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Molecular Modeling Studies of the DCCD-Treated Cytochrome bc_1 Complex: Predicted Conformational Changes and Inhibition of Proton Translocation

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Dicyclohexylcarbodiimide (DCCD) binds covalently to an acidic amino acid located in the cd loop connecting membrane-spanning helices C and D of cytochrome *b* resulting in an inhibition of proton translocation in the cytochrome bc_1 complex with minimal effects on the steady state rate of electron transfer. Single turnover studies performed with the yeast cytochrome bc_1 complex indicated that the initial phase of cytochrome *b* reduction was inhibited 25–45% in the DCCD-treated cytochrome bc_1 complex, while the rate of cytochrome c_1 reduction was unaffected. Simulations by molecular modeling predict that binding of DCCD to glutamate 163 located in the cd2 loop of cytochrome *b* of chicken liver mitochondria results in major conformational changes in the protein. The conformation of the cd loop and the end of helix C appeared twisted with a concomitant rearrangement of the amino acid residues of both cd1 and cd2 loops. The predicted rearrangement of the amino acid residues of the cd loop results in disruptions of the hydrogen bonds predicted to form between amino acid residues of the cd and ef loops. Simultaneously, two new hydrogen bonds are predicted to form between glutamate 272 and two residues, aspartate 253 and tyrosine 272. Formation of these new hydrogen bonds would restrict the rotation and protonation of glutamate 272, which may be necessary for the release of the second electrogenic proton obtained during ubiquinol oxidation in the bc_1 complex.

KEY WORDS: bc_1 complex; cytochrome *b*; DCCD; mitochondria; proton pumping; molecular modeling $2Fe_2S$.

The cytochrome bc_1 complex is an integral multiprotein complex of the inner mitochondrial membrane, which catalyzes the transfer of electrons from ubiquinol to cytochrome *c* coupled to the translocation of protons across the membrane (Brandt and Trumpower, 1994; Trumpower and Gennis, 1994). 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. All of these bc complexes contain three similar polypeptides with redox centers including cytochrome *b*, a single polypeptide containing two *b*-type hemes (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 (Ohnishi *et al.*, 1989; Saraste, 1984; Schägger *et al.*, 1986). Cytochrome *b*, a hydrophobic protein with eight membrane-spanning helices (Beattie *et al.*, 1994; Yun *et al.*, 1991), forms the core of the bc_1 complex and plays a key role in the electron transfer and proton pumping activities of the complex (Brandt and Trumpower, 1994). The other two redox-proteins, cytochrome c_1 and the iron–sulfur protein, are anchored to the membrane by an alpha helix with the bulk of the protein protruding from the outer surface of the inner membrane.

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Key to abbreviations: cytochrome bc_1 complex, ubiquinol: cytochrome *c* oxidoreductase; DBH_2, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol; DCCD, dicyclohexylcarbodiimide; DM, dodecyl maltoside; EEDQ, N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline.

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Dicyclohexylcarbodiimide (DCCD), the well-known carboxyl-modifying reagent, has been widely used to study the mechanism of proton translocation in various enzyme complexes including the F_1F_0-ATPase where DCCD blocks proton movements by binding covalently to a glutamate or aspartate residue in the c-subunit of F_0 (Zhang and Fillingame, 1994). DCCD was shown to cause similar inhibitory effects on proton translocation in the bc_1 complex studied in intact rat liver (Clejan *et al.*, 1984b) and beef heart mitochondria (Degli-Esposti *et al.*, 1982; Lorusso *et al.*, 1983). In our laboratory, we noted that a low molar ratio of DCCD to the cytochrome bc_1 complex (50 nmol DCCD/nmol cytochrome *b*) caused an uncoupling of proton translocation and electron transfer in rat liver mitochondria (Clejan *et al.*, 1984b) and bc_1 complexes isolated from yeast (Clejan and Beattie, 1983) and *Rhodobacter sphaeroides* (Wang *et al.*, 1998) as well as in the b_6f complex isolated from spinach chloroplasts (Wang and Beattie, 1991). These low concentrations of DCCD blocked 50% the rate of proton movements and 10% the rate of electron transfer in the isolated bc_1 and bf complexes. Similar conclusions were reached in an in vivo study, using the algae *Chlorella sorkiniana* in which low concentrations of DCCD were reported to induce a partial decoupling of the protonmotive Q cycle (Joliot and Joliot, 1998).

To better understand the mechanism of DCCD inhibition of proton movements in the bc_1 complex, we investigated the site of DCCD binding to the bc_1 complex. Radioactive DCCD, at the low concentrations that uncoupled proton movements and electron transfer, was bound exclusively to cytochrome *b* in the isolated yeast bc_1 complex (Beattie *et al.*, 1984). Higher concentrations of DCCD resulted in the nonspecific labeling of additional subunits. Subsequent studies in our laboratory indicated that treating the isolated complex with low concentrations of DCCD resulted in the covalent and specific binding of the radioactive DCCD to Asp-160 of cytochrome *b* in the yeast bc_1 complex (Wang *et al.*, 1995), to either Asp-155 or Glu-166 of cytochrome b_6 in the chloroplast bf complex (Wang and Beattie, 1992), and to Asp-187 of the bc_1 complex from *R. sphaeroides* (Wang *et al.*, 1998). The location of these acidic amino acids in the cd2 loop connecting membrane-spanning helices C and D of cytochrome *b*, which face the positive side of the membrane, suggested a potential role for these amino acids as proton carriers.

A possible explanation for the decoupling of electron transport and proton translocation by these reagents is that the covalent binding of DCCD to an acidic amino acid blocks the proton channel from the Q_o site to the positive side of the membrane, thus forcing the proton to move along another pathway to the opposite side of the membrane (Brandt and Trumpower, 1994). A similar explanation was offered to explain the partial decoupling of proton pumping and electron transfer observed in the yeast bc_1 complex containing a spontaneous mutation in which the conserved glycine-137 of cytochrome *b* was mutated to glutamate (Bruel *et al.*, 1995).

The recent resolution of the crystal structure of the cytochrome bc_1 complex has raised questions about the previous suggestions that acidic amino acids localized in the cd2 loop may play a direct role in proton movements (Iwata *et al.*, 1998; Xia *et al.*, 1997; Zhang *et al.*, 1998). The cd loop of cytochrome *b* is not located sufficiently close to the Q_o site or to a potential proton channel connected to that site to permit acidic amino acids to function as proton carriers (Iwata *et al.*, 1998). Moreover, recent experimental evidence has suggested that glutamate-272 located in the conserved PEWY region of the ef loop of cytochrome *b* may be directly involved in transfer of the second proton derived from ubiquinol oxidation (Crofts *et al.*, 1999a,b).

In this study, we have used molecular modeling to predict the conformational changes that might occur when DCCD is bound to cytochrome *b*, specifically to Glu-163 of the chicken bc_1 complex. The molecular modeling studies suggest that the binding of DCCD to cytochrome *b* results in a conformational change such that the ability of Glu-272 to function as a proton donor is severely restricted.

MATERIALS AND METHODS

Growth of Yeast Cells, Preparation of Mitochondria, Purification of bc_1 Complex, Enzyme Reactions, and Labeling of Cytochrome *b* With DCCD

Yeast cells were grown to midlogarithmic phase in a medium containing 1% yeast extract, 2% peptone, and 2% galactose ($A_{650nm} = 1.0$–1.4) and mitochondria prepared as described previously (Fu and Beattie, 1991). The cytochrome bc_1 complex was isolated from submitochondrial particles solubilized with dodecyl maltoside (DM) followed by chromatography on DEAE-Biogel A and Hi-Trap Q column using an FPLC (Ghosh *et al.*, 2001). The activity of the cytochrome bc_1 complex was determined by the reduction of 40 μM horse heart cytochrome *c* at 550 nm using the ubiquinol analog decylbenzoquinol (DBH_2) as electron donor and an extinction coefficient of 21.5 mM^{-1} cm^{-1} for cytochrome *c*.

Single turnover measurements were performed at room temperature by rapid scanning stopped flow spectroscopy, using an OLIS modernized Cary 17 spectrophotometer equipped with an OLIS stopped flow. As the dead time of the instrument was 5 ms, this time was chosen as time 0, after which point data were collected. Reactions were started by mixing purified cytochrome bc_1 complex (containing 2 μM of cytochrome *b* in a degassed 50 mM Tris-HCl buffer pH 7.5, containing 100 mM KCl, 80 mM sucrose, 0.2 mM EDTA, 1 μM antimycin) and 30 μM DBH_2 (Saribas *et al.*, 1999). The rate of cytochrome *b* and c_1 reduction was determined at 563 nm and 552 nm, respectively. For each experiment, at least six datasets were averaged, and the standard deviation was calculated.

To study the effects of DCCD, the bc_1 complex was incubated with DCCD in a ratio of 50 nmol DCCD/nmol cytochrome *b* in a buffer containing 5 mM K-Hepes, 100 mM KCl, pH7.5, at 12°C for 60 min. Excess DCCD was removed by filtration, using a Microcon Centrifuge filter YM-100 with three washes as described previously (Wang *et al.*, 1995). The control bc_1 complex was incubated with the same amount of ethanol under the same conditions.

Molecular Modeling

Molecular modeling was performed with an Octane Silicon Graphics workstation. The X-ray crystal structure for the native cytochrome bc_1 complex isolated from *Gallus gallus* (PDB 1BCC) was downloaded from the Brookhaven Protein Data Bank, as the structure for the yeast bc_1 complex was not available at the time of these studies. DCCD was assembled and linked to the glutamate-163 residue of cytochrome *b*, using the Biopolymer module from Insight II software from Molecular Simulations. With the exception of the Q_o site including residues 124–182 and 234–298 of cytochrome *b* and DCCD, the entire molecule was fixed before molecular minimization and molecular dynamics calculations were performed. The portion surrounding Glu-272 was soaked with a water sphere of 16 Å radius. Changes in the conformation of the cd loop and ef loop region were examined as follows: Minimization was performed using a constant valence force field (CVFF) and the method of steepest and conjugate descents with a nonbond cutoff of 12 Å until the gradient was less than 1 $kcal \cdot mol^{-1} \cdot Å^{-1}$. Molecular dynamics were examined for 80 ps at 300 K using 1 fs time steps with an equilibration period of 0.1 ps by continued interactions with the same constraints. Differences between the DCCD labeled and control conformations were examined by visual comparison (overlays) and distance measurements between reference atoms.

RESULTS

Initial Rates of Cytochrome *b* and c_1 Reduction

The previous conclusions that DCCD treatment results in a decoupling of electron transfer and proton pumping in the DCCD-treated bc_1 complex were based on steady-state measurements, which may be limited by interactions of the complex with exogenous or endogenous substrates. Hence, we determined the effects of DCCD on the initial rates of reduction of cytochromes *b* and c_1 using stopped flow measurements in which the reaction was started by the addition of the substrate, DBH_2. The reduction of cytochrome *b* followed the triphasic pattern previously observed in which three different rate constants can be determined, while the rate of cytochrome c_1 reduction was monophasic (Fig. 1). In the DCCD-treated complex,

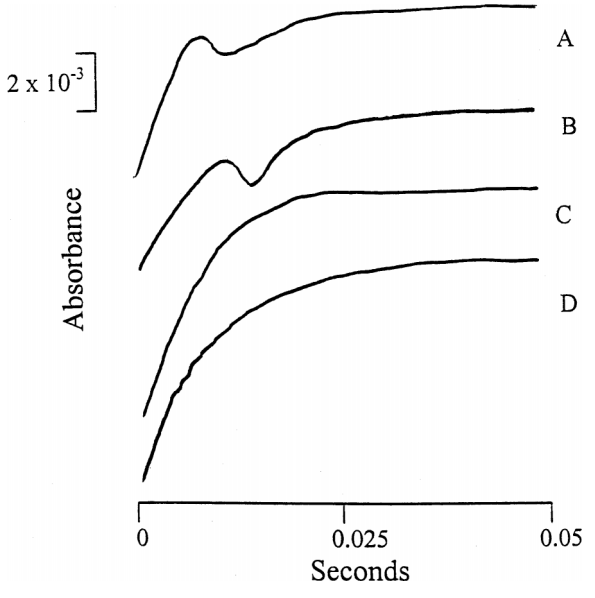

Fig. 1. Initial rates of reduction of cytochrome *b* and c_1. Single turnover measurements were performed at room temperature by rapid scanning stopped flow spectroscopy, using an OLIS modernized Cary 17 spectrophotometer equipped with an OLIS stopped flow. Reactions were started by mixing purified cytochrome bc_1 complex (containing 2 μM cytochrome *b* in 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 80 mM sucrose, 0.2 mM EDTA, 1 μM antimycin) and 30 μM DBH_2. The rate of cytochrome *b* and c_1 reduction was determined at 563 nm and 552 nm respectively. A. (untreated bc_1 complex) and B. (DCCD-treated bc_1 complex). Rate of reduction of cytochrome *b*. C. (untreated bc_1 complex) and D. (DCCD-treated bc_1 complex). Rate of reduction of cytochrome c_1.

Table I. Initial Rates of Cytochrome *b* and c_1 Reduction

	Cytochrome *b* reduction			
	Phase I	Phase II	Phase III	Cytochrome c_1 reduction
Untreated bc_1 complex	16.48 ± 2.90	-5.16 ± 1.72	4.56 ± 1.80	16.28 ± 3.39
DCCD treated bc_1 complex	10.29 ± 1.69^a	-4.87 ± 1.20	6.19 ± 1.35^b	16.42 ± 3.67

Note. The initial rate of cytochrome *b* reduction (nmol cytochrome *b* reduced min^{-1} μmol ctyochrome b^{-1}) was measured at 563 nm and the initial rate of cytochrome c_1 reduction (nmol cytochrome c_1 reduced $min^{-1}\mu$mol cytochrome b^{-1}) was measured at 552 nm in an OLIS-modernized Cary 17 equipped with an OLIS stopped flow as described in Materials and Methods. The rates of Phases I, II, and III of cytochrome *b* reduction were determined from the slopes of individual experiments (see Fig. 1). For each experiment, at least six datasets were averaged and the standard deviation was calculated.

[a] Difference is statistically significant.

[b] Difference is not statistically significant.

the initial rapid phase in which cytochrome *b* is partially reduced was inhibited 37%; however, the partial reoxidation phase and the slow rereduction phase of cytochrome *b* were not significantly affected in the DCCD-treated complex. Similarly, the initial rate of cytochrome c_1 reduction was unaffected by DCCD treatment of the bc_1 complex (Table I).

Molecular Modeling

In previous studies in our laboratory using carefully defined conditions, we established that DCCD binds specifically to Asp-160 of the yeast mitochondrial cytochrome bc_1 complex (Wang *et al.*, 1995) and to Asp-187 of the *R. sphaeroides* complex (Wang *et al.*, 1998). The conditions for specific labeling involved incubation of the bc_1 complex with 50 nmol DCCD/nmol of cytochrome *b* at 12°C (Beattie *et al.*, 1984). The specificity of these previous results indicated that molecular modeling studies might suggest an explanation for the uncoupling of proton movements and electron transfer observed under these experimental conditions. In the chicken cytochrome bc_1 complex used for the molecular modeling studies, DCCD is expected to bind to Glu-163 located in the cd2 loop of cytochrome *b*. This same residue was previously implicated in the binding of radioactive DCCD to cytochrome *b* in a bc_1 complex isolated from beef heart mitochondria (Beattie, 1993; Clejan *et al.*, 1984a). When the simulated molecular model of native cytochrome *b* is superimposed on the simulated model of cytochrome *b* with DCCD bound to Glu-163, a series of conformational changes in cytochrome *b* are observed. The backbone of the cd1 loop appears twisted resulting in changes in the arrangement of the amino acid side chains, while the cd2 helix is twisted and shortened (Fig. 2). Consequently, the cd1 loop and the top of membrane-spanning C helix are twisted resulting in a rotation of the backbone of the ef loop (Fig. 3). The movement of the cd1 loop, helix C, and the rotation of the ef loop results in a rearrangement of the positions of the side chains of the amino acids present in these regions of cytochrome *b*. Three of the five hydrogen bonds predicted between residues of the cd helices and the ef loop

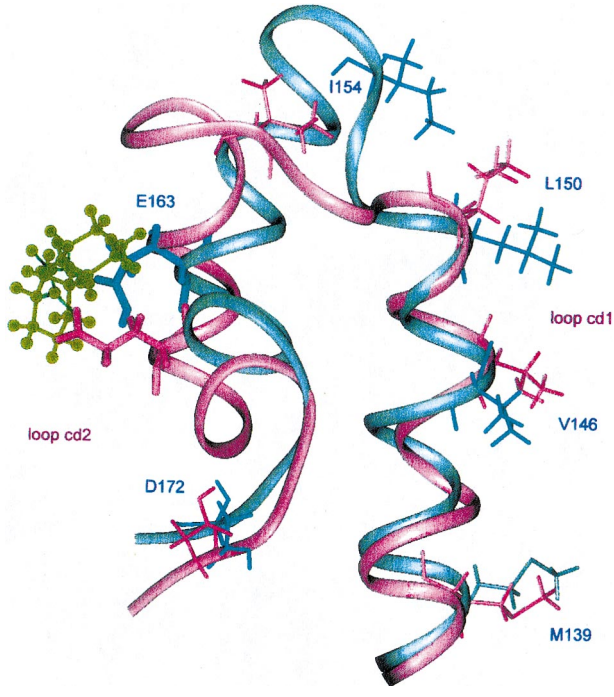

Fig. 2. Binding of DCCD to Glu-163 located in the cd loop connecting membrane-spanning helices C and D of cytochrome *b*. The blue ribbon diagram corresponds to the cd loop in the untreated bc_1 complex and the red ribbon diagram to the cd loop of the DCCD-treated bc_1 complex. The molecular simulation of DCCD is shown in green. Stick diagrams of the indicated amino acid side chains are presented to demonstrate the rearrangements of amino acid residues, which occur when DCCD is bound to cytochrome *b*. Blue indicates residues in the untreated bc_1 complex and red indicates residues in the DCCD-treated bc_1 complex.

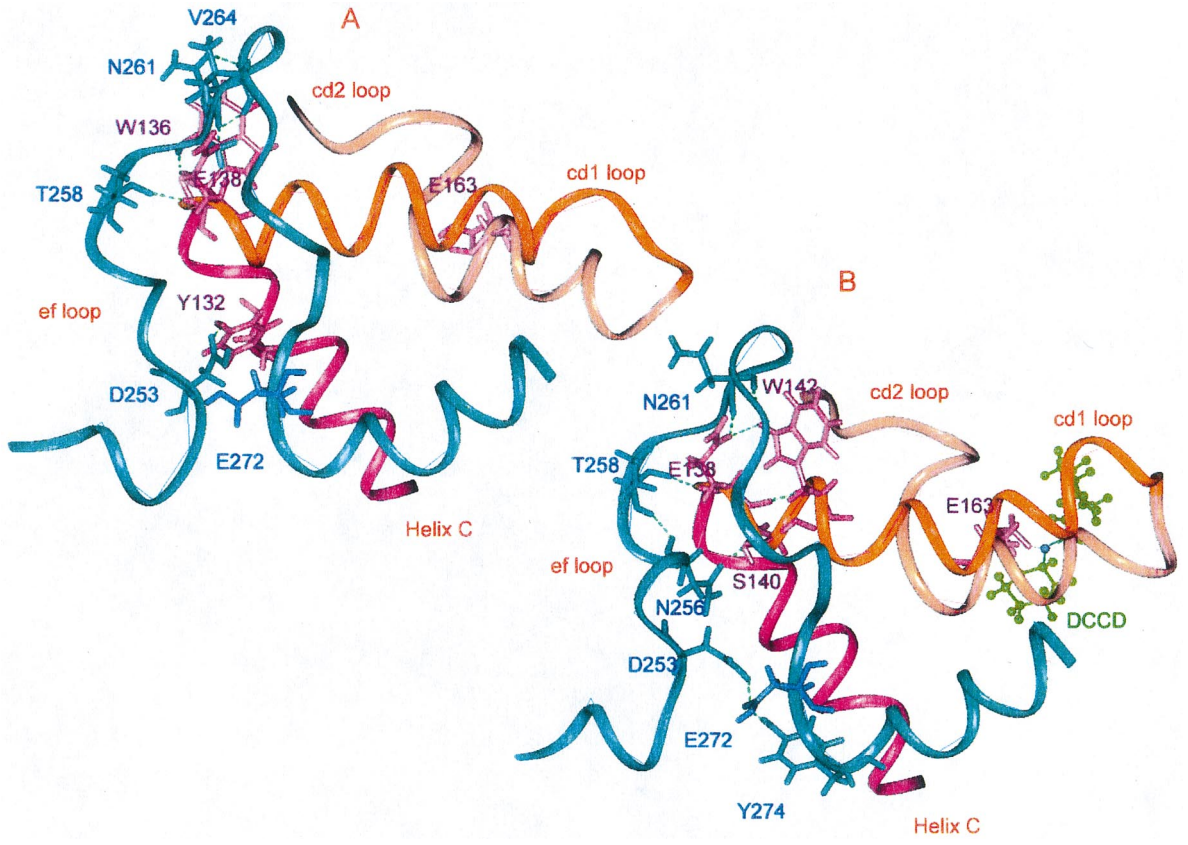

Fig. 3. Changes in the predicted hydrogen bonds between the cd and ef loops of cytochrome *b* upon binding of DCCD to Glu-163 of cytochrome *b*. The side chains of the highlighted amino acid residues are those predicted to form hydrogen bonds linking the cd and ef loops of cytochrome *b*. The cd1 loop is shown as yellow, the cd2 loop as beige, the ef loop as blue and the end of the membrane-spanning helix C as pink. A. Untreated bc_1 complex. B. DCCD-treated bc_1 complex with the simulated DCCD molecule shown in green.

in the native cytochrome *b*, Y-132:D-253, Q-138:V-264, and A-260:W-136, have been lost as a consequence of the conformational changes induced by DCCD binding to Glu-163 (Fig. 3 and Table II). Concomitantly, five new hydrogen bonds are predicted to form at S-140:N-256, W-142:N-256, D-253:E-272, and Y-274:E-272 (Fig. 3 and Table II).

Of considerable importance to proton translocation is the predicted formation of the two new predicted hydrogen bonds involving Glu-272, recently implicated as a proton donor during the oxidation of the semiquinone of ubiquinol at the Q_o site of cytochrome *b* (Crofts *et al.*, 1999a,b). The OE2 atom of the carboxylate group of Glu-272 is moved 3.04 Å, the atom HD2 of Asp-253 is

Table II. Hydrogen Bonds Predicted Between Amino Acid Residues in the ef and cd Loops of the DCCD Labeled and Native Cytochrome *b*

Native cytochrome *b*			DCCD labeled cytochrome *b*		
Electron Donor	Electron Acceptor	Distance (Å)	Electron Donor	Electron Acceptor	Distance (Å)
Q138:HN	T258:O	2.43	Q138:HN	T258:O	2.43
N261:HN	Q138:OE1	1.96	N261:HN	Q138:OE1	1.96
Y132:HH	D253:OD1	2.13	S140:HN	N256:OD1	2.09
Q138:HE22	V264:O	2.23	W142:HE1	N261:O	2.29
A260:HN	W136:O	2.39	D253:HD2	E272:OE2	2.25
			Y274:HH	E272:OE1	1.77

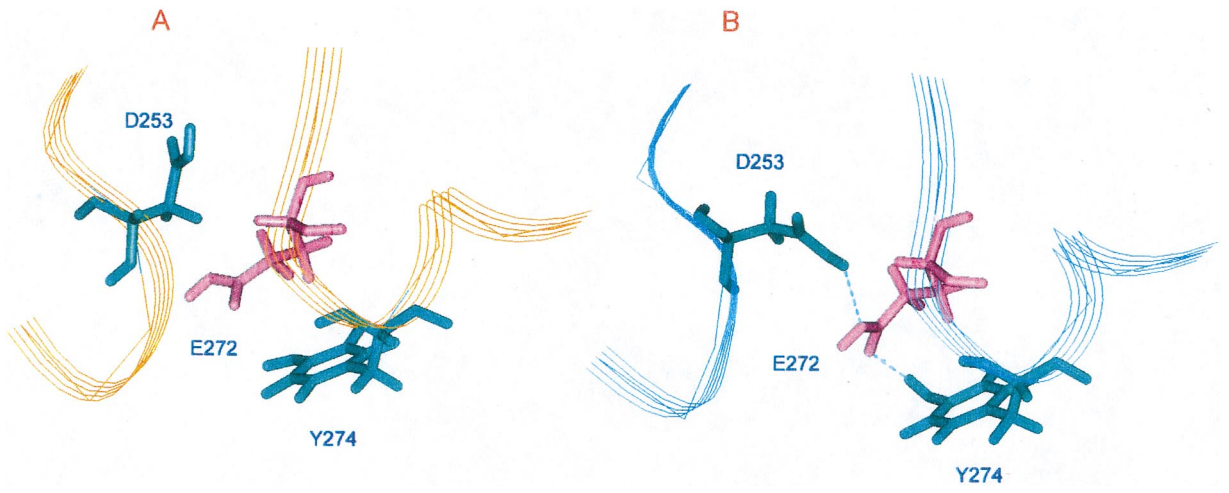

Fig. 4. The microenvironment surrounding Glu-272 of cytochrome *b*. A. Ribbon diagram of the ef loop (in yellow) in the native bc_1 complex. B. Ribbon diagram of the ef loop (in blue) in the bc_1 complex treated with DCCD. Two hydrogen bonds are predicted to from between Glu-272 (shown in red) with Asp-253 and Tyr-274 in the DCCD-treated bc_1 complex.

moved 3.41 Å toward Glu-272, which results in the predicted formation of a hydrogen bond between Glu-272 and Asp-253. Simultaneously, the HH atom of Tyr-274 is moved 1.05 Å toward Glu-272 resulting in the predicted formation of a hydrogen bond between Glu-272 and Tyr-274 (Fig. 4 and Table II). It should be noted that no hydrogen bonds are predicted for Glu-272 in the wild type enzyme. The predicted hydrogen bonds involving Glu-272 in the DCCD-treated bc_1 complex may impede the ability of Glu-272 to accept a proton and to rotate as predicted and thus act as a proton carrier (Fig. 4).

DISCUSSION

The results of this study provide an explanation for the observation that the binding of DCCD to acidic amino acids in cytochrome *b* results in a decoupling of steady state electron transfer reactions and proton pumping in the cytochrome bc_1 complexes of mitochondria and bacteria and the cytochrome *bf* complex of chloroplasts (Beattie, 1993; Brandt and Trumpower, 1994). According to the modified Q cycle, the binding of ubiquinol to the Q_o site of cytochrome *b* results in the release of a proton through stabilization of the quinol anion by the protein. Transfer of the first proton occurs through H^+ transfer from quinol to the iron–sulfur protein, followed by movement of the extrinsic domain of the iron–sulfur protein from its binding site on cytochrome *b* to the binding site on cytochrome c_1 where the proton is released. The second proton of ubiquinol is released after proton transfer from the semiquinone to Glu-272 to form the neutral acid side chain, which subsequently rotates with transfer of the proton to the exterior. This model is based on the different positions of the inhibitors that are structurally similar to ubiquinol, stigmatellin (distal), and myxothiazol (proximal), observed in the crystal structure of the bc_1 complex. In the structure observed with stigmatellin in the distal site, the side chain of Glu-272 is rotated out of the Q_o site and acts to constrict the proximal site. Rotation of the side chain of Glu-272 must occur for the semiquinone to move from the distal to the proximal domain before oxidation (Crofts *et al.*, 1999a,b). In addition, Crofts *et al.* (1999a,b) have suggested that protonation and the subsequent deprotonation of the side chain of Glu-272 must occur to form the semiquinone anion, which would move into the proximal domain and donate an electron to heme b_L. Moreover, the suggested rotation of the Glu-272 side chain would result in positioning its polar carboxyl group into a channel present in cytochrome *b* leading to the aqueous phase. This putative channel may form a water chain to facilitate transfer of the proton from Glu-272 to the exterior.

The molecular modeling studies suggest that the hydrogen bonds predicted to form between Glu-272 and two other amino acids, Asp-253 and Tyr-274, when DCCD binds to Glu-163, would restrict the movement of the side chain of Glu-272. The restricted movement would thus interfere with the protonation and rotation of Glu-272, which are necessary for the subsequent transfer of the second proton produced during ubiquinol oxidation. This suggestion provides an explanation for the observation that DCCD blocked by 50% proton pumping in the bc_1 complexes of beef heart (Degli-Esposti *et al.*, 1982; Lorusso *et al.*,

1983), rat liver (Clejan *et al.*, 1984b), and yeast (Clejan and Beattie, 1983) mitochondria as well as the bc complex of *R. sphaeroides* (Wang *et al.*, 1998) and the bf complexes of spinach (Wang and Beattie, 1991) and algae (Joliot and Joliot, 1998) chloroplasts. The conformational changes predicted to occur in the cd and ef loops of cytochrome *b* when DCCD is bound would prevent the involvement of Glu-272 in proton transfer, but would not affect the release of the first proton concomitant with reduction of the iron–sulfur protein.

The observed decrease in the initial rate of cytochrome *b* reduction in the DCCD-treated bc_1 complex may also be explained by this mechanism. The additional hydrogen bonds formed with Glu-272 may impede the initial rapid rate of electron transfer but not the steady state rate as reflected in the rates of cytochrome *c* reduction in the DCCD-treated mitochondria or isolate complexes. The additional hydrogen bonds to Glu-272, however, appear to block completely the proton channel to the exterior of the inner membrane such that the second proton may be released to the inside of the membrane for electron transfer to continue.

Recently, our previous conclusions obtained with DCCD were criticized with the report that DCCD inhibited equally both electrogenesis and electron transfer in chromatophores isolated from *R. sphaeroides* (Shinkarev *et al.*, 2000). In these experiments, very high concentrations of DCCD were incubated with intact chromatophores at room temperature. In our laboratory, low concentrations of DCCD (50 nmol/nmol cytochrome *b*), previously shown to cause maximum inhibition of proton pumping and labeling of cytochrome *b*, were incubated at 8–10°C with isolated bc_1 complexes reconstituted into proteoliposomes, which were shown earlier to be impermeable to protons (Beattie and Villalobo, 1982). Under these conditions, only cytochrome *b* was labeled with radioactive DCCD (Beattie *et al.*, 1984) and only one amino acid residue of cytochrome *b* contained significant radioactivity (Wang *et al.*, 1995). The use of higher concentrations of DCCD could have resulted in nonspecific effects including binding of DCCD to more than one acidic amino acid residue present in cytochrome *b* or to acidic amino acids present in other subunits of the bc_1 complex.

The molecular modeling studies presented in this paper provide an explanation for the observed effects of DCCD on the electron transfer and proton pumping activities of the cytochrome bc_1 complex. The changes in the conformation of cytochrome *b* predicted by molecular modeling to occur when DCCD binds to Glu-163 of the protein cannot be proven absolutely; however, the effects of these predicted changes on the steady state rates of proton pumping and electron transfer as well as on the initial rates cytochrome *b* reduction are consistent with the conformational changes predicted in the molecular modeling studies.

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