Topographical Organization of Cytochrome *b* in the Yeast Mitochondrial Membrane Determined by Fluorescence Studies with *N*-Cyclohexyl-*N*′-[4-(dimethylamino)naphthyl]carbodiimide†

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ABSTRACT: In previous studies, we reported that dicyclohexylcarbodiimide (DCCD) inhibited proton translocation in the cytochrome bc_1 complex from yeast mitochondria and was bound selectively to cytochrome *b*. Extensive trypsin digestion of [^{14}C]DCCD-labeled cytochrome *b* isolated from a cytochrome bc_1 complex treated with DCCD yielded a single radiolabeled 7.0 kDa peptide with the N-terminus VTLWNVG, indicating that trypsin cleavage had occurred at arginines-110 and -178. This segment of cytochrome *b* contains one acidic residue, aspartate-160, localized in amphiphilic, non-membrane-spanning, helix cd. To explore the environment of amphiphilic helix cd, we employed a fluorescent derivative of DCCD, *N*-cyclohexyl-*N*′-[4-(dimethylamino)naphthyl]carbodiimide (NCD-4). After incubation of NCD-4 with a cytochrome bc_1 complex isolated from yeast mitochondria, a fluorescent compound was formed with a 340 nm excitation peak and a 441 nm emission peak. NCD-4 was selectively bound to cytochrome *b* and inhibited proton translocation with only a minimal inhibitory effect on electron transfer in the cytochrome bc_1 complex reconstituted into proteoliposomes. Competition experiments and trypsin digestion of NCD-4-labeled cytochrome *b* indicated that NCD-4 and DCCD were bound to the same site on cytochrome *b*. The fluorescence of NCD-4 bound to the cytochrome bc_1 complex was quenched equally by CAT-16, an amphiphilic spin-label that intercalates at the membrane surface, and 5-doxylstearic acid, a nitroxide derivative of stearic acid, and to a lesser extent by 7-doxylstearic and 12-doxylstearic acids. At all concentrations studied, the hydrophilic membrane impermeant quenchers, CAT-1 and D-569, did not quench the fluorescence of NCD-4; however, TEMPO, which freely permeates the membrane, effectively quenched the NCD-4 fluorescence. These results suggest that the non-membrane-spanning helix cd containing aspartate-160 is localized within the membrane but near the surface of the membrane.

The cytochrome bc_1 complex present in the mitochondrial inner membrane catalyzes electron transfer from ubiquinol to cytochrome *c* with the concomitant translocation of protons from the matrix to the cytosolic side of the membrane such that an electrogenic proton gradient is generated across the membrane (Brandt & Trumpower, 1994; Beattie, 1993). Similar functions are performed by analogous *bc* complexes found in bacterial respiratory and photosynthetic electron transport chains as well as in the photosynthetic electron transfer chains localized in the thylakoid membranes of green plants (Cramer et al., 1987; Hauska et al., 1983).

Recently, we reported that dicyclohexylcarbodiimide (DCCD),[1] the well-established carboxyl-modifying reagent, inhibited proton translocation in the cytochrome bc_1 complex isolated from yeast mitochondria reconstituted into proteoliposomes without significant effect on ubiquinol:cytochrome *c* oxidoreductase activity either in the reconstituted or soluble complex or in rat liver mitochondria (Beattie & Villalobo, 1982; Beattie et al., 1984; Clejan et al., 1984). Similarly, DCCD blocked electrogenic proton movements in the cytochrome *bf* complex reconstituted into proteoliposomes with a minimal inhibitory effect on the cytochrome *c* reductase activity of either the soluble or the reconstituted complex (Wang & Beattie, 1991). These results suggest that the primary effect of DCCD is on the proton-translocating device of both the cytochrome bc_1 and the cytochrome *bf* complexes.

Subsequently, it was shown in this laboratory that DCCD binds selectively to cytochrome *b* in the bc_1 complex (Beattie et al., 1984) and to cytochrome b_6 in the *bf* complex (Wang & Beattie, 1991), suggesting that these proteins provide a similar function in proton translocation at this site in their respective electron transfer chains. Cytochrome *b*, the most hydrophobic subunit of the mitochondrial bc_1 complex, spans the inner membrane in eight transmembrane α helices (Figure 1). In this model, helix cd, originally designated as a membrane-spanning helix in the nine-helix model (Widger et al., 1984; Saraste, 1984), is now suggested to be localized outside the membrane (Brasseur, 1988). In the current study, we report that DCCD binds to aspartate-160, localized in

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[1] Abbreviations: bc_1 complex, cytochrome bc_1 complex or ubiquinol: cytochrome *c* oxidoreductase; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; NCD-4, *N*-cyclohexyl-*N*′-[4-(dimethylamino)naphthyl]carbodiimide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); CAT-1, 4-(trimethylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; D-569, 4-[*N*,*N*-dimethyl-*N*-(3-sulfopropyl)ammonio]-2,2,6,6-tetramethylpiperidine-1-oxyl; CAT-16, 4-(*N*,*N*-dimethyl-*N*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; 5-DSA, 5-doxylstearic acid; 7-DSA, 7-doxylstearic acid; 12-DSA, 12-doxylstearic acid; DQH_2, duroquinol; Q_6, coenzyme Q_6; Q_6H_2, reduced coenzyme Q_6.

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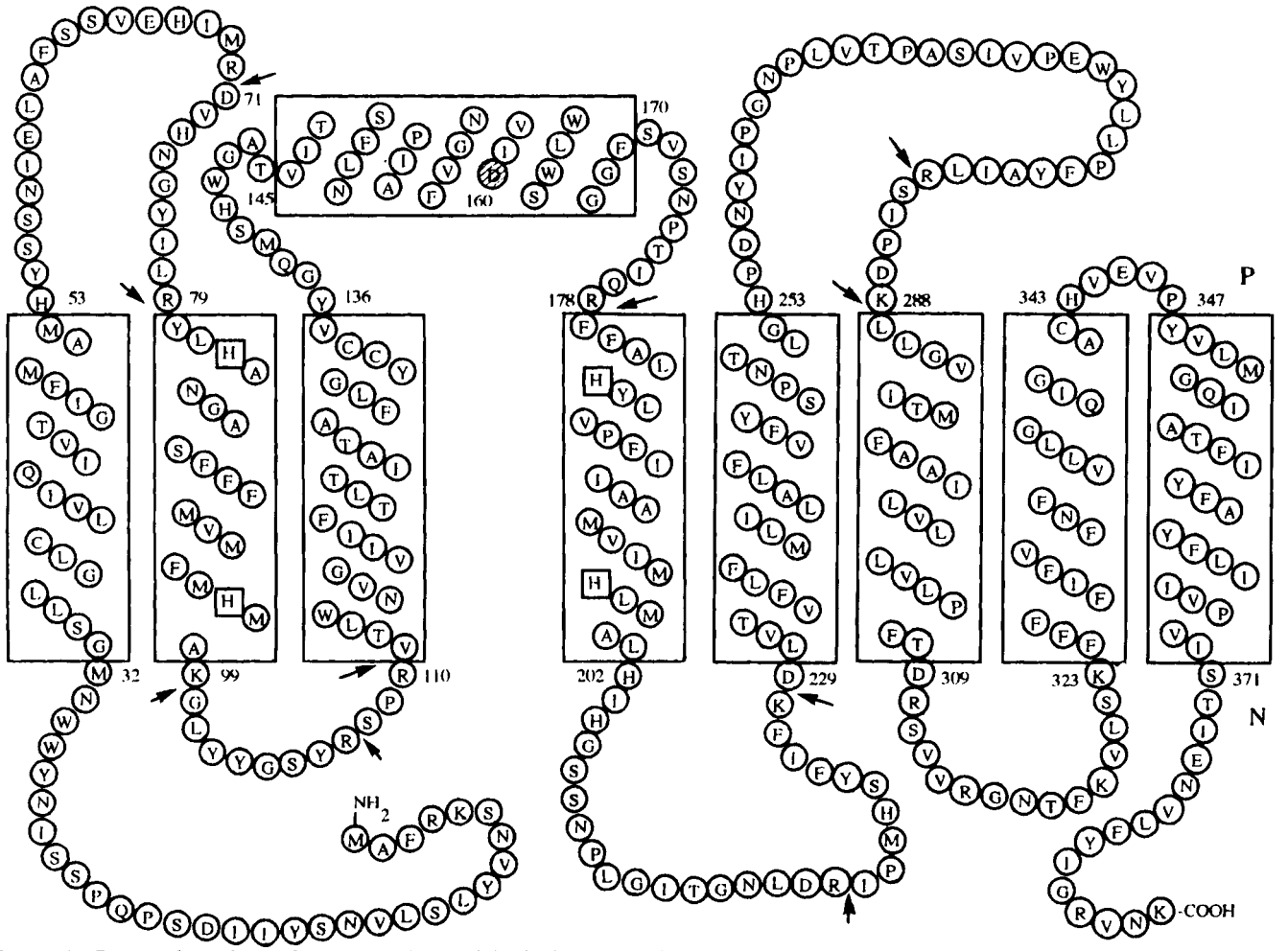

FIGURE 1: Proposed topology of yeast cytochrome *b* in the inner mitochondrial membrane (Brandt & Trumpower, 1994). The conserved histidines involved in binding the two hemes are indicated by squares. Aspartate-160, identified as the binding site for DCCD, is indicated by a shaded circle. The solid arrows indicate the predicted sites of trypsin cleavage.

helix cd of cytochrome *b*. Similarly, the binding site for DCCD on cytochrome b_6 was recently determined to be aspartate-155 and/or glutamate-166 also localized in helix cd connecting membrane-spanning helixes C and D (Wang & Beattie, 1992). These results suggest that the acidic amino acids in the *b* cytochromes may be involved in proton pumping on the positive side of the membrane.

In order to characterize the environment surrounding aspartate-160, we have used *N*-cyclo-*N'*-[4-(dimethylamino)-naphthyl]carbodiimide (NCD-4),[1] a fluorescent analogue of DCCD initially introduced by Chadwick and Thomas (1983). NCD-4 was bound specifically to cytochrome *b* and inhibited proton translocation with only minimal inhibitory effects on electron transfer in the cytochrome bc_1 complex reconstituted into proteoliposomes. Fluorescence quenching experiments using spin-labels suggested that helix cd containing the NCD-4, and hence the DCCD, binding site is localized within the membrane as the fluorescence of NCD-4 was equally quenched by amphiphilic and membrane intercalating probes.

EXPERIMENTAL PROCEDURES

Preparation of the Cytochrome bc_1 Complex, Labeling with DCCD and NCD-4, and Incorporation into Proteoliposomes. The cytochrome bc_1 complex was isolated from yeast mitochondria by the procedure of Ljungdahl et al. (1980) as described previously (Beattie & Marcelo-Baciu, 1991). The isolated complex was suspended in a medium containing 100 mM KCl, 200 mM sucrose, and 50 mM K-Hepes, pH 6.2, and incubated either with [^{14}C]DCCD, dissolved in methanol, at a molecular ratio of 50 nmol of DCCD/nmol of cytochrome *b* at 12 °C for 1 h, or with NCD-4 (dissolved in a 100 mM stock solution in ethanol) at a molar ratio of 150 nmol of NCD-4/nmol of cytochrome *b* overnight at 4 °C or at room temperature for 1 h. The incubation mixture was passed through a Diaflo YM 100 membrane to remove any unbound [^{14}C]DCCD or NCD-4 followed by washing the complex 3 times with a solution containing 30 mM Tris−succinate, 0.5% sodium cholate, 30 mM octyl glucoside, and 0.1% soybean lecithin, pH 6.5 (Wang & Beattie, 1992). The washing was complete when the counts due to [^{14}C]DCCD in the eluate were decreased to background level.

The NCD-4-labeled cytochrome bc_1 complex was incorporated into liposomes as described previously for the incorporation of the cytochrome *bf* complex labeled with [^{14}C]DCCD (Wang & Beattie, 1992). For the competition studies, the purified bc_1 complex was suspended in a medium containing 100 mM KCl, 50 mM Hepes, pH 6.2, 0.2 M sucrose, and 100 nmol of DCCD/nmol of cytochrome *b* in the bc_1 complex for 1.5 h at 12 °C. The unbound DCCD was removed by filtration as described above. NCD-4 (150 nmol/nmol of cytochrome *b*) was then added to either the control or the DCCD-labeled bc_1 complex and the fluorescence measured at 10 min intervals using 340 nm for excitation and 441 nm for the maximum emission.

Proteolytic Digestion of the Cytochrome bc_1 Complex with Trypsin. The [^{14}C]DCCD-labeled cytochrome *b* was isolated from the DCCD-treated bc_1 complex dissociated with 5% SDS and 2-mercaptoethanol overnight prior to electrophoresis using the continuous elution PAGE Model 491 PrepCell (BioRad). The eluted cytochrome *b* was digested with trypsin at a ratio of 1:20 (trypsin/cytochrome *b*) at 4 °C for 7 and 9 days and analyzed by SDS−PAGE in a 15% gel without glycine (Schagger et al., 1986). One lane was cut into 2 mm slices for counting and the other blotted electrophoretically to a poly(vinylidene difluoride) (PVDF) protein sequencing membrane in a medium containing 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid]/10% methanol, pH 11.0. The peptides were sequenced by Dr. John Hempel in the Protein Sequence Facility at the University of Pittsburgh School of Medicine, Pittsburgh, PA.

Cytochrome *b* was isolated from the cytochrome bc_1 complex labeled with NCD-4 and digested with trypsin for 7 days at 4 °C as described above for the DCCD-labeled cytochrome *b*. After SDS−PAGE of the digested sample, the gel was fixed, exposed to ultraviolet light, photographed, and stained with Coomassie blue. The negative of the gel photograph was scanned to determine the fluorescence intensity density of each peak on the gel.

Enzyme Assays. Proton pumping and electron transfer were measured simultaneously as described previously (Wang & Beattie, 1991). The reaction buffer contained 1 mM Na-Hepes, pH 6.9, 50 mM NaCl, 6 μM cytochrome *c*, 0.12 μM cytochrome *b* present in the bc_1 complex (with or without NCD-4), and 21 μM duroquinol (DQH_2) in a total volume of 2.4 mL.

Fluorescence Experiments. The cytochrome bc_1 complex (0.2 μM) was suspended in 2 mL of medium containing 100 mM KCl, 200 mM sucrose, and 50 mM Hepes-K, pH 7.0. NCD-4 was added to the solution at a molecular ratio of 1:150 (protein/NCD-4) and the mixture incubated for 1 h at room temperature prior to determining the absolute, uncorrected fluorescence spectra and fluorescence quenching in a Shimadzu RF 5000 U spectrofluorometer at 25 °C (Wang & Beattie, 1993). Where indicated, the substrates Q_6, and Q_6H_2 were added from a 4 mM stock solution in ethanol. Alternatively, the same substrates were added to the suspension before NCD-4.

Miscellaneous Procedures. In general, SDS−PAGE was performed as described by Laemmli (1970) in gels of 15% acrylamide. To isolate the tryptic peptides of cytochrome *b*, the modified system of Schagger et al. (1986) was used with a 16% acrylamide gel of 0.75 mm thickness containing Tris−Tricine in the upper buffer and Tris-HCl in the lower buffer. The apparent specific radioactivity was calculated by dividing the cpm in the radioactive peak, after subtraction of the background, by an arbitrary number representing the staining intensity of the peptide band.

Materials. DCCD was obtained from Schwarz/Mann; radioactive [^{14}C]DCCD (55 mCi/mmol), obtained from Amersham, in toluene was evaporated to dryness under a stream of nitrogen and redissolved in methanol just prior to each experiment. NCD-4 and the following spin-labels were obtained from Molecular Probes Inc., Eugene, OR: CAT-1, D-569, CAT-16, 5-DSA, 7-DSA, and 12-DSA. Q_6 was obtained from Sigma. All of the other chemicals were of the highest purity available commercially.

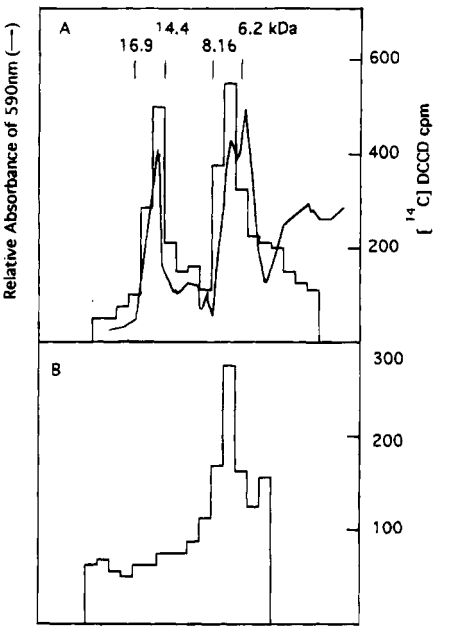

FIGURE 2: Trypsin digestion of [^{14}C]DCCD-labeled cytochrome *b*. The ^{14}C]DCCD-labeled cytochrome *b* was isolated from the [^{14}C]DCCD-labeled bc_1 complex as described under Experimental Procedures. Cytochrome *b* was digested for 7 and 9 days (Figure 2A,B, respectively) with trypsin and analyzed by SDS−PAGE. Part of the gel was stained with Coomassie blue (—) and then was cut into 2 mm slices for liquid scintillation counting. The 7 kDa peptide was blotted to a PVDF membrane for amino acid sequencing.

RESULTS

Tryptic Digestion of [^{14}C]DCCD-Labeled Cytochrome b. To establish the site of DCCD binding to cytochrome *b*, exhaustive digestion of [^{14}C]DCCD-labeled cytochrome *b* with trypsin was performed. SDS−PAGE of the low molecular weight digestion fragments revealed the presence of two discrete bands with apparent molecular masses of 15 and 7 kDa (Figure 2A). With increasing time of digestion, the radioactivity present present in the larger peptide completely disappeared while significant radioactivity remained associated with the smaller peptide (Figure 2B). Some overall loss of radioactivity was observed during the long incubation times. The 7 kDa peptide was removed from the gel by blotting and sequenced to give the N-terminal sequence VTLWNVG, suggesting that this peptide contains the 68 amino acid residues extending from valine-111 to arginine-178 resulting from trypsin cleavage at arginine-110 and arginine-178 (Figure 1). This peptide contains a single acidic amino acid, aspartate-160.

Effects of NCD-4 on the Cytochrome bc_1 Complex. The effect of NCD-4 on both electron transfer and proton translocation in the cytochrome bc_1 complex was measured simultaneously as described previously for the DCCD-treated cytochrome *bf* complex (Wang & Beattie, 1991). Increasing concentrations of NCD-4 incubated with the bc_1 complex resulted in increasing inhibitory effects on the proton translocation activity, reaching a maximum inhibitory effect of 65% at 250 nmol of NCD-4/nmol of cytochrome *b* (Figure 3); however, the rate of electron transfer was only inhibited 20% by the same concentration of NCD-4. These results suggest that NCD-4 has similar effects on the energy transduction steps during electron transfer through the cytochrome bc_1 complex as was previously observed with DCCD (Clejan & Beattie, 1983). The lack of stoichiometric binding to the bc_1 complex by NCD-4 was not unexpected

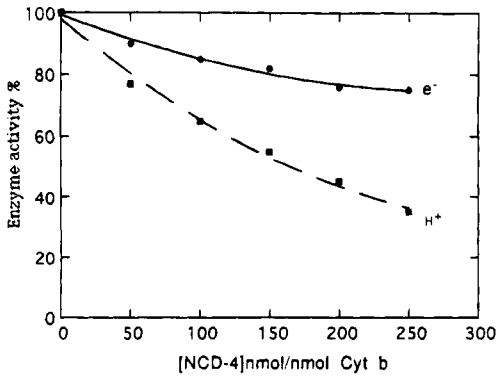

FIGURE 3: Concentration dependence of the inhibitory effects of NCD-4 on proton translocation and electron transfer of the cytochrome bc_1 complex reconstituted into protoliposomes. Proton translocation (- - -) and electron transfer (●—●) were measured simultaneously using a pH electrode placed in the cuvette in the spectrophotometer.

as considerable nonspecific interactions of the carbodiimides occur with both proteins and phospholipids (Beattie et al., 1983).

Analysis of the NCD-4-labeled cytochrome bc_1 complex by SDS−PAGE revealed that the fluorescent label was mainly associated with cytochrome *b* (Figure 4A). When the gel was exposed to ultraviolet light, strong fluorescence was associated with cytochrome *b* and weak fluorescence with core protein II; however, no fluorescence above the background level was observed to be associated with any of the other subunits such as core protein I, cytochrome c_1, the iron−sulfur protein, and the smaller subunits of the bc_1 complex. These results suggest that NCD-4 is covalently and selectively bound to cytochrome *b* but not to any other subunits of the cytochrome bc_1 complex as was previously reported for DCCD (Beattie et al., 1984).

The location of the binding site of NCD-4 on cytochrome *b* was investigated by trypsin digestion of NCD-4-labeled cytochrome *b*. Exposure of the tryptic peptides to ultraviolet light after SDS−PAGE revealed that a single peptide with a molecular mass of 7.0 kDa was fluorescent (Figure 4B). This peptide corresponds in molecular mass to that of the peptide obtained after trypsin digestion of [^{14}C]DCCD-labeled cytochrome *b* (Figure 2). The binding of NCD-4 and DCCD to a peptide of identical molecular mass suggests that NCD-4 and DCCD bind to the same site on cytochrome *b*. This conclusion was strengthened by competition experiments with DCCD (Figure 4C). Increasing the time of incubation of the bc_1 complex with NCD-4 resulted in an increase in fluorescence intensity; however, when the bc_1 complex was preincubated with DCCD prior to the addition of NCD-4, no increase in fluorescence intensity was observed, suggesting that DCCD and NCD-4 bind to the same site on the protein.

Fluorescence Studies with NCD-4-Labeled Cytochrome bc_1 Complex. Solutions of NCD-4 in either water or organic solvents do not possess any intrinsic fluorescence. After incubation of NCD-4 with the cytochrome bc_1 complex at room temperature for 1 h, the fluorescence emission at 441 nm was observed after excitation at 340 nm. The maximum emission at 441 nm is consistent with the formation of an *N*-acylurea derivative of NCD-4 coupled to a carboxyl group (Chadwick & Thomas, 1983). These workers reported that the *N*-acylurea formed from NCD-4 and glacial acetic acid had an emission maximum of 425 nm in 100% ethanol and

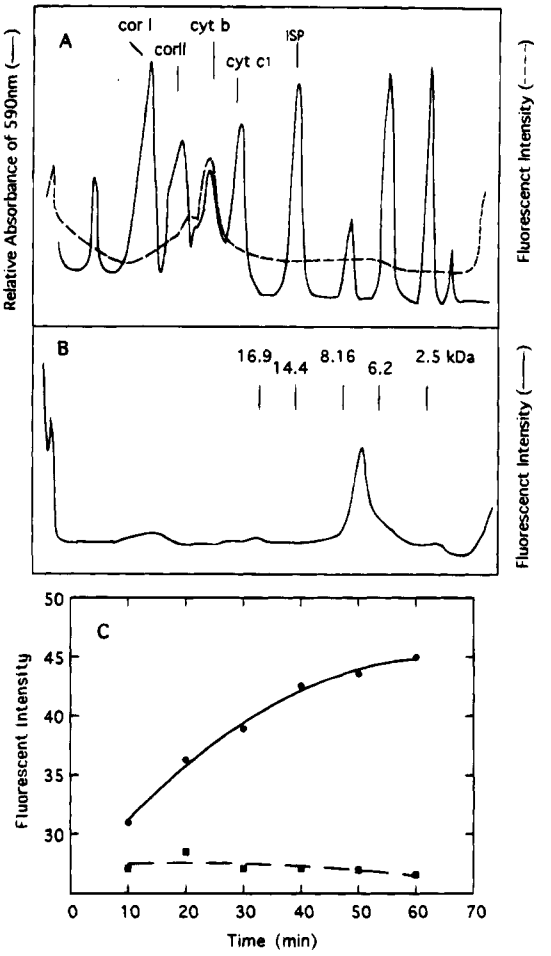

FIGURE 4: Fluorescence of the NCD-4-labeled cytochrome bc_1 complex. (A) The NCD-4-labeled bc_1 complex was subjected to SDS−PAGE in a 12.5% acrylamide gel according to Laemmli (1970). After electrophoresis, the gel was fixed and exposed to ultraviolet light, photographed, and then stained with Coomassie blue. The intensity of the protein bands was determined by scanning densitometry of the stained gel (—). The fluorescence of each band was determined by scanning densitometry of the negative of the photograph obtained under ultraviolet light (- - -). (B) Cytochrome *b* was purified from the NCD-4-treated bc_1 complex and digested with trypsin for 7 days as described under Experimental Procedures. After SDS−PAGE, the gel was fixed and exposed to ultraviolet light and photographed. The negative of the photograph was analyzed by scanning densitometry. (C) NCD-4 was incubated with the bc_1 complex for increasing times as described under Experimental Procedures (●—●). The bc_1 complex was preincubated for 1.5 h with DCCD prior to incubation with NCD-4 for the times indicated (- - -).

440 nm in 50% ethanol and concluded that any fluorescence emission observed in the presence of NCD-4 resulted from protein-bound *N*-acylurea derivatives. The emission spectrum observed after the reaction of NCD-4 with the cytochrome bc_1 complex suggests that NCD-4 forms an *N*-acylurea derivative with an aspartate or glutamate localized in a moderately hydrophobic environment of the cytochrome bc_1 complex.

In order to obtain information on the localization of the binding site of the fluorescent probe, NCD-4, relative to the surface of the membrane, paramagnetic fluorescence quenching experiments were performed (London & Feigensen, 1981; Blatt et al., 1984; Wang & Beattie, 1993). The amino acid residue of cytochrome *b* to which DCCD binds, aspartate-160, is localized in a putative extramembranous, yet hydrophobic, α helix (Brasseur, 1988). We employed the following compounds as spin-label quenchers: doxyl

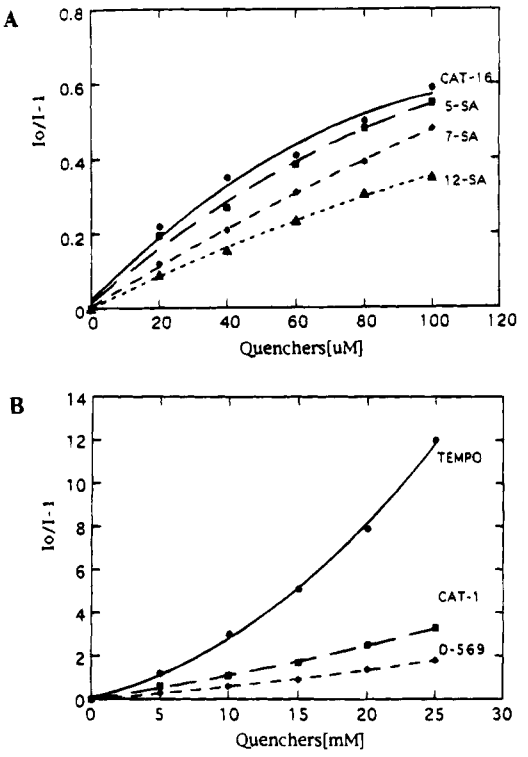

FIGURE 5: Fluorescence quenching of NCD-4-labeled cytochrome bc_1 complex by spin-label probes. (A) The NCD-4-labeled bc_1 complex incorporated into proteoliposomes was suspended in 2 mL of reaction buffer containing 0.12 μM cytochrome *b* in the cytochrome bc_1 complex. The hydrophobic quenchers, identified on the right side of the figure, were added from a 20 mM stock solution directly to the assay cuvettes to the final concentration indicated in the figure. After 5 min equilibration time, the fluorescence intensity was recorded. The plot was calculated using the Stern−Volmer equation described under Results. (B) The hydrophilic quenchers, identified on the right side of the figure, were added from a 100 mM stock solution directly to the assay cuvettes to the final concentrations indicated in the figure. The fluorescence intensity was recorded after excitation at 340 nm and emission at 441 nm (−). Each point is the average of four or more experiments.

derivatives of stearic acid such as 5-DSA, 7-DSA, and 12-DSA which are the most common lipid spin-label probes; CAT-16, a cationic amphiphilic spin-label that partitions so that the polar group and the spin-label are at the membrane surface; TEMPO, a probe that freely permeates the membrane; plus CAT-1 and D-569 which are the most polar spin-labels. Dynamic collision, or Stern−Volmer, quenching obeys the relationship (Lacowicz, 1983):

$$I_0/I = 1 + K_D[Q]$$

where I_0 and I are the fluorescent intensities in the absence and presence of the quencher, [Q] is the concentration of quencher, and K_D is the Stern−Volmer quenching constant. The quenching observed in the current study using a multisubunit enzyme complex may not follow the simple relationship expressed in the equation; however, the equation provides a useful basis for analysis of the data obtained. Figure 6A,B shows the quenching effects of the various spin-labels on the fluorescence of the NCD-4-treated cytochrome bc_1 complex using the relationship to plot the results:

$$I_0/I - 1 = K_D[Q]$$

where $I_0/I - 1$ is plotted against [Q]. The observed order of quenching efficiency was CAT-16 = 5-DSA > 7-DSA > 12-DSA (Figure 5A). The slight downward curvature observed at higher concentrations of some probes may reflect that a fraction of the NCD-4 is inaccessible to the quencher (Pringle & Taber, 1985). The polar spin-labels CAT-1 and D-569 did not effectively quench the fluorescence even at high concentrations; however, the freely membrane-permeant spin-label TEMPO was able to effectively quench the fluorescence of NCD-4 in a concentration-dependent manner (Figure 5B). The concave-upward concentration of the quencher TEMPO has been observed previously in studies of the NCD-4-labeled H^+-ATPase (Pringle & Taber, 1985) and in quenching experiments using NCP, a fluorescent derivative of DCCD, bound to CF_1CF_o (Mitra & Hammes, 1990). These results suggest that the site of NCD-4 binding to cytochrome *b* is located between the polar region of the membrane and carbon-5 of the fatty acid side chain of the membrane phospholipids at an approximate distance of 2−3 Å from the membrane surface. These conclusions are based on the observation that the fluorescence of NCD-4 was quenched most effectively by the amphiphilic spin-label CAT-16 and by the hydrophobic probe 5-DSA.

In order to further characterize the site of NCD-4 binding to cytochrome *b*, the effects of both the oxidized and reduced substrates, Q_6 and Q_6H_2, on the fluorescent emission spectrum were investigated. Addition of oxidized Q_6 had no effect on the emission spectrum; however, addition of reduced coenzyme Q, Q_6H_2, resulted in an 8−9 nm blue shift of the emission spectrum after addition of NCD-4 to the cytochrome bc_1 complex concomitant with an increase in the fluorescence intensity. Similar results were obtained whether the NCD-4 was added to the bc_1 complex before the Q_6 and/or the Q_6H_2 or whether the substrates were added to the cytochrome bc_1 complex before the NCD-4.

DISCUSSION

The results of the current study suggest that radioactive DCCD binds to aspartate-160 of cytochrome *b* from yeast mitochondria. This conclusion is based on the partial N-terminal amino acid sequence of a 7.0 kDa peptide labeled with [^{14}C]DCCD obtained after exhaustive digestion of a [^{14}C]DCCD-labeled cytochrome *b* with trypsin. Aspartate-160 is localized in helix cd, originally designated as a membrane-spanning helix (Widger et al., 1984; Saraste, 1984), but now considered to be extramembranous based on hydrophobicity and amphipathy calculations (Brasseur, 1988). Indirect evidence supporting this localization of helix cd has been provided by the identification of amino acid residues in the cytochrome *b* polypeptide which when mutated confer resistance to inhibitors of electron transfer in the *bc* complexes of yeast mitochondria (Colson, 1983), the photosynthetic bacteria *Rhodobacter capsulatus* (Gennis et al., 1993), and mammalian mitochondria (Howell, 1990). Mutations conferring resistance to the inhibitors of quinol oxidation, such as myxothiazol, mucidin, and stigmatellin, map at the outer surface of the inner membrane, while mutations conferring resistance to the inhibitors of quinone reduction such as antimycin A and Diuron were observed at residues clustered at the inner surface of the membrane (Colson, 1993). Biochemical evidence supporting this model has been obtained by *phoA* gene fusion experiments (Yun et al., 1991) and by the susceptibility to trypsin digestion of both cytochrome *b* (Beattie et al., 1994) and cytochrome b_6 (Wang & Beattie, 1992).

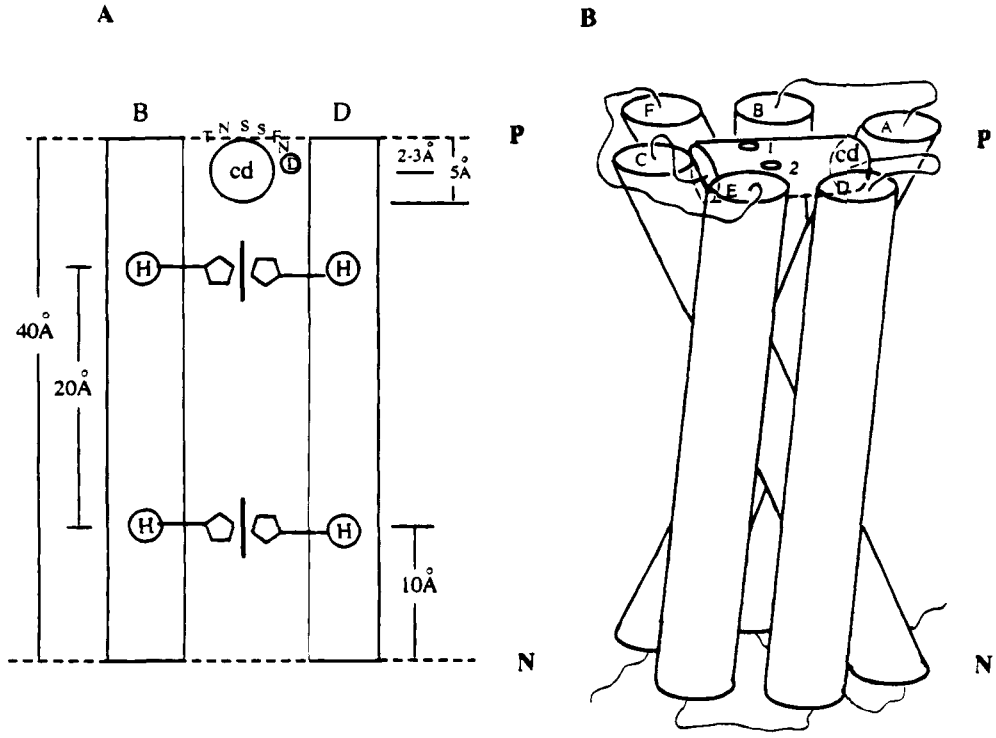

FIGURE 6: Model for the topographical organization of helix cd connecting membrane-spanning helices C and D of yeast mitochondrial cytochrome *b*. (A) The relative position of helix cd in the membrane, indicating the depth of 5-DSA at 6.25 Å from the surface of the membrane, the 5-Å diameter of an α helix, and the position of the two hemes separated by 20 Å in the membrane. (B) A three-dimensional view of helix cd relative to the membrane-spanning helices of cytochrome *b*. Circle 1 on helix cd represents the relative position of the DCCD binding site on cytochrome b_6 of the *bf* complex (aspartate-155 and/or glutamate-166), while circle 2 represents the relative DCCD binding site on cytochrome *b* of the bc_1 complex (aspartate-160).

The results obtained in the present study indicate that NCD-4, the fluorescent analogue of DCCD, interacts with the cytochrome bc_1 complex isolated from yeast mitochondria in the same manner as does DCCD. For example, both NCD-4 and DCCD bind specifically to cytochrome *b* and not to any of the other subunits of the bc_1 complex. In addition, NCD-4 inhibited proton translocation in the bc_1 complex reconstituted into proteoliposomes without a significant effect on the rate of electron transfer. Digestion of NCD-4-labeled cytochrome *b* with trypsin produced a fluorescent peptide with a molecular mass of 7.0 kDa identical in size to that labeled with [^{14}C]DCCD (Figures 2 and 4B). These results suggest that NCD-4 and DCCD are bound to the same residue of cytochrome *b*.

Further information about the topographical localization of aspartate-160 in helix cd connecting the membrane-spanning helices C and D of cytochrome *b* in the inner mitochondrial membrane has been obtained with the fluorescent derivative of DCCD. The results of the spin-label quenching experiments suggest that the binding site for NCD-4 on helix cd is buried in the membrane where it is shielded from the external medium. This conclusion is based on the observation that CAT-16, the amphiphilic probe that intercalates at the membrane surface, and 5-DSA, the stearic acid derivative with the spin group on carbon-5, were almost equally effective in quenching the fluorescence of NCD-4. Significant quenching of NCD-4 fluorescence was observed with the other membrane probes, 7-DSA and 12-DSA, which are further buried in the membrane. In addition, the polar probes such as CAT-1 and D-569 were ineffective quenchers of the NCD-4 fluorescence even at high concentrations, suggesting that the NCD-4 moiety bound to aspartate-160 is not in contact with the hydrophilic surface of the membrane.

Depiction of helix cd in an Edmundson wheel projection indicates that all of the hydrophobic amino acid residues are localized on one side of the helix while the four hydrophilic amino acids tyrosine, asparagine, and the two serines are localized on the other side of the wheel (Figure 6A). In this projection, aspartate-160 is localized in a hydrophobic region of the helix close to the hydrophilic patch. Examination of helical wheel projections of *b* cytochromes from several species indicates a similar orientation of the hydrophobic amino acid residues on one side of the membrane and a similar hydrophilic patch (Beattie, 1993). Interestingly, the glutamate and aspartate residues to which DCCD binds on cytochrome b_6 of spinach chloroplasts are localized within the hydrophilic patch as are the acidic amino acids in cytochrome *b* of bovine, mouse, and *Aspergillus*, while the analogous aspartate in human cytochrome *b* is localized near the hydrophobic residues.

A model that incorporates these observations proposes that helix cd containing aspartate-160 is localized with its axis parallel to the surface of the membrane such that the four hydrophilic amino acids are oriented facing the surface of the membrane while the remainder of the helix is within the membrane itself (Figure 6A). Such a model would place aspartate-160 2−3 Å from the surface of the membrane based on the predictions that the 5-doxyl group on 5-DSA is 6.25 Å from the surface. Thus, the NCD-4 bound to aspartate-160 should be equally accessible to a spin probe at the surface of the membrane and the hydrophobic probe on carbon-5 of stearic acid. Moreover, this proposal places aspartate-160 midway between the surface of the membrane and the heme group at center o which has been suggested to be localized 10 Å from the surface of the membrane (Saraste, 1984). We suggest that the protons released by quinol oxidation at center o may be translocated to the surface of the membrane with

the involvement of aspartate-160, the only hydrophilic amino acid in the hydrophobic stretch of amino acids.

Earlier models for the orientation of cytochrome *b* in the membrane based on analyses of the amino acid residues conferring antibiotic resistance had suggested that four different membrane-spanning α helices of cytochrome *b*, helices A−D, might form an α-helical bundle (Link et al., 1993). The presence of several mutations conferring resistance to inhibitors of quinol oxidation at center o in helix F suggests that this helix may also be involved in the formation of the α-helical bundle. A role for the loop connecting helices E and F, the so-called PEWY loop, in the reactions at center o has also been suggested on the basis of the presence of invariant amino acids in this segment of the protein (Lemesle-Meunier et al., 1993). We suggest that the six α helices of cytochrome *b* (A, B, C, D, E, and F) may be oriented at an angle relative to the surface of the membrane and thus provide a well for the insertion of helix cd (Figure 6B). This model also accommodates the two hemes bound to invariant histidines on helices B and D.

The changes in NCD-4 fluorescence observed after addition of the substrate, Q_6H_2, provide support for this model. The binding domains for ubiquinol on bovine cytochrome *b* involve several amino acids present in helix cd (He et al., 1994). We suggest that the interaction of Q_6H_2 with center o, the quinol-oxidizing site, may distort the orientations of the helical bundles relative to amphiphilic helix cd, such that the fluorescence of NCD-4 is enhanced. Alternatively, the binding of the substrate to the bc_1 complex may cause a conformational change in the protein, leading to the change in fluorescence. The proposed interaction of ubiquinol with helix cd is consistent with the results of photoaffinity labeling of cytochrome *b* with azido derivatives of ubiquinol (He et al., 1994).

This model also helps explain the observation that DCCD and NCD-4 inhibit proton translocation without significant inhibitory effects on electron transfer. According to the Q cycle, the generally accepted mechanism to explain the pathways of electron transfer through the bc_1 complex, electron transfer and proton movements should be coupled in an obligatory manner (Brandt & Trumpower, 1994). We suggest that the bulky DCCD, or NCD-4, group bound to helix cd may block the exit of protons without affecting the transfer of electrons from the quinol to the iron−sulfur protein. The proton may then diffuse back across the membrane to the negative side, thus uncoupling proton movements from electron flow. A similar mechanism has been suggested by Miki et al. (1994) to explain the inhibitory effects of DCCD on reversed electron flow through the bc_1 complex.

The assignment of the DCCD binding site to an aspartate in an α helix that does not span the membrane is unusual. Previously, it was reported that DCCD binds to acidic amino acid residues present in membrane-spanning helices of hydrophobic proteins such as the proteolipid subunit of the F_1/F_o proton-translocating ATPase (Fillingame, 1992; Yoshida et al., 1981, 1982), the plasma membrane H^+-ATPase of *Neurospora crassa* (Sussman et al., 1987), subunit III of cytochrome *c* oxidase (Casey et al., 1980; Prochaska et al., 1981), and the transhydrogenase of bovine heart mitochondria (Wakabayaski & Hatefi, 1987). The localization of the acidic amino acid to which DCCD binds in an amphiphilic helix within the membrane suggests possible alterative routes for the movement of protons in membranes.

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