Topographical Organization of Cytochrome b_6 in the Thylakoid Membrane of Spinach Chloroplasts Determined by Fluorescence Studies with *N*-Cyclohexyl-*N'*-[4-(dimethylamino)naphthyl]carbodiimide†

Yudong Wang and Diana S. Beattie*

Department of Biochemistry, West Virginia University School of Medicine, Morgantown, West Virginia 26506-9142

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ABSTRACT: In a recent study [Wang & Beattie (1992) *Biochemistry 31*, 8445–8459], we reported that dicyclohexylcarbodiimide (DCCD) was bound to either aspartate-155 or glutamate-166 localized in an amphiphilic, non-membrane-spanning, helix of cytochrome. Moreover, DCCD inhibits proton translocation in a cytochrome *bf* complex reconstituted into proteoliposomes without significant inhibition of electron transfer, suggesting that the helix containing aspartate-155 and glutamate-166 may play a role in proton movements. In order to explore the environment of this amphiphilic helix, we employed a fluorescent derivative of DCCD, *N*-cyclohexyl-*N'*-[4-(dimethylamino)naphthyl]carbodiimide (NCD-4). After incubation of NCD-4 with a cytochrome *bf* complex isolated from spinach chloroplasts, a fluorescent compound was formed with a 331-nm excitation peak and 440-nm emission peak. NCD-4 was selectively bound to cytochrome b_6 and inhibited proton translocation with only a minimal inhibitory effect on electron transfer in the cytochrome *bf* complex reconstituted into proteoliposomes. Exhaustive digestion of the NCD-4-labeled cytochrome b_6 with trypsin resulted in the formation of a single 6-kDa fluorescent peptide with similar properties to the peptide labeled with radioactive DCCD. The fluorescence of NCD-4 bound to the cytochrome *bf* complex reconstituted into proteoliposomes was quenched by CAT-16, an amphiphilic spin label that intercalates at the membrane surface, as well as by nitroxide derivatives of stearic acid in the order 5-doxylstearic acid > 7-doxylstearic acid > 12-doxylstearic acid. At higher concentrations, the hydrophilic membrane-impermeant quenchers, CAT-1 and D-569, also quenched the fluorescence of NCD-4. These results suggest that the non-membrane-spanning helix containing aspartate-155 and glutamate-166 is localized within the membrane but near the surface of the membrane where it is shielded from the external aqueous environment.

The cytochrome *bf* complex, an intrinsic membrane-bound protein complex localized in the thylakoid membrane of chloroplasts of higher plants, mediates electron transfer between photosynthesis I and II (Cramer et al., 1987). As electrons move from plastoquinol through the cytochrome *bf* complex to plastocyanine, protons are translocated across the thylakoid membrane into the lumen of the thylakoid such that an electrogenic proton gradient is generated across the membrane (Hauska et al., 1983). A similar function is catalyzed by the cytochrome bc_1 complex of the mitochondrial inner membrane, which transfers electrons from ubiquinol to cytochrome *c* with the translocation of protons in the opposite direction across the mitochondrial membrane to the cytosolic side of the inner membrane (Hauska et al., 1983).

Recently, we reported that dicyclohexylcarbodiimide (DCCD),[1] the well-established carboxyl-modifying reagent, inhibited proton translocation in the cytochrome *bf* complex isolated from spinach chloroplasts reconstituted into proteoliposomes (Wang & Beattie, 1991). Similar inhibitory effects of DCCD on proton translocation by the cytochrome bc_1 complex isolated from yeast mitochondria reconstituted into proteoliposomes were reported earlier (Beattie & Villalobo, 1982; Clejan & Beattie, 1983). In addition, concentrations of DCCD which blocked electrogenic proton movements in both complexes had little or no inhibitory effect on electron transfer measured as cytochrome *c* reductase activity in either of the isolated complexes (Clejan & Beattie, 1983; Wang & Beattie, 1991) or in rat liver mitochondria (Clejan, 1984). 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 cytochrome bc_1 complex (Beattie et al., 1984) and to cytochrome b_6 of the *bf* complex (Wang & Beattie, 1991), suggesting that these proteins provide a similar function in proton translocation at this site in the electron-transfer chain.

The actual binding site for DCCD on cytochrome b_6 was recently determined to be either aspartate-155 and/or glutamate-166 by partial sequence analysis of tryptic fragments of cytochrome b_6 labeled with [^{14}C]DCCD (Wang & Beattie, 1992). Recent studies of the topographical orientation of cytochrome b_6 in the thylakoid membrane (Szczepaniak & Cramer, 1990; Wang & Beattie, 1992) have indicated that both of these amino acids are localized in helix cd, a putative extramembranous, yet hydrophobic, helix of cytochrome b_6.

In order to characterize the environment surrounding aspartate-155 and glutamate-166, we explored the effects of *N*-cyclohexyl-*N'*-[4-(dimethylamino)naphthyl]carbodiimide (NCD-4), a fluorescent analogue of DCCD, initially introduced by Chadwick and Thomas (1983), on proton

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; NCD-4, *N*-cyclohexyl-*N'*-[4-dimethylamino)naphthyl]carbodiimide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 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.

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translocation by the cytochrome *bf* complex incorporated into proteoliposomes. NCD-4 has been reported to inhibit Ca^{2+} binding to the Ca^{2+}-ATPase (Chadwick & Thomas, 1983, 1984; Munkonge et al., 1989) and proton translocation by the F_1/F_0 H^+-ATPase (Pringle & Taber, 1985) while binding to the same carboxyl groups on the protein to which DCCD binds (Pick & Weiss, 1985). In the current study, we have demonstrated that NCD-4 inhibits proton translocation in a *bf* complex incorporated into proteoliposomes with only minimal inhibitory effects on electron transfer. In addition, NCD-4 is bound specifically to cytochrome b_6 by an *N*-acylurea linkage to a single 6-kDa peptide, suggesting that NCD-4 binds to the same amino acid as DCCD. Fluorescent quenching experiments using spin labels have been used to provide information on the localization of helix cd containing the NCD-4 binding (or DCCD binding) site relative to the membrane.

EXPERIMENTAL PROCEDURES

Preparation of Cytochrome bf Complex. The cytochrome *bf* complex was isolated from spinach chloroplasts by the procedure of Hurt and Hauska (1981) as described previously (Hauska, 1986; Wang & Beattie, 1991).

Labeling of Cytochrome bf Complex with NCD-4 and Incorporation into Proteoliposomes. The cytochrome *bf* complex isolated from spinach chloroplasts was suspended in a medium containing 100 mM KCl, 200 mM sucrose, and 50 mM K-Hepes, pH 6.2, and incubated with NCD-4 generally in a molecular ratio of 150 nmol of NCD-4/nmol of cytochrome b_6 overnight at 4 °C or for 1 h at room temperature. The addition of NCD-4 was from a 100 mM stock solution in ethanol. The unbound reagent was removed by filtration through a Diaflo ultrafilter YM 30 membrane and followed by washing the complex three 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 NCD-4-labeled cytochrome *bf* complex was incorporated into liposomes as described previously for the incorporation of the cytochrome *bf* complex labeled with [^{14}C]-DCCD (Wang & Beattie, 1992).

Digestion of NCD-4-Labeled Cytochrome b_6 by Trypsin. Cytochrome b_6 was isolated from the *bf* complex previously labeled with NCD-4 by electroelution prior to digestion with trypsin in a ratio of 1/20 (trypsin/protein) at 4 °C for 3–7 days. After SDS–PAGE of the digested sample, the gel was fixed, exposed to ultraviolet light, photographed, and then stained with Coomassie blue.

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_6 present in the NCD-4-labeled cytochrome *bf* complex, and 21 μM duroquinol (DQH_2) in a total volume of 2.4 mL.

Fluorescence Experiments. The fluorescence spectra were determined in an Shimadzu RF 5000U spectrofluorimeter at 25 °C. Fluorescence quenching was performed in 2 mL of reaction buffer containing 100 mM KCl, 50 mM K–Hepes, pH 6.9, and 0.15 mg of protein of the NCD-4-labeled cytochrome *bf* complex incorporated into liposomes. The quenchers, CAT-16, 5-DSA, 7-DSA, and 12-DSA, were maintained in 20 mM stock solution in ethanol and were added to the reaction mixtures at final concentrations of 10, 30, 50, 70, and 100 μM. CAT-1 and D-569 were maintained in 100 or 200 mM stock solution and were added to the reaction mixtures at final concentrations of 1, 5, 10, 15, and 20 mM. The reactions were incubated at room temperature for 5 min prior to measurement of fluorescence intensity. Excitation was at 331 nm, and emission was determined at 440 nm.

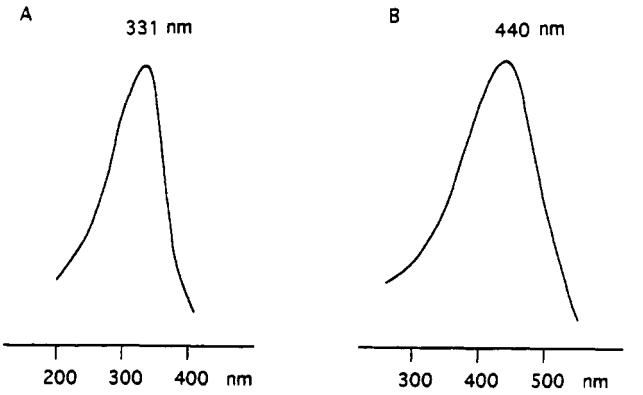

FIGURE 1: Fluorescence excitation and emission spectra for cytochrome *bf* incubated with NCD-4. The cytochrome *bf* complex was incubated with NCD-4 for 1 h at 25 °C in a molar ratio of 150 nmol of NCD-4/nmol of cytochrome b_6 in the cytochrome *bf* complex in 6 mL of reaction buffer as described under Experimental Procedures. After filtration and washing of the complex, the fluorescence spectrum was recorded at 25 °C on an RF 5000U spectrofluorimeter. (A) The excitation spectrum. (B) The emission spectrum.

Materials. DCCD was obtained from Schwarz-Mann; 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. All of the other chemicals were of the highest purity available commercially.

RESULTS

Solutions of NCD-4 in either water or organic solvents do not possess any intrinsic fluorescence. After incubation of NCD-4 with the cytochrome *bf* complex at room temperature for 1 h, fluorescent products were observed as indicated in Figure 1. Maximal excitation was observed at 331 nm and maximal emission at 440 nm. The emission spectrum at 440 nm is consistent with the formation of an *N*-acetylurea derivative of NCD-4 coupled to a carboxyl group (Chadwick & Thomas, 1983). These workers reported that the *N*-acetylurea formed from NCD-4 and glacial acetic acid had an emission maximum of 425 nm in 100% ethanol and 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 and the cytochrome *bf* complex suggests that NCD-4 forms an *N*-acylurea derivative with an aspartate or glutamate localized in a moderately hydrophobic environment of the cytochrome *bf* complex.

Analysis of the NCD-4-labeled cytochrome *bf* complex by SDS–polyacrylamide gel electrophoresis revealed that the fluorescent label was mainly associated with cytochrome b_6 (Figure 2). When the gel was exposed to ultraviolet light, strong fluorescence was associated with cytochrome b_6 and weak fluorescence with cytochrome *f*. No fluorescence was observed associated with either the iron–sulfur protein or subunit IV. These results suggest that NCD-4 is covalently and selectively bound to cytochrome b_6 but not any other subunits of the cytochrome *bf* complex. Similar results were reported previously for the labeling of the cytochrome *bf* complex with radiolabeled DCCD (Wang & Beattie, 1991).

To investigate further the site of binding of the NCD-4 on cytochrome b_6, the NCD-4-labeled cytochrome b_6 was isolated from the *bf* complex and subjected to exhaustive digestion by

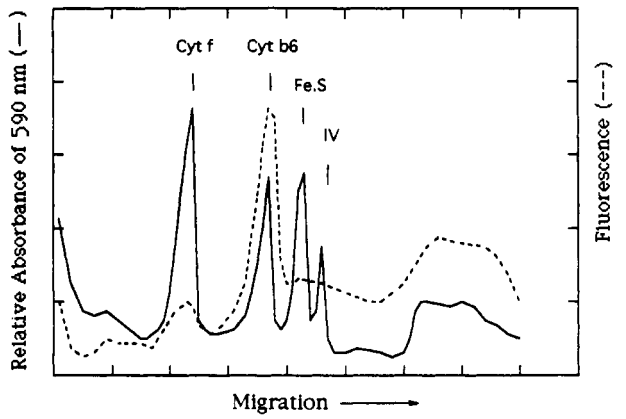

FIGURE 2: Fluorescent labeling of the subunits of the cytochrome *bf* complex after labeling with NCD-4. The NCD-4-labeled cytochrome *bf* complex was analyzed by SDS–PAGE on a 12.5% polyacrylamide gel performed according to Laemmli (1970). After electrophoresis, the gel was fixed and exposed to ultraviolet light, photographed, and then stained with Coomassie blue. The intensity of the protein bands was determined by densitometry of the stained gel (—). The fluorescence was determined by scanning densitometry of the negative of the photograph (- - -).

trypsin. Exposure of the tryptic peptides to ultraviolet light after SDS–PAGE revealed two fluorescent protein bands (Figure 3). The larger band corresponds in molecular weight to undigested cytochrome b_6 while the smaller band (labeled P in Figure 3) has an apparent molecular mass of 6 kDa. Increasing the time of digestion with trypsin resulted in an enhancement of the fluorescence of the 6-kDa peptide and a decrease in the fluorescence of cytochrome b_6. These results indicate that only one peptide of cytochrome b_6 is labeled with NCD-4. Moreover, the apparent molecular mass of the fluorescent peptide is identical to that of the tryptic peptide containing [^{14}C]DCCD (Wang & Beattie, 1992), suggesting that NCD-4 and [^{14}C]DCCD are bound to the same fragment of cytochrome b_6.

The effects of NCD-4 on both electron transfer and proton translocation were measured simultaneously in the Aminco DW-2 spectrophotometer as described previously for the DCCD-treated cytochrome *bf* complex (Wang & Beattie, 1991). Increasing the concentration of NCD-4 incubated with the *bf* complex resulted in a greater inhibition of proton translocating activity, reaching a maximum inhibitory effect of 70% at 300 nmol of NCD-4/nmol of cytochrome b_6 (Figure 4); however, the rate of electron transfer was not inhibited significantly 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 *bf* complex as were previously observed with DCCD. Moreover, these results also indicate that NCD-4 binds to the same site, either aspartate-155 or glutamate-166, as DCCD on cytochrome b_6.

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). The amino acid residues of cytochrome b_6 to which DCCD binds, aspartate-155 or glutamate-166, are localized in a putative extramembranous, yet hydrophobic, α-helix (Brasseur, 1988). We wished to establish the localization of this helix relative to the membrane and, thus, employed the following compounds as spin label quenchers: doxyl 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; and CAT-1 and D-569, which are the most polar spin labels. Dynamic collisional, 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. Figures 5 and 6 show the quenching effects of the various spin labels on the fluorescence of the NCD-4-treated cytochrome *bf* complex using the following 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 5). The polar spin labels CAT-1 and D-569 did not effectively quench the NCD-4 bound to the cytochrome *bf* complex at low concentrations; however, at higher concentrations both CAT-1 and D-569 were effective quenchers (Figure 6). These results suggest that NCD-4 is bound to a region of the protein close to the surface of the membrane where its fluorescence is quenched most effectively either by the amphiphilic spin label, CAT-16, or by the polar spin labels CAT-1 and D-569.

The hydrophobic quenchers, 12-DSA, 7-DSA, 5-DSA, and CAT-16, at the concentrations which interfered with the NCD-4 fluorescence, had no effect on the rate either of proton uptake or of electron transfer in the *bf* complex reconstituted into liposomes. The only exception was CAT-16, the amphiphilic probe, which resulted in a doubling of the observed rate of cytochrome *c* reduction without any change in the rate of proton movements, perhaps reflecting a change in the binding of the electron acceptor to the membrane. In addition, these quenchers did not cause any changes in the fluorescence spectrum due to the hemes of the cytochrome *bf* complex, suggesting that the low concentrations of the quenchers used in these experiments do not change the structure of the complex.

DISCUSSION

The results of the current study indicate that NCD-4, the fluorescent analogue of DCCD, interacts with the cytochrome *bf* complex isolated from spinach chloroplasts in the same manner as does DCCD. For example, both molecules bind specifically to cytochrome b_6 and not to the other proteins of the *bf* complex. In addition, increasing concentrations of NCD-4 inhibited both the rate and extent of proton translocation in a cytochrome *bf* complex reconstituted into proteoliposomes but had a minimal effect on the rate of electron transfer. It should be noted that the maximum inhibition observed with NCD-4 occurred at concentrations double those of DCCD required to achieve comparable inhibitions (Wang & Beattie, 1990). The greater bulk of the NCD-4 molecule compared to DCCD may be responsible for this concentration effect.

The digestion of NCD-4-labeled cytochrome b_6 by trypsin produced only one fluorescent peptide (P) with a molecular weight of 6 kDa. Similarly, the digestion of [^{14}C]DCCD-labeled cytochrome b_6 by trypsin produced a radioactive peptide (B_2) which has the same molecular mass (Wang & Beattie, 1992). These results suggest that NCD-4 and DCCD are bound to the same position, aspartate-155 and/or glutamate-166 of cytochrome b_6.

The results of this study also provide additional information about the topographical organization of cytochrome b_6 in the

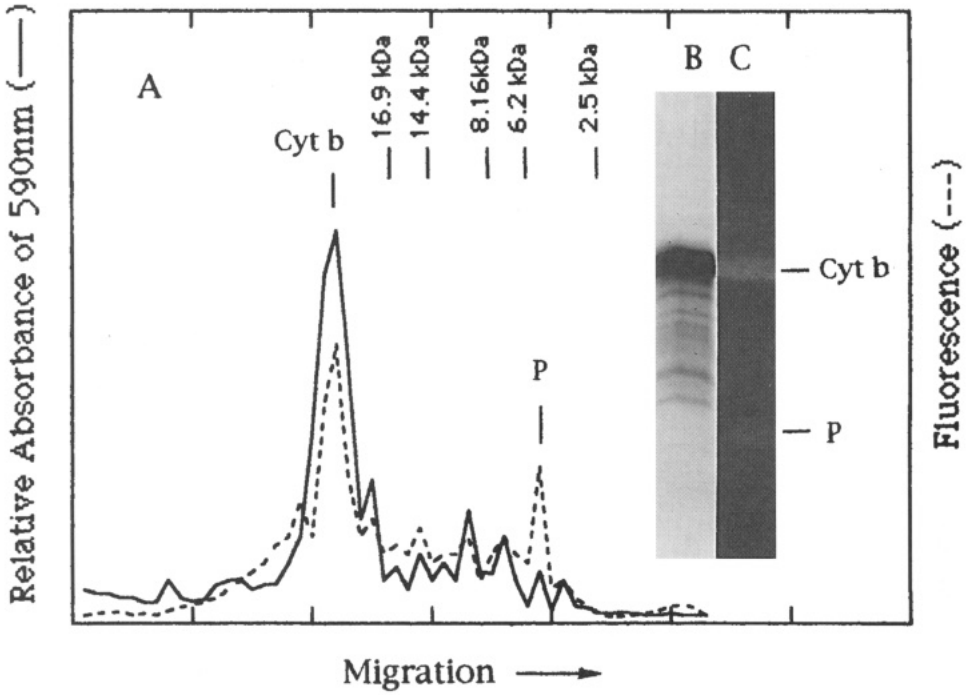

FIGURE 3: NCD-4-labeled cytochrome b_6 was digested by trypsin at a ratio of 20/1 (protein/trypsin) for 3–7 days at 4 °C. (A) After SDS–PAGE, the gel was fixed, exposed to ultraviolet light, photographed and then stained with Coomassie blue prior to scanning densitometry (—). The fluorescence intensity was determined by scanning densitometry of the negative of the photograph (– – –). (B) Photograph of stained gel by Coomassie blue. (C) Photograph of fluorescent gel.

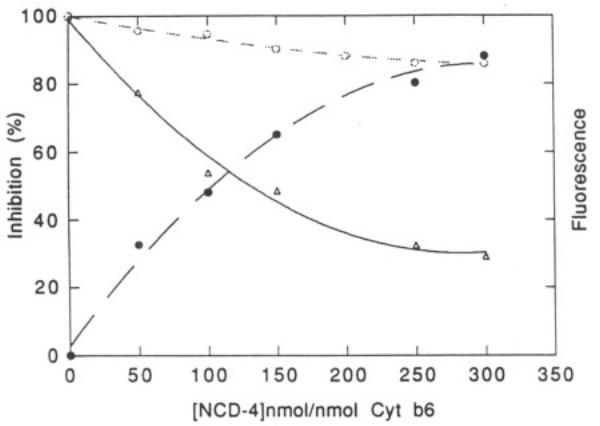

FIGURE 4: Concentration dependence of the inhibitory effects of NCD-4 on proton translocation and electron transfer of the cytochrome *bf* complex reconstituted into proteoliposomes and the fluorescence intensity of the NCD–4 bound to the complex. Proton translocation (Δ—Δ) and electron transfer (O– – –O) were measured simultaneously using a pH electrode placed in the cuvette in the spectrophotometer. The control activity was measured with an untreated cytochrome *bf* complex reconstituted into proteoliposomes as described under Experimental Procedures. The fluorescence intensity was recorded after 1-h incubation of the cytochrome *bf* complex at 25 °C with the concentrations of NCD-4 indicated in the figures using an excitation of 331 nm and determining the emission at 440 nm (● – – ●). Each point is the average of four or more experiments.

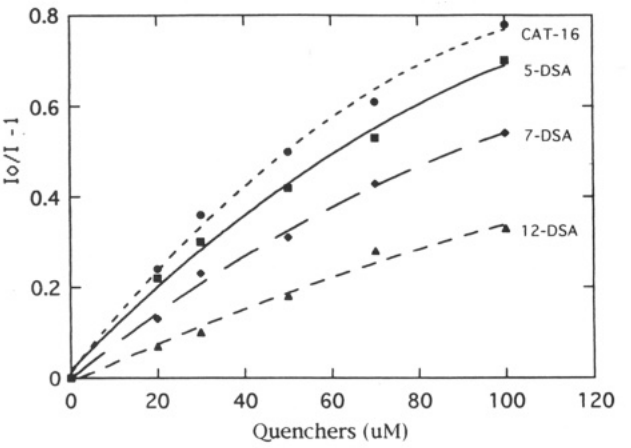

FIGURE 5: Fluorescence quenching of NCD-4-labeled cytochrome *bf* complex by hydrophobic spin labels. The NCD-4-labeled cytochrome *bf* complex incorporated into proteoliposomes was suspended in 2 mL of reaction buffer containing 0.12 μM cytochrome b_6 in the cytochrome *bf* complex. The quenchers, identified 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 as noted under Experimental Procedures and in the legend to Figure 4. The plot was calculated using the Stern–Volmer equation described under Results.

thylakoid membrane. The initial analysis of the hydrophobicity of cytochrome b_6 using the amino acid sequence deduced from the gene suggested the presence of 5 hydrophobic regions predicted to be membrane-spanning helices (Widger et al., 1984). Moreover, computer analyses of protein structure using several different programs predicted that each of these hydrophobic regions would form an α-helix (Chou & Fasman, 1978; Garnier et al., 1978). Subsequently, the 5-helix model for cytochrome b_6 and the related 9-helix model for mitochondrial cytochrome *b* were challenged by studies in which the locus of mutations in cytochrome *b* conferring resistance to antimycin and myxothiazol (or related antibiotics) was mapped (diRago & Colson, 1988; diRago et al., 1989). These antibiotics bind to different sites on cytochrome *b* in the cytochrome bc_1 complex such that myxothiazol blocks quinol oxidation and antimycin blocks quinone reduction (Trumpower, 1990). To accommodate the location of the antibiotic-resistant mutants with similar biochemical properties to the same side of the membrane, it was necessary to remove the most amphipathic helix, originally called helix IV, from the membrane into an extramembranous location. Determination of the hydrophobic moment of this helix provided additional evidence for this localization (Brasseur, 1988). As a conse-

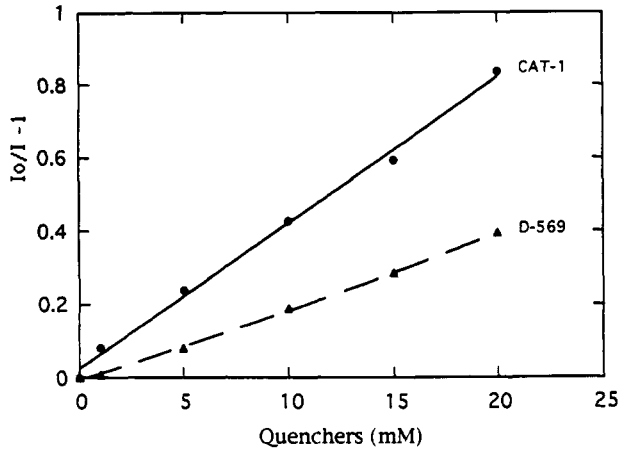

FIGURE 6: Fluorescence quenching of NCD-4-labeled cytochrome *bf* complex by hydrophilic spin labels. The NCD-4-labeled *bf* complex incorporated into proteoliposomes was suspended in 2 mL of reaction buffer as described in the legend to Figure 5. The quenchers, identified on the right side of the figure, were added from a 100 mM stock solution directly to the assay cuvettes to the final concentrations indicated in the figure. The plot was calculated using the Stern–Volmer equation described under Results.

quence of the proposed removal of helix IV from the membrane, the folding pattern predicted 8 membrane-spanning helices for cytochrome *b* and 4 membrane-spanning helices for cytochrome b_6. Recent biochemical investigations have provided evidence supporting the 4-helix model for cytochrome b_6 (Szcaepaniak & Cramer, 1990; Wang & Beattie, 1992).

The 4-helix model of cytochrome b_6 places aspartate-155 and glutamate-166, the residues to which DCCD and NCD-4 bind, in helix IV, or, as it is now called, helix cd, to indicate its localization between membrane-spanning helices C and D (Figure 7A). In the topographical predictions from the most recent data, helix cd has been placed in an extramembranous position. Determination of the exact localization of helix cd relative to the membrane and the membrane-spanning helices of cytochrome b_6 is important for a greater understanding of the mechanism of proton translocation in this segment of the electron transport chain because of the suggested involvement of aspartate-155 or glutamate-166 in proton movements. Interestingly, depiction of helix cd in an Edmundson wheel projection indicates that all the hydrophobic amino acid residues are localized on one side of the helix, while the 4 hydrophilic residues, tyrosine-151, serine-162, aspartate-155, and glutamate-166, are all localized on the other side of the helix (Figure 7A).

The results of the spin label quenching experiments suggest that helix cd is localized on the surface of the membrane but shielded from the external medium. These conclusions are based on the observation that CAT-16, the amphiphilic probe, was the most effective quencher at low concentrations and that the spin-labeled doxylstearic acid derivatives, especially 5-DSA, were also effective quenchers. The observed quenching by the hydrophilic spin label quenchers, CAT-1 and D-569, at higher concentrations suggests that helix cd is close to the hydrophilic medium at the surface of the membrane.

A model that incorporates these observations proposes that the helix cd containing aspartate-155 and glutamate-166 is localized with its axis parallel to the surface of the membrane such that the 4 hydrophilic amino acids are oriented facing the surface of the membrane while the remainder of the helix is within the membrane itself. Hence, the fluorescent NCD-4 bound to either aspartate-155 or glutamate-166 must lie close to the surface of the membrane but still be accessible to the

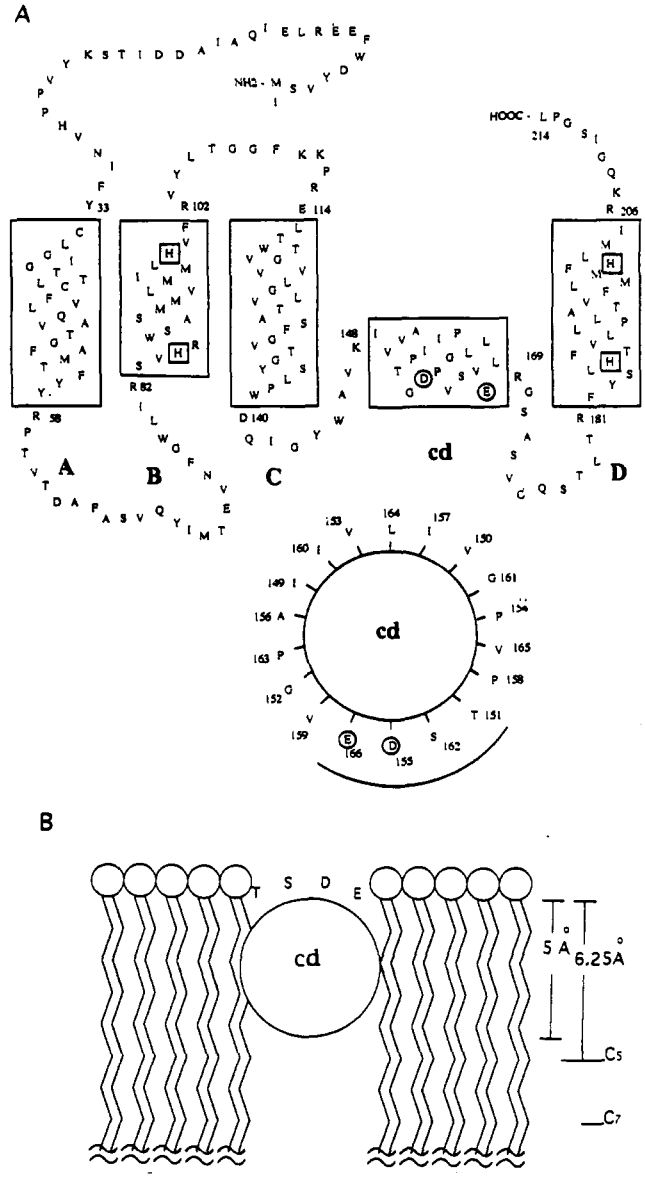

FIGURE 7: A model for the topographical organization of cytochrome b_6 in the thylakoid membrane. (A) The 4 membrane-spanning helices, A, B, C, and D, with amphiphilic helix cd containing helices C and D at the surface of the membrane. According to Edmundson wheel, helix cd containing D-155 and E-166 has 4 hydrophobic amino acid residues, T-151, S-162, D-155, and E-166, localized on one side of the helix (underlined in the diagram), while the hydrophobic amino acid residues are arranged on the other side of the helix. (B) The relative position of helix cd in the membrane, indicating the depth of 5-DSA derivative at 6.25 Å from the surface of the membrane and the 5-Å diameter of an α-helix.

5-DSA derivative within the membrane (Figure 7B). This proposed localization for the carboxylic acid that binds DCCD contrasts to that of other proton-translocating enzyme complexes where the site of DCCD binding has been shown to be an aspartate or glutamate residue localized in a hydrophobic membrane-spanning helix. Examples of this localization include the proteolipid subunit of the F_1/F_0 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 site of DCCD binding on cytochrome b_6 to a helix close to the surface of the membrane has implications for the pathway of proton movement from

the quinol oxidation site localized within the membrane either to the bulk phase outside the membrane or to a localized proton gradient. The Q_0 binding site, where the oxidation of the quinol occurs, may be comprised of amino acids both in the membrane-spanning helices C and D as well as in the extramembranous regions of the protein including the amphipathic helix cd, which has a similar arrangement of hydrophobic and hydrophilic residues as well as many conserved residues in all cytochromes *b* (Beattie, 1993). We suggest that the aspartate or glutamate in helix cd to which DCCD binds may act to facilitate the movement of protons from the quinol localized within the hydrophobic region of the membrane to the surface of the membrane, where it contributes to the overall protonmotive force. Perhaps the DCCD-sensitive component acts as a proton channel in a manner similar to that proposed for the F_0 portion of the F_1/F_0 ATPase (Hassinen & Vuokila, 1993). Experiments are currently underway in our laboratory to establish more precisely the localization of the amino acid(s) involved in the binding of DCCD.

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