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Minireview

A Proposed Pathway of Proton Translocation through the *bc* Complexes of Mitochondria and Chloroplasts

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The cytochrome *bc* complexes of the electron transport chain from a wide variety of organisms generate an electrochemical proton gradient which is used for the synthesis of ATP. Proton translocation studies with radiolabeled N,N′-dicyclohexylcarbodiimide (DCCD), the well-established carboxyl-modifying reagent, inhibited proton-translocation 50–70% with minimal effect on electron transfer in the cytochrome bc_1 and cytochrome *bf* complexes reconstituted into liposomes. Subsequent binding studies with cytochrome bc_1 and cytochrome *bf* complexes indicate that DCCD specifically binds to the subunit *b* and subunit b_6, respectively, in a time and concentration dependent manner. Further analyses of the results with cyanogen bromide and protease digestion suggest that the probable site of DCCD binding is aspartate 160 of yeast cytochrome *b* and aspartate 155 or glutamate 166 of spinach cytochrome b_6. Moreover, similar inhibition of proton translocating activity and binding to cytochrome *b* and cytochrome b_6 were noticed with N-cyclo-N-(4-dimethylamino-napthyl)carbodiimide (NCD-4), a fluorescent analogue of DCCD. The spin-label quenching experiments provide further evidence that the binding site for NCD-4 on helix cd of both cytochrome *b* and cytochrome b_6 is localized near the surface of the membrane but shielded from the external medium.

KEY WORDS: Proton translocation; cytochrome bc_1 and cytochrome *bf* complexes; DCCD; NCD-4.

INTRODUCTION

The cytochrome bc_1 complex of the mitochondrial electron transport chain catalyzes electron transfer from ubiquinol to cytochrome *c* coupled to electrogenic proton translocation across the inner mitochondrial membrane. This electrochemical gradient can be used for the synthesis of ATP by the F_1/F_0 proton-translocating ATPase localized in the mitochondrial membrane as well as for ion and substrate transport across the membrane. 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 (Hauska *et al.*, 1983). All of these *bc* complexes contain three similar polypeptides with redox centers including cytochrome *b*, a single polypeptide containing two *b*-type hemes (cytochrome b_L and b_H), a *c*-type cytochrome (cytochrome c_1 or cytochrome *f*), and an iron-sulfur protein containing the Rieske 2Fe-2S cluster. These three proteins with their distinctive metal centers catalyze the electron transfer reactions through the *bc* complexes. Considerable structural similarities including amino acid sequence homology have been reported for these three redox proteins isolated from various species, suggesting the universality of the electron transfer reactions catalyzed by the *bc* complexes. Consequently, our knowledge of the mechanism of electron flow through this segment of the electron transfer chain has increased significantly during the past decade as these proteins have been further characterized.

Despite our rapidly increasing understanding of the proteins involved in electron transfer reactions in

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the diverse bc complexes, much less is known about the mechanism of electrogenic proton translocation that accompanies electron transfer. During the transfer of electrons from a reduced quinol to cytochrome c (or an analogous electron acceptor) in all of these complexes, an observed proton/electron stoichiometry (H^+/e^- ratio) of 2 has been reported (Alexandre *et al.*, 1980; Leung and Hinkle, 1975; Guerrieri and Nelson, 1975; Beattie and Villalobo, 1982). In earlier studies, a H^+/e^- ratio of 1 had been reported during electron transfer from photosystem II to photosystem I (the cytochrome bf complex) of the photosynthetic electron transfer chain, suggesting that the bf complex functioned only as a plastoquinol: plastocyanin oxidoreductase without the formation of an electrochemical gradient (Cramer *et al.*, 1987; Hauska and Trebst, 1975). More recently, H^+e^- ratios approaching 2 have been observed during electron transfer between the two photosystems, suggesting that the bf complex is involved in the electrogenic transfer of a H^+ into the lumen of the thylakoid during electron transfer (Rich, 1988; Hope and Rich, 1989) and thus functions in an analogous manner to the other bc complexes.

The currently accepted mechanism for the generation of an electrochemical proton gradient during electron transfer in the cytochrome bc_1 complex is the modified Q cycle initially proposed by Mitchell (1976). The central feature of the Q cycle mechanism is the presence of two separate quinone binding sites in the bc_1 complex. One of these sites, Q_o, is involved in the oxidation of ubiquinol at the positive side of the membrane and a second site, Q_i, is involved in the reduction of ubiquinone at the negative side of the membrane (Trumpower, 1990). The Q cycle proposes that during oxidation of a quinol at the Q_o site, one electron is transferred to the iron-sulfur protein with the generation of a transient semiquinone which is then immediately oxidized by cytochrome b_L, the low-potential cytochrome b localized near the positive side of the membrane. The iron-sulfur protein is, in turn, oxidized by cytochrome c_1, while cytochrome b_L transfers electrons to cytochrome b_H, the high-potential cytochrome b localized near the opposite (negative) side of the membrane. Quinone is then reduced to a stable semiquinone at the Q_i site by transfer of an electron from cytochrome b_H. The second electron necessary to reduce the semiquinone initially formed at the Q_i site to a quinol is obtained by a second oxidation of a quinol at the Q_o site.

Experimental evidence for the modified Q cycle has been obtained from studies of the effects of various inhibitors of electron transfer in the cytochrome bc_1 complex (Rich, 1984; von Jagow and Link, 1986). One set of inhibitors, exemplified by myxothiazol and other methoxyacrylates, blocks quinol oxidation at the Q_o site and binds specifically to cytochrome b_L (von Jagow and Ohnishi, 1985), while a second set of inhibitors, such as antimycin, blocks quinone reduction at the Q_i site and binds specifically to cytochrome b_H (von Jagow *et al.*, 1984). Kinetic studies of electron transfer through the bc_1 complex have established the site of action of these inhibitors on cytochrome b. Moreover, structural information about the Q_o and Q_i sites has been derived from studies of mutations which confer resistance to these inhibitors in bc complexes from yeast (diRago and Colson, 1988; diRago *et al.*, 1989), photosynthetic bacteria (Daldal *et al.*, 1989; Knaff, 1992), and mammals (Howell *et al.*, 1987; Howell and Gilbert, 1988). All of the mutations involve conservative amino acid substitutions on the cytochrome b protein, mapping either on the quinol-oxidizing side for myxothiazol-type inhibitors or on the quinone-reducing side for antimycin-like inhibitors. To accommodate the data obtained by mapping the site of these amino acid substitutions conferring antibiotic resistance, the topographical orientation of cytochrome b in the mitochondrial membrane has been revised to an eight-helical model from the nine-helical model (diRago and Colson, 1988; diRago *et al.*, 1989) originally proposed based on hydropathy plots (Widger *et al.*, 1984; Saraste, 1984).

For the past few years, the focus of studies in our laboratory has been the mechanism of proton translocation in the cytochrome bc_1 complex isolated from yeast mitochondria and the cytochrome bf complex isolated from spinach chloroplasts. We have used dicyclohexylcarbodiimide[2] (DCCD), the well-established carboxyl-modifying reagent, to study the proton-translocation device in both the bc_1 and the bf complexes. DCCD was originally reported to block

[2] Abbreviations used: DCCD, *N,N'*-dicyclohexylcarbodiimide; NCD-4, *N*-cyclohexyl-*N'*-(4-dimethylaminonaphthyl)carbodiimide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CAT-1, 4-trimethylammonium-2,2,6,6-tertramethylpiperidine-1-oxyl iodide; D0-569, 4-(N,N-dimethyl-N-(3-sulfopropyl)) ammonium-2,2,6,6-tetramethyl piperidine-1-oxyl; CAT-16 or D-531, 4-(*N,N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; 5-DSA, 5-doxylstearic acid; 7-DSA, 7-doxylstearic acid; 12-DSA, 12-doxylstearic acid.

proton translocation in the F_1/F_0 proton-translocating ATPases by covalently binding to an essential glutamate or aspartate residue localized in a hydrophobic region of a membrane-spanning alpha helix of the F_0 proteolipid (Fillingame, 1980). Similar inhibitions of proton translocation across membranes by DCCD have been reported for 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 and Hatefi, 1987).

This review will discuss results obtained in our laboratory indicating that DCCD inhibits proton translocation in both the cytochrome bc_1 and *bf* complexes reconstituted into proteoliposomes without significant effect on quinol:cytochrome *c* reductase activity in the complex or in isolated mitochondria. The lack of inhibition of electron flow by DCCD suggests that the primary effect of DCCD is on the proton-translocating device of both the cytochrome bc_1 and cytochrome *bf* complexes. In addition, we have observed that radioactive and fluorescent derivatives of DCCD bind selectively to cytochrome *b* of the bc_1 complex and to cytochrome b_6 of the *bf* complex, suggesting that these proteins provide a similar function in proton translocation in these two complexes.

EFFECTS OF DCCD ON PROTON TRANSLOCATION AND ELECTRON TRANSFER IN THE CYTOCHROME bc_1 AND CYTOCHROME *bf* COMPLEXES

An enzymatically active cytochrome bc_1 complex has been isolated from yeast mitochondria by either ammonium sulfate fractionation of cholate-solubilized submitochondrial particles (Sidhu and Beattie, 1982) or by column chromatography of mitochondria solubilized by dodecylmaltoside (Ljungdahl *et al.*, 1986). When the bc_1 complex isolated by either procedure was reconstituted into proteoliposomes prepared from phospholipids containing a small amount of diphosphatidylglycerol (cardiolipin), respiratory control ratios greater than 4 were observed in the presence of an uncoupler, suggesting that an electrogenic membrane potential had been established (Beattie and Villalobo, 1982; Beattie and Marcelo-Baciu, 1991). Proton ejection in these bc_1 complexes

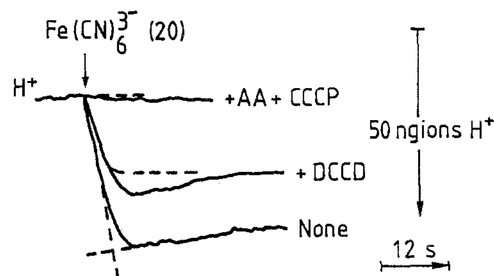

Fig. 1. Effect of DCCD on proton ejection by the cytochrome bc_1 complex reconstituted into proteoliposomes. Proton ejection was measured with a small pH-sensitive electrode in the medium described in Beattie and Villalobo (1982). Proton ejection was recorded upon addition of a pulse consisting of 20 nmol of potassium ferricyanide. DCCD indicates that the bc_1 complex was preincubated with DCCD prior to reconstitution. The dashed line indicates proton ejection in control liposomes in the presence of the uncoupler, CCCP. AA refers to antimycin A.

was also measured directly with a sensitive pH electrode in a low-buffer capacity medium using the decyl analogue of ubiquinol as substrate and catalyic amounts of cytochrome *c* as electron acceptor (Beattie and Villalobo, 1982). Under these conditions, H^+/e^- ratios approaching 1.9 were observed upon the addition of a pulse of ferricyanide to the medium (Fig. 1). The control H^+/e^- ratio of 1.0 observed in the presence of an uncoupler indicates the release to the external medium of the scalar protons from the reduced quinol. Preincubation of the bc_1 complex with DCCD at 12°C, to minimize nonspecific binding of DCCD to phospholipids, resulted in a decreased rate and extent of electrogenic proton ejection with an observed H^+/e^- ratio of 1.2 (Fig. 1) suggesting that the interaction of DCCD with the bc_1 complex had resulted in a profound inhibition of electrogenic proton translocation.

In these initial measurements of H^+/e^- ratios, it was noted that the rate and extent of proton ejection in the presence of an uncoupler, a measure of the rate of oxidation of the quinol substrate, was approximately the same in the liposomes reconstituted with either the control or the DCCD-treated bc_1 complex (Beattie and Villalobo, 1982). This result suggested that DCCD had a minimal effect on the control rates of electron transfer in the bc_1 complex. Table I summarizes a number of experimental approaches which have confirmed this observation using the rate of proton ejection and the rate of cytochrome *c* reduction as measures of electron transfer activity (Clejan and

Table I. Effect of DCCD on the Enzymatic Activity of the bc_1 Complex from Yeast Mitochondria[a]

Experiment 1	Control: $H^+/2e^-$ ratio	Control: Rate of H^+ ejection	+CCCP: $H^+/2e^-$ ratio	+CCCP: Rate of H^+ ejection
Control	3.78	50.3	2.08	33.9
+DCCD (200 nmol/nmol cytochrome *b*)	2.67	33.6	2.07	28.8
Decrease (%)	62	33	0	15
Range of decrease (%)	60–83	30–60		15–25
Experiment 2	**Cytochrome *c* reductase activity: −Cholate**	**+Cholate**	**Free**	
Control	3.42	4.43	4.4	
+185 nmol DCCD per nmol cytochrome *b*	2.77	4.03	4.1	
Decrease (%)	19	9.0	6.8	

[a]The bc_1 complex was incorporated into liposomes, treated with DCCD, and the unbound DCCD removed by chromatography on Sephadex. Proton ejection was measured with a pH electrode and enzymatic activities as the rate of cytochrome *c* reduction with DBH_2 as substrate (Clejan and Beattie, 1983).

Beattie, 1983; Beattie and Marcelo-Baciu, 1991). Similarly, incubation of a tightly coupled rat liver mitochondria with DCCD under a variety of experimental conditions had no discernible effect on the rate of electron flow through the bc_1 region of the electron transfer chain while inhibiting completely the electrogenic translocation of protons (Clejan *et al.*, 1984b).

The effects of DCCD on electron flow and proton translocation have now been extended to the cytochrome *bf* complex isolated from spinach chloroplasts (Wang and Beattie, 1991). A H^+/e^- ratio of 1.7 was observed in the *bf* complex reconstituted into proteoliposomes with reduced duroquinol as substrate. Incubation of the *bf* complex with DCCD for 1 hour at 12°C prior to incorporation of the complex into liposomes resulted in a 20% decrease in the rate of electron transfer and a 60% decrease in the rate of proton pumping. The H^+/e^- ratio observed with the DCCD-treated *bf* complex was 1.0 and represented the scalar proton obtained by oxidation of the quinol substrate (Fig. 2). In similar studies with intact chloroplasts, we reported that DCCD blocked proton translocation with minimum effects on electron transfer (Sprague *et al.*, 1988).

An unexplained conclusion from these studies is that the interaction of DCCD with the cytochrome *bc* region in the electron transfer chains of both mitochondria and chloroplasts results in an uncoupling of proton translocation from electron transfer. The

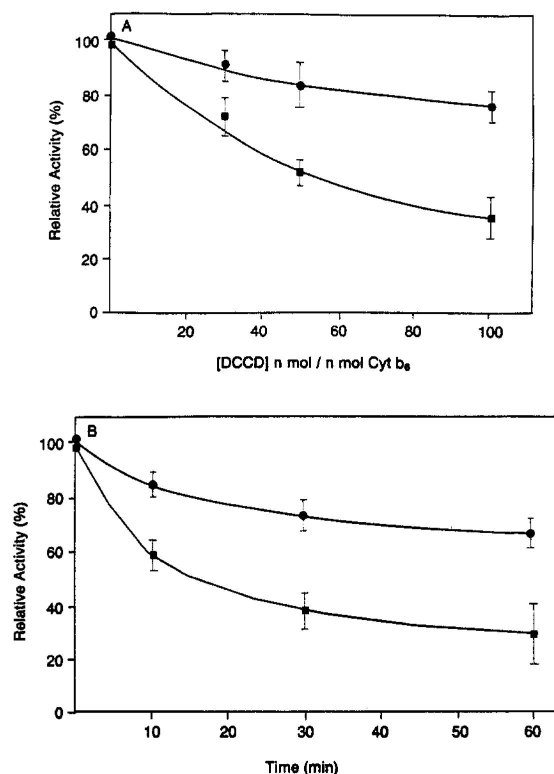

Fig. 2. Concentration and time dependence of the inhibition by DCCD of electron transfer and proton translocation by the *bf* complex reconstituted into proteoliposomes. (A) Effects of DCCD concentration. (B) Time course of the inhibition by DCCD. Electron transfer (●); Proton translocation (■).

inhibitory effects of DCCD suggest that one or more of the proteins of the *bc* complex may be involved in proton movements independently of the oxidation–reduction reactions. It has been difficult to reconcile these results, repeated many times by different workers in several laboratories, with the currently accepted Q-cycle which proposes an obligatory coupling between electron transfer and proton movements. This subject will be discussed in greater detail later in this article.

BINDING OF RADIOACTIVE DCCD TO THE CYTOCHROME bc_1 AND CYTOCHROME *bf* COMPLEXES

The specific inhibitory effects of DCCD on proton translocation in the cytochrome bc_1 region of the electron transport chain prompted investigations of the possibility that a covalent linking of DCCD to one subunit of the enzyme might occur as had been demonstrated for other proton-translocating enzyme complexes. Incubation of the cytochrome bc_1 complex isolated from yeast mitochondria with 50–100 nmol of DCCD/nmol of cytochrome *b* at 12°C did not result in any changes in the appearance of the high-molecular-weight subunits of the complex, although a slight broadening of the low-molecular-weight subunits was noted (Fig. 3). No evidence, however, of crosslinking between subunits of the complex was observed. The radioactive DCCD was bound selectively to cytochrome *b* and to a broad band with an apparent low molecular weight which was removed by extraction of the complex with chloroform:methanol and was subsequently shown to contain either free [^{14}C]DCCD or cardiolipin (Beattie *et al.*, 1984). The preferential binding of DCCD to cytochrome *b* in the bc_1 complex from yeast mitochondria suggested that cytochrome *b* may play an important role in proton translocation at this site of the respiratory chain (Beattie and Clejan, 1982).

In similar studies with a bc_1 complex isolated from beef heart mitochondria, we reported that radioactive DCCD was bound with similar kinetics to cytochrome *b* and to a low-molecular-weight subunit plus phospholipids in this complex (Clejan *et al.*, 1984a). To obtain these results, the bc_1 complex was reisolated after the incubation with DCCD by centrifugation through sucrose or by chromatography on Sephadex G-50; however, the use of harsher treatments such as precipitation with ammonium sulfate or

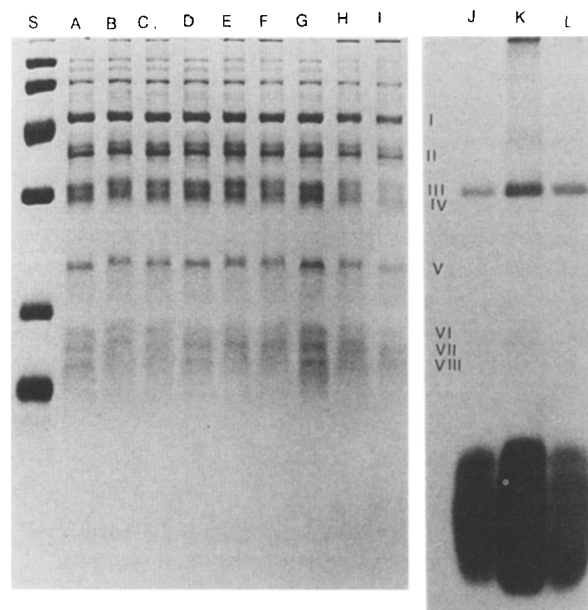

Fig. 3. Effect of temperature and time of incubation with DCCD on the electrophoretic mobility and labeling of the subunits of the bc_1 complex. High-molecular-weight standards: 94,000, 67,000, 43,000, 30,000, 20,000, and 14,400 (lane S); bc_1 complex (40 μg) incubated with DCCD (100 nmol/nmol of cytochrome *b*) for 0, 30, and 60 min, respectively, at 12°C (lanes A–C), 20°C (lanes D–F), and 35°C (lanes G–I). Right, fluorogram of the gel of complex III treated with DCCD for 1 h at 12°C (lane J), 25°C (lane K), and 20°C (lane L).

trichloroacetic acid to reisolate the complex after the incubation with DCCD resulted in nonspecific labeling of all the subunits of the complex and increased labeling of the low-molecular-weight subunit of the complex relative to cytochrome *b*, suggesting that additional chemical reactions with DCCD may have occurred (Beattie *et al.*, 1985). These results may explain some of the discrepancies reported in the literature on the site of binding of DCCD in the bc_1 complex from beef heart mitochondria (Esposti *et al.*, 1983; Lorusso *et al.*, 1983; Nalecz *et al.*, 1983).

Recently, the site of DCCD binding to the cytochrome *bf* complex isolated from spinach chloroplasts was examined (Wang and Beattie, 1991). Radiolabeled DCCD was bound selectively in a time- and concentration-dependent manner to cytochrome b_6 of an enzymatically active *bf* complex (Fig. 4). Incubation with 50 nmol of DCCD/nmol of cytochrome b_6 resulted in approximately 65% of the total radioactive label on cytochrome b_6 with a lower degree of labeling of cytochrome *f*, the iron-sulfur protein, and the 17-kDA polypeptide. These results suggest

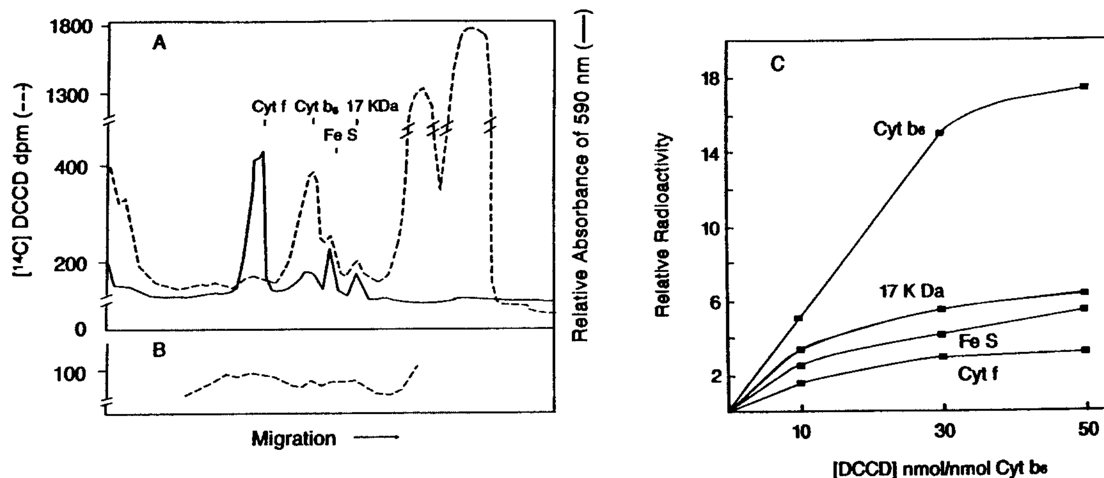

Fig. 4. Labeling of subunits of purified cytochrome *bf* complex by [^{14}C]DCCD. (A) SDS-PAGE-labeled cytochrome *bf* complex in a gel of 15% acrylamide. Densitometric pattern of the Coolmassie blue-stained bands (———). The radioactivity present in 2-mm gel slices (----). (B) Radioactivity in gel slices after treatment of the *bf* complex with 10 nmol DCCD/nmol cytochrome b_6, performed as in (A). (C) The autoradiogram of gels obtained after incubation of the *bf* complex with three concentrations of DCCD was scanned by laser scanning densitometry. The Coomassie blue-stained gel prior to autoradiography was also scanned by laser scanning densitometry. The area under each curve of the autoradiogram was divided by the area under the peak of each subunit in the gel stained with Coomassie blue to give the relative intensity.

that cytochrome b_6 plays a similar role in proton translocation through the *bf* complex as does cytochrome *b* in the bc_1 complex.

SITE OF BINDING OF DCCD ON *b*-TYPE CYTOCHROMES

DCCD reacts with carboxyl groups in proteins to form an O-acylurea derivative which in hydrophobic regions of the protein may rearrange to form a stable *N*-acylurea derivative (Senior, 1983). In other proton-translocating proteins, DCCD has been shown to bind to an aspartate or glutamate in a hydrophobic region of the protein (Fillingame, 1980). An examination of the primary sequence of cytochrome *b* from yeast mitochondria deduced from the gene and analyzed by hydropathy plots revealed two aspartate residues in hydrophobic regions to which DCCD might covalently bind. Aspartate residues are present at positions 160 and 229 in hydrophobic alpha helices of yeast cytochrome b (Fig. 5). A comparison of the sequences of several *b*-type cytochromes revealed that the aspartate at 160 is not conserved in all species; however, a glutamate or an aspartate residue is present in the helix in proximity to position 160 in all of the species examined with the exception of *Rhodobacter capsulata* (Hauska *et al.*, 1988). By contrast, the aspartate at position 229 is present in cytochrome *b* from all species with the exception of cytochrome b_6 in chloroplasts. In the *bf* complex, an equivalent aspartate residue is present in another subunit of the complex with a molecular mass of 17 kDa and with considerable homology to the carboxyl region of the mitochondrial cytochrome *b*.

Our initial approach was to isolate cytochrome *b* from a bc_1 complex labeled with radioactive DCCD under conditions previously shown to label cytochrome *b* specifically and to inhibit proton translocation. The extremely hydrophobic nature of cytochrome *b* caused this protein to adhere tenaciously to polyacrylamide gels and thus prevented its efficient removal by electrophoretic elution in a number of solvents. Recently, we have successfully separated the subunits of the bc_1 complex, including cytochrome *b*, in high yields by using preparative gel electrophoresis. Cyanogen bromide cleavage of the labeled cytochrome *b* at tryptophan residues has yielded one labeled peptide with an apparent molecular weight of 2300 kDa. Examination of the primary structure of cytochrome *b* suggested that cleavage of the tryptophans at positions 143 and 164 would result in a single peptide with that apparent molecular weight. The remaining peptides obtained by cleavage at tryptophan residues are predicted to have molecular weights greater than 3100, with most of the peptides larger than 9000. The 2300 molecular weight peptide containing radioactive DCCD after the cleavage with

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                                    160                     229
Y    ...L F S A I P F V G N D I V S W L W.....F K D L V T...
B    ...L L S A I P Y I G T D L V Q W I W.....I K D I L G...
H    ...L L S A I P Y I G T D L V Q W I W.....I K D A L G...
M    ...L L S A I P Y I G T T L V E W I W.....I K D I L G...
A    ...L M S A I P W I G Q D I V E F I W.....F K D L I T...
Sp   ...V P D A I P V I G S P L V E L L R.....P N D L L Y...
Rc   ...L F G A I P G I G P S I Q A W L L.....I K D L F A...
Rs   ...L F G A I P G I G H S I Q T E L L.....I K D V F A...
P    ...G L F A I P G V D E A I Q T W L L.....I K D L F A...
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Fig. 5. Predicted amino acid sequences for the regions of cytochrome *b* with aspartate or glutamate residues in hydrophobic regions of the protein. For ease of comparison, the numbering system for cytochrome *b* from yeast was used. Y, yeast; B, bovine; H, human; M, mouse, A, *Aspergillus nidulans*; Sp, spinach; Rc, *Rhodobacter capsulatus*; Rs, *Rhodobacter sphaeroides*; P, *Paracoccus denitrificans.*

cyanogen bromide contains aspartate 160 initially predicted as a probable site of DCCD binding. Currently, attempts are under way to sequence this hydrophobic peptide.

To establish the site of binding of DCCD on cytochrome b_6, the *bf* complex labeled with [^{14}C]DCCD was selectively digested with chymotrypsin and trypsin (Wang and Beattie, 1992). A 17-kDA fragment containing radioactive DCCD and the heme moiety was obtained after chymotrypsin digestion, while a 12.5-kDa fragment containing both radioactive DCCD and the heme moiety was obtained after trypsin digestion, suggesting that the site of DCCD binding might be on aspartate-140, aspartate-155, or glutamate-166. Extensive trypsin digestion of cytochrome b_6 containing [^{14}C]DCCD yielded two radioactive peptides with molecular masses of 6.0 and 6.5 kDA which were sequenced. A comparison of the sequence obtained from the two peptides to the gene sequence suggested that DCCD binds to either residue 155 or 166 on cytochrome b_6. Unfortunately, the conditions required for hydrolysis and sequencing of the peptide resulted in the loss of radioactive DCCD from the peptide so that the exact amino acid residue binding DCCD could not be determined.

The initial projections of the secondary structure of the *b*-type cytochromes predicted nine membrane-spanning helices for cytochrome *b* and five for cytochrome b_6 (Widger *et al.* 1984; Saraste, 1984). In these projections, aspartate 160 of yeast cytochrome *b* and both aspartate 155 and glutamate 166 of cytochrome b_6 are localized in a membrane-spanning helix. As discussed above, the initial topographical model for cytochrome *b* was revised after analysis of hydrophobic moments according to Eisenberg *et al.* (1982) and the sequencing of antibiotic-resistant mutants (Brasseur, 1988). The resulting model predicts eight membrane-spanning helixes for cytochrome *b* and four for cytochrome b_6 with the original amphipathic helix IV, currently called helix cd, removed from the membrane into a superficial alignment close to the surface of the membrane (Fig. 6). This projection places aspartate-160 of yeast cytochrome *b* and both aspartate-155 and glutamate-166 of spinach cytochrome b_6 in this extramembranous, yet hydrophobic, helix of these proteins and as such has implications for the pathway of protons from the site of quinol oxidation localized within the membrane either to the bulk phase outside the membrane or to a localized proton gradient.

BINDING OF FLUORESCENT DERIVATIVE OF DCCD TO CYTOCHROMES *b* AND b_6

The effects of *N*-cyclo-*N*-(4-dimethylamino-naphthyl)carbodiimide (NCD-4), a fluorescent analogue of DCCD on proton translocation in the bc_1 and *bf* complexes, were studied in order to characterize the environment surrounding the binding site of DCCD on both cytochrome *b* and cytochrome b_6. NCD-4 has been reported to inhibit the binding of Ca^{2+} to the Ca^{2+}-ATPase (Chadwick and Thomas, 1983, 1984; Munkonge *et al.*, 1989) and proton translocation by the F_1/F_0H^+-ATPase (Pringle and Taber, 1985) while binding to the same site on the protein as does DCCD (Pick and Weiss, 1985).

After incubation of the *bf* complex isolated from spinach chloroplasts with NCD-4, a fluorescent compound was formed with a 331 nm excitation peak and a 440 nm emission peak (Wang and Beattie, 1993a). It should be noted that solutions of NCD-4 in water or organic solvents do not possess any intrinsic fluorescence. The emission spectrum at 440 nm is consistent with the formation of an *N*-acylurea derivative of NCD-4 coupled to a carboxyl group (Chadwick and Thomas, 1983) and suggests that NCD-4 forms such a derivative with an acidic amino acid residue in a hydrophobic environment of the *bf* complex.

Analysis of the NCD-4 labeled cytochrome *bf* complex by SDS-polyacrylamide gel electrophoresis revealed that the flourescent label was mainly associated with cytochrome b_6. NCD-4 also inhibited the

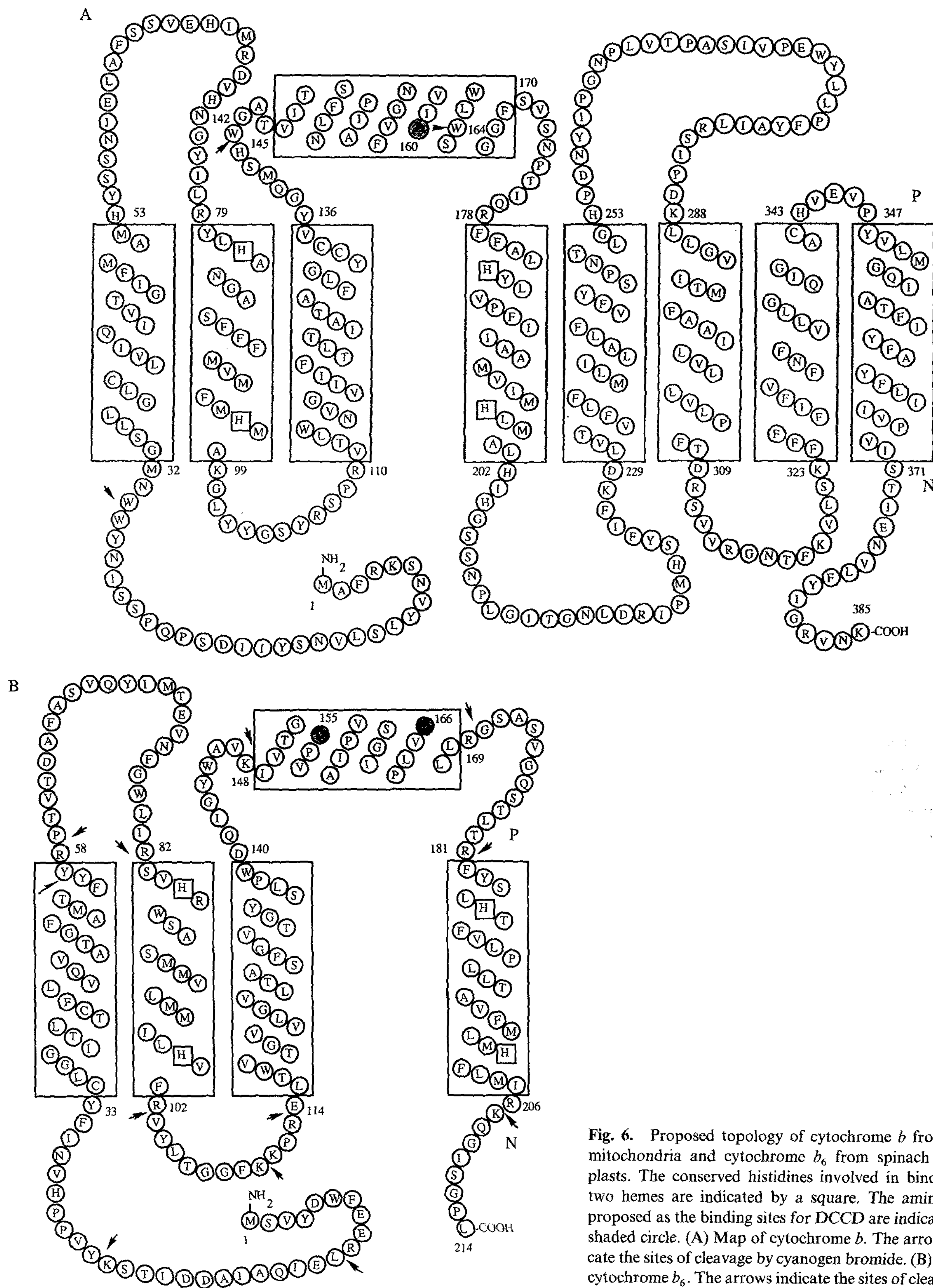

Fig. 6. Proposed topology of cytochrome *b* from yeast mitochondria and cytochrome b_6 from spinach chloroplasts. The conserved histidines involved in binding the two hemes are indicated by a square. The amino acids proposed as the binding sites for DCCD are indicated in a shaded circle. (A) Map of cytochrome *b*. The arrows indicate the sites of cleavage by cyanogen bromide. (B) Map of cytochrome b_6. The arrows indicate the sites of cleavage by trypsin and chymotrypsin.

proton translocating activity of the *bf* complex reconstituted into proteoliposomes in a time and concentration-dependent manner without significant inhibitory effects on electron transfer. These results suggest that NCD-4 binds to the same site, either aspartate-155 or glutamate-166, as DCCD on cytochrome b_6 with identical effects on the enzymatic and proton-translocating activities of the complex.

In similar studies (Wang and Beattie, 1993b), the effects of NCD-4 on the cytochrome bc_1 complex isolated from yeast mitochondria were examined. Incubation of the bc_1 complex with NCD-4 resulted in a maximal emission of 410 nm characteristic of an *N*-acylurea derivative in an even more hydrophobic environment than that suggested for the binding site of NCD-4 on cytochrome b_6. The fluorescent NCD-4 was observed to bind specifically to cytochrome *b* in the bc_1 complex while inhibiting proton translocation in the lipsome-associated complex, indicating that the effects on NCD-4 on the bc_1 complex were identical to those reported for DCCD.

Information on the localization of the binding site of NCD-4, the fluorescent derivative of DCCD, relative to the surface of the membrane was obtained by performing paramagnetic fluorescence quenching experiments with appropriate spin label quenchers (London and Feigensen, 1981; Blatt *et al.*, 1984). In order to establish the localization of helix cd, the proposed binding site for DCCD on the *b* cytochromes, relative to the membrane, we employed polar spin label quenchers such as CAT-1 and D-569, nonpolar and lipid spin label probes including several doxyl derivatives of stearic acid such as 5-DSA, 7-DSA, and 12-DSA, and a cationic amphiphilic spin label, CAT-16, which is proposed to partition so that the polar group and the spin label are at the membrane surface. In Fig. 7 the quenching effects of the various spin labels on the fluorescence of the NCD-4 treated bc_1 and *bf* complexes are plotted according to the Stern–Volmer equation where $I_o/I - 1$ is plotted against [Q] (Lacowicz, 1983).

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

The observed order of quenching efficiency was CAT-16 > 5-DSA > 7-DSA > 12-DSA. The polar spin labels CAT-1 and D-569 did not quench the NCD-4 bound to either the bc_1 or *bf* complexes.

The results of the spin-label quenching experiments suggest that the binding site for NCD-4 on helix cd of both cytochrome *b* and cytochrome b_6 is localized near the surface of the membrane but shielded from the external medium. These conclusions are based on the observation that the fluorescence of NCD-4 was not quenched by the polar probes, indicating that helix cd does not form a loop external to the membrane where the NCD-4 binding site would be exposed to the medium. The amphiphilic probe proposed to intercalate with its polar groups at the surface of the membrane but with its fatty acid chain within the membrane produced the most significant quenching, suggesting that the amino acid binding NCD-4 may be localized near this site; however, the quenching observed with the stearic acid derivatives, especially 5-DSA, indicate that helix cd may be localized partially within the membrane based on previous reports that 5-DSA partitions into the membrane such that the doxyl group is 6.5 Å from the surface of the membrane (Mitra and Hammes, 1990). The data also indicate that the lipid spin label probes, 5-DSA and 7-DSA, were more effective in quenching NCD-4 bound to cytochrome *b* than to cytochrome b_6 (Fig. 7), suggesting that aspartate 160 of cytochrome *b* is positioned at a greater depth in the membrane than either aspartate 155 or glutamate 166 of cytochrome b_6.

Examination of helix cd, the proposed binding site of DCCD and NCD-4 in both cytochrome *b* and cytochrome b_6, in an Edmundson wheel projection provides further support for the suggestion that this helix is associated with the membrane. A comparison of the helical wheel projections for helix cd of six cytochromes *b* indicates that all of the hydrophobic amino acid residues are localized on one side of the membrane, while the hydrophilic amino acid residues are localized on the other side of the helix (Fig. 8). The large hydrophobic surface of this helix with its many conserved amino acids may associate with the hydrophobic regions of the membrane such that the hydrophilic patch with the four polar amino acids is intercalated with the hydrophilic surface of the membrane. The aspartate or glutamate proposed as a binding site for DCCD is localized within or close to the hydrophilic patch on the helical wheel representing helix cd. The proposed alignment of the hydrophilic patch toward the surface of the membrane would place the binding site for NCD-4 on the spinach cytochrome b_6 closer to the surface than that on yeast cytochrome *b* where aspartate 160 is not localized in the hydrophilic patch.

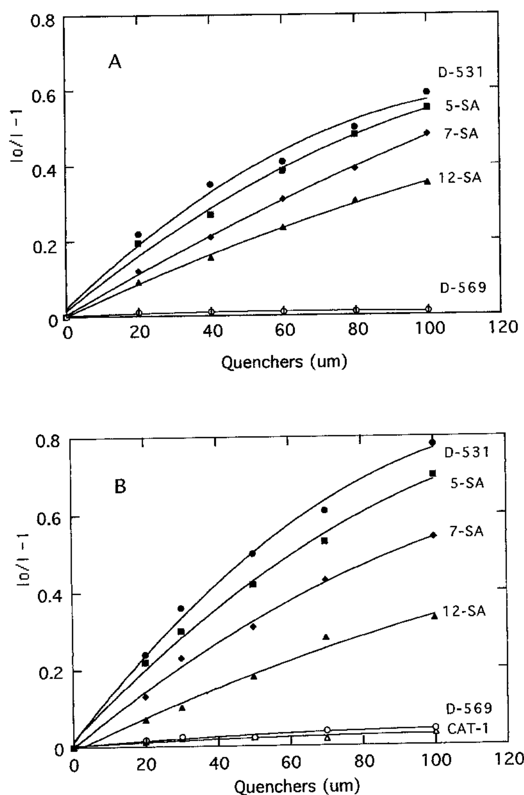

Fig. 7. Fluorescence quenching of NCD-4-labeled cytochrome bc_1 (A) and cytochrome *bf* (B) complexes by spin labels. The quenchers identified on the right side of each figure were added to the assay cuvettes containing the bc_1 or *bf* complexes reconstituted into proteoliposomes and the fluorescence determined and compared to that obtained without the spin quencher.

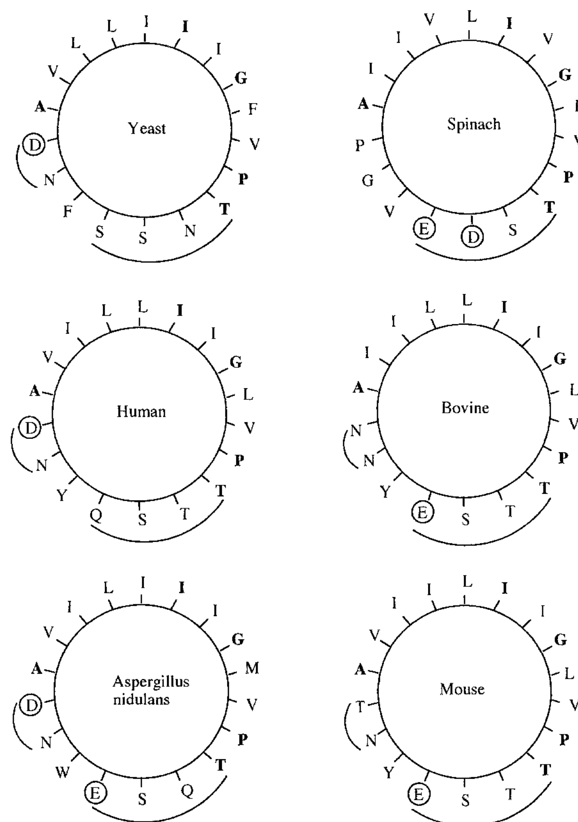

Fig. 8. Helical wheel diagrams for helix *cd* of cytochrome *b* from six species. The conserved amino acids are indicated in bold lettering and the hydrophilic amino acids are outlined by a curved line. The suggested binding sites for DCCD are indicated by a circle.

CONCLUSIONS AND FUTURE DIRECTIONS

The carboxyl-modifying reagent, DCCD, has provided interesting and valuable information about the mechanism of proton translocation through the cytochrome *bc* complexes. The effects of DCCD in diverse systems from the isolated bc_1 and *bf* complexes reconstituted into liposomes to isolated rat liver mitochondia and intact chloroplasts demonstrate that proton movements can be uncoupled from electron transfer through this region of the electron transfer chain. Moreover, helix cd, an amphiphilic and non-membrane-spanning helix, has been implicated in the pathway of proton movements as DCCD is covalently bound to an aspartate or glutamate residue localized in this helix.

A closer examination of this alpha helix may help in our understanding of the putative role of this acidic residue in proton translocation and, in addition, may partially explain some of the apparent discrepancies. Several hydrophobic and hydrophilic amino acids of helix cd are conserved in cytochrome *b* of all species, while many conservative substitutions as valine, isoleucine, or leucine are observed. As discussed in two of the other articles in this issue (Gennis *et al.*, 1993; Colson, 1993), several mutations conferring resistance to center O inhibitors result from changes in amino acids in helix cd. Moreover, mutations leading to decreased enzymatic activity have resulted from changes in the amino acids in the region of the protein connecting helixes C and cd as well as in helix cd itself. These results suggest that the Q_o binding site, where the oxidation of the quinol occurs, consists of amino acids both in the membrane-spanning alpha helices and in the extramembranous regions of the protein including amphiphilic helix cd. 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.

This model may also be useful in our attempts to explain the observed uncoupling of proton pumping and electron flow in the *bc* complexes by DCCD. Perhaps, when the pathway for proton movements through the acidic residue in helix cd is blocked by the bulky DCCD group, the conformation of Q_o, the quinol oxidizing site, is distorted such that both electrons pass directly to the iron-sulfur protein without the return of one electron through cytochrome b_H to Q_i, the quinone reducing site on the other surface of the membrane. Conformational changes induced by DCCD treatment of either the isolated bc_1 complex or rat liver mitochondria have been implicated by the changes in the red shift observed after addition of the Q_i and Q_o inhibitors, antimycin or myxothiazol, to the reduced complex. The spectral shifts resulting from the binding of these specific inhibitors to cytochrome *b* indicate conformational changes in the vicinity of the heme. Hence, changes induced by DCCD in these spectral shifts provide further evidence for conformational perturbations.

Another serious problem with the proposed model is that DCCD does not inhibit proton pumping in all *bc* complexes especially the complexes isolated from bacteria such as *Paracoccus* and *Rhodobacter*. It is possible that in these organisms an analogous acidic amino acid residue which may facilitate proton movements is localized in an environment that does not allow the formation of a covalent bond with DCCD. Alternatively, acidic amino acid residues in other extramembranous regions of cytochrome *b* may play a similar role. For example, the PEWY region localized between residues 294–297 of cytochrome *b* has been implicated in the enzymatic activity of the *bc* complex from *Rhodobacter spheroides* (Crofts *et al.*, 1992).

Further studies using site-directed mutagenesis coupled with the analysis of enzymatic activity as well as structural determinations using fluorescent probes should help in elucidating further the mechanism of proton movements.

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