Chemical Modification of the Bovine Mitochondrial bc_1 Complex Reveals Critical Acidic Residues Involved in the Proton Pumping Activity[†]

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ABSTRACT: Bovine heart ubiquinol−cytochrome *c* reductase (bc_1 complex) was modified with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), which is a selective reagent for buried carboxyl groups. EEDQ treatment caused a loss of the proton pumping activity of liposome-reconstituted bc_1 complex, without effect on the passive proton conductivity of the proteoliposomes. Although the decoupling effect produced on proton translocation was similar to that elicited by *N*,*N*′-dicyclohexylcarbodiimide (DCCD) modification of cytochrome *b* and subunit IX, EEDQ modified different subunits, namely the Core protein II and the iron−sulfur protein (ISP). A time-dependent increase of the labeling of both subunits was observed which was kinetically comparable with the decrease of the H^+/e^- ratio. Trypsin treatment of the complex showed that the EEDQ-modified carboxyl group in the ISP belongs to the protruding moiety of the protein, holding the Fe/S cluster. The results obtained show that critical acidic residues, located in different subunits of the bc_1 complex, at both sides of the membrane, contribute to its proton pumping activity.

Ubiquinol−cytochrome *c* oxidoreductase (bc_1 complex) of the mitochondrial respiratory chain catalyzes electron transfer from ubiquinol to ferricytochrome *c* and translocates protons from the matrix to the intermembrane space. The electrochemical gradient thus generated can be used to drive ATP synthesis by the F_0F_1-ATPsynthase or substrate and ion transport across the inner membrane.

The number of protons translocated for each electron transferred by the complex, i.e., the H^+/e^- ratio, is two under level-flow conditions, that is, under conditions of negligible proton-motive force (Δp).[1] Only one of these two protons is vectorially translocated from the negative (N) to the positive (P) phase of the membrane, the other being released directly at the P site as derived formally from the oxidation of quinol by ferricytochrome *c*.

The H^+/e^- ratio has been shown to decrease under conditions where the electron-transfer activity is unaffected. This effect, which is referred to as the decoupling effect, was first shown after covalent modification of bc_1 complex subunit(s) with DCCD (*1*−*4*). Similarly, a decoupling effect was produced upon limited proteolysis of the polypeptide subunits of the bc_1 complex isolated from bovine heart (*5*, *6*). A decoupling of the bc_1 complex in *Saccharomyces cerevisiae* has been shown to be caused by the point mutation G137E in the cytochrome *b* subunit (*7*).

This intrinsic property of the bc_1 complex to be decoupled, which is apparently shared also by the cytochrome-*c* oxidase (*8*), has been observed in more physiological conditions. Our group has shown that the respiration-dependent transmembrane pH difference (the chemical component of the proton-motive force) affects the proton translocation activity of the bc_1 complex at the steady-state respiration. The H^+/e^- ratio was found to be in linear inverse correlation with the extent of the measured transmembrane pH gradient (*9*, *10*). An interesting observation within this work was that anions of weak acids such as azide or arachidonate (whose p*K*s are around 4.5) reversed to a substantial extent the decoupling effect exerted by ΔpH on the proton pump of the bc_1 complex (*11*). These results support the general concept that protonation/deprotonation events associated with oxidoreduction of the quinone catalytic center require proton conductive pathways linking the aqueous bulk phases to the redox center (*12*−*14*), and indicate that the protonation of the residue(s) involved represents a critical event in the vectorial proton translocation process. The weak acids may mimic the protolytic residue(s) in facilitating the protonation of the redox quinone couple, thus acting as protein internal protonophores. On the basis of these and analogous findings in point-mutated bacteriorhodopsin (*15*) as well as in the bacterial reaction center (*16*, *17*), the attention was focused on acidic residues as directly involved in the mechanism of energy-linked proton translocation in membrane proteins and in particular in the bc_1 complex (*13*, *18*).

Hence we were interested in extending the study of the modification of carboxyl residues in the subunits of the bc_1 complex which may be involved in the proton pumping

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[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; AMF, 4′-((aminoacetamido)methyl)fluorescein. ISP, iron−sulfur protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DQH_2, duroquinol; Δp, transmembrane proton-motive force; ΔpH, transmembrane pH gradient; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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activity. Beattie and co-workers have suggested that aspartate 160, in the non-membrane-spanning helix *cd* of yeast cytochrome *b*, is most likely the residue whose modification causes decoupling of the bc_1 complex (*19*). This group has furthermore proposed that aspartate-155 or glutamate-166 in the cytochrome b_6 may play an analogous role in the proton translocation mechanism of the cytochrome *bf* complex of the thylakoid membranes (*20, 21*). As far as the bovine enzyme is concerned, evidence was provided that decoupling by DCCD was correlated with the modification of the 8 kDa subunit IX (*3, 22*), thereafter referred to as the DCCD-binding protein (*23*). Subsequently, DCCD modification of residue(s) in both cytochrome *b* and subunit IX was shown to accompany decoupling of the complex (*24*).

Here we report on the modification of the bovine bc_1 complex by EEDQ. It is shown that EEDQ treatment caused decoupling of the complex, although the subunits which underwent modification are different from those modified by DCCD. The results show that critical acidic residues, located in different subunits of the bc_1 complex, at both sides of the membrane, contribute to the proton pumping activity.

MATERIALS AND METHODS

Purification of Cytochrome-c Reductase and Cytochrome-c Oxidase Complexes. The cytochrome-*c* reductase and cytochrome-*c* oxidase complexes were isolated from bovine heart mitochondria according to Rieske (25) and Errede et al. (26), respectively.

Preparation of bc_1 Vesicles. Reconstitution of the bc_1 complex into phospholipid vesicles was performed by the cholate dialysis method of Leung and Hinkle (*27*) as described by Cocco et al. (*11*).

Measurement of Cytochrome-c Reductase and Proton-Motive Activities in bc_1 Vesicles. Reductase activity of liposome-reconstituted bc_1 complex was measured with a dual-wavelength spectrophotometer (Johnson Research Foundation, Philadelphia) at the wavelength couple 550−540 nm in a basic reaction mixture containing 1 mM K-Hepes, pH 7.4, 100 mM KCl, 1 mM KCN, 7.5 μM ferricytochrome *c*. Duroquinol was used as substrate.

Redox-linked proton translocation was measured essentially as described by Lorusso et al. (*3*). Spectrophotometric determination of cytochrome *c* reduction and electrometric determination of proton translocation were carried out simultaneously on the same sample of vesicle suspension in the following medium: 1 mM K-Hepes, pH 7.4, 100 mM KCl, 1 mM KCN, 7.5 μM ferricytochrome *c*, and 1 μg/mL valinomycin.

Modification of bc_1 Complex with EEDQ and DCCD. The bc_1 complex (20 mg of protein/mL) in 10 mM K-Hepes, pH 7.0, 0.02% Tween 80, was incubated at 0 °C with methanolic solutions of EEDQ and DCCD at the concentrations specified in the legends to the figures and, where indicated, in the presence of 16 mM 4′-((aminoacetamido)methyl)fluorescein (AMF). Control samples were treated only with the organic solvent. After incubation, aliquots of the bc_1 complex suspension were directly added to a sonicated phospholipid suspension or precipitated in 90% cold acetone. The pellets were solubilized in 5% SDS, 15% glycerol, 50 mM Tris, pH 6.8, 2% β-mercaptoethanol and subjected to SDS−PAGE.

Electrophoretic Analysis of the bc_1 Complex. SDS−PAGE analysis was performed on slab gels (10 cm long, 0.1 cm thick) according to Schagger and von Jagow (*28*) using a Tris/Tricine buffer system at pH 6.8. Slab gels were run at 30 V for 2 h and at 90 V overnight. Gels were either stained with Coomassie Brilliant Blue G and destained or soaked in 50% methanol for 6 h and then photographed on a dark surface under an ultraviolet light source.

Binding of [^{14}C]DCCD to bc_1 Polypeptides. The bc_1 complex (20 mg/mL), preincubated 30 min at 0 °C in the absence or in the presence of EEDQ, was further incubated 30 min with [^{14}C]DCCD. Aliquots of the bc_1 suspension were withdrawn and precipitated in 90% cold acetone, and the resulting pellets were processed for electrophoresis. After destaining, the gels were dried and the autoradiography was performed using a Kodak X-OMAT-AR film at −70 °C for 10 days.

Spectrophotometric Determination of Redox Changes of Cytochrome b and c_1. Redox changes of cytochrome *b* were followed with a double-beam dual-wavelength spectrophotometer, at the wavelength couples 562−575 and 566−575 nm. Redox changes of cytochrome c_1 were followed at 552−540 nm. For steady-state experiments, bc_1 vesicles (0.9 μM cytochrome c_1), suspended in a medium containing 1 mM K-Hepes, pH 7.4, 100 mM KCl, were supplemented with 0.2 μM soluble cytochrome-*c* oxidase and 0.2 μM ferricytochrome *c*. Duroquinol (200 μM) was used as substrate.

For pre-steady-state measurements, bc_1 vesicles (0.4 μM cytochrome c_1) were suspended in the assay mixture containing 1 mM K-Hepes, pH 7.4, 100 mM KCl, 1 mM KCN, and 1 μg/mL valinomycin. Temperature was 10 °C.

Measurement of Membrane Potential. The membrane potential generated by respiration in bc_1 vesicles was monitored following the fluorescence quenching of externally added safranin at the excitation and emission wavelengths of 525 and 575 nm, respectively. bc_1 vesicles (0.2 μM cytochrome c_1) were suspended in a mixture containing 1 mM K-Hepes, 100 mM KCl, 0.1 μM soluble cytochrome-*c* oxidase, 200 μM duroquinol, and 1 μg/mL nigericin. Respiration was started by the addition of ferricytochrome *c*.

Materials. EEDQ and DCCD were obtained from Sigma Chemical Co. AMF was obtained from Molecular Probes Inc. [^{14}C]DCCD (54 mCi/mmol) was obtained from Amersham in toluene and was evaporated to dryness under a stream of nitrogen and redissolved in methanol just prior to the experiment. All other reagents were of the highest purity grade commercially available.

RESULTS

Proton Translocation Activity. EEDQ is a carboxyl group-specific reagent (*29, 30*) widely used to modify acidic residues in proteins. Modification by EEDQ of cytochrome-*c* oxidase isolated from bovine heart resulted in about 70% inhibition of vectorial proton translocation activity, similarly to what was produced by DCCD modification (*31*). The effect of EEDQ modification on proton translocation activity of the bc_1 complex is shown in Figure 1. Treatment of the bc_1 complex with EEDQ for 1 h at 0 °C, before reconstitution into phospholipid vesicles, caused a concentration-dependent decrease of both the rate and the extent of proton translo-

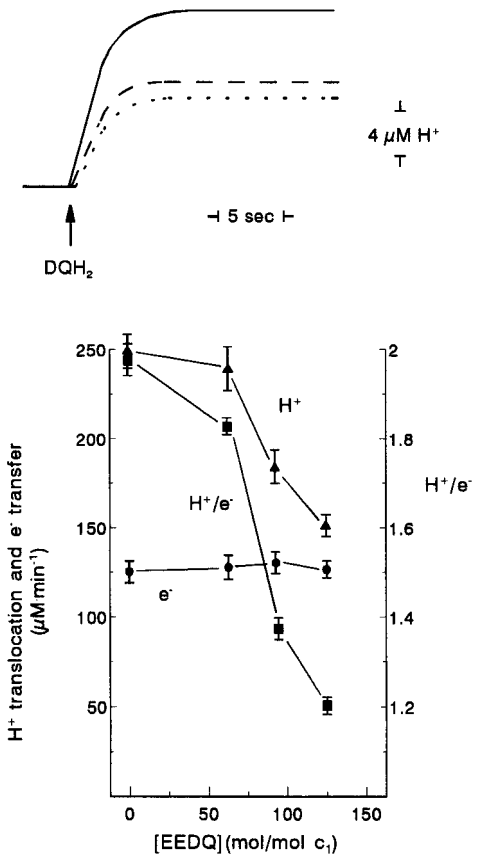

FIGURE 1: Effect of EEDQ modification on proton translocation activity of bc_1 complex. The bc_1 complex was treated with EEDQ for 1 h prior to the reconstitution into phospholipid vesicles. The bc_1 vesicles were suspended at a concentration of 0.7 μM cytochrome c_1 in the reaction mixture described under Materials and Methods. The reaction was started by the addition of 11 μM duroquinol: (—) control; (- - -) bc_1 complex treated with 10 mM EEDQ (125 mol/mol of cytochrome c_1); (···) plus 3 μM CCCP. Rates of proton release in the upper traces of the figure are 245 and 152 μM min^{-1} in the control and in the EEDQ-treated enzyme, respectively. The rate of antimycin-insensitive proton release and cytochrome *c* reduction amounted to about 2% of the values obtained in the absence of the inhibitor.

cation, whereas the rate of electron-transfer activity was unaffected. At 125 mol/mol of cytochrome c_1 the level flow H^+/e^- ratio was decreased by about 50%, indicating suppression of the electrogenic proton pump activity, the remaining release of 1 H^+/e^- resulting from the scalar oxidation of the quinol by the electron acceptor ferricytochrome *c*. The EEDQ-dependent decoupling effect is further substantiated by the experiments reported in Figure 2. It is shown (Figure 2A) that treatment of the bc_1 complex with EEDQ completely suppressed the respiratory control in bc_1 vesicles, causing an enhancement of the coupled rate of the electron transfer (uncoupler absent) of the modified enzyme to a value attained by the control enzyme under uncoupled conditions. Tracing of fluorescence quenching of safranin (Figure 2B) added to the bc_1 vesicles respiring at the steady-state, in the presence of cytochrome *c* and soluble cytochrome-*c* oxidase, indicates that EEDQ treatment almost completely abolished the capability of the bc_1 complex to set up the membrane potential across the liposomal membrane. Accordingly, the typical responses of the steady-state redox level of the cytochrome *b* to the collapse of either of the components of the proton-motive force (*12*) were almost

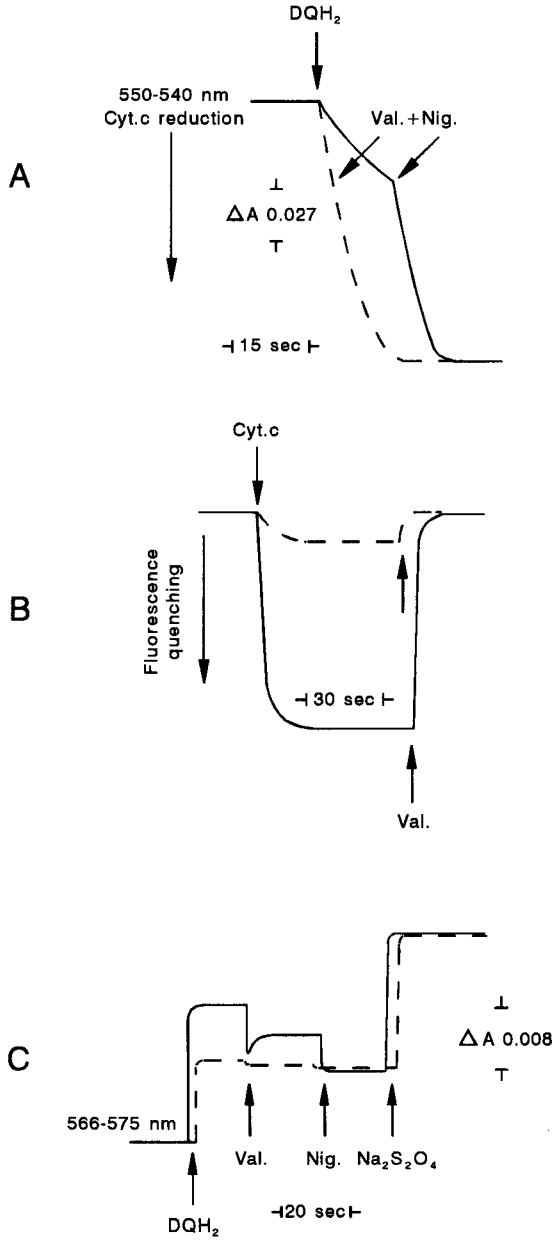

FIGURE 2: Effect of EEDQ modification on respiratory control, steady-state membrane potential, and steady-state redox level of cytochrome *b* in bc_1 vesicles. The bc_1 complex was treated with EEDQ (125 mol/mol of cytochrome c_1) for 1 h and reconstituted into phospholipid vesicles. (A) Cytochrome-*c* reductase activity was measured as described under Materials and Methods using 10 μg of protein/mL. Where indicated, 33 μM duroquinol and 0.1 μg/mL each of valinomycin and nigericin were added. (B) Membrane potential generated by duroquinol respiration was monitored following the fluorescence quenching of externally added 3 μM safranin. Where indicated, 0.2 μM ferricytochrome *c* and 1 μg/mL valinomycin were added. (C) Steady-state redox level of cytochrome *b* in turning-over bc_1 vesicles was measured as described under Materials and Methods. The reaction was started by addition of 200 μM duroquinol. Where indicated, 1 μg/mL each of valinomycin and nigericin were added, followed by few grains of sodium dithionite: control (solid lines); EEDQ-treated bc_1 complex (dashed lines).

completely suppressed in vesicles containing the EEDQ-modified bc_1 complex (Figure 2C). Treatment of the bc_1 complex with DCCD (50 mol/mol of cytochrome c_1) prior to the reconstitution into vesicles caused the same effects (not shown).

FIGURE 3: Autoradiogram of [^{14}C]DCCD treated bc_1 complex after SDS−PAGE. The bc_1 (20 mg/mL) complex preincubated with and without EEDQ (125 mol/mol of cytochrome c_1), was treated with 50 mol of [^{14}C]DCCD/mol of cytochrome c_1. After incubation the samples were precipitated in 90% cold acetone. The pellets were solubilized, and the samples were subjected to SDS−PAGE. Autoradiography was performed as described under Materials and Methods: (a) control; (b) bc_1 complex pretreated with EEDQ.

To exclude any impairment in the liposome insertion of the bc_1 complex, caused by EEDQ treatment, the following control experiments were carried out: (i) the vesicles containing either the control or the EEDQ-treated bc_1 complex were passed through an AcA-34 column, and this showed that the enzyme was strictly associated to phospholipids; (ii) oxidation by ferricyanide of the duroquinol-reduced bc_1 complex showed that vesicles, containing either the control or the EEDQ-treated enzyme, were 90% right-side oriented; (iii) measurement of valinomycin-mediated proton diffusion across the bc_1 vesicle membrane showed that the suppression of redox-linked proton translocation, caused by EEDQ-treatment of the bc_1 complex, was not due to enhancement of the rate of passive proton back-diffusion.

bc_1 Complex Subunits Modified by EEDQ. To identify those bc_1 complex subunits whose modification may be correlated with the observed decoupling effect, we have followed two independent approaches: (i) difference of labeling by [^{14}C]DCCD of the bc_1 complex preincubated with and without EEDQ and (ii) direct measurement of the binding of EEDQ in the presence of the hydrophobic nucleophile 4′-((aminoacetamido)methyl)fluorescein (AMF) which labels EEDQ- (and DCCD-) activated carboxyl residues by formation of an amide bond (*32*, *33*). Figure 3 shows the labeling by a saturating concentration of [^{14}C]DCCD of the subunits of the bc_1 complex. As previously reported (*3*, *24*), 2 out of the 11 subunits of the complex, namely the cytochrome *b* and subunit IX, appeared labeled by [^{14}C]DCCD. Preincubation of the bc_1 complex with EEDQ (125 mol/mol of cytochrome c_1) did not prevent the labeling of both the subunits; on the contrary, a slight increase of the band intensities could even be observed. In the same experiment, samples of the enzyme suspension were withdrawn for reconstitution into liposomes and measurement of the proton translocating activity. It was observed that both reagents, added either separately or together, caused the H^+/e^- ratio to drop to 1.0−1.2 without affecting the rate of electron transfer. It thus appears that EEDQ and DCCD, though giving rise to the same decoupling effect, modify different subunits, as the binding of each is not competitive.

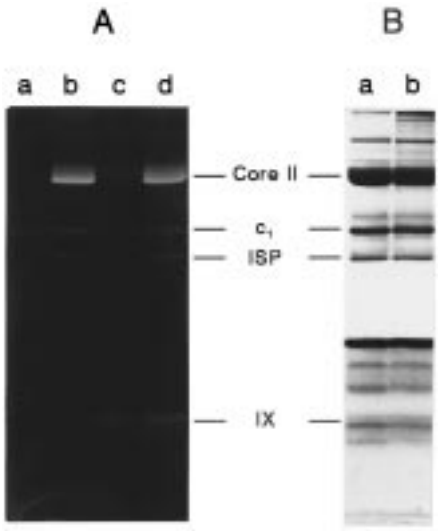

FIGURE 4: Binding of EEDQ and DCCD to bc_1 complex subunits. (A) The bc_1 complex was treated for 1 h without (a) and with 125 mol of EEDQ/mol of cytochrome c_1 (b), for 30 min with 50 mol of DCCD/mol of cytochrome c_1 (c), and for 30 min with EEDQ (125 mol/mol of cytochrome c_1) followed by a further 30 min incubation with 50 mol of DCCD/mol of cytochrome c_1 (d). AMF (16 mM, dimethylformamide solution) was also present in the incubation mixture of all samples (a−d). After electrophoresis the gel was exposed to ultraviolet light and photographed. (B) Coomassie-stained gel of the control (a) and EEDQ-treated (b) bc_1 complex.

This is further substantiated by the experiment illustrated in Figure 4. Here the incubation of the bc_1 complex with EEDQ (lane b), DCCD (lane c), and DCCD and EEDQ added together (lane d) was carried out in the presence of fluorescent AMF, which specifically requires a carboxyl residue modified by either reagent to bind to. In the control sample (lane a), AMF does not bind to any subunit and the only fluorescent band refers to the cytochrome c_1 which is intrinsically fluorescent. The appearance of the AMF fluorescence shows, for the reasons specified above, that EEDQ reacts with carboxyl residues located in the Core protein II and ISP, whereas DCCD reacts with a carboxyl residue only in subunit IX. Where present together in the incubation medium, EEDQ and DCCD appear to react independently of each other and Core protein II, ISP, and subunit IX are all labeled, with no band being subdued. Again, as indicated above (Figure 3), the binding of DCCD to the subunit IX seems to be somewhat increased by the preincubation of the enzyme with EEDQ. Interestingly, the *b* cytochrome, although labeled by direct binding of [^{14}C]-DCCD (Figure 3), does not appear to be labeled by fluorescent AMF. The Coomassie Blue stained gel of the bc_1 complex shows that EEDQ modification did not cause any apparent change in the electrophoretic pattern of the enzyme (Figure 4B).

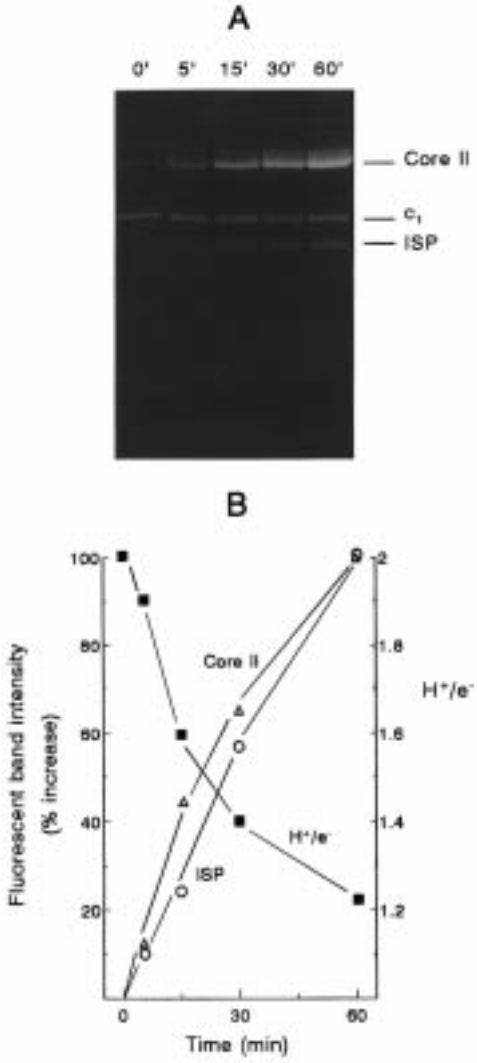

FIGURE 5: Time course of the effect of EEDQ binding to bc_1 complex subunits. The bc_1 complex was treated with EEDQ (125 mol/mol of cytochrome c_1) in the presence of 16 mM AMF. At the times indicated, aliquots of the bc_1 suspension were added to sonicated phospholipid suspension or precipitated in acetone and subjected to SDS−PAGE. After electrophoresis, the gel was exposed to ultraviolet light and photographed (A) The fluorescence of the bands was determined by scanning densitometry of the negative of the photograph and plotted as the percentage of the 60 min band intensities (open symbols) (B) The time course of the effect of EEDQ on the H^+/e^- ratio is also reported (■). The values reported represent the mean of three experiments. For other experimental conditions see Materials and Methods.

The time course of carboxyl residue modification in the Core protein II and ISP by EEDQ is reported in Figure 5. A time-dependent increase in the labeling of both subunits was observed over a 60 min incubation, with comparable kinetics. At longer incubation times, no further detectable increase of the labeling was observed. Measurement of proton translocation activity showed a time-dependent decrease of the H^+/e^- ratio, which correlates fairly well with the labeling of the two subunits.

To establish the binding site of EEDQ on the ISP, the bc_1 complex was reacted with EEDQ, in the presence of AMF, after limited proteolysis with trypsin. Tryptic digestion causes cleavage of the first 10 N-terminal residues of Core protein II and also generates a fragment of apparent M_r of 25 kDa which remains attached to the complex (*34*). The

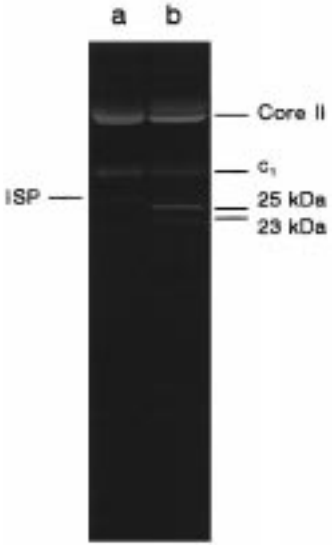

FIGURE 6: EEDQ binding on trypsinized bc_1 complex. The bc_1 complex (5 mg/mL) in 20 mM Tris, pH 8.2, was incubated with trypsin (1:100, w/w) at 25 °C for 1 h. Protease digestion was stopped by the addition of a 5-fold excess of trypsin inhibitor. The suspension was centrifuged as described by Cocco et al. (6), and the pellets were resuspended at a concentration of 20 mg/mL in 10 mM Hepes, pH 7.0, 0.02% Tween 80. Treatment of the control (a) and of trypsinized bc_1 complex (b) with EEDQ was carried out as described in the legend to Figure 4 and under Materials and Methods.

ISP is also digested by trypsin and two cleavage products, both containing the Fe/S cluster, are formed. They differ from one another in 20 residues, and none of these is a carboxyl residue. The larger product (apparent M_r 23 kDa) is retained by the complex (*6*, *34*). A similar proteolytic fragment was recently isolated, crystallized, and shown to retain the native spectroscopic and redox properties (*35*). The crystal structure of this fragment was furthermore determined (*36*). Figure 6 shows that this 23 kDa cleavage product of the ISP contains the residue modified by EEDQ. The 25 kDa cleavage product of the Core protein II also retained the EEDQ modified site.

Kinetics of Cytochrome b and c_1 Reduction. Figure 7A shows the effect of EEDQ modification on pre-steady-state reduction kinetics of cytochrome *b* and c_1 by duroquinol in bc_1 vesicles supplemented with antimycin. A definite and reproducible 30% inhibition of the rate of cytochrome *b* reduction was observed, whereas the rate of cytochrome c_1 reduction measured at 552−540 nm was quite unaffected. With myxothiazol present, no effect on the rate of reduction of cytochrome *b* was observed. Thus it appears that the reaction of EEDQ with the bc_1 complex inhibits, partly, the antimycin-insensitive pathway of cytochrome *b* reduction, the quinol oxidation site of the Q-cycle model. Consistently, as shown in Figure 7B, EEDQ treatment also caused a significant inhibition of the antimycin-promoted oxidant-induced extrareduction of cytochrome *b*, whereas no effect was observed on cytochrome c_1 oxidation by ferricyanide.

Antimycin titration of the electron-transfer activity showed that EEDQ treatment promoted a sigmoidal titration curve for the inhibitory action of antimycin, with its I_{50} (the concentration giving 50% inhibition) being shifted to a value some 30% higher than that for the control enzyme (results not shown).

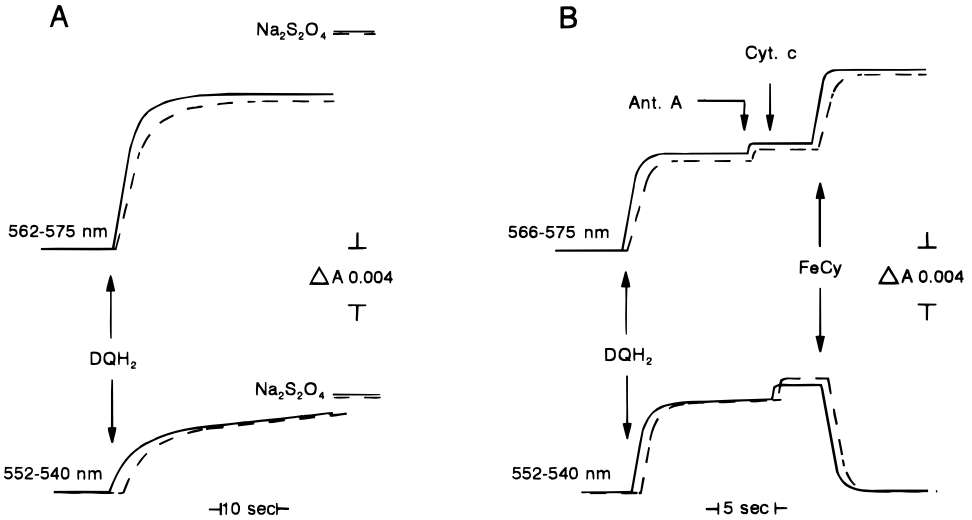

FIGURE 7: Effect of EEDQ on pre-steady-state reduction of cytochrome *b* and c_1 and oxidant-induced extra-reduction of cytochrome *b* in bc_1 vesicles. The bc_1 complex was treated with EEDQ (125 mol/mol of cytochrome c_1) and reconstituted into liposomes. The vesicles were suspended (final concentration of 0.4 μM cytochrome c_1) in the reaction mixture described under Materials and Methods. (A) The mixture was supplemented with 2.5 μM antimycin, and the reaction was started by the addition of 17 μM duroquinol. (B) The reaction was started by the addition of duroquinol (33 μM) followed, where indicated, by antimycin (2.5 μM), ferricytochrome *c* (0.07 μM), and ferricyanide (FeCy) (40 μM): control (solid lines); EEDQ-treated bc_1 complex (dashed lines).

DISCUSSION

The reaction of the bc_1 complex with EEDQ gives rise to a decoupling effect, with suppression of the vectorial proton pump activity. The effect is similar to that produced by DCCD modification (*1*−*4*). EEDQ is a selective reagent for buried carboxyl groups (*29*), whereas DCCD reacts predominantly with carboxyl and sulfhydryl groups as well as with tyrosines (*32, 37*). Upon reaction of DCCD (or EEDQ) with protein carboxyl groups, the product undergoes, in the presence of an excess of a hydrophobic nucleophile, further reaction giving rise to an amide derivative product (*37, 38*). [^{14}C]DCCD binds, as reported, to cytochrome *b* and subunit IX (*3, 22, 24*) (Figure 3). The nucleophile AMF did not label the DCCD-modified cytochrome *b* (Figure 4). The subunit IX, modified by DCCD, was, on the contrary, labeled by AMF, and this serves as an internal positive control. It is then possible that the residue labeled by DCCD in the bovine mitochondrial cytochrome *b* is not a carboxyl residue. Wang et al. (*19*), making use of a fluorescent derivative of DCCD, have, on the other hand, indicated aspartate-160 in the yeast cytochrome *b* as the residue to which DCCD binds. It has to be noted here that neither the aspartate-160 of yeast cytochrome *b* nor the suggested counterpart of the bovine cytochrome *b*, glutamate-163, is a conserved residue (*39*). On the contrary, in the 7 kDa proteolytic fragment of the yeast cytochrome *b*, constituted by the helix C and the *cd* loop, where the aspartate-160 was located (*19*), a highly-conserved tyrosine residue is present (Y-132) (*39*) to which DCCD might bind. The possibility, however, that the DCCD-modified residue may be inaccessible to AMF could also be considered.

EEDQ modifies carboxyl residues in the Core protein II and ISP, whose labeling correlates fairly well kinetically with the decrease of the proton pumping activity (Figure 5). A location of the Core proteins on the negative side of the membrane, protruding into the matrix space, was revealed by two-dimension electron microscopy (*40*). Recent X-ray diffraction data (*41*) have confirmed this location and further assigned a role to Core proteins to provide dimer interactions (mainly Core protein II) across the two-fold symmetry axis and to contribute, together with subunit VI, a possible proton access pathway to the quinol reduction site.

On the opposite side of the membrane, the quinone oxidation site appears to be contributed by the ends of helices B, C, and F and the connecting loops *cd* and *ef* of cytochrome *b*, by subunits IX and, probably, XI, and by domains of ISP on the C-terminal side, holding the Fe/S cluster (*42*). Crucial for cytochrome *b*/ISP interaction appear to be the residues glycine-137 and asparagine-256 of the cytochrome *b* in the bc_1 complex of *S. cerevisiae* (*43*). This site of the protein complex is apparently involved in the described chemical modifications. In fact, (i) the fragment of yeast cytochrome *b*, constituted by helix C and loop *cd*, contains the residue modified by DCCD (*19*); (ii) acidic residues in the protruding moiety of the ISP holding the Fe/S cluster are suggested to be involved in salt bridge/hydrogen bond network conferring stability to the protein (*36*), and one of these, i.e., aspartate-166, buried (*36*) and highly conserved (*44*), could be the target residue for EEDQ modification; (iii) the 8 kDa subunit IX, which is the targeting presequence of the ISP and is retained as a constitutive subunit of the complex (*45*), contains a residue which is modified by DCCD under decoupling conditions; (iv) point mutation G137E in cytochrome *b* of *S. cerevisiae* causes decoupling of the bc_1 complex (*7*).

The present results thus show that modification of residues in different subunits, on both sides of the membrane, leads to decoupling. The suggested possibility that an acidic residue on the N side of the membrane, Aspartate-229 of cytochrome *b*, may be involved in proton uptake from the matrix (*11, 46*) and the finding that S206L mutation in the *de* loop of *S. cerevisiae* cytochrome *b*, situated on the N side of the membrane, leads to the same decoupling effect as the G137E mutation on the opposite side does (*7*), are consistent with our conclusions.

As far as the mechanism of decoupling is concerned, various authors have suggested that, upon modification and/or mutation of residues critical for proton pumping, protons might diffuse back to the negative side (*7*, *18*, *19*, *47*). Anomalous redox reactions of quinones, caused by residue modification, might contribute to the decoupling effect. Upon carboxyl residue modification by EEDQ, we have in fact observed (Figure 7) an inhibition of the pre-steady-state reduction and of the oxidant-induced extrareduction of cytochrome *b*, without effect on the pre-steady-state reduction of cytochrome c_1 or on the overall rate of cytochrome *c* reduction.

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