Carboxyl Residues in the Iron−Sulfur Protein Are Involved in the Proton Pumping Activity of *P. denitrificans* bc_1 Complex†

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ABSTRACT: A study is presented on chemical modification of the three subunit *Paracoccus denitrificans* bc_1 complex. *N*-(Ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) treatment caused a loss of the proton pumping activity of liposome-reconstituted bc_1 complex. A similar effect, which is referred to as the decoupling effect, resulted upon reaction of *N*,*N*′-dicyclohexylcarbodiimide (DCCD) with the complex. Direct measurement of the binding of EEDQ to the complex subunits, performed in the presence of the fluorescent hydrophobic nucleophile 4′-[(aminoacetamido)methyl]fluorescein (AMF), showed that the iron−sulfur protein (ISP) and cytochrome c_1 were labeled by EEDQ, whereas cytochrome *b* was not. Tryptic digestion and sequencing analysis of the fluorescent fragment of the ISP revealed this to consist of a segment with six acidic residues, among which the highly conserved aspartate 160 is present. Analogous experiments on DCCD binding showed that all the three subunits of the complex were labeled. However, DCCD concentration dependence of carboxyl residue modification in the individual subunits and of proton pumping activity showed that the decrease of the H^+/e^- ratio correlated only with the modification of the ISP. Tryptic digestion of labeled ISP and sequencing analysis of the fluorescent fragment gave results superimposable upon those obtained with EEDQ. Chymotryptic digestion and sequencing analysis of the single fluorescent fragment of cytochrome *b* showed that this fragment contained glutamate 174 and aspartate 187. We conclude that, in the *P. denitrificans* bc_1 complex, carboxyl residues in cytochrome *b* do not appear to be critically involved in the proton pump mechanism of the complex.

The cytochrome bc_1 complex (ubiquinol−cytochrome *c* oxidoreductase) is localized in the inner membrane of mitochondria, where it catalyzes electron transfer from ubiquinol to cytochrome *c* and coupled proton translocation from the matrix to the intermembrane space. Analogous bc_1 complexes are integral part in the electron transport chains of chloroplasts (the *bf* complex) and of aerobic and photosynthetic bacteria. The various complexes differ from one another in the number of constituent subunits, the mammalian enzyme being made up of eight supernumerary subunits in addition to those holding the redox centers, namely, the *b* and c_1 cytochromes and the Rieske Fe/S protein (ISP).[1] The bacterial bc_1 complexes, on the other hand, contain only three to four polypeptides.

For each electron transferred to cytochrome *c* by the mitochondrial bc_1 complex, one proton is taken up at the negative phase (N) of the inner membrane and vectorially released at the positive phase (P). A second proton, which derives formally from the quinol oxidation, is directly released at the P site, thus giving an overall H^+/e^- ratio of 2. This ratio has been shown to decrease under conditions not affecting the electron-transfer activity. This effect, which is referred to as the decoupling effect, has been demonstrated in mammalian (*1*−*5*) and yeast (*6*−*8*) mitochondria, in chloroplast (*9*−*10*) and membrane preparations of *Rhodobacter sphaeroides* (*11*).

The decoupling effect exerted by the amino acid modifier DCCD led to suggest the involvement of acidic residues in the pump mechanism of the complex. Evidence was actually provided indicating that the decoupling effect by DCCD was correlated with the modification of the bovine bc_1 complex 8 kDa subunit IX (*2*, *12*), thereafter referred to as the DCCD binding protein (*13*). Subsequently Cocco et al. (*14*) showed that glutamate 53 is the target for DCCD modification of this subunit. Beattie and co-workers (*15*) have reported that aspartate 160 in the non-membrane-spanning helix cd of yeast cytochrome *b* and its counterparts, aspartate 155 (or glutamate 166) in the cytochrome b_6 of the thylakoid membrane cytochrome *bf* (*16*−*17*) and aspartate 187 of

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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; DBH, 2,3-dimethoxy-5-methyl-6-undecyl-1,4-benzoquinol; CCCP, carbonyl cyanide (*m*-chlorophenyl)hydrazone; PVDF, poly(vinylidene difluoride).

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cytochrome *b* in *Rb. sphaeroides* (*11*) are modified by DCCD. These observations led this group to ascribe the decoupling effect of DCCD to modification of these cytochrome *b* residues. However, it should be noted that these are residues not conserved in cytochrome *b* of all species. Furthermore, Shinkarev et al. (*18*) recently reported that the change of aspartate 187 of *Rb. sphaeroides* cytochrome *b* to asparagine did not alter the effect of DCCD on the functions of the bc_1 complex.

Cocco et al. (*19, 14*) have introduced EEDQ, which specifically modifies buried acidic residues, into the study of the proton pump mechanism of the bc_1 complex from bovine heart. The results obtained suggested that acidic residues in the core protein II and in ISP may be involved in the pump function of the complex. In particular, ISP aspartate 166, which is a conserved residue, was indicated as a residue critically involved in this function.

Because of the apparent involvement of acidic residues residing in supernumerary subunits of mammalian bc_1 complex (the core protein II and the 8 kDa subunit IX) in addition to those belonging to the redox subunits (the cytochrome *b* and ISP), it appeared of interest to study the effect of DCCD and EEDQ modification on the proton pump function of a three-subunit bacterial bc_1 complex.

Here we report a detailed study on acidic residue modification in *Paracoccus denitrificans* bc_1 complex, which was previously reported to be not susceptible to undergoing decoupling after DCCD treatment (*20*). The results we present show that (i) the *P. denitrificans* bc_1 complex was indeed decoupled by treatment with either DCCD or EEDQ and (ii) acidic residues in the ISP and cytochrome *b* were modified. Among these, residues in the ISP do, while glutamate 174 (or aspartate 187) in the cytochrome *b* do not, appear to be critically involved in the proton pump mechanism of the complex.

EXPERIMENTAL PROCEDURES

Cell Growth and Purification of P. denitrificans bc_1 Complex. MK10, a *P. denitrificans* strain overexpressing the bc_1 complex (*21*), was grown at 32 °C on succinate as the major carbon source (*22*).

Disruption of cells, isolation of membranes, protein determination, solubilization of membranes, and one-step protein purification by ion-exchange chromatography were performed as described earlier (*21, 23, 24*).

Preparation of bc_1 Vesicles. Reconstitution of *P. denitrificans* bc_1 complex into phospholipid vesicles was performed essentially as described by Yang and Trumpower (*20*), with some modifications. Acetone-washed soybean phospholipids were suspended in 50 mM KCl and 50 mM K-HEPES, pH 7.2, to yield a final concentration of 30 mg/mL phospholipids with 15% (w/v) cardiolipin. The suspension was first vortexed and then sonicated under N_2 with a microtip sonicator for about 10 min until the mixture turned clear. Purified bc_1 complex (0.04 mL of 30 mg of protein/mL) was gently mixed with lauryl maltoside (0.06 mL of 200 mg/mL), after which 1.90 mL of 30 mg/mL sonicated phospholipids was added. This mixture was incubated on ice for 1 h and then dialyzed against 1 L of 50 mM KCl and 50 mM K-HEPES, pH 7.2, for 3 h and against 1 L of 99.6 mM KCl and 1 mM K-HEPES, pH 7.2, overnight.

Measurement of Cytochrome c Reductase Activity and Protonmotive Activity in bc_1 Vesicles. Reductase activity of liposome-reconstituted bc_1 complex was measured with a dual-wavelength spectrophotometer (Johnson Research Foundation, Philadelphia, PA) following the cytochrome *c* reduction at the wavelength couple 550−540 nm ($\epsilon = 19.1$ $mM^{-1}\cdot cm^{-1}$) in a basic reaction mixture containing 1 mM K-HEPES, pH 7.2, 100 mM KCl, 1 mM KCN, and 7.5 μM ferricytochrome *c*. DBH (2,3-dimethoxy-5-methyl-6-undecyl-1,4-benzoquinol, 30 μM) was used as substrate. Respiratory control ratio, measured as described (*20, 25*), gave values not lower than 4.

In the oxidant pulse experiments, bc_1 vesicles, suspended in the last dialysis medium also containing 1 mM KCN, 1 μM ferricytochrome *c*, and 1 μg/mL valinomycin, were reduced with 50 μM DBH. The reaction was started by adding 3 μM ferricyanide. Acidification of the external medium was followed electrometrically (*20, 25*).

In the reductant pulse experiments (*2, 25*), bc_1 vesicles suspension was supplemented with 1 mM KCN, 7.5 μM ferricytochrome *c*, and 1 μg/mL valinomycin. The reaction was started by adding 10 μM DBH. Spectrophotometric determination of cytochrome *c* reduction at 550−540 nm and electrometric determination of proton release were carried out simultaneously on the same sample of vesicle suspension.

Modification of bc_1 Complex with EEDQ and DCCD. Cytochrome bc_1 complex (20 mg of protein/mL) in 10 mM K-HEPES, pH 7.0, and 0.02% Tween 80 was incubated at 0 °C with methanolic solutions of EEDQ and DCCD at the concentrations specified in the figure captions. For labeling studies the incubation mixture also contained 16 mM 4′-[(aminoacetamido)methyl]fluorescein (AMF) (*14, 19*). After 1 h of 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, and 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 (*26*) with a 12% separating gel. Slab gels were run at 30 V for 2 h and at 90 V for a further 5 h and either stained with Coomassie Brilliant Blue G and then destained or soaked in 50% methanol for 6 h and then photographed on a dark surface under an ultraviolet light source.

Proteolytic Digestion of EEDQ- and DCCD-Labeled bc_1 Complex Subunits and Sequence Analysis. EEDQ- and DCCD-labeled ISP and DCCD-labeled cytochrome *b* bands were cut from the gel and electroeluted. The proteins, at 0.5 mg/mL, were digested with the indicated proteolytic enzymes at a ratio of 1:20 (proteolytic enzyme:protein w/w) at 4 °C and then analyzed by a modified Laemmli low molecular weight polypeptide SDS−PAGE system, in a 20.1% T−0.5% C separating gel, pH 9.3, and a 9.4% T−4.8% C stacking gel, pH 6.8 (*14*). Gels were either stained with Coomassie Brilliant Blue G and then destained or soaked in 50% methanol for 6 h and then photographed on a dark surface under an ultraviolet light source. For sequence analysis, gels were blotted electrophoretically onto a poly(vinylidene difluoride) (PVDF) protein sequencing membrane, in a medium containing 10 mM CAPS [3-(cyclohexylamino)-1-

propanesulfonic acid] and 10% methanol, pH 11.0. Peptides were sequenced with a fully automatic peptide sequencer (model 473A of Applied Biosystems).

MATERIALS

DBH, EEDQ, and DCCD were obtained from Sigma Chemical Co.; AMF was obtained from Molecular Probes Inc. Trypsin, trypsin inhibitor, and chymotrypsin were from Boehringer Mannheim. All other reagents were of the highest purity grade commercially available.

RESULTS

Protonmotive Activity Measurement. The effect of EEDQ on the redox and protonmotive activity of *P. denitrificans* bc_1 complex was tested by treating the enzyme with the reagent before reconstitution into phospholipid vesicles. Measurement of proton translocation coupled to electron-transfer activity by the liposome containing bc_1 complex is shown in Figure 1. Original traces of a representative experiment are reported in Figure 1a showing that addition of ferricyanide to bc_1 vesicles incubated in the presence of DBH, valinomycin, and cytochrome *c* gave rise to a rapid ejection of protons from the vesicles, whose extent was almost halved in the presence of the uncoupler CCCP and drastically reduced in EEDQ-reacted bc_1 complex-containing vesicles. The H^+/e^- ratio, obtained from the extent of H^+ release and the amount of added ferricyanide, dropped from around 2.0 in the control to 1.20 for the treated enzyme. Subtracting from these values 1 H^+/e^- which is uncoupler-insensitive, around 80% inhibition of the proton pump activity occurred after EEDQ modification. It has to be noted that in these experiments the rate of proton leak is definitely negligible. This is quite expected for the liposome reconstituted system, in which the passive proton permeability is much lower than in the native membrane (*27*).

In Figure 1b the results of reductant pulse experiments are reported, in which bc_1 vesicles, incubated in the presence of valinomycin and 7.5 μM cytochrome *c*, were pulsed with DBH. EEDQ treatment of the enzyme caused a slight decrease of the rate of electron-transfer activity and a more marked decrease of the rate of proton release. A statistical evaluation of the effect of EEDQ treatment on the H^+/e^- ratio for the uncoupler-sensitive proton translocation is reported in Figure 1c. EEDQ caused a concentration-dependent decrease of the H^+/e^- ratio measured with either method, under conditions slightly affecting the reductase activity.

Complex Subunits Modified by EEDQ. Direct measurement of the binding of EEDQ to the complex was performed in the presence of the fluorescent hydrophobic nucleophile AMF, which specifically requires an EEDQ-modified carboxyl residue to bind to, by formation of an amide bond (*28*, *29*). Of the three constituent subunits of the complex, cytochrome c_1 and ISP were labeled by AMF whereas cytochrome *b* was not (Figure 2a). An analogous experiment carried out on the bovine enzyme (*19*), did not shown any labeling of the cytochrome c_1 subunit. However, it should be noted that *P. denitrificans* cytochrome c_1 presents in its structure, when compared with the counterpart in the bovine enzyme, an additional glutamate-rich sequence (*30*); thus its labeling by AMF was quite expected. The concentration dependence of carboxyl residue modification (Figure 2b) was similar for the two subunits being labeled.

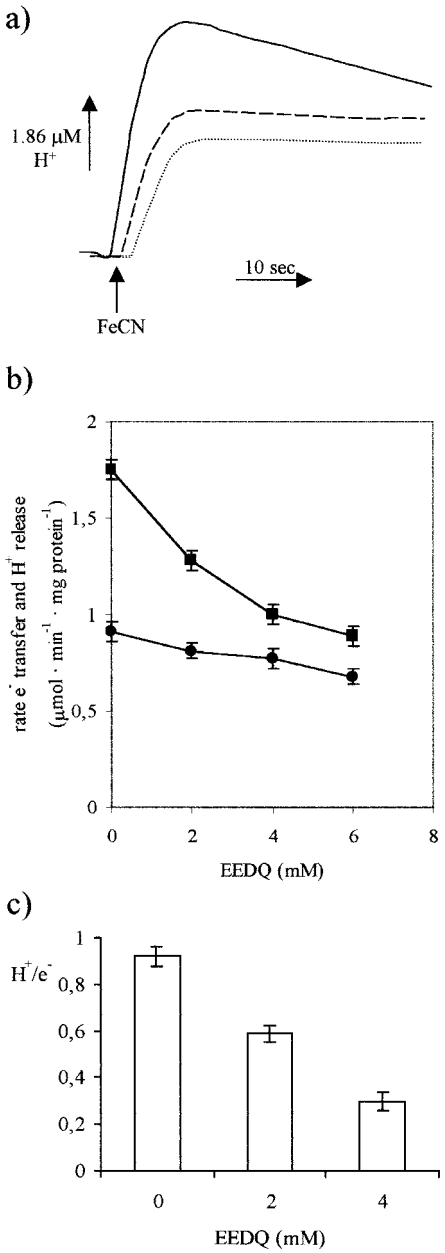

FIGURE 1: Effect of EEDQ modification on proton translocation activity of the bc_1 complex. Cytochrome bc_1 complex at 20 mg of protein/mL (80 nmol of cytochrome c_1/mL) was treated, prior to the reconstitution into phospholipid vesicles with EEDQ concentrations ranging from 2 to 6 mM, this resulting in a EEDQ/c_1 ratio of 25−75 mol/mol. (a) Oxidant pulse experiment: bc_1 vesicles were suspended at a concentration of 0.6 μM cytochrome c_1 in the reaction mixture described under Materials and Methods. The reaction was started by the addition of 3 μM ferricyanide. (—) Control; (---) 4 mM EEDQ-treated bc_1 complex; (···) plus 3 μM CCCP. (b) Reductant pulse experiment: bc_1 vesicles were suspended at a concentration of 0.6 μM cytochrome c_1 in the reaction mixture described under Materials and Methods. The reaction was started by adding 10 μM DBH. (●) Electron transfer; (■) proton release. (c) H^+/e^- ratio for uncoupler-sensitive proton translocation. The values reported are the means ± SD of measurements from nine different experiments.

To identify the carboxyl residues in the ISP that were labeled by EEDQ/AMF, the fluorescent band was excised from the gel and electroeluted, and the resulting peptide was digested with trypsin. The digested sample was then sub-

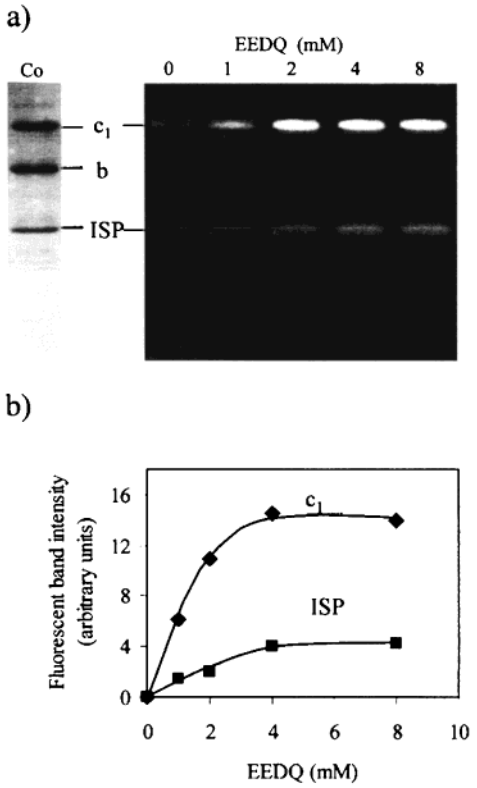

FIGURE 2: Binding of EEDQ to bc_1 complex subunits. Cytochrome bc_1 complex was treated with the indicated concentrations of EEDQ in the presence of 16 mM AMF. After 1 h of incubation, aliquots of the bc_1 suspension were precipitated in acetone and the solubilized pellets were subjected to SDS−PAGE. (a) Gel exposed to ultraviolet light. Lane Co shows the Coomassie-stained gel. (b) Fluorescence of the bands was determined by scanning densitometry of the photograph and plotted, as arbitrary units. The c_1 fluorescent bands were corrected for the intrinsic fluorescence of the cytochrome, in the control.

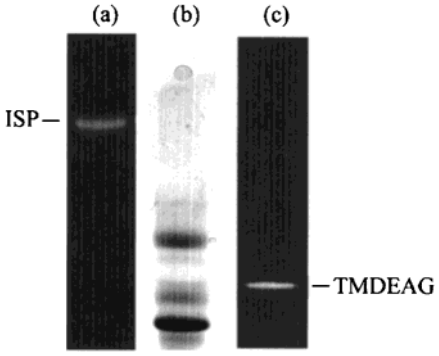

FIGURE 3: Trypsin digestion of EEDQ-labeled ISP. EEDQ-labeled ISP fluorescent band (lane a) was cut from the gel and electroeluted. The sample (0.5 mg of protein/mL) was digested with trypsin for 24 h and the reaction was stopped by adding a 5-fold excess of trypsin inhibitor. The gel was either stained with Coomassie Brilliant Blue G and destained (lane b) or soaked in 50% methanol for 6 h and then photographed on a dark surface under an ultraviolet light source (lane c). For sequence analysis, gels were blotted electrophoretically to a PVDF protein sequencing membrane and the fluorescent peptide was sequenced with a fully automatic peptide sequencer. The N-terminal sequence of the tryptic fluorescent peptide is reported.

jected to SDS−PAGE (Figure 3, lane b). Only one fragment out of seven produced displayed AMF fluorescence (Figure 3, lane c). Sequencing analysis of this fragment, of estimated molecular mass 4.5 kDa, gave the sequence TMDEAG. This suggests that the cleavage product contains the residues from Thr-118 to Arg-164 (see Figure 6). Six carboxyl residues are contained in this fragment. Glu-124, Asp-146, and Asp-160 are also present in the bovine ISP (see Figure 6). It remains to be established which of these residues is actually modified by EEDQ. However, determination of solvent-accessible area of the bovine counterparts (*31*, *32*) has revealed that Asp-160 (Asp-166 in the bovine numbering) is the only buried residue, as requested by the chemistry of EEDQ modification.

Studies with DCCD. Yang and Trumpower have reported that DCCD treatment does not inhibit the proton translocation activity of bc_1 complex purified from *P. denitrificans* (*20*). In light of the results obtained with EEDQ modification, we have reexamined the effect of DCCD on the *P. denitrificans* complex. As compared to EEDQ, we had to use 1 order of magnitude higher concentrations of DCCD in order for the effect to be elicited. With regard to the experimental conditions used in ref *20*, the incubation mixture of the present experiments contained 0.02% Tween 80, with the DCCD to cytochrome c_1 ratios being quite similar. Figure 4a shows the results of an experiment in which binding of DCCD to the subunits of the complex was measured in the presence of AMF. All the three subunits could indeed be labeled. However, the binding of DCCD to the individual subunits showed a markedly different concentration dependence. Cytochrome c_1, whose intrinsic fluorescence is evident in the control, was strongly labeled by AMF, with the signal appearing largely saturated at 10−20 mM DCCD (see Figure 4b). Cytochrome *b* was also labeled by AMF, with the relative intensity band being independent of the DCCD concentration, in the range 10−40 mM. The ISP, on the other hand, appeared to react with DCCD only when the concentration of the reagent was raised to 40 mM (Figure 4a,b). The Coomassie blue-stained gel (Figure 4a, right panel) of the control and DCCD-treated enzyme shows that DCCD did not cause any cross-linking side reactions between the subunits.

The effect of the reaction of DCCD on the protonmotive activity of the complex was also followed, and the results are reported in Figure 4c. The H^+/e^- ratio for vectorial proton translocation measured in the liposome-reconstituted bc_1 complex, with both oxidant and reductant pulses, dropped only when the concentration of DCCD was raised to 40 mM, that is, at concentrations causing modification of ISP subunit. Under these conditions, DCCD treatment caused only a small, if any, decrease of the rate of electron transfer (not shown); the redox activity was, however, completely sensitive to antimycin. It thus appears from these experiments that modification by DCCD of either *b* and c_1 cytochromes does not correlate with the observed decoupling effect. On the other hand, a role of carboxyl residue(s) in the ISP, already suggested by the experiment with EEDQ, is further evidenced.

Tryptic digestion of DCCD-labeled ISP and subsequent SDS−PAGE of the digestion sample revealed the presence of a single fluorescent band. After sequencing analysis, this fragment resulted to be exactly the same as that obtained by tryptic digestion of the EEDQ-treated enzyme (not shown).

The electroeluted fluorescent cytochrome *b* was digested with chymotrypsin and the resulting peptides were subjected

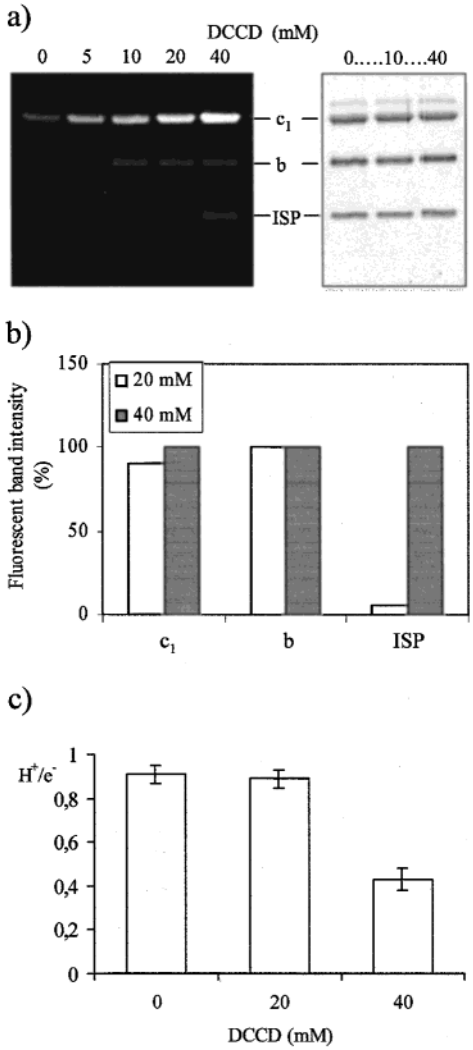

FIGURE 4: Binding of DCCD to bc_1 complex subunits and effect on the protonmotive activity. Cytochrome bc_1 complex was treated with the indicated concentrations of DCCD as described under Materials and Methods. After 1 h of incubation, aliquots of the bc_1 suspension were precipitated in acetone and subjected to SDS−PAGE or added to a sonicated phospholipid suspension. (a) Gel exposed to ultraviolet light. Lanes on the right refers to the Coomassie-stained gel. (b) Fluorescence of the bands was determined by scanning densitometry of the photograph and plotted as a percentage of the fluorescence intensity measured at 40 mM DCCD. (c) Effect of DCCD on bc_1 vesicle protonmotive activity. Proton translocation and electron-transfer activities were measured with both oxidant and reductant pulse experiments, as described under Materials and Methods and in the caption to Figure 1.

to SDS−PAGE (Figure 5). One out of six fragments displayed fluorescence. Sequencing analysis of this fragment, whose apparent molecular mass was about 5 kDa, gave the sequence GQMSFW, thus suggesting it contains the residues from Gly-152 to His-198. Two carboxyl residues are present in this sequence, Glu-174 and Asp-187. Their counterparts in the bovine enzyme reside in the non-membrane-spanning cd_2 helix and in the cd loop, respectively (see Figure 6).

DISCUSSION

When experiments on DCCD modification of the bc_1 complex are performed, DCCD treatment may result in inhibition of both proton translocation and electron transfer to almost the same extent (*18*, *33*, *34*), with no change in

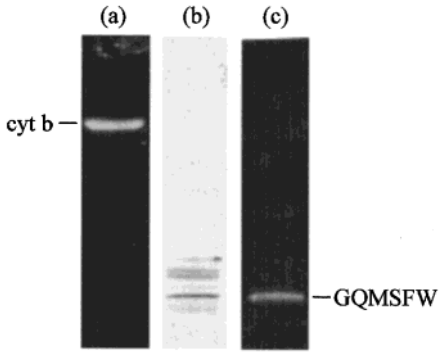

FIGURE 5: Chymotrypsin digestion of DCCD-labeled cytochrome *b*. DCCD-labeled cytochrome *b* fluorescent band (lane a) was cut from the gel and electroeluted. The sample (0.5 mg of protein/mL) was digested with chymotrypsin for 7 days; afterward the digestion was stopped by the addition of 2 mM phenylmethanesulfonyl fluoride (PMSF). Gels were either stained with Coomassie Brilliant Blue G and destained (lane b) or soaked in 50% methanol for 6 h and then photographed on a dark surface under an ultraviolet light source (lane c). For sequence analysis, gels were blotted electrophoretically to a PVDF protein sequencing membrane and the fluorescent peptide was sequenced with a fully automatic peptide sequencer. The N-terminal sequence of the tryptic fluorescent peptide is reported.

the H^+/e^- ratio. We too observed such an effect in bovine as well as in *P. denitrificans* bc_1 complex. To our experience, multiple DCCD modifications, involving extended segments of most of the complex subunits, do occur under these conditions. On the contrary, if experimental conditions are chosen resulting in a pure decoupling effect, then the results obtained allow us to get insights into the involvement of the individual subunits, and of residues in these, in the proton pumping activity of the enzyme.

Here we have shown that *P. denitrificans* bc_1 complex shares with mammalian, yeast, chloroplast, and other aerobic or photosynthetic bacteria the property to undergo decoupling. The use of the fluorescent probe AMF ensures that carboxyl residues are indeed the target of the modification. Parallel experiments on DCCD concentration dependence of carboxyl residue modification, in the individual subunits, and of proton pumping activity by the liposome-reconstituted complex (Figure 4), show an involvement of the ISP in the proton pump mechanism. We consider the binding of EEDQ and DCCD to the *P. denitrificans* cytochrome c_1 subunit as a consequence of the presence of a glutamate-rich N-terminal sequence, absent in the eukaryotic counterpart. It should be noted that binding of DCCD and EEDQ to the eukaryotic cytochrome c_1 has never been reported; neither has an involvement of this subunit in the proton pump function been suggested.

Ten times higher concentrations of DCCD than those usually used for modification of the bovine complex had to be used to modify the *P. denitrificans* complex. This is likely the reason why DCCD was not found to modify the bovine enzyme ISP (*19*). However, the bovine enzyme was still decoupled after modification of Glu-53 in the 8 kDa subunit IX (*14*). This observation would indicate the presence in eukaryotic complex of different critical residues located in different subunits.

A role of carboxyl residues in the non-membrane-spanning helix cd and in the cd loop (see Figure 6) of cytochrome *b* in the proton pumping activity has been repeatedly suggested

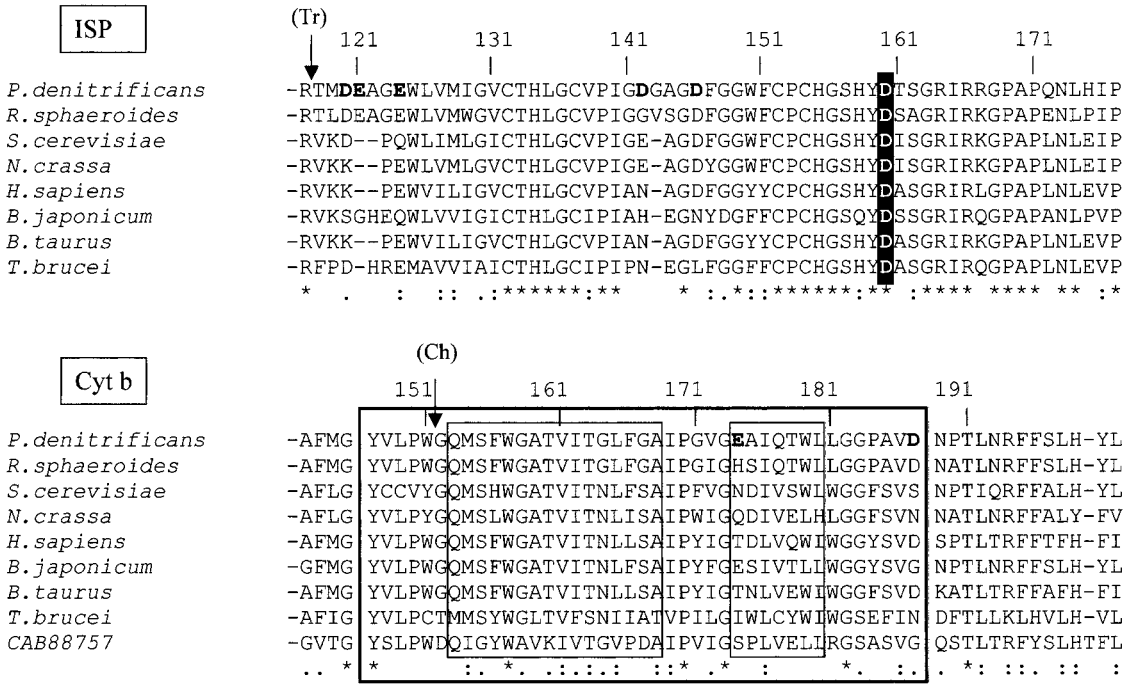

FIGURE 6: Sequence alignment of the carboxyl region of the ISP protein and of the cd loop region of cytochrome *b*. The arrows indicate the trypsin (Tr) and chymotrypsin (Ch) cleavage sites. The conserved residue Asp-160 in the ISP fragment is highlighted. The nonconserved acidic residues present in the *P. denitrificans* fluorescent fragment are in boldface type. The cd_1 and cd_2 non-membrane-spanning helices (bovine sequence) of cytochrome *b* are in light boxes; the cd loop is in a bold box. The nonconserved acidic residues present in the *P. denitrificans* cytochrome *b* fluorescent fragment are in boldface type. CAB88757, cytochrome b_6 of spinach thylakoids *bf* complex. (∗)-identical or conserved residues in all sequences in the alignment; (:) conserved substitutions; (.) semiconserved substitutions. Numbering refers to the *P. denitrificans* sequence. Sequences were obtained from SWISSPROT database (Rel. 39), and multiple sequence alignment was performed with CLUSTALW software (www.ch.embnet.org).

(*11*, *15*−*17*). From the present data, it appears that modification by DCCD of this residue(s) does not cause decoupling of the *P. denitrificans* enzyme (Figure 4). Furthermore, as noted by Crofts et al. (*35*), Asp-187 in the *cd* loop of *Rb. sphaeroides* cytochrome *b* does not appear close enough to the proton extrusion pathway site to have an obvious functional role. Finally, substitution of this residue with asparagine did not hamper the bc_1 complex functions, nor did it modify the effect caused by DCCD (*18*).

The experiments reported here on the use of EEDQ as a carboxyl residue modifier produced results superimposable upon those obtained with DCCD. The same carboxyl residue-containing region in the ISP was modified under conditions in which the two reagents caused decoupling of the complex. Asp-160, present in the fluorescent fragments, is homologous with Asp-166 in the ISP of the bovine enzyme, which was previously suggested to be involved in the proton pump function of the complex (*14*, *19*). In the bovine enzyme it was found that, under decoupling conditions, carboxyl residues in the core protein II were also modified by EEDQ (*14*, *19*). Thus, in addition to the ISP subunit, which appears to contribute with its carboxyl residue(s) to the proton pump mechanism of both bacterial and eukaryotic enzymes, the supernumerary subunits core protein II and the 8 kDa subunit IX seem to play a role in this process, in the bovine complex. In the description of the crystal structure of the enzyme from bovine heart (*36*, *37*), core proteins enclose a large cavity also housing subunit IX, in an arrangement that is suggested to contribute a possible proton access pathway at the N side of the membrane and, at the same time, to prevent proton leakage back to the negative phase of the membrane. It is tempting to speculate that these supernumerary subunits may play a role in the optimization of the proton uptake process at the N site of the membrane and of the coupling efficiency of the process.

On the basis of the present and related observations (*38*, *10*), it is proposed that modification by DCCD or EEDQ of carboxyl residue(s) in the ISP blocks the proton-conducting channel delivering at the quinol oxidation center the protons generated upon quinol oxidation. It has to be noted that aspartate 166 of the bovine bc_1 complex ISP (and its counterpart Asp-160 in the *P. denitrificans* enzyme) is a buried residue involved in an internal bridge/hydrogen-bond network essential for Fe/S cluster stability in the protein (*39*). Furthermore, its engagement, through hydrophobic−hydrophilic interactions, with other residues in the cluster domain suggests a role in the proton outlet at the quinol oxidation side (*40*).

It is worthwhile emphasizing that the H^+/e^- ratio for vectorial proton translocation in the bovine bc_1 complex is decreased by the steady-state transmembrane pH difference. The ratio value was indeed found to be in linear inverse correlation with the extent of the transmembrane pH gradient (*41*, *42*). It is conceivable that this decoupling between electron flow and proton translocation, which is observed also in the bovine cytochrome oxidase (*43*), may have a role in lowering, at high value of the protonmotive force, the oxygen concentration and the reduction level of electron transport carriers, thus preventing superoxide anion generation (*44*, *45*).

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