Localization of acidic residues involved in the proton pumping activity of the bovine heart mitochondrial bc_1 complex

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Abstract Chemical modification of carboxyl residues in polypeptide subunits of the mitochondrial bc_1 complex causes a decoupling effect, that is inhibition of the proton pumping activity, without affecting the rate of electron transfer to ferricytochrome c. The study presented here is aimed at localizing and identifying the residues whose modification results in decoupling of the complex. Glutamate-53 in subunit IX (the DCCD-binding protein) and aspartate-166 in the Rieske iron-sulfur protein are the residues modified by *N*,*N*′-dicyclohexylcarbodiimide (DCCD) and *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), respectively. The results obtained also suggest that the carboxy-terminal sequence of the Core protein II, which is fairly rich in acidic residues, may also play a role in the vectorial proton translocation activity of the complex.

Key words: Mitochondria; bc_1 complex; Proton transport; Carboxyl residue; Chemical modification; Decoupling

1. Introduction

The cytochrome bc_1 complex is an integral multisubunit enzyme of the inner mitochondrial membrane, which catalyzes electron transfer from ubiquinol to ferricytochrome c. Coupled to electron transfer through the complex, there is an outwardly oriented proton pumping process, so that an electrogenic gradient, positive at the cytosolic site, is generated across the membrane.

The complex from high eukaryotic species consists of 11 subunits, of which the b and c_1 cytochromes and the Rieske iron-sulfur protein (ISP) are the only subunits carrying redox centers. The other supernumerary subunits are the two Core proteins and six additional polypeptides whose molecular mass ranges from 14 to 6.4 kDa [1]. The primary structure of all the component polypeptides, as well as the membrane topology, protein-protein interactions, the position of the redox centers and the binding sites of substrates and inhibitors, as revealed by X-ray diffraction analysis [2–4], have been settled. However, the mechanism by which protons are translocated through the complex still waits for a definite elucidation.

When the bc_1 complex either in the native membrane or reconstituted into phospholipid vesicles is pulsed with reduced ubiquinone analogues, one H^+ is vectorially translocated at the positive (P) side of the membrane for every electron transferred to the acceptor cytochrome c. This H^+/e^- ratio of 1 appears to decrease at the steady-state, in the presence of a respiration driven transmembrane pH difference (ΔpH, the chemical component of the protonmotive force). The H^+/e^- ratio value was indeed found to exhibit a linear inverse correlation with the extent of the measured pH gradient [5,6]. This effect, commonly referred to as decoupling effect, was reversed by the presence in the reaction medium of anions of weak acids, such as azide or arachidonate, whose p*K*'s are around 4.5 [7]. These results suggest that ΔpH controlled protonation-deprotonation processes of residues placed along proton conduction pathways linking the aqueous bulk phases to the redox centers, represent a critical step in the vectorial proton pump mechanism [8–10]. On the basis of these and related findings in point-mutated bacteriorhodopsin [11] and in the bacterial reaction center [12,13], our attention was drawn to acidic residues possibly involved in the energy-linked proton pump mechanism.

Evidence for such an involvement of acidic residues in proton pumping had, in fact, been provided by studies on covalent modification of bc_1 complex subunit(s) with DCCD. This reagent, which reacts predominantly with carboxyl and sulfhydryl groups as well as tyrosines, modifies residues in the heart mitochondrial cytochrome b and the 8 kDa subunit IX, causing decoupling of the bc_1 complex activity [14–16]. More recently, a highly specific reagent for buried carboxyl groups [17,18] was used and acidic residues were modified belonging to Core protein II and the iron-sulfur protein [19].

In this paper a study is presented aimed at identifying the carboxyl residues whose modification by EEDQ and DCCD results in decoupling of the complex.

2. Materials and methods

2.1. Materials

EEDQ and DCCD were obtained from Sigma Chemical Co.; AMF was obtained from Molecular Probes Inc. All other reagents were of the highest purity grade commercially available.

2.2. Purification of cytochrome c reductase complex

The cytochrome c reductase complex was isolated from bovine heart mitochondria according to Rieske [20].

2.3. 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 [21] as described by Cocco et al. [7].

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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; ΔpH, transmembrane pH gradient

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2.4. Measurement of cytochrome c reductase and protonmotive activities in bc_1 vesicles

Redox-linked proton translocation was measured essentially as described by Lorusso et al. [14]. Spectrophotometric determination of cytochrome c reduction, measured with a dual wavelength spectrophotometer (Johnson Research Foundation, Philadelphia, USA) at the wavelength couple 550–540 nm 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.

2.5. Modification of bc_1 complex with EEDQ and DCCD

bc_1 complex (20 mg protein/ml) in 10 mM K-HEPES, pH 7.0, 0.02% Tween 80, was incubated at 0°C with methanolic solutions of EEDQ (125 mol/mol cytochrome c_1) and DCCD (50 mol/mol cytochrome c_1) and, where indicated, in the presence of 16 mM 4′-((aminoacetamido)methyl)fluorescein (AMF) for 1 h and 30 min respectively. After incubation, aliquots of the bc_1 complex suspension were directly added to a sonicated phospholipid suspension for reconstitution or precipitated in 90% cold acetone. The pellets were solubilized and subjected to SDS-PAGE as described in [19].

2.6. Tryptic digestion of EEDQ- and DCCD-labelled bc_1 complex subunits and sequence analysis

EEDQ-labelled Core protein II and ISP and DCCD-labelled subunit IX were cut from the gel and electroeluted. The proteins at 0.5 mg/ml were digested with trypsin at a ratio of 1:20 (trypsin: protein, w/w) at 4°C for 24 h and then analyzed by modified Laemmli low molecular weight polypeptide SDS-PAGE system [22] in a 20.1% T, 0.5% C, pH 9.3 separating gel and a 9.4% T, 4.8% C, pH 6.8 stacking gel. 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. 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]/10% methanol, pH 11.0. Peptides were sequenced with a fully automatic peptide sequencer (model 473A of Applied Biosystems).

3. Results

Our group has reported that treatment of the bc_1 complex from bovine heart with either EEDQ or DCCD prior to reconstitution into liposomes, causes a decrease of the rate and the extent of proton translocation activity, without affecting the rate of electron transfer to ferricytochrome c [8,19]. As a result, the H^+/e^- ratio for proton pumping drops from 1, in the control, to around 0.2 and 0.3 for the EEDQ and DCCD treated enzyme respectively (Fig. 1A). Direct measurements of the binding of EEDQ and DCCD were performed in the presence of the fluorescent hydrophobic nucleophile, AMF, which specifically requires a carboxyl residue modified by either reagent to bind to, by formation of an amide bond [23,24]. Fig. 1B shows that EEDQ reacts with carboxyl residues in the Core protein II and ISP, whereas DCCD modifies only acidic residues in the 8 kDa subunit IX. The fluorescent labelling did not change whether EEDQ and DCCD were present together in the incubation medium, that is the two modifying reagents appear to react independently of each other (not shown). It has to be noted that although labelled by ^{14}C-DCCD [16,19], the modified cytochrome b did not react with AMF. This observation raises the question whether the DCCD-binding residue in the b cytochrome is really an acidic residue. As an alternative explanation, it can be considered that the DCCD-modified residue may be inaccessible to the fluorescent AMF.

In order to identify the carboxyl residues which react with AMF, the fluorescent bands were excised from the gels, electroeluted and the resulting peptides digested with trypsin. The digested samples were subjected to SDS-PAGE and the fluorescent fragments were removed from the gel by blotting and sequenced.

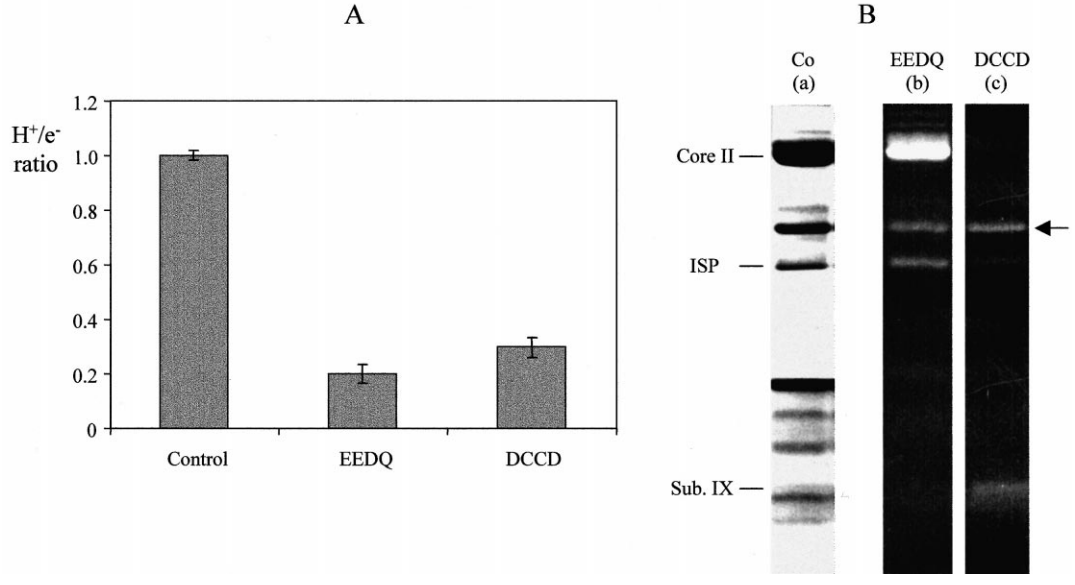

Fig. 1. Binding of EEDQ and DCCD to bc_1 complex subunits and their effect on proton translocation activity in bc_1 vesicles. A: bc_1 complex, treated with EEDQ or with DCCD, was reconstituted into phospholipid vesicles. bc_1 vesicles were suspended at a concentration of 0.7 μM cytochrome c_1 in the reaction mixture described in Section 2. The reaction was started by the addition of 11 μM duroquinol. B: bc_1 complex was treated with EEDQ (b) and DCCD (c) in the presence of AMF (16 mM, dimethylformamide solution). After electrophoresis the gel was exposed to ultraviolet light and photographed (b and c). The fluorescent band indicated by the arrow refers to the cytochrome c_1 which is intrinsically fluorescent. Lane (a) refers to the Coomassie stained gel.

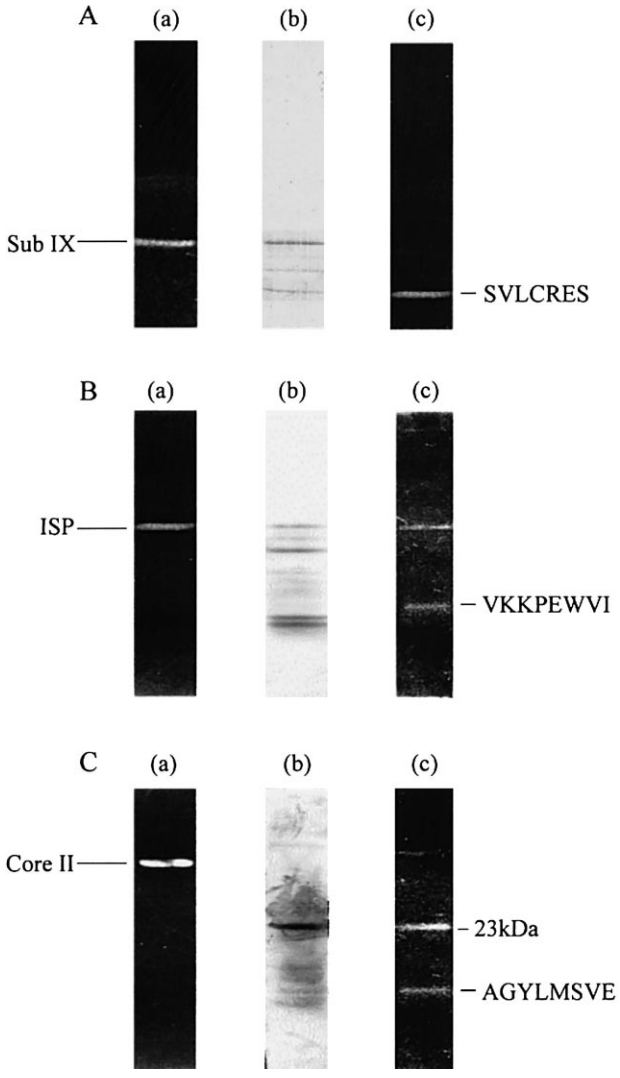

Fig. 2. Digestion by trypsin of isolated EEDQ- and DCCD-labelled subunits of the bc_1 complex. DCCD-labelled subunit IX (A, lane a) and EEDQ-labelled ISP (B, lane a) and Core protein II (C, lane a) were cut from the gel and electroeluted. The proteins at 0.5 mg/ml were digested with trypsin and then analyzed by SDS-PAGE system. Gels were either stained with Coomassie Brilliant Blue G and destained (lanes b) or soaked in 50% methanol for 6 h and then photographed on a dark surface under an ultraviolet light source (lanes c). For sequence analysis gels were blotted electrophoretically onto a poly(vinylidene difluoride) (PVDF) protein sequencing membrane and fluorescent peptides sequenced with a fully automatic peptide sequencer. The N-terminal sequences of the tryptic fluorescent peptides are reported.

The 8 kDa subunit IX is the so-called DCCD-binding protein, because of the correlation found between its chemical modification and the decoupling of the complex [14–16]. Tryptic digestion of this subunit gave rise to three peptides (Fig. 2A, lane b), only one of these, of apparent molecular mass of 3.5 kDa, was fluorescent (lane c). This fragment was sequenced and the resulting N-terminal sequence, SVLCRES, suggested it to contain the C-terminal sequence of the protein, starting from Ser-48. A single acidic residue is present in this fragment, glutamate-53.

After the digestion of the ISP only one fragment, out of about ten produced (Fig. 2B, lane b), displayed the AMF fluorescence (lane c). Sequencing analysis of this fragment, of apparent M_r of 5.5 kDa, gave the sequence VKKPEWVI, this suggesting that the cleavage product contains the amino acid residues from Val-127 to Arg-172 or Lys-173. Three carboxyl residues are contained in this fragment, Glu-131, Asp-152 and Asp-166 (see Fig. 3). Determination of solvent accessible area of the individual residues [25,26] reveals, however, that aspartate-166 is, of the three acidic residues, the only buried residue, as requested by EEDQ modification chemistry [17,18].

Tryptic digestion of labelled Core protein II and subsequent SDS-PAGE of the low molecular weight digestion fragments (Fig. 2C, lane b) revealed the presence of two fluorescent bands whose molecular masses are around 23 and 5 kDa respectively (lane c). It has to be noted that by increasing the digestion time, the larger peptide band tended to disappear with concomitant reinforcement of the fluorescence intensity associated with the 5 kDa band. Under these conditions many intermediate molecular mass fragments appeared (Fig. 2C, lane b), however, none of these displayed fluorescence. This, together with the fact that this gel system allowed the detection of fragments up to 1 kDa of molecular mass [22], led us to consider that the EEDQ-modified carboxyl residues reside into the 5 kDa fragment. Sequence analysis indicated that it contains the amino acid residues from Ala-366 to Lys-415 or his neighbor Lys-416. The fragment appears to be fairly rich in acidic residues, containing three glutamate and four aspartate residues. Again, from solvent accessible area measurements [25,26], Glu-373 and Glu-381 residues ap-

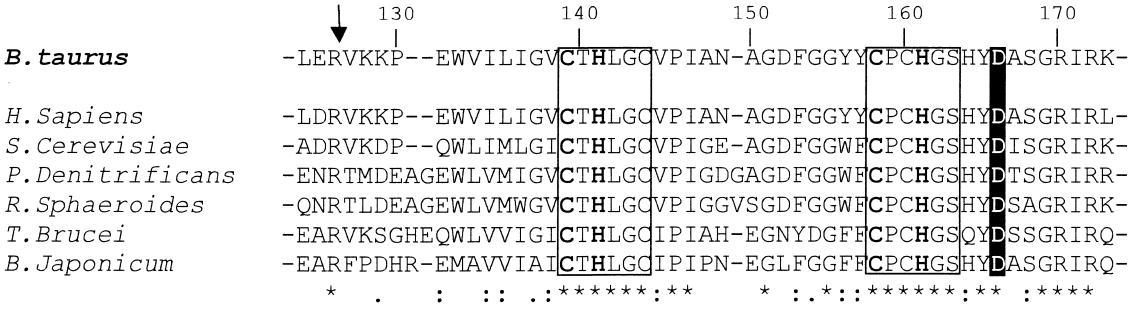

Fig. 3. Sequence alignment of the carboxyl region of the ISP protein. Conserved regions, containing residues which serve as ligands to the iron-sulfur cluster (in bold), are in boxes. The residue Asp-166 is highlighted. The arrow indicates the trypsin cleavage site. (*) indicates identical or conserved residues in all sequences in the alignment, (:) conserved substitutions, (.) semi-conserved substitutions. Sequences were obtained from SWISSPROT data base (Rel.37) and multiple sequence alignment was performed using EBI (European Bioinformatics Institute) CLUSTALW software (www2.ebi.ac.uk/).

pear highly hydrophobic, the other being definitely hydrophilic.

4. Discussion

EEDQ as well as DCCD reacted bc_1 complex, while retaining the electron transfer activity, loses the capacity to pump protons. In this study we have localized the modified carboxyl residues in the Core protein II, subunit IX and ISP, that is in subunits which are located either at the matrix side (Core II, subunit IX) or at the cytosolic side (ISP) of the membrane.

As reported by Xia et al. [2] and by Iwata et al. [4] in the description of the crystal structures of the enzyme from bovine heart mitochondria, Core proteins enclose a large cavity also housing the 8 kDa subunit IX, which is the targeting presequence of the ISP and is retained as a constitutive subunit of the complex long way apart from the N-terminal transmembrane anchor of the ISP subunit [27]. The arrangement of these subunits contributes, together with subunit VI, a possible proton access pathway at the matrix quinone reduction site and prevents proton leakage back at the negative side of the membrane [4]. The decoupling ensuing from the modification of acidic residues in the carboxyl tail of Core protein II and of glutamate-53 in the strictly associated subunit IX may be caused either by decreased efficiency of proton uptake at the matrix inlet or by a dissipative leakage of protons, at the same side, through the complex.

The EEDQ modification of the ISP did also correlate kinetically with the decoupling effect [19]. Aspartate-166 is most likely the target for EEDQ modification and, noteworthy, with regard to the other two acidic residues present in the tryptic fragment we obtained, is the only highly conserved one [28] (Fig. 3). This residue is buried and involved in an internal bridge/hydrogen-bond network essential for the [2Fe-2S] cluster stability [29]. In fact, in a yeast mutant D166Q no FeS cluster could be observed [30]. Furthermore, aspartate-166 is also engaged, through hydrophobic-hydrophilic interactions, with other residues in the cluster domain, so that it may take part, directly or indirectly, in the proton outlet at the quinol oxidation center. A role of the ISP cluster in proton exit has indeed been recently suggested [31,32].

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