots (0.6 ml) were assayed spectrally as described in the Experimental section, for succinate- (left) or DBH_2- (right) reduced cytochromes *b* and c_1, respectively. After reaching anaerobiosis (15 min) the spectrum of the reduced sample versus the oxidized reference was scanned at room temperature. Subsequently both the antimycin-A induced reduction and red shift were recorded as outlined in the Experimental section. The total reducible cytochromes *b* and c_1 was obtained by adding a few grains of dithionite to the sample cuvette.

maximum reduction of cytochrome *b* was observed after 5 min. Addition of 250 μM DBH_2 initially reduced approximately 15% of the total cytochrome *b* in this experiment. No further reduction was observed after 10 min. Increasing the concentration of DBH_2 to 500 μM did not result in greater reduction of cytochrome *b*, but caused a dramatic increase in the amount of cytochrome c_1 reduced.

Effect of Proteolytic Digestion on the Reduction of Cytochromes b *and* c_1 *and on Succinate Dehydrogenase Activity*

Digestion of either mitoplasts or submitochondrial particles with trypsin had no effect on the total dithionite-reducible cytochrome *b* (Table II). This result was anticipated as previous studies had indicated that trypsin digestion of an isolated Complex III had no effect on cytochrome *b* as analyzed by SDS-gels (Ball *et al.*, 1977). By contrast, treatment of mitoplasts for 90 min with either chymotrypsin or *S. aureus* protease resulted in the loss of 24.3% and 14.3% respectively of the total reducible cytochrome *b*. These proteolytic enzymes had no appreciable effect on the cytochrome *b* content when submitochondrial particles were similarly treated. Table II also indicates that the content of cytochrome c_1 was more drastically reduced when mitoplasts rather than submitochondrial particles were digested with chymotrypsin.

In a similar experimental approach, trypsin or chymotrypsin digestion of

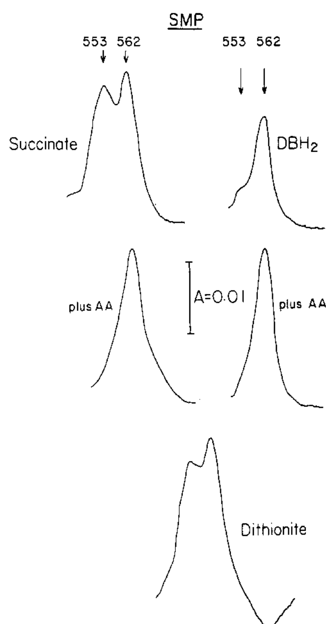

Fig. 5. Spectra of cytochrome *b* and cytochrome c_1 reduced with succinate or DBH_2 in submitochondrial particles. Cytochrome *b* and cytochrome c_1 were reduced with succinate (left) or DBH_2 (right) in submitochondrial particles suspended at pH 6.3 (Meunier-Lemesle *et al.*, 1980) at a protein concentration of 1.6 mg/ml. Spectra were determined as described in the legend to Fig. 3. Total reducible cytochrome *b* (0.662 nmol mg liter and c_1 (0.592 nmol mg liter) were obtained by dithionite addition.

submitochondrial particles at room temperature resulted in a continuous loss of succinate dehydrogenase activity throughout a 2-hr period (Table III). Only 30% of the original enzyme activity remained after this period. By contrast, digestion of mitoplasts or mitochondria with either trypsin or chymotrypsin had no effect on succinate dehydrogenase.

Discussion

A model for the topology of Complex III in the inner mitochondrial membrane consistent with the data of this study is shown in Fig. 7. The localization of cytochrome c_1 and the ISP on the *c* side of the inner membrane is based on the labeling patterns observed with [^{125}I]DABS in mitochondria and the lack of labeling of these proteins in submitochondrial particles. By contrast, subunits I and II of Complex III with molecular weights of 49,000 and 39,000 (the so-called core proteins) plus subunit III, the 31,000-dalton polypeptide corresponding to cytochrome *b*, are labeled with [^{125}I]DABS in both mitochondria and submitochondrial particles, indicating that these proteins span the inner membrane extending from both surfaces. Previously, a similar localization of these polypeptides in Complex III of beef heart mitochondria was reported by Bell et al. (1979). In this study, however, core protein I appeared less accessible to the hydrophilic reagent in submitochondrial particles than in our study.

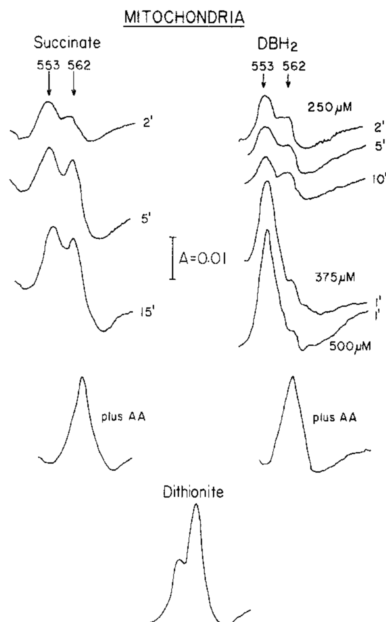

Fig. 6. Time course of the reduction of cytochrome *b* and cytochrome c_1 by succinate or DBH_2 in mitochondria. Mitochondria were suspended at 2.6 mg/ml in the medium described by Meunier-Lemesle *et al.* (1980). Succinate (30 mM) was added to the sample cuvette, and the spectra were recorded after 2, 5, and 15 min with the oxidized sample as reference. DBH_2 (250 μM) was added, and the spectra were recorded 2, 5, and 10 min later. Two 125-μM aliquots of DBH_2 were added to the sample cuvette, and the spectrum was recorded after 1 min.

Further confirmation of the opposite orientation of mitoplasts and submitochondrial particles of yeast mitochondria was obtained in studies of the effect of trypsin and chymotrypsin digestion on succinate dehydrogenase activity. The activity of this enzyme was only decreased after proteolytic digestion of submitochondrial particles but not of mitoplasts, indicating that in submitochondrial particles the succinate dehydrogenase enzyme complex faces the medium where it is accessible to digestion by the exogenous proteases.

The [^{125}I]DABS-labeled immunoprecipitates obtained with both the antibodies against Complex III and cytochrome *b* suggest that cytochrome *b* spans the membrane. A greater intensity of labeling with [^{125}I]DABS of this protein in the mitochondria, however, was observed as compared to submitochondrial particles despite the observation that more proteins are heavily labeled with [^{125}I]DABS in the submitochondrial particles than in the mitochondria. One explanation might be that the polypeptide chain of cytochrome *b* is more exposed on the cytoplasmic side than on the matrix side of the membrane. Alternatively, fewer tyrosine or histidine residues available for reaction with [^{125}I]DABS may be present in that part of the cytochrome *b* chain facing the matrix. The former suggestion is supported by previous reports that an antibody to cytochrome *b* inhibits electron transport only in mitochondria but not in submitochondrial particles (di Jeso *et al.*, 1978) and

Table II. Effect of Proteolytic Digestion on the Dithionite-Reducible Cytochrome *b* and c_1 Content of Mitoplasts and Submitochondrial Particles[a]

Treatment	Cytochrome *b* nmol mg^{-1}	Cytochrome *b* % Control	Cytochrome c_1 nmol mg^{-1}	Cytochrome c_1 % Control
Mitoplasts				
Control	0.297	—	0.260	—
Trypsin	0.297	100	N.D.	N.D.
Chymotrypsin	0.222	75.7	0.135	52
S. aureus protease	0.260	85.7	0.244	94
Submitochondrial particles				
Control	0.234	—	0.266	—
Trypsin	0.234	100	N.D.	N.D.
Chymotrypsin	0.214	92	0.223	84%
S. aureus protease	0.224	96	0.236	87%

[a]The controls were incubated at room temperature for 90 min. Mitoplasts and submitochondrial particles were prepared and treated with the three proteases as described in the Experimental section. A few grains of dithionite were added to each sample and the spectra recorded immediately.

that the hemes of both cytochrome b_{562} and b_{566} are close to the outside of the membrane when determined by electron spin resonance spectroscopy (Case and Leigh, 1976). The significant loss of dithionite-reducible cytochrome *b* after chymotrypsin digestion of mitoplasts coupled with the lack of chymotrypsin effect in submitochondrial particles also supports this proposed orientation of cytochrome *b* in the membrane.

Table III. Effect of Proteolytic Digestion on Succinate Dehydrogenase Activity of Mitoplasts and Submitochondrial Particles[a]

Mitoplast digestion time	Control[b]	Trypsin	% Control	Chymotrypsin	% Control
Zero time	22.1	—	100	25.4	100
60 min	22.3	19.2	86	31.6	124
120 min	21.2	18.8	89	31.1	122

SMP digestion time	Control[c]	Trypsin	% Control	Chymotrypsin	% Control
Zero time	12.5	—	100	—	100
30 min	—	8.75	70	—	—
60 min	13.6	5.86	43	9.25	74
120 min	11.0	3.46	31	7.00	56

[a]Mitoplasts and submitochondrial particles were prepared and treated with the two proteases as described in the Experimental section. Controls were incubated with or without trypsin plus trypsin inhibitor for the same period of time.
[b]Control incubated at room temperature.
[c]Control incubated at room temperature with trypsin and trypsin inhibitor.
[d]Succinate dehydrogenase activity is in nmol min^{-1} mg^{-1}.

Despite the labeling of cytochrome *b* by the hydrophilic reagent DABS, the bulk of the protein is apparently imbedded in the lipid layer of the membrane where it is inaccessible to digestion by trypsin. In this context, it is interesting to note that cytochrome *b* is insensitive to trypsin digestion of Complex III isolated from either beef heart or yeast mitochondria (Ball *et al.*, 1977; Sidhu and Beattie, unpublished observations). Furthermore, an intramembranous localization of cytochrome *b* would explain the lack of iodination of cytochrome *b* catalyzed by lactoperoxidase (Weiss and Ziganke, 1976). The use of lipophilic probes such as arylazidophospholipids (Girdlestone *et at.*, 1981) will provide further evidence for the suggestion that cytochrome *b* is present in the interior of the membrane.

An asymmetric localization of Complex III in the mitochondrial membrane is also indicated by investigations of the steady-state reductions of cytochromes *b* and c_1 with the coenzyme Q analogue, DBH_2. This substrate leads to a completely different pattern of reduction of cytochromes *b* and c_1 depending on its addition to either mitochondria or submitochondrial particles. The results obtained with DBH_2 as substrate also suggest that the bound ubisemiquinone reported to be present in Complex III (Ohnishi and Trumpower, 1980; Yu *et al.*, 1980; Packham *et al.*, 1980) may be buried in the membrane where it is not readily accessible to the externally added DBH_2. For example, additions of the reduced quinone to mitochondria or

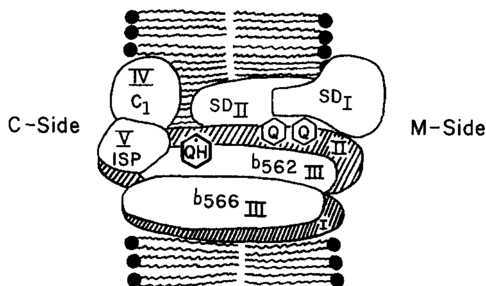

Fig. 7. A model for the orientation of Complex III in the mitochondrial membrane. ISP is iron-sulfur protein or Subunit V of Complex III; c_1 is cytochrome c_1 or subunit IV or Complex III; b_{566} and b_{562} are the two proposed forms of cytochrome *b* or subunit III of Complex III; I and II are the two "core" proteins or subunits I and II of Complex III; SD_I and SD_{II} are the subunits of succinate dehydrogenase, and the two Q's are the ubiquinones associated with Complex II (Girdlestone *et al.*, 1981). C side refers to the cytoplasmic or outer side, and M side refers to the matrix or inner side of the inner membrane.

mitoplasts resulted in the reduction of less than half the cytochrome *b* reducible with succinate; however, almost all of the dithionite-reducible c–c_1 was reduced after addition of DBH_2. These data suggest that setting the $QH_2/QH^{\cdot}$ redox poise by addition of DBH_2 on the c side of the membrane may prevent the formation of sufficient bound ubisemiquinone in the membrane to maintain cytochrome *b* in the reduced state, although the ISP and *c* cytochromes are reduced under these conditions. The subsequent addition of antimycin resulted in 90% reduction of the total cytochrome *b*, indicating the presence of a functional Complex III.

By contrast, when the reduced quinone was added to the matrix side of the membrane, completely different results were obtained. Cytochrome *b* was reduced to the same extent by DBH_2 and succinate in submitochondrial particles; however, almost undetectable levels of cytochrome c_1 were reduced by DBH_2, suggesting that insufficient bound ubisemiquinone is formed to reduce the ISP and consequently cytochrome c_1. Again, addition of antimycin resulted in the extra-reduction of cytochrome *b* to the same extent in the presence of DBH_2 as succinate.

Further evidence for the inaccessibility of the bound ubisemiquinone of Complex III to added quinones was provided in the studies of cytochrome *c* reductase activities with either DBH_2 or succinate as substrate. The activity measured in both mitochondria and submitochondrial particles was apparently assayed at the maximum velocity as determined by measurements of K_m and V_{max}; however, addition of cholate to either mitochondria, mitoplasts, or submitochondrial particles caused a large increase in the rate of cytochrome *c* reduction. These effects were not observed with succinate as substrate, suggesting that solubilization with cholate removes a physical constraint on the maximum rate of electron transport with DBH_2 as substrate.

An asymmetric arrangement of Complex III in the mitochondrial membrane is proposed in this study based on both a structural and functional approach. Such an arrangement is consistent with current mechanisms proposed for the vectorial translocation of protons during electron transport in this segment of the respiratory chain. Mitchell's Q cycle (Mitchell, 1975; Trumpower, 1981) proposes an asymmetric distribution of the $QH_2/QH^{\cdot}$ and $QH^{\cdot}/Q$ couples in the membrane as a mechanism to move protons from the matrix to the cytosolic side of the membrane. In addition, the Q cycle proposes that the two forms of cytochrome *b* are localized on different sides of the membrane. Another recently 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 and Engel, 1980). This model also would be consistent with the localization of both cytochrome *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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