Membrane Potential-linked Reversed Electron Transfer in the Beef Heart Cytochrome bc₁ Complex Reconstituted into Potassium-loaded Phospholipid Vesicles

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Toshiaki Miki*, Machi Miki†, and Yutaka Orii‡

From the *Department of Public Health, Faculty of Medicine, Kyoto University, Kyoto 606 and the †Department of Hygiene, Faculty of Medicine, Kinki University, Osaka 589, Japan

The cytochrome bc₁ complex purified from beef heart mitochondria was incorporated into potassium (K⁺)-loaded phospholipid vesicles by a cholate dialysis method to study the reverse reaction of electron transfer in the complex. The reduction of cytochrome b in the presence of sodium ascorbate was observed on addition of valinomycin to the K⁺-loaded proteoliposomes in a medium containing no external KCl; it was followed by the gradual oxidation. Nigericin accelerated the reoxidation of reduced cytochrome b, indicating that a K⁺ diffusion potential (negative inside) induced the reduction of cytochrome b. The extent of the cytochrome b reduction depended on the magnitude of the diffusion potential across the liposomal membranes, and its maximal reduction was attained at more than 210 mV of the diffusion potential. It was cytochrome b₅₆₂ that was reduced during the establishment of the K⁺ diffusion potential in the presence of ascorbate, and about 90% of cytochrome b₅₆₂ was estimated to be reduced. Antimycin A and myxothiazol inhibited the diffusion potential-induced reduction of cytochrome b₅₆₂, and ubiquinone was proved to be essential for the reversed electron transfer. The K⁺ diffusion potential also induced the partial reduction of cytochrome b₅₆₆ when cytochrome b₅₆₂ had previously been reduced with ascorbate plus tetramethyl-p-phenylenediamine. These results were interpreted well based on the Q cycle scheme which assumed the energy-dependent reduction of ubiquinone at center o. Dicyclohexylcarbodiimide, which did not perturb the ability of proteoliposomes to generate the K⁺ diffusion potential, inhibited the energy-dependent reduction of cytochrome b₅₆₂ without a significant loss in the catalytic activity of the complex. The half-inhibition was brought about by 200 mol of dicyclohexylcarbodiimide/mol of cytochrome c₁. These results strongly suggest the coupling of a proton flow with the reversed electron transfer in the bc₁ complex.

The cytochrome bc₁ complex catalyzing the electron transfer from ubiquinol to a c-type cytochrome plays a central role in the process of oxidative phosphorylation taking part in mitochondria, chloroplasts, or other analogous redox systems. The electron transfer mechanism of the bc₁ complex is described consistently by a Q cycle scheme originally proposed by Mitchell (1) and subsequently refined by Trumpower (2). In the protonotive Q cycle (2), ubiquinol is oxidized at center o. A first electron from ubiquinol is transferred to iron-sulfur protein, which then reduces cytochrome c₁. The second electron transferred from ubisemiquinone to cytochrome b₅₆₂ recycles through the bc₁ complex; reduced cytochrome b₅₆₂ gives an electron to cytochrome b₅₆₂, which then reduces ubiquinone at center i.

In contrast to the electron transfer pathway in the bc₁ complex, the mechanism by which the electron transfer gives rise to a transmembrane proton electrochemical potential is not clear. According to the original Q cycle (1), it is the vectorial transmembrane electron transfer from cytochrome b₅₆₂ to cytochrome b₅₆₂ that generates a membrane potential. However, later studies have shown that electron transfer between the two b hemes can account for only approximately 40% of the membrane potential generated by the bc₁ complex (3-5). Therefore, the mechanism generating the remaining 60% of the membrane potential has been expected to be elucidated. Robertson and Dutton (6) indicated that this portion of the membrane potential was associated with the electron transfer from cytochrome b₅₆₂ to ubiquinone at center i in the bc₁ complex of the photosynthetic bacterium Rhodobacter sphaeroides. On the other hand, recent experiments by Drachev et al. (7) using the bc₁ complex of R. sphaeroides chromatophores showed that the electrogenic step was the transfer of protons associated with the oxidation and reduction of ubiquinone at both center o and i rather than the vectorial transfer of electrons. Konstantinov (8) also postulated the vectorial movement of protons for the electrogenic mechanism in the mitochondrial bc₁ complex.

The process of energy transduction in the bc₁ complex has also been investigated by measurement of the back reaction; the electron transfer from a component with a more negative redox potential to a component with a more positive potential driven by an electrochemical potential gradient of protons. The reversed electron transfer in the bc₁ complex has been studied with mitochondria or submitochondrial particles energized with ATP, and two kinds of redox response of cytochrome b to addition of ATP have been reported, the reduction of both cytochrome b components (b₅₆₂ and b₅₆₆) in the absence of antimycin A (9-11), and the oxidation of a reduced cytochrome b₅₆₂ in the presence of antimycin A (3, 12, 13). But few systematic studies on the reverse reaction of electron transfer in the bc₁ complex have been carried out by using the purified enzyme reconstituted into phospholipid vesicles.

Here, we report that the K⁺ diffusion potential induces the reduction of cytochrome b components in the bc₁ complex reconstituted into phospholipid vesicles in the absence of anti-
depleted enzyme was incorporated into liposomes in the same way after the reactivation of the enzyme. DCCD treatment of cytochrome bc1 vesicles was carried out as follows. A portion of the proteoliposomes was mixed with an appropriate amount of DCCD in ethanol, and the mixture was incubated for 16–20 h at 0 °C. Control vesicles were treated in the same way except that ethanol was added in place of the DCCD solution. The final concentration of ethanol was always 1%.

Spectroscopic measurements were carried out with a Unisoku multilength spectrophotometer equipped with a thermostatted cell holder. The catalytic activity of the reconstituted bc1 complex was assayed at 20 °C in a medium containing 10 mM MOPS/NaOH (pH 7.4), 100 mM KCl, 50 μmol cytochrome c, and 25 μmol quinone. The initial rate of cytochrome c reduction was corrected against non-enzymatic reduction of cytochrome c by ubiquinol in the absence of bc1 vesicles. Proteoliposomes reconstituted with the bc1 complex exhibited the oxidation control ratio (18) ranging from 7 to 9, which was determined as the ratio of the enzyme activity in the presence and absence of 0.2 μmol of valinomycin plus 2 μg of nigericin. The ability of K+-loaded proteoliposomes containing the bc1 complex to form a K+ diffusion potential was checked spectrophotometrically using safranine as an optical probe for the membrane potential (21) as follows. A 5-μl portion of proteoliposomes containing the bc1 complex was added to 1 ml of a medium containing 4.8 μmol safranine, 10 mM MOPS/NaOH (pH 7.4), 100 mM NaCl. The reaction was initiated by adding valinomycin, and the absorbance change of safranine was followed at 525 nm (22) or at 530–578 nm (21). A ubiquinone-10 content of the purified bc1 complex was estimated according to the reported method (23). The concentration of ubiquinone was determined spectrophotometrically using an extinction coefficient of 12.25 mM−1 cm−1 for the difference between the oxidized and reduced quinone at 275 nm (24). Other extinction coefficients used were Δε650 nm (reduced-oxidized) = 19.2 mM−1 cm−1 for cytochrome c (14) and Δε665–639 nm (reduced-oxidized) = 17.5 mM−1 cm−1 for cytochrome c1 (14). Protein was estimated according to Lowry et al. (25).

RESULTS

Diffusion Potential-Induced Reduction of Cytochrome b in the bc1 Complex Reconstituted into K+-loaded Liposomes—Valinomycin induces a vectorial flow of potassium ions (K+) from inside to outside K+-loaded vesicles, resulting in the formation of a potassium diffusion potential (negative inside) across the liposomal membranes. Fig. 1 shows the response of the reduction level of cytochrome b in the reconstituted bc1 complex to the valinomycin-induced K+-diffusion potential. In these experiments, cytochrome c1 and iron-sulfur protein in the bc1 complex were reduced first with 5 mM sodium ascorbate in a medium containing no external KCl (Fig. 1A). As shown in
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Fig. 2. Dependence of cytochrome b reduction on the magnitude of the membrane potential. A 50-μl aliquot of bc\textsubscript{1} vesicles containing 4.0 μM cytochrome b was suspended in 0.95 ml of 10 mM MOPS/NaOH (pH 7.4) containing various concentrations of KCl and NaCl. Total concentration of NaCl plus KCl was always 100 mM. Diffusion potential values (Δψ) were calculated from the Nernst equation assuming the internal KCl concentration of 100 mM. The reduction of cytochrome b was initiated by adding 0.4 μg of valinomycin in the presence of 5 mM ascorbate. Other conditions were as in Fig. 1.

Fig. 1B, a rapid increase in absorbance at 562 nm relative to 575 nm, the wavelength pair selected to monitor the redox state of cytochrome b components, was observed upon addition of valinomycin. The maximum reduction level was achieved 3 min after addition of valinomycin, followed by its gradual decrease. Nigericin, which collapsed the K\textsuperscript{+} gradient, accelerated the rapid reduction of cytochrome bcl vesicles suspended in a medium containing 100 mM KCl (Fig. 1D). These results clearly indicate that the valinomycin-mediated K\textsuperscript{+} diffusion potential induces the reduction of cytochrome b in the ascorbate-reduced bc\textsubscript{1} complex reconstituted into liposomes.

Fig. 2 shows the dependence of cytochrome b reduction on the magnitude of the valinomycin-induced K\textsuperscript{+} diffusion potential (Δψ) formed across liposomal membranes. The diffusion potential was varied by changing the concentration of KCl in the reaction medium and calculated from the Nernst equation, Δψ (mV) = 60-log(1/K\textsubscript{eq}/K\textsubscript{int}), based on the assumed internal KCl concentration of 100 mM. No reduction of cytochrome b\textsubscript{562} was observed below the expected value of 30 mV, and the reduction level of cytochrome b increased sigmoidally with increasing the diffusion potential above 30 mV. The maximal reduction was attained at more than 210 mV (the initial concentration of external KCl was less than 30 μM).

Fig. 3 shows the spectral analysis of Δψ-induced reduction of cytochrome b. When a difference spectrum was taken between prereduced by ascorbate-reduced cytochrome b\textsubscript{562} vesicles, absorption maximum appeared at 562 nm (solid line in Fig. 3). On the contrary, a peak was found at 565 nm along with a shoulder around 558 nm in a difference spectrum between Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}-reduced and diffusion potential-imposed bc\textsubscript{1} vesicles (the spectrum is not included in Fig. 3). These results indicate that cytochrome b\textsubscript{562} in the reconstituted bc\textsubscript{1} complex is the main component that is reduced upon generation of the K\textsuperscript{+} diffusion potential in the presence of ascorbate.

We estimated the extent of the cytochrome b\textsubscript{562} reduction using ascorbate and TMPD as electron donors. As reported with the purified complex from beef heart mitochondria (26), cytochrome b\textsubscript{562} in the reconstituted bc\textsubscript{1} complex can be fully reduced with ascorbate plus TMPD (data not shown, but see Fig. 8). By comparing a difference spectrum between Δψ-imposed minus ascorbate-reduced bc\textsubscript{1} vesicles with that between the (ascorbate and TMPD)-reduced minus ascorbate-reduced vesicles, about 90% of cytochrome b\textsubscript{562} was estimated to be reduced during the formation of the diffusion potential. This result is important (see "Discussion").

Effect of Electron Transfer Inhibitors—The effect of electron transfer inhibitors on the Δψ-induced reduction of cytochrome b\textsubscript{562} was examined with the reconstituted bc\textsubscript{1} complex supplemented with either antimycin A or myxothiazol (Fig. 4). In these experiments, the reconstituted bc\textsubscript{1} complex was pre-reduced with 5 mM sodium ascorbate for 30 min at 20 °C. Sodium ascorbate alone reduced cytochrome b very slowly in the reconstituted bc\textsubscript{1} complex (see Fig. 1D) as well as in the purified enzyme (data not shown). About 10% of total cytochrome b was finally in the reduced form during incubation for 30 min in the presence of 5 mM ascorbate. Spectral examination indicated that the cytochrome b species reduced by ascorbate was cytochrome b\textsubscript{562} (data not shown). When antimycin A at a molar ratio of 2.1 mol/mol cytochrome c\textsubscript{1} was added to the reconstituted bc\textsubscript{1} complex, generation of the diffusion potential promoted the reoxidation of cytochrome b\textsubscript{562} pre-reduced by ascorbate (Fig. 4B). On the contrary, myxothiazol at a ratio of 8.3 mol/mol cytochrome c\textsubscript{1} lowered the reduction level of cytochrome b\textsubscript{562} to 12% of the control level (Fig. 4C). Either 0.5 nmol of antimycin A or 2.0 nmol of myxothiazol did not affect the time course of safranine response (data not shown), thus eliminating the possibility for those inhibitors to perturb the formation of the diffusion potential.

Effect of Ubiquinone—When the ubiquinone (Q)-depleted bc\textsubscript{1} complex (0.03 mol of Q\textsubscript{10}/mol of cytochrome c\textsubscript{1}) was incorporated into K\textsuperscript{+}-loaded liposomes, only 7% of total cytochrome b was reduced upon generation of the K\textsuperscript{+} diffusion potential (Fig. 5). Therefore, ubiquinone-2 (Q\textsubscript{2}) was added to ascorbate-reduced proteoliposomes reconstituted with the Q-depleted bc\textsubscript{1} complex before generation of the diffusion potential to examine the effect of ubiquinone. The addition of Q\textsubscript{2} restored the Δψ-induced reduction of cytochrome b\textsubscript{562} up to the 42% reduction of total cytochrome b at a molar ratio of 0.75 mol of Q\textsubscript{2}/mol of cytochrome c\textsubscript{1}. On the other hand, Q\textsubscript{2} externally added to proteoliposomes reconstituted with the native bc\textsubscript{1} complex enhanced the energy-dependent reduction of cytochrome b\textsubscript{562} maximally by 10% (Fig. 5). Although the maximum level of the recovered cytochrome b\textsubscript{562} reduction in the Q-depleted bc\textsubscript{1} complex is lower than that observed with the native complex, this result indicates that ubiquinone is an essential component for the reverse transfer of electrons. With increasing concentra-
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**Fig. 4. Effect of electron transfer inhibitors on Δψ-induced reduction of cytochrome b₅₆₂.** Ascorbate-reduced bc₁ vesicles containing 0.24 nmol of cytochrome c₁ were prepared as described in Fig. 3. The reduction of cytochrome b₅₆₂ was initiated by adding 0.2 μg of valinomycin after addition of none (A), 0.5 nmol of antimycin A (B), or 2.0 nmol of myxothiazol (C). Other experimental conditions were as in Fig. 1.

**Fig. 5. Effect of ubiquinone on Δψ-induced reduction of cytochrome b₅₆₂.** Proteoliposomes (0.1 ml) containing either 0.48 nmol of the purified bc₁ complex (●) or 0.40 nmol of the Q-depleted enzyme (○) were suspended in 10 mM MOPS/NaOH (pH 7.4), 100 mM NaCl, and reduced with 5 mM sodium ascorbate at 20 °C for 30 min. After 1 μl of Q₂ in ethanol was added to the reaction mixture to give a Q₂/cytochrome c₁ ratio indicated in the figure, Δψ-induced reduction of cytochrome b₅₆₂ was initiated by adding 0.1 μg of valinomycin. Other conditions were as in Fig. 4.

**Fig. 6. Effect of DCCD on the reversed electron transfer and the diffusion potential formation in K⁺-loaded bc₁ vesicles.** Proteoliposomes (0.2 ml) containing 4.5 μmol cytochrome c₁ were mixed with 2 μl of ethanol containing either none (dashed line) or 200 mM DCCD (solid line) and incubated at 0 °C for 16 h. A, a 0.1-ml aliquot of the control and the DCCD-treated vesicles was used to measure the Δψ-induced reduction of cytochrome b₅₆₂ as described in Fig. 1. Where indicated, 2 μmol of ascorbate (asc), 0.1 μg of valinomycin (val), and sodium dithionite were added. B, a 5-μl portion of the proteoliposomes was used to check their ability to form the K⁺ diffusion potential as described under "Materials and Methods." The absorbance change of safranine was followed at 530–578 nm. Additions were 5 μg of valinomycin (val.) and 1 μg of nigericin (nig.).

**Fig. 7. Dose-dependent effect of DCCD on K⁺-loaded bc₁ vesicles.** Proteoliposomes (0.1 ml) containing 5.2 μmol of cytochrome c₁ were incubated for 16 h at 0 °C with DCCD at a molar ratio indicated in the figure. A 50-μl and a 5-μl portion of the DCCD-treated vesicles were used to test the reversed electron transfer (●) and the diffusion potential formation (△) as described in Fig. 6, respectively. The enzyme activity (●) were assayed in the presence of 0.1 μg of valinomycin plus 2 μg of nigericin as described under "Materials and Methods." The 100% value represents 0.014 for ΔA₅₆₂-₅₇₈ nm (cytochrome b₅₆₂ reduction), 0.082 for ΔA₄₃₀-₄₇₈ nm (safranine response), and 392 mol of cytochrome c mol⁻¹ (mol cytochrome c₁)⁻¹ for the enzyme activity.

The proteoliposomes to form the diffusion potential (Figs. 6B and 7). Furthermore, the enzyme activity was inhibited by only 10% during a 16-h incubation at 0 °C with DCCD at a molar ratio of 481 mol/mol cytochrome c₁. The energy-dependent reduction of cytochrome b₅₆₂ decreased with increasing concentration of DCCD in a dose-dependent manner, an apparent half-inhibition concentration being 200 mol of DCCD/mol of cytochrome c₁. Contrary to the inhibitory effect of DCCD on the reversed electron transfer, it exerted no effect on the ability of the proteoliposomes to form the diffusion potential (Figs. 6B and 7).
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8 shows the effect of the diffusion potential on the reconstituted bc₁ complex when ascorbate and TMPD were used as electron donors. In contrast to brown adipose tissue mitochondria, in which ascorbate plus TMPD led to the partial reduction of cytochrome b₅₆₂ (9% reduction of total cytochrome b) (11), cytochrome b₅₆₂ in the reconstituted bc₁ complex was fully reduced by the ascorbate/TMPD system (Fig. 8A), as reported with the purified complex from beef heart mitochondria (26). After the complete reduction of cytochrome b₅₆₂ with ascorbate plus TMPD, addition of valinomycin induced a further increase in absorbance at 565 minus 575 nm (Fig. 8A). And nigericin caused the slow oxidation of the cytochrome b component which had been reduced on generation of the diffusion potential (Fig. 8A). As shown in Fig. 8B, a characteristic spectrum of cytochrome b₅₆₆ with a peak at 566 nm and a shoulder around 555 nm was obtained when the diffusion spectrum was taken before and after addition of valinomycin (solid line). These results indicate that the K⁺ diffusion potential induced the reduction of cytochrome b₅₆₆ in the reconstituted bc₁ complex in the presence of ascorbate and TMPD. The extent of cytochrome b₅₆₆ reduction was estimated to be about 30% of the cytochrome.

**DISCUSSION**

Energy-linked reduction of cytochrome b has been studied with mitochondria or submitochondrial particles prepared from different sources (9-11). Originally, cytochrome b₅₆₂ was reported to be reduced when ATP was added under anaerobic conditions to beef heart submitochondrial particles (9) or pigeon heart mitochondria (10) in which cytochrome b₅₆₂ had been fully reduced with a substrate such as succinate. Later, it was shown that energization of brown adipose tissue mitochondria from guinea pig with ATP led to the antimycin A-sensitive reduction of cytochrome b₅₆₂ as well as cytochrome b₅₆₆ in the presence of ascorbate plus TMPD (11), suggesting that ATP was capable of inducing reduction of both cytochrome b components (b₅₆₂ and b₅₆₆). We confirmed the energy-dependent reduction of cytochrome b₅₆₂ and b₅₆₆ using ascorbate and ascorbate/TMPD, respectively, in the reconstituted system with energy provided by a K⁺ diffusion potential (negative inside). Another type of energy-linked redox response of cytochrome b is the oxidation of prereduced cytochrome b₅₆₂ in the presence of antimycin A (3, 12, 13), Beattie and Villalobo (19) demonstrated, using proteoliposomes reconstituted with the yeast bc₁ complex, that the K⁺ diffusion potential (negative inside) in the presence of antimycin A promoted the oxidation of cytochrome b prereduced by the Qₚ/QH₂ mixture. Therefore, we believe that the results presented here are the first report demonstrating and characterizing the energy-linked reduction of cytochrome b in the reconstituted proteoliposome system.

The present study has shown that ubiquinone is an essential component for the energy-linked reduction of cytochrome b₅₆₂ (Fig. 5), which is inhibited by electron transfer inhibitors like antimycin A and myxothiazol (Fig. 4). Cytochrome b₅₆₂ is also promoted to be reduced on generation of the K⁺ diffusion potential in the presence of ascorbate and TMPD (Fig. 8). These results can be consistently interpreted in terms of the Q cycle scheme which assumed the energy-dependent reduction of ubiquinone at center a. The pathway of the reversed electron transfer in the bc₁ complex can be summarized as follows (Fig. 8B). Step 1: ascorbate reduces both cytochrome c₁ and iron-sulfur protein. Step 2: the next step is the reduction of ubiquinone to ubiquinol at center o. Electrons to reduce ubiquinone are supplied from the iron-sulfur center which is reduced by ascorbate via cytochrome c₁. The membrane potential is required to enable electrons to move at center o from iron-sulfur protein with a more positive redox potential of +280 mV (29) to the ubiquinone/ubisemiquinone couple with a more negative potential as low as −230 mV or to the ubisemiquinone/ubiquinol couple with a potential of +190 mV (30). This step is sensitive to myxothiazol. There was no sign of the transient reduction of cytochrome b₅₆₂ even when the ΔΨ-induced reduction of cytochrome b₅₆₂ in the presence of ascorbate was investigated with a stopped-flow and rapid-scan technique. Moreover, antimycin A inhibited the ΔΨ-induced reduction of cytochrome b₅₆₂ (Fig. 4). We, therefore, conclude that ubisemiquinone, a possible intermediate during ubiquinone reduction, is not likely to give an electron to cytochrome b₅₆₆ in the reverse reaction of electron transfer. Step 3: reduced ubiquinone moves from center o to center i. Step 4: ubiquinol reduces cytochrome b₅₆₂ at center i, and antimycin A inhibits this reaction. Step 5: the diffusion potential can induce electron transfer from cytochrome b₅₆₂ to cytochrome b₅₆₆ when cytochrome b₅₆₂ is in the fully reduced form (Fig. 8).

The main question in the present work is the effect of DCCD on the reversed electron transfer in the reconstituted bc₁ complex. We observed that DCCD prevented the reversed electron transfer from cytochrome c₁ to cytochrome b₅₆₂ without a significant loss in the catalytic activity of the reconstituted bc₁ complex (Figs. 6 and 7). Similar results have been reported by Beattie and Villalobo (19), in which the energy-linked oxidation of prereduced cytochrome b in the presence of antimycin A was inhibited by the DCCD treatment of the reconstituted bc₁ complex.

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1. Y. Orii, and T. Miki, unpublished observation.
plex from yeast mitochondria with only minimal effects on its electron transfer activity. Moreover, DCCD is the well-known reagent to block proton translocation in the $b_{c_1}$ complex.

One possible model incorporating the proton flow into the Q cycle is shown in Fig. 9, which is a modified version of the scheme proposed by Konstantinov (8). This model postulates the reversible flow of protons between the outer medium and center o (Fig. 9, A and B). Important features of the proton flow are as follows. First, protons move electromagnetically from center o to the outer phase in the catalytic reaction (Fig. 9A). Second, the membrane potential promotes the uptake of protons from the outer medium in the reverse reaction (Fig. 9B), and the reduction of ubiquinone at center o (Step 2) is tightly coupled to the membrane potential-induced uptake of protons. Third, DCCD specifically blocks the reversible flow of protons between center o and the outer medium without affecting any step of the electron transfer pathway.

According to this model, the effect of DCCD on the $b_{c_1}$ complex can be interpreted as follows. In the reverse reaction (Fig. 9B), DCCD inhibits the $\Delta\psi$-dependent uptake of protons and, consequently, blocks the reverse transfer of electrons because the reversed electron transfer from the iron-sulfur cluster to ubiquinone is closely coupled to the $\Delta\psi$-dependent uptake of protons. In the catalytic reaction (Fig. 9A), however, DCCD does not inhibit the electron transfer activity of the $b_{c_1}$ complex (Fig. 7) in spite of its specific effect on the proton translocation activity of the complex (19). These observations are interpreted well by the Q cycle when the pathway of the proton flow in the DCCD-treated $b_{c_1}$ complex is different from that in the intact complex. We, therefore, postulated in the catalytic reaction (Fig. 9A) that protons produced by the $Q_{H_2}$ oxidation at center o might go back electrochemically from center o to the inner space through the lipid phase (dotted line in Fig. 9A) because the normal electrogenic pathway for the proton ejection is blocked by DCCD. In this case, the enzyme activity of the $b_{c_1}$ complex would not be affected by the DCCD treatment because DCCD does not block any step of the electron transfer pathway.

Generation of the $K^+$ diffusion potential induced the reduction of about 90% of cytochrome $b_{562}$ (Fig. 2), which was interpreted to be reduced by ubiquinol (Step 4). But $Q_{H_2}$ can reduce only 58% of cytochrome $b_{562}$ (35% of total cytochrome b) in reaction of the reconstituted $b_{c_1}$ complex with 10 $\mu$M $Q_{H_2}$ (data not shown), in line with the result observed in the purified complex from beef heart mitochondria (34). These results suggest that the electron transfer step from ubiquinol to cytochrome $b_{562}$ is somewhat an up-hill reaction. Using the $b_{c_1}$ complex of R. sphaeroides chromatophores, Drachev et al. (7) also showed that the transfer of protons associated with the reduction of ubiquinone at center i was the electrogenic step (see also Ref. 38). We, therefore, assumed the reversible proton flow between the inner phase and center i in analogy with Step 2. The energy-dependent reduction of cytochrome $b_{562}$ (Step 4) seems to proceed along with the inward flow of protons.

It has been generally accepted (3, 35) that membrane potential-induced reversed electron transfer from cytochrome $b_{562}$ to $b_{566}$ (Step 5) is due to equilibria shifts associated with membrane potential-induced change in electron distributions between the two cytochrome b components which are arranged transmembranously in the $b_{c_1}$ complex (36). The main point of our present work is that the reversed transport of electrons is closely coupled to the reverse flow of protons, and one possible model is presented based on the Q cycle (Fig. 9). This model also accounts for the specific effect of DCCD on the $b_{c_1}$ complex. Beattie and co-workers have reported that DCCD binds to the cytochrome b subunit of the $b_{c_1}$ complex purified from various sources (19, 28), and the binding site of DCCD has been reported to be Asp$^{160}$ in the cytochrome b subunit of the yeast $b_{c_1}$ complex (28). The aspartate residue is present in the $\alpha$-helical segment which lies on the outer surface of mitochondrial (lipo-somal) membranes (37). These results suggest that the cytochrome b subunit is significantly involved in the proton flow through the $b_{c_1}$ complex, presumably by forming a proton channel, and that DCCD might block the outlet part of the proton channel (inlet of the proton uptake). Further studies are required for elucidating the role of the cytochrome b subunit in proton translocation coupled to the electron transfer reaction of the $b_{c_1}$ complex.
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