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Inhibition of Reversed Electron Transfer and Proton Transport in the Beef Heart Cytochrome bc_1 Complex by Chemical Modification

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Chemical modification of the bovine heart cytochrome bc_1 complex with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) has been reported to inhibit the proton pumping activity without affecting the rate of electron transfer to ferricytochrome *c*. This study aims to examine the effect of EEDQ on energy-linked reversed electron transfer in the bc_1 complex reconstituted into potassium-loaded phospholipid vesicles. Generation of a valinomycin-mediated potassium-diffusion potential induced the reduction of cytochrome *b* in the reconstituted bc_1 complex in the presence of sodium ascorbate. The time course of the cytochrome *b* reduction was well correlated with that of the absorbance change of safranine, an optical probe for measuring membrane potential. Treatment of the bc_1 complex with EEDQ caused a decrease in the potential-induced reduction of cytochrome *b* as well as in the proton translocation activity. But a significant loss in the ubiquinol–cytochrome *c* reducing activity was not observed in the EEDQ-treated bc_1 complex. The time- and concentration-dependent effect of EEDQ on the reversed electron transfer was well correlated with that of the proton translocation activity of the bc_1 complex. These findings strongly support the idea that the potential-induced reversal of electron transfer is coupled to the reverse flow of protons in the cytochrome bc_1 complex.

Key words: chemical modification, cytochrome bc_1 complex, proton transport, respiratory chain, reversed electron transfer.

Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; FCCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone; [2Fe–2S], iron–sulphur cluster; ISP, Rieske iron–sulphur protein; MOPS, 4-morpholinepropanesulfonic acid; PMS, phenazine methosulphate; Q_2 and Q_2H_2, the oxidized and reduced form of ubiquinone-2; SMP, submitochondrial particles; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

The cytochrome bc_1 complex (commonly known as ubiquinol–cytochrome *c* reductase or Complex III) is an integral multisubunit enzyme that catalyses electron transfer from ubiquinol to ferricytochrome *c* in the mitochondria respiratory chain. This catalytic reaction is coupled to the translocation of protons from the matrix side to the intermembrane space across the mitochondrial inner membrane to generate a proton gradient and membrane potential for ATP synthesis or substrate and ion transport (*1*, *2*). The enzyme from high eucaryotic origins is composed of 11 subunits, of which cytochrome *b*, cytochrome c_1 and the Rieske iron–sulphur protein (ISP) are redox proteins. Two *b*-type hemes, heme b_L ($Em = -34$ mV) and heme b_H ($Em = +90$ mV), are bound to cytochrome *b*, one *c*-type heme (heme c_1, $Em = +230$ mV) is attached to cytochrome c_1, and ISP houses a [2Fe–2S] cluster ($Em = +280$ mV). In addition to these four prosthetic groups, ubiquinone-10 is usually found in purified enzyme prepared from mammalian mitochondria (*3*).

The mechanism of electron transfer and proton translocation in the bc_1 complex has been best described by the modified version (*4*) of the Q-cycle (*5*, *6*). The key feature of the model is the presence of two distinct ubiquinol/ubiquinone-binding sites, namely a ubiquinol oxidation site (Qo site) near the intermembrane space side, and a ubiquinone reduction site (Qi site) near the matrix side. According to the modified Q cycle (*4*), electrons are delivered into a bifurcated pathway at the Qo site for the coupling to proton translocation. The first electron of ubiquinol is transferred initially to the [2Fe–2S] cluster of ISP, resulting in the formation of a ubisemiquinone intermediate at the Qo site. The electron in ISP is subsequently transferred to cytochrome c_1 and then to cytochrome *c* (the higher potential chain). The second electron from ubiquinol is transferred to a lower potential chain consisting of heme b_L and heme b_H, which span the inner membrane and delivers electrons to the Qi site on the matrix side. At the Qi site, ubiquinone is re-reduced to semiquinone by heme b_H to which the electron has been transferred from heme b_L across

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mitochondrial membranes. Oxidation of two equivalents of ubiquinol at the Qo site is thus required for reduction of one equivalent of ubiquinone at the Qi site. In a complete catalytic cycle, the sequential oxidation of two ubiquinol molecules releases four protons into the intermembrane space, while two protons are taken up from the matrix during the reduction of one ubiquinone molecule. The X-ray crystal structure of the mitochondrial complex from various sources has been solved to an atomic resolution of 2.5–3 Å in the presence or absence of different inhibitors (*7*–*10*). These different structures suggest that the hydrophilic head domain of ISP housing the [2Fe–2S] cluster moves between a position where the [2Fe–2S] cluster is close to heme b_L (the *b* position) and a position where the [2Fe–2S] cluster and heme c_1 reside proximally (the c_1 position). Mobility of the ISP head domain has been the subject of much recent work, and biochemical and genetic studies have provided strong evidence that movement is required for the function of the bc_1 complex (*11*, *12*). Reduction of ISP occurs at the *b* position, followed by the movement of ISP toward the c_1 position, which facilitates electron transfer to cytochrome c_1.

We were the first to demonstrate that the electron transfer reaction in the bc_1 complex can be reversed with energy provided by the formation of a membrane potential using the bovine bc_1 complex reconstituted into potassium-loaded phospholipid vesicles (*13*). A potassium-diffusion potential (negative inside) generated across the liposomal membranes promoted the reduction of heme *b* components at the expense of the transient oxidation of cytochrome c_1, indicating electron transfer from cytochrome c_1 with a more positive redox potential to cytochrome *b* components with a more negative potential. Two important features were also demonstrated for the reversed electron transfer reaction. First, ubiquinone is an essential component of the energy-linked reversed electron transfer reaction. Second, DCCD, which inhibits the proton translocation activity of the bc_1 complex without a significant loss in the electron transfer activity, also decreases the potential-induced reduction of cytochrome *b* components, suggesting that the potential-induced reversed electron transfer is coupled to the reverse flow of protons. These properties of reversed electron transfer were confirmed later to be in common with the enzyme purified from photosynthetic bacteria *Rhodobacter sphaeroides* (*14*).

Recently, Cocco *et al*. reported that *N*-(ethoxycarbonyl)-2-ethoxyl-1,2-dihydroquinoline (EEDQ), a specific reagent for buried carboxyl groups of proteins, inhibited specifically the proton translocation activity of the bc_1 complex from bovine heart mitochondria as well as from *Paracoccus denitrificans* with a minimal loss in the electron transfer activity (*15*, *16*). They showed that EEDQ modified core protein II and ISP in the bovine heart cytochrome bc_1 complex, while DCCD was bound to the cognate cytochrome *b* subunit and to subunit IX. In the three-subunit *P. denitrificans* bc_1 complex, ISP and cytochrome c_1 were labelled by EEDQ. Furthermore, tryptic digestion and sequence analysis of EEDQ-modified *Paracoccus* ISP revealed that EEDQ was bound to the region containing amino acid residues from Thr-118 to Arg-164 in which six acidic residues are found. Based on these binding experiments, they argued that the ISP subunit contributes with its carboxyl residue(s) to the proton translocation mechanism of both bacterial and eucaryotic complexes, and that the conserved Asp-160 of *P. denitrificans* ISP (equivalent to Asp-166 of the bovine ISP) is a plausible candidate of the site of specific modification, although the sites of EEDQ modification have not been unambiguously established in both cases (*15*, *16*). Later, Ebert *et al*. (*17*) confirmed that EEDQ also blocked electrogenic proton pumping in the yeast bc_1 complex reconstituted into proteoliposomes without affecting its electron transfer activity, and that EEDQ was bound to the iron–sulphur protein and to the core protein II of the yeast enzyme.

In this study, we examined the effect of EEDQ on both proton translocation and reversed electron transfer in the bc_1 complex. Treatment of the bc_1 complex with EEDQ resulted in the simultaneous inhibition of proton translocation and potential-induced reversed electron transfer in the bovine bc_1 complex. Correlation of the specific inhibitory effect of the chemical modifier on these energy-linked phenomena supports the idea that the reversed electron transfer is coupled to an energy-linked reverse flow of protons in the bc_1 complex. A model for the potential-induced reversed electron transfer reaction is constructed based on the modified Q cycle, and a possible mechanism of the inhibitory effect of EEDQ on the bc_1 complex is discussed.

MATERIALS AND METHODS

Materials—Valinomycin, FCCP and cytochrome *c* (horse heart, type III) were purchased from Sigma (St Louis, MO, USA). MOPS was a product of Dojindo (Kumamoto, Japan). Safranine, sodium ascorbate, TMPD and EEDQ were obtained from Wako Chemicals (Osaka, Japan). A resource PHE pre-packed column (6 ml of column volume) for hydrophobic interaction chromatography was a product of GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Asolectin (soybean phospholipids) was purchased from Associate Concentrates (Woodside, NY, USA) and partially purified as reported (*18*). Ubiquinone-2 was a kind gift from Eisai (Tokyo, Japan) and ubiquinol-2 (Q_2H_2) was prepared as described previously (*19*). The cytochrome bc_1 complex was purified from beef heart muscle and assayed according to a previous report (*20*). The purified bc_1 complex contained 4.2–4.9 nmol of cytochrome c_1/mg of protein and 0.36–0.42 mol ubiquinone-10/mol cytochrome c_1.

Chemical Modification and Reconstitution of the bc_1 Complex—EEDQ treatment of the bc_1 complex was carried out as follows (*15*). The purified bc_1 complex (20 mg protein/ml) was diluted in the range of 50–55 μM cytochrome c_1 with 10 mM MOPS/NaOH–100 mM KCl, and incubated at 0°C with a methanol solution of EEDQ at an appropriate molar ratio as specified in the figure legend. Then, the EEDQ-treated bc_1 complex was mixed with a sonicated solution of 40 mg/ml asolectin in 10 mM MOPS/NaOH (pH 7.4), 100 mM KCl,

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2% sodium cholate to give a final concentration of 5 μM cytochrome c_1 for reconstitution of proteoliposomes. Reconstitution of the bc_1 complex into K^+-loaded phospholipid vesicles was carried out by the cholate dialysis method (*13*) with a minor modification as follows. The mixture of phospholipids and bc_1 complex was dialysed against 10 mM MOPS/NaOH (pH 7.4), 100 mM KCl to remove cholate. After reconstitution was completed, KCl outside the vesicles was replaced with NaCl by further extensive dialysis against 10 mM MOPS/NaOH (pH 7.4)–100 mM NaCl. Final dialysis was carried out against 0.5 mM MOPS/NaOH (pH 7.4) containing 100 mM NaCl for 4 h.

Optical Measurement—All optical measurements were carried out with a Unisoku multi-wavelength spectrophotometer or an Ocean Optics spectrophotometer (model USB2000). Proton movements were measured spectrophotometrically at 20°C using phenol red as a pH indicator as follows (*21*). A 50-μl portion of bc_1 vesicles was suspended in 0.95 ml of 0.1 mM MOPS/NaOH (pH 7.4) containing 50 μM phenol red and 100 mM KCl, followed by the addition of 25 nmol of Q_2H_2, 0.5 nmol of cytochrome *c* and 0.1 μg of valinomycin. After a pre-incubation period of ~10 min, the reaction was initiated by a pulse of potassium ferricyanide. Absorbance change of phenol red was followed at a wavelength setting of 557 minus 504.5 nm. In each experiment, known amounts of HCl were added for calibration of the absorbance change of phenol red. Proton permeability of vesicle membranes was estimated for convenience with phenol red by adding 0.1 μg of valinomycin to 50 μl of K^+-loaded proteoliposomes suspended in 0.95 ml of 0.1 mM MOPS/NaOH (pH 7.4), 100 mM NaCl (*22*). The ubiquinol–cytochrome *c* reductase activity of the reconstituted bc_1 complex was determined at room temperature in 1 ml of 10 mM MOPS/NaOH (pH 7.4) containing 100 mM KCl, 50 μM cytochrome *c*, 25 nmol of Q_2H_2, 0.1 μg/ml valinomycin and 1 μg/ml FCCP. The initial rate of cytochrome *c* reduction was corrected against non-enzymatic reduction of cytochrome *c* by ubiquinol in the absence of proteoliposomes. The ability of K^+-loaded proteoliposomes to form a potassium-diffusion potential was checked spectrophotometrically using safranine as an optical probe for the membrane potential (*13*). The absorbance change of safranine was monitored at the wavelength pair of 530 minus 578 nm (*23*).

Separation of the Purified bc_1 Complex into ISP and the ISP-Depleted bc_1 Complex—The ISP-depleted bc_1 complex was prepared according to a previous report (*24*) as follows. The purified bc_1 complex (around 2.5 mg of protein) was applied to a Resource PHE column equilibrated with 25 mM HEPES/NaOH (pH 8.0) containing 1 mM DTT, 1 mM sodium EDTA, 0.25% sodium deoxycholate, 0.2 M NaCl and 20% glycerol, and washed with 20 column volumes of the same buffer. A fraction containing ISP was eluted with 25 mM HEPES/NaOH (pH 8.0) containing 1 mM DTT, 1% sodium deoxycholate and 20% glycerol. Finally, a fraction containing the ISP-depleted bc_1 complex was eluted with a buffer containing 25 mM HEPES/NaOH (pH 8.0), 3% Tween 20, 1 mM DTT and 20% glycerol.

Analytical Methods—The cytochrome c_1 concentration was determined from the difference spectrum of the ascorbate-reduced *versus* ferricyanide-oxidized enzyme, using an extinction coefficient of 17.5/mM cm at 553 minus 540 nm, and the extinction coefficient used for cytochrome *c* was 19.2/mM cm at 550 nm after dithionite reduction (*20*). Potassium ferricyanide was determined by the extinction coefficient of 1.04/mM cm at 420 nm (*25*). Protein was estimated by a modified Lowry method (*26*).

RESULTS

Correlation of Reversed Electron Transfer with the Diffusion Potential Formation in K^+-loaded bc_1 Vesicles—Valinomycin, a potassium ionophore, increases the K^+ conductance of the liposomal membranes of K^+-loaded phospholipid vesicles, resulting in the formation of a potassium-diffusion potential across the membranes. Figure 1A shows the concentration effect of valinomycin on the time course of the absorbance change of safranine, which was used as an optical probe to monitor the formation of a membrane potential. When valinomycin at a ratio of 2.0 μg/mg phospholipids was added to bc_1 vesicles, a rapid decrease in safranine absorbance was observed, followed by its gradual increase to the original level (trace 1 in Fig. 1A). The decrease in safranine absorbance corresponds to the generation of a potassium-diffusion potential, and its formation was quite transient at the valinomycin concentration of 2.0 μg/mg phospholipids. As the valinomycin concentration was lowered to 0.5 and 0.02 μg/mg phospholipids (traces 2 and 3), the duration of the decreased level of safranine absorbance was extended to 2 and 12 min, respectively. The amplitude of the initial absorbance decrease, however, was comparable to that observed at a ratio of 2.0 μg/mg phospholipids. Ethanol, a solvent for valinomycin, did not induce any change in safranine absorbance (trace 4).

We previously reported that the valinomycin-mediated potassium-diffusion potential promoted the reduction of cytochrome *b* in the reconstituted bc_1 complex in the presence of sodium ascorbate (*13*). The concentration effect of valinomycin on the time course of cytochrome *b* reduction in the ascorbate-reduced bc_1 complex is also shown in Fig. 1B. In these experiments, bc_1 vesicles were first pre-incubated with 5 mM sodium ascorbate for 10 min at 20°C to reduce cytochrome c_1 and ISP. As shown in trace 1 of Fig. 1B, the addition of valinomycin at 2.0 μg/mg phospholipid induced the transient reduction of cytochrome *b*, followed by its reoxidation. As the valinomycin concentration was decreased from 0.5 to 0.02 μg/mg phospholipids, cytochrome *b* in the reconstituted bc_1 complex stayed in the reduced form for a longer period (traces 2 and 3). Ethanol alone caused no change in the reduction level of cytochrome *b* (trace 4). Even though the final concentration of proteoliposomes for the safranine method (0.2 mg phospholipid/ml) is much lower than that used for measurements of reversed electron transfer (5 mg phospholipid/ml), the time course of cytochrome *b* reduction correlated well with that of safranine response,

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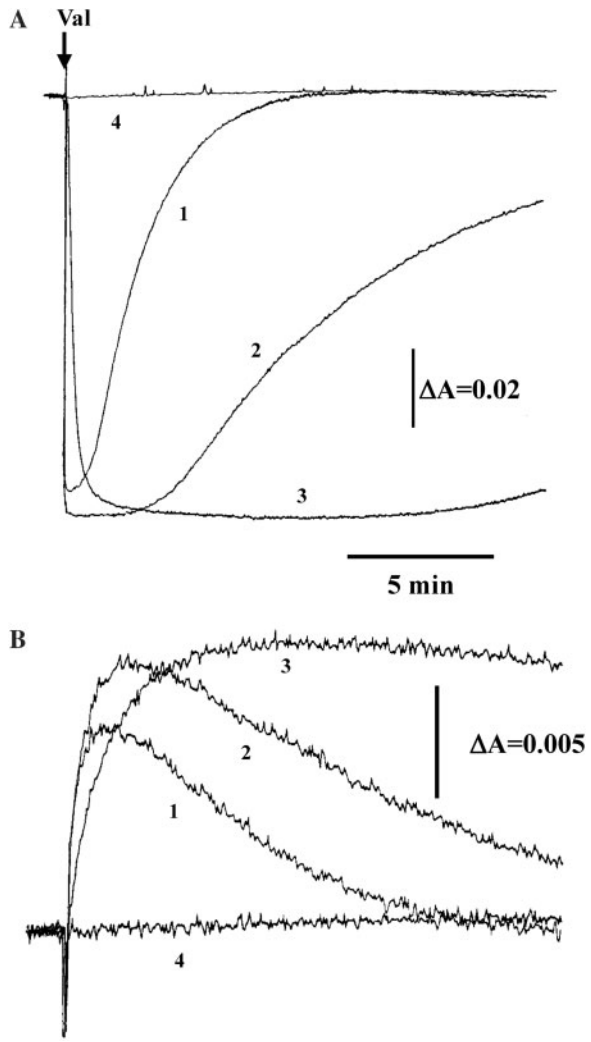

Fig. 1. **Concentration effect of valinomycin on time courses of safranine response and potential-induced cytochrome *b* reduction in K^+-loaded bc_1 vesicles.** (A) (Safranine response) a 5-μl portion of bc_1 vesicles containing 23 pmol of cytochrome c_1 was suspended in 1 ml of 10 mM MOPS/NaOH (pH 7.4), 4.8 μM safranine and 100 mM NaCl. Absorbance change of safranine was followed at a wavelength setting of 530 minus 578 nm. (B) (Cytochrome *b* reduction) a 50-μl portion of bc_1 vesicles was suspended in 0.35 ml of 10 mM MOPS/NaOH (pH 7.4), 100 mM NaCl and pre-incubated for 10 min in the presence of 5 mM sodium ascorbate. Absorbance change of cytochrome *b* was measured at 562 minus 575 nm. The reactions were initiated by adding valinomycin at (1) 2.0, (2) 0.5, (3) 0.02, (4) 0 μg/mg phospholipid, respectively.

as long as the valinomycin concentration was expressed as the ratio to phospholipids. These results indicate that the formation of a potassium-diffusion potential induced the reduction of cytochrome *b* in the reconstituted bc_1 complex. The optimal amount of valinomycin to induce the maximal reduction of cytochrome *b* was between 0.02 and 0.5 μg/mg phospholipids. About 50–55% of total dithionite-reducible cytochrome *b* was usually reduced, and heme b_H was the main component that was reduced on generation of a diffusion potential in the presence of sodium ascorbate, as reported previously (*13*). It should also be noted that the potential-induced reduction of

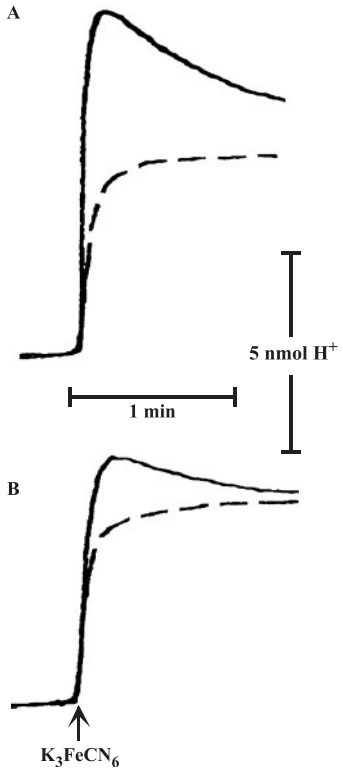

Fig. 2. **Effect of EEDQ on proton ejection by bc_1 vesicles**. The purified bc_1 complex containing 2.6 nmol of cytochrome c_1 was incubated with either methanol alone (A) or EEDQ in methanol at a molar ratio of 48 mol EEDQ/mol of cytochrome c_1 (B) for 2 h at 0°C prior to reconstitution. A 50-μl portion of bc_1 vesicles were suspended in 0.95 ml of 0.1 mM MOPS/NaOH (pH 7.4) containing 50 μM phenol red, 100 mM KCl, 25 μM Q_2H_2 and 0.5 μM cytochrome *c*, followed by the addition of 0.1 μg of valinomycin. The reaction was started by adding 5 nmol of potassium ferricyanide (K_3FeCN_6). Dashed lines indicate pH change in the presence of 1 μg of FCCP.

cytochrome *b* can be observed even without the external addition of ubiquinone-2, provided that the purified preparation of the bc_1 complex contains a small amount of ubiquinone-10 (Q_{10}), i.e. around 0.4 mol Q_{10}/mol cytochrome c_1 (see section 'DISCUSSION').

Effect of Chemical Modification on Energy-linked Reduction of Cytochrome b and Proton Translocation in the Reconstituted bc_1 Complex—EEDQ, a chemical reagent to modify acidic amino acid residues in proteins, is reported to inhibit proton translocation in the cytochrome bc_1 complex and cytochrome *c* oxidase (*15*, *16*). Figure 2 shows the inhibitory effect of EEDQ on the proton translocation activity of the reconstituted bc_1 complex. In these experiments, bc_1 vesicles were suspended in a medium containing 100 mM KCl to eliminate the effect of the potassium-diffusion potential, and valinomycin was also present for counter movement of K^+ ions as charge compensating cations. When 5 nmol of ferricyanide was pulsed to intact bc_1 vesicles incubated with Q_2H_2 and a small amount of cytochrome *c*, rapid acidification was observed with a subsequent slow rate of H^+ back flow (Fig. 2A, solid line). The maximal amount of protons appearing outside vesicles was estimated to be 1.9 mol H^+/mol of e^- transferred. In the

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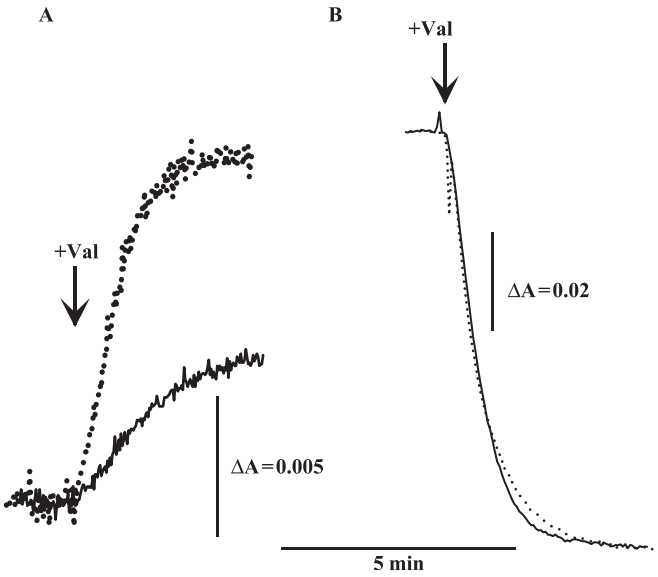

Fig. 3. **Effect of EEDQ on potential-induced reduction of cytochrome *b* in K^+-loaded bc_1 vesicles.** The bc_1 complex was treated with EEDQ in methanol (solid line) or methanol alone (dotted line) as in Fig. 2. The potential-induced reduction of cytochrome *b* (A) and safranine response (B) were measured as described in Fig. 1. Where indicated, 0.1 μg (A) or 5 ng (B) of valinomycin was added to initiate the reaction.

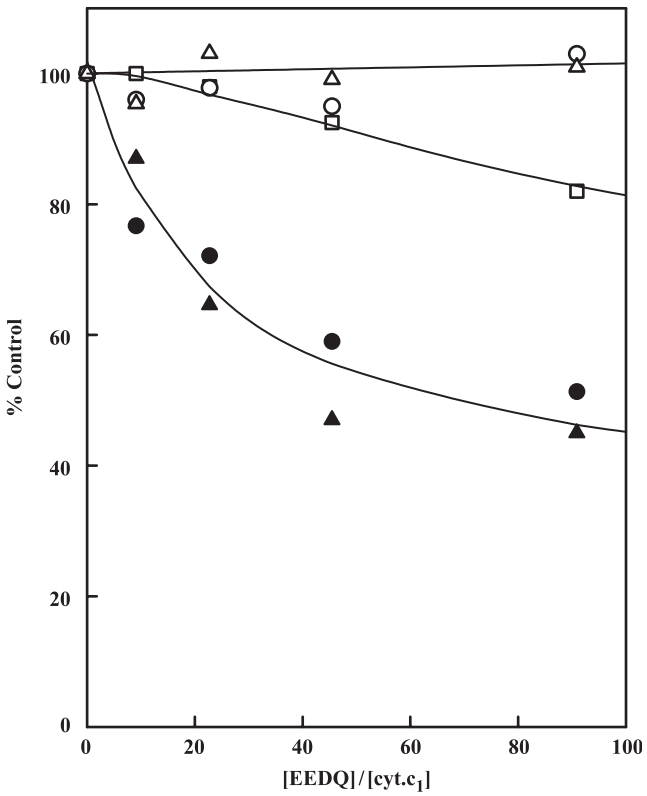

Fig. 4. **Concentration effect of EEDQ on bc_1 vesicles.** The purified bc_1 complex (2.7 nmol cytochrome c_1) was pretreated for 2 h at 0°C with EEDQ at the molar ratios indicated in the Figure. After reconstitution into liposomes, the potential-induced reduction of cytochrome *b* (closed circle), diffusion potential formation (open circle) and proton ejection (closed triangle) were assayed as in Figs 1 and 3. The catalytic activity (open square) and proton permeability (open triangle) of bc_1 vesicle membranes were measured as described in section 'MATERIALS AND METHODS'.

presence of FCCP (dashed line), a H^+/e^- ratio of 1.0 was observed, indicating the scalar release of protons by ubiquinol oxidation. Thus, the net H^+/e^- ratio for proton translocation in the intact bc_1 complex was calculated to be 0.9. The proton pumping activity of the EEDQ-treated bc_1 complex is shown in Fig. 2B. In the experiments, the purified bc_1 complex was pre-incubated for 2 h at 0°C at a molar ratio of 48 mol EEDQ/mol cytochrome c_1 prior to reconstitution into liposomes. The proton translocation activity of the bc_1 complex liposomes was significantly affected by the EEDQ treatment, as indicated by a decreased extent of acidification (solid line), and by the value of the H^+/e^- ratio which was lowered to 0.36 by the EEDQ treatment (40% of control vesicles). These results indicate that EEDQ inhibited proton translocation in the bc_1 complex as reported previously (*15*).

Figure 3 shows the inhibitory effect of EEDQ on the potential-induced reduction of cytochrome *b* in the reconstituted bc_1 complex. As shown in Fig. 3A, the EEDQ treatment of the bc_1 complex contributed to a lowering of the potential-induced reduction of cytochrome *b* to 38% of that observed in control vesicles. Figure 3B shows that the absorbance change of safranine in proteoliposomes containing the EEDQ-treated bc_1 complex is the same as that in control vesicles, indicating that the diffusion potential formation in bc_1 vesicles was insensitive to treatment of the bc_1 complex with EEDQ.

The inhibitory effect of different concentrations of EEDQ on both proton translocation and reversed electron transfer in the bc_1 complex is shown in Fig. 4. Potential-induced cytochrome *b* reduction was inhibited parallel with the proton pumping activity of the reconstituted bc_1 complex. Contrary to the inhibitory effect of EEDQ on proton translocation and on reversed electron transfer, EEDQ treatment caused no changes in the passive proton movement across liposomal membranes and the ability of proteoliposomes to generate a diffusion potential formation. The results eliminate the possibility for EEDQ to perturb the proton and potassium permeability of liposomal membranes. The decrease in the ubiquinol–cytochrome *c* reductase activity by EEDQ treatment was only 17% even at 91 mol EEDQ/mol of cytochrome c_1.

Figure 5 shows a time course of the effect of EEDQ on bc_1 vesicles. Inhibition of proton translocation by EEDQ treatment accompanied a parallel loss in the extent of potential-induced reduction of cytochrome *b* with increasing incubation time. No significant effect of EEDQ on the electron transfer activity and on the diffusion potential formation was observed during the incubation time.

Is Reduction of Heme b_H by Ascorbate Plus TMPD Truly Reverse Electron Transfer?—It is well known that the heme b_H component in the cytochrome bc_1 complex is readily reduced in the presence of both ascorbate and TMPD (*27*). This reduction process is sometimes referred to as 'reverse electron transfer' in the bc_1 complex (*28*, *29*). This term implies that the ascorbate and TMPD system reduces first both cytochrome c_1

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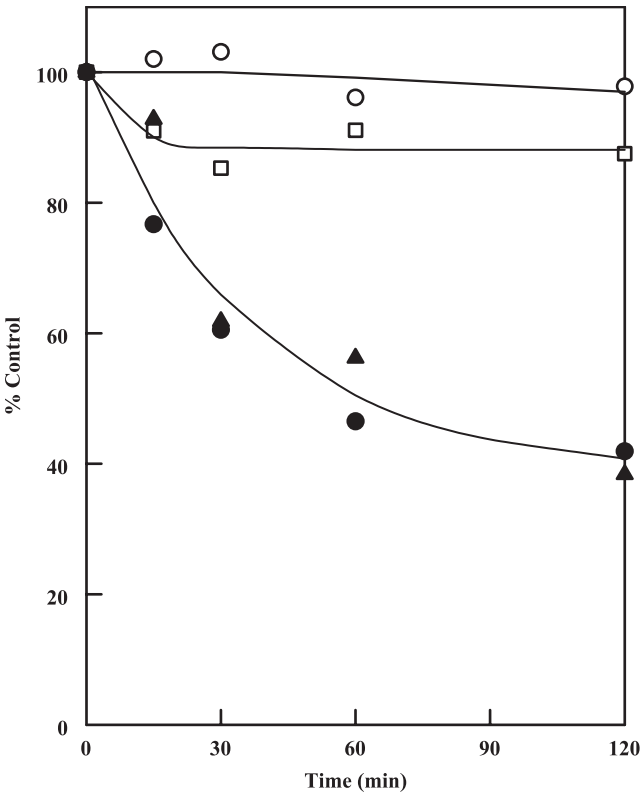

Fig. 5. **Time courses of EEDQ inhibition.** The bc_1 complex (2.7 nmol cytochrome c_1) was incubated with 125 nmol EEDQ at 0°C. The electron transfer activity (open square), proton ejection (closed triangle), the potential-induced reduction of cytochrome *b* (closed circle) and diffusion potential formation (open circle) were measured as described in Fig. 4.

and ISP, then an electron is delivered from ISP to heme b_L via heme b_H (*28*). Ubiquinone was also reported not to be required for the 'reverse electron transfer' in the presence of ascorbate and TMPD (*28*). The result is in contrast to our previous report demonstrating that ubiquinone is an essential component in the potential-induced reversed electron transfer reaction (*14*). We therefore, prepared the ISP-depleted bc_1 complex to examine whether the reduction of heme b_H by ascorbate and TMPD is truly due to reverse electron transfer from ISP to heme b_H (Fig. 6). The purified bc_1 complex (lane 1 in Fig. 6A) was separated into the ISP-depleted complex (lane 2) and ISP (lane 3) by hydrophobic interaction chromatography, as originally reported by Shimomura *et al*. (*24*). We also confirmed the successful reconstitution of the active enzyme from the ISP-depleted complex and separated ISP (data not shown), in accordance with the original report (*24*). As shown in Fig. 6B, the reduction of heme b_H was observed on addition of ascorbate plus TMPD to the ISP-depleted complex reconstituted into liposomes. The extent of heme b_H reduction in the ISP-depleted complex was the same as that in the intact bc_1 complex, although the initial rate of the heme b_H reduction in the ISP-depleted complex was 1.8-fold faster than that in the intact complex. The difference between the initial reduction rates might be ascribed to some conformational changes caused by the depletion of ISP from the bc_1 complex. The ascorbate and PMS system gave a similar result (data not shown). These results clearly indicate that ISP is not involved in the reduction of heme b_H by the ascorbate plus TMPD system. It is more conceivable that TMPD, a membrane-permeable redox mediator (*30*), could give an electron directly to heme b_H.

DISCUSSION

We previously showed that a potassium-diffusion potential generated across liposomal membranes promoted the reverse reaction of electron transfer in the reconstituted bc_1 complex in the presence of an appropriate reductant like sodium ascorbate (*13*). We also demonstrated here that the time course of cytochrome *b* reduction was identical to that of the diffusion potential formation, as long as the valinomycin concentration was expressed as a ratio to the amount of phospholipids (Fig. 1). This result indicates that the reduction of cytochrome *b* is maintained during the formation of a diffusion potential. Extensive dialysis to remove external KCl outside proteoliposomes was required to obtain a good correlation between the time course of the cytochrome *b* reduction and that of safranine response. At a very low concentration of external KCl, a diffusion potential would be generated and decayed in a similar time course both in the safranine method and in the cytochrome *b* reduction.

In our previous report, we demonstrated the essential role of ubiquinone in the reversed electron transfer reaction (*13*). When a small amount of ubiquinone-10 (around 0.4 mol Q_{10}/mol cytochrome c_1) found in the purified preparation of the bc_1 complex was removed by repeated ammonium sulphate precipitation in the presence of sodium cholate, potential-induced reduction of cytochrome *b* was decreased to 7% of total cytochrome *b*. The addition of ubiquinone-2 to the Q-depleted complex recovered the potential-dependent reduction of cytochrome *b* up to 42% of total cytochrome *b* (*13*). The essentiality of ubiquinone for energy-linked reversed electron transfer was confirmed later by Tolkatchev *et al*. (*14*). Contrary to our results, Matsuno-Yagi *et al*. (*28*, 29) recently reported that ubiquinone was not required for 'reverse electron transfer' from cytochrome c_1 to heme b_H in bovine heart submitochondrial particles (SMP). It should be noted, however, that in their experiments the reduction of heme b_H by ascorbate plus either TMPD or PMS was referred to as 'reverse electron transfer' in the bc_1 complex. They demonstrated that heme b_H was readily reduced by the ascorbate/TMPD or the ascorbate/PMS system in SMP, ubiquinone-depleted SMP, and ubiquinone-replenished SMP, thus concluding that ubiquinone is not essential in 'reverse electron transfer' (*28*, *29*). But TMPD and PMS have been used as membrane-permeable redox mediators, although ascorbate is impermeable to mitochondrial membranes as well as to artificial liposomal membranes (*30*). Therefore, it might be probable that the reduced form of TMPD or PMS can react directly with heme b_H in the mitochondrial bc_1 complex. In fact, we observed that ascorbate plus TMPD can reduce heme b_H both in the intact and

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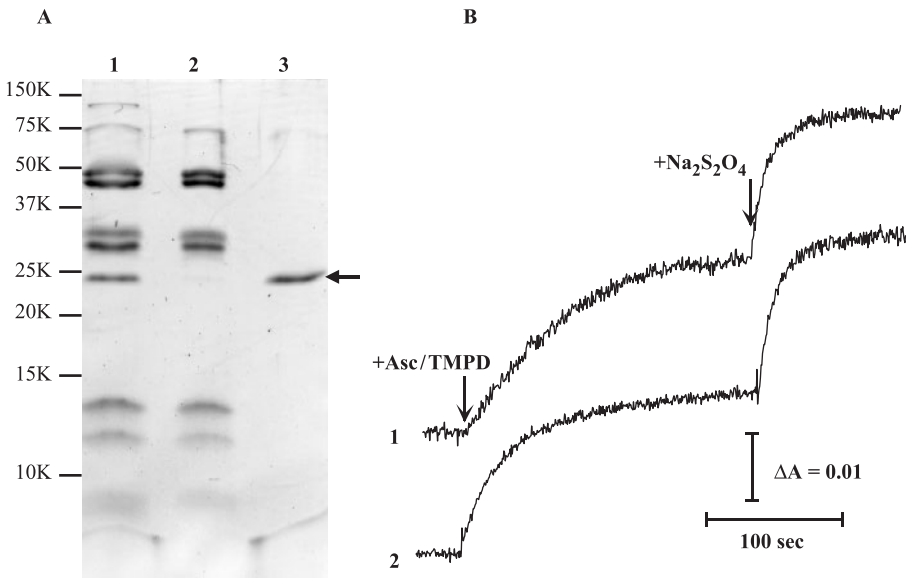

Fig. 6. **Reduction of heme b_H by ascorbate plus TMPD in ISP-depleted bc_1 complex.** (A) SDS–PAGE of the original bc_1 complex as prepared and the ISP-depleted bc_1 complex. The original bc_1 complex (lane 1) was separated into the ISP-depleted complex (lane 2) and the iron–sulphur protein (lane 3) as described in section 'MATERIALS AND METHODS'. An arrow in the figure indicates the Rieske iron–sulphur protein. (B) Reduction of heme b_H by ascorbate plus TMPD. The intact (trace 1) and the ISP-depleted enzyme (trace 2) were incorporated into liposomes, and suspended in 10 mM MOPS/NaOH (pH 7.4) and 100 mM KCl at a final concentration of 0.6 μM cytochrome c_1. Additions indicated in the figure were 10 mM sodium ascorbate plus 100 μM TMPD (Asc/TMPD) and a small amount of solid sodium dithionite ($Na_2S_2O_4$). The redox state of heme *b* was followed at 562 minus 575 nm.

in the ISP-depleted bc_1 complex reconstituted into liposomes (Fig. 6). Since the distance of two iron atoms between heme c_1 and heme b_L is 34.4 Å and an iron atom of heme b_H is a further 20.6 Å away from that of heme b_L in the crystal structure of the bovine heart bc_1 complex (*31*), it seems unlikely that an electron is transferred directly from heme c_1 to heme b_L or heme b_H in the ISP-depleted enzyme. It is more conceivable that the reduced form of the membrane-permeable redox mediator, TMPD or PMS, gives an electron to heme b_H at the Qi site of the bc_1 complex. Starting from the situation where heme b_H is in the reduced form with ascorbate and TMPD, a potassium-diffusion potential generated across liposomal membranes promotes the further reduction of the heme b_L component, as reported previously (*13*).

DCCD has been used to specifically inhibit the proton translocation activity of the bc_1 complex with a minimal loss in the electron transfer activity (*36*). We also demonstrated that DCCD treatment of the bc_1 complex reconstituted in the phospholipids vesicles resulted in the specific inhibition of the reversed electron transfer reaction without a significant effect on the catalytic activity (*13*). Based on the specific inhibitory effect of DCCD, we presented a model in which the reverse flow of protons from the outer medium to the Qo site through cytochrome *b* subunit is coupled to reversed electron transfer in the bc_1 complex, and showed that DCCD specifically blocks the reversible proton flow by modification of the cytochrome *b* subunit (*13*).

EEDQ, a reagent that also reacts with carboxyl groups in proteins, was recently reported to have the similar inhibitory effect on the proton translocation activity of the bc_1 complex and cytochrome *c* oxidase without a significant loss in their electron transfer activity (*15*, *32*). Here, we confirmed the specific inhibitory effect of the chemical modifier on the proton translocation activity of the purified preparation of the bovine bc_1 complex reconstituted into phospholipids vesicles. Moreover, we presented the first report that chemical modification of the bc_1 complex with EEDQ also resulted in the inhibition of the diffusion potential-dependent reduction of cytochrome *b*. Both the proton translocation activity and the energy-linked reduction of cytochrome *b* are inhibited by EEDQ at the same concentration and in the same time-dependent manner (Figs 4 and 5). These results strongly support the idea that the reversed electron transfer proceeds coupled to the reverse flow of protons through the bc_1 complex.

The key step in the reversed electron transfer reaction is the reduction of ubiquinone to ubiquinol at the Qo site with energy provided by a potassium diffusion potential, as proposed previously (*13*). In this reaction, two protons must be delivered from the outer medium to the Qo site to generate ubiquinol from ubiquinone. According to the modified Q cycle (*4*, 34), proton conduction at the Qo site under the normal catalytic cycle is described as follows. When the [2Fe–2S] cluster is reduced by ubiquinol, the imidazole nitrogen on His161 of ISP is protonated by accepting a proton from ubiquinol. After the [2Fe–2S] cluster accepts an electron from ubiquinol, ISP is able to move to the c_1 position where it is oxidized. Thus, the redox reaction is formally a hydrogen transfer from the ubiquinol (non-elecetogenic), since ISP acts

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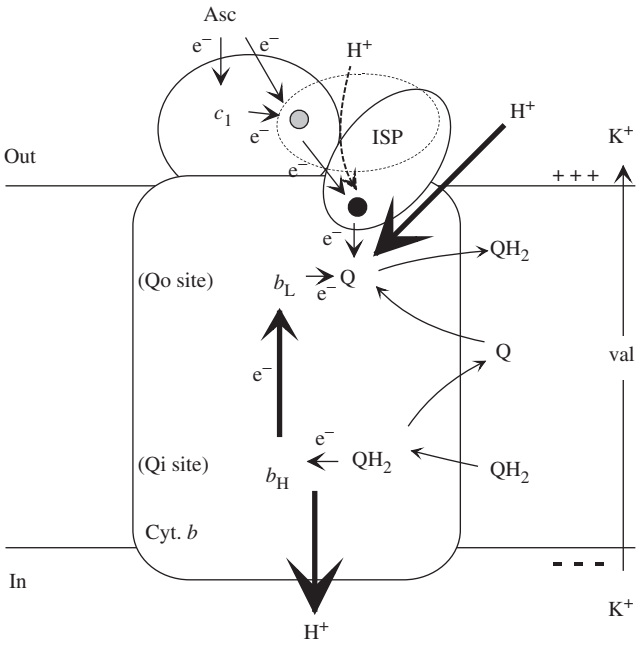

Fig. 7. **A model for EEDQ inhibition of reversed electron transfer in the reconstituted bc_1 complex.** Only the membrane-spanning cytochrome *b* subunit (cytochrome *b*) containing hemes b_L and b_H, the catalytic domains of the iron–sulphur protein (ISP) and cytochrome c_1 (c_1) are illustrated for simplicity. The *b* and c_1 position of the ISP catalytic domain are indicated by a solid and dotted line, respectively, and filled small circles in the ISP catalytic domain represent the [2Fe–2S] cluster. Sodium ascorbate (Asc) reduces both cytochrome c_1 and the [2Fe–2S] cluster. Three potential-dependent steps are shown by bold arrows, and the dashed arrow is postulated to be an EEDQ-sensitive step in reversed electron transfer. Valinomycin (val) mediates a passive flow of potassium (K^+) ions, resulting in the formation of a diffusion potential (negative inside) across liposomal membranes. See text for more explanation.

as both an electron and a proton carrier. When the iron–sulphur cluster is oxidized by cytochrome c_1, the proton of the imidazole nitrogen is released to the inter-membrane space. Although it is less clear how the second proton is pumped out, Glu-271 of cytochrome *b* is speculated to be the acceptor of the second proton from ubiquinol (*34*), suggesting another proton-conducting channel in the cytochrome *b* subunit. Therefore, two protons to generate ubiquinol at the Qo site in the reversed electron transfer would be delivered through ISP and cytochrome *b*, respectively.

Figure 7 shows a model for the potential-induced reversed electron transfer which is constructed on the basis of the modified Q cycle, and is a revised version of the scheme proposed in previous reports (*13*, *14*). In this model, a small part of ubiquinone-10 is postulated to be in a reduced form in the ascorbate-reduced bc_1 complex. Ubiquinol reduces heme b_H at the Qi site to produce ubisemiquinone. Then, a potassium-diffusion potential drives an electron from heme b_H to heme b_L, and ubisemiquinone can reduce heme b_H again. During the oxidation of ubiquinol at the Qi centre, protons are released to the inside of liposomes depending on a diffusion potential. The release of protons might proceed along proton uptake pathways suggested for the ubiquinone reduction at the Qi site in the yeast bc_1 complex (*37*). Ubiquinone generated at the Qi site can freely move to the Qo site, and the reduced form of heme b_L gives an electron to ubiquinone at the Qo site. Simultaneously, a diffusion potential promotes the uptake of a proton from the outer medium to the Qo site, presumably through Glu-271 of the cytochrome *b* subunit. The second electron and the second proton necessary to completely reduce ubiquinone at the Qo site come from the outer medium through cytochrome c_1 and ISP. Then, ubiquinol generated at the Qo site diffuses back to the Qi site of the bc_1 complex in which heme b_H is in the oxidized form. As a result of multiple turnovers of the ubiquinol oxidation at the Qi site and the ubiquinone reduction at the Qo site, there is a gradual reduction of cytochrome *b* induced by a diffusion potential. Thus, three potential-dependent processes consisting of proton uptake from the outside medium, proton release to the inside of liposomes, and electron movement from heme b_H to heme b_L are involved in the reversed electron transfer reaction.

Cocco *et al*. (*15*, 16) showed that EEDQ modified Core protein II and ISP in the bovine heart cytochrome bc_1 complex, whereas in the three-subunit *P. denitrificans* bc_1 complex, ISP and cytochrome c_1 became labeled with EEDQ. Furthermore, they argued that the conserved Asp-160 of the *P. denitrificans* ISP (equivalent to Asp-166 of the bovine ISP) is a plausible candidate of the site of specific modification, although the sites of EEDQ modification have not unambiguously been established in both cases. On the basis of the binding experiments, Cocco *et al*. (*16*) proposed that the ISP subunit contributes with its carboxyl residue(s) to the proton translocation mechanism of both bacterial and eucaryotic complexes, thus lowering the H^+/e^- ratio of the proton translocation in the bc_1 complex. According to their proposal, the inhibitory effect of EEDQ on the reversed electron transfer reaction could be explained as follows (Fig. 7). In the experiments of the reversed electron transfer reaction, both cytochrome c_1 and ISP were reduced first with sodium ascorbate. X-ray diffraction studies have indicated that ISP is largely in the *b* position where the [2Fe–2S] cluster is located near heme b_L, when both cytochrome c_1 and ISP are in the reduced form (*35*). When ISP resides in the *b* position, energy provided by a diffusion potential would be required to promote the uptake of protons from the outer medium to the Qo site through the ISP subunit. EEDQ bound to acidic amino acid residue(s) of ISP might block the diffusion potential-dependent uptake of protons through ISP, resulting in inhibition of reversed electron transfer in the bc_1 complex. However, no proton pathway has yet been documented in the water-soluble head domain of ISP thus far. A possible way to supply protons to the Qo site might be the entry part of 'the proton leakage pathway' described by Gurung *et al*. (*33*). They reported the generation and characterization of *R. sphaeroides* strains expressing cytochrome bc_1 complexes with substitutions at one or both the histidine ligands of the [2Fe–2S] cluster. These mutants lacked the [2Fe–2S] cluster because of missing histidine ligands and,

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thus, possessed no bc_1 activity. However, when the mutant complex lacking the [2Fe–2S] cluster was co-reconstituted in phospholipid vesicles together with the intact bovine mitochondrial bc_1 complex or cytochrome *c* oxidase, proton ejection, normally observed in intact reductase or oxidase vesicles during the oxidation of their corresponding substrates, disappeared. These results indicated that elimination of the [2Fe–2S] cluster in mutant bc_1 complexes opened up a 'proton leakage' pathway within the bc_1 complex, and the authors argued that the [2Fe–-2S] cluster may function as a proton-exiting gate in the normal catalytic cycle of the bc_1 complex (*33*).

We postulated that an electron and a proton are delivered separately to the Qo site through ISP in the EEDQ-modified complex to account for the role of ISP in the specific inhibitory effect of EEDQ on proton translocation and reversed electron transfer (Fig. 7). In the Rieske protein family, however, the oxidation state and the histidine protonation status were reported to be coupled (*39*, 40), suggesting that the two histidine ligands are expected to be protonated, whenever the [2Fe–2S] cluster is reduced. If this is the case for the ascorbate-reduced bc_1 complex, another mechanism could be suggested for the inhibitory effect of EEDQ on the bc_1 complex. In the reversed electron transfer reaction, a proton of the protonated histidine ligand (His-161) is supposed to be transferred to ubisemiquinone together with an electron of the reduced [2Fe–2S] cluster to generate ubiquinol at the Qo site (*34*). Another proton of the histidine ligand (His-141) would also be released at the Qo site upon oxidation of the reduced [2Fe–2S] cluster, and the released proton is expected to go to the inside of liposomes (through an unknown pathway) depending on a potassium-diffusion potential (negative inside). If EEDQ modification of ISP leads to the inhibition of the process of the proton release to the inside of liposomes, the reversed electron transfer might also be blocked due to the tight coupling between deprotonation of the histidine ligands and the oxidation of the [2Fe–2S] cluster. Addressing the lack of a significant loss in the electron transfer activity in the chemically modified bc_1 complex despite the decreased H^+/e^- ratio, it has been suggested that protons might diffuse back to the negative side of membranes upon modification (*13*, *15*, *36*). In the case of EEDQ modification, however, EEDQ is supposed to be bound to the hydrophilic domain of ISP. The only chance for protons in ISP to diffuse back to the negative side would be when ISP is located at the b position. In the EEDQ-modified complex, a proton transferred from ubiquinol to ISP might be trapped somewhere in ISP until ISP moves to the b position in the next turnover.

Cocco *et al*. (*16*) argued that Asp-160 of *Paraccocus* ISP (counterpart of Asp-166 of bovine ISP) is a possible binding site of EEDQ, suggesting that the aspartate residue plays a key role in the proton translocation of the bc_1 complex. In the crystal structure of the bovine bc_1 complex, Asp-166 of the ISP subunit is located near the protein surface and is adjacent to the conserved Tyr-165 that forms a hydrogen-bond to Cys-139, the cysteine ligand of the [2Fe–2S] cluster. Guergova-Kuras *et al*. (*38*) reported that the site-directed mutant strain of *R. sphaeroides* cytochrome bc_1 complex—where Tyr156 (equivalent to Tyr-165 of the bovine ISP) was mutated to Trp—showed a decrease in Em and an increase in pK (from 7.6 to 8.5) and modified EPR properties of ISP. We, therefore, performed EPR experiments for the EEDQ-treated and untreated bc_1 complex to examine whether the modification with EEDQ could affect the EPR spectrum of the [2Fe–2S] cluster. The EPR spectra at 12 K of the dithionite-reduced bc_1 complex treated with EEDQ were, however, very similar to those of the intact complex (data not shown). The *g* values of the g_x and g_y signals were found to be 1.77 and 1.90, respectively, in both the EEDQ-modified and the unmodified complex. Moreover, stigmatelline, a Qo site inhibitor, shifted the g_x and g_y signals to $g = 1.79$ and $g = 1.89$, respectively, in both the EEDQ-treated and the untreated complex. These results suggest that EEDQ modification of the bc_1 complex does not affect the physical properties of the [2Fe–2S] cluster, although the EPR experiments do not exclude the possibility that EEDQ is bound to Asp166 of bovine ISP.

Ebert *et al*. (*17*) recently reported that when Asp-186 of yeast ISP, the residue analogous to Asp-166 in bovine ISP, was mutated to alanine, the mutant bc_1 complex (D186A) can still pump protons with a H^+/e^- ratio of 1.0 identical to that of the wild type. Moreover, EEDQ is bound to both wild type and D186A iron–sulphur proteins, and EEDQ had no effect on electron transfer activity, but did inhibit the rate of proton pumping across the membrane in both the wild type and the D186A mutant (*17*). These results suggest that EEDQ must bind to a carboxyl group other than Asp-186 in yeast ISP and that this residue may be involved in the proton translocation reactions under normal circumstances. Ebert *et al*. (*17*) speculated Asp152, Asp123 or Glu105 of bovine ISP as a possible candidate(s) for the EEDQ modification site, since these acidic residues are conserved across most species. It has to also be noted that Cocco *et al*. and Ebert *et al*. have attempted to detect the EEDQ-modified subunits or peptide fragments by using 4′-[(amino-acetamido)methyl] fluorescein (AMF), which forms an amide bond with a carboxyl-specific reagent such as EEDQ and can thus be identified by its fluorescence (*15–17*). With such an approach, albeit its convenience, one might simply fail to detect EEDQ-modified sites (or subunits) to which AMF is inaccessible. Further studies are required to elucidate the inhibitory effect of EEDQ on proton translocation coupled to the electron transfer reaction of the bc_1 complex.

Supplementary data are available at JB online.

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