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MODIFICATION OF THE CATALYTIC FUNCTION OF THE MITOCHONDRIAL CYTOCHROME b-c_1 COMPLEX BY DICYCLOHEXYLCARBODIIMIDE

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N,N'-Dicyclohexylcarbodiimide (DCCD) induces a complex set of effects on the succinate-cytochrome c span of the mitochondrial respiratory chain. At concentrations below 1000 mol per mol of cytochrome c_1, DCCD is able to block the proton-translocating activity associated to succinate or ubiquinol oxidation without inhibiting the steady-state redox activity of the b-c_1 complex either in intact mitochondrial particles or in the isolated ubiquinol-cytochrome c reductase reconstituted in phospholipid vesicles. In parallel to this, DCCD modifies the redox responses of the endogenous cytochrome b, which becomes more rapidly reduced by succinate, and more slowly oxidized when previously reduced by substrates. At similar concentrations the inhibitor apparently stimulates the redox activity of the succinate-ubiquinone reductase. Moreover, DCCD, at concentrations about one order of magnitude higher than those blocking proton translocation, produces inactivation of the redox function of the b-c_1 complex. The binding of [^{14}C]DCCD to the isolated b-c_1 complex has shown that under conditions leading to the inhibition of the proton-translocating activity of the enzyme, a subunit of about 9500 Da, namely Band VIII, is the most heavily labelled polypeptide of the complex. The possible correlations between the various effects of DCCD and its modification of the b-c_1 complex are discussed.

Introduction

DCCD is a powerful protein reagent which preferentially acts by modifying carboxyl groups located in nonpolar environments [1]. In spite of its rather high reactivity to proteins, DCCD is able to modify specifically H^+-ATPase complexes [2] by stoichiometrically binding to a hydrophobic polypeptide in the membrane sector of the enzymes [3]. More recently, other proton-translocating membrane-bound enzymes have been found to be modified by this reagent, like cytochrome oxidase [4,5], NADH-$NADP^+$ transhydrogenase [6,7], and ubiquinol-cytochrome c reductase [8–11]. The concentrations of the inhibitor required for the inactivation of these enzymes, however, are much higher than those inhibiting H^+-ATPases, and this can raise severe uncertainties about the specificity of DCCD modification and the correlation between the binding and the functional effects [5].

One important piece of experimental evidence may contribute to the solution of this problem. Either in cytochrome oxidase or in NADH-$NADP^+$ transhydrogenase the inhibition of elec-

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, N',N'-dicyclohexylcarbodiimide; $\Delta\Psi$, transmembrane potential; Q_1, ubiquinone-1; Q_2H_2, ubiquinol-2; DCIP, 2,6-dichlorophenolindophenol; Hepes, *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

tron transfer usually occurs at DCCD concentrations higher than those affecting the proton-translocating activities [4,5,7], indicating a differential modification of the two coupled functions of the enzymes. Such an observation is much more evident in the case of ubiquinol-cytochrome *c* reductase (b-c_1 complex), where the DCCD titer required for the inhibition of the redox activity is about one order of magnitude higher than that blocking proton translocation [8,9].

The above evidence has prompted us to undertake a systematic study of the effects induced by the diimide on various activities in the succinate-cytochrome *c* span of the mitochondrial respiratory chain, and their possible relationships with the modification of the b-c_1 complex. Preliminary accounts of some aspects of this work have been published elsewhere [8,9].

Materials and Methods

Preparations. Bovine heart mitochondria [12], submitochondrial particles [13], rat liver mitochondria [14] and rat heart mitochondria [15] were prepared as described in the references cited. Cytochrome *c*-depleted mitochondria were prepared as detailed in Ref. 16.

Isolated b-c_1 complex was purified either by the method of Hatefi [17] or the method of Rieske [18], and routinely contained 3 nmol cytochrome c_1 per mg protein (determined as in Ref. 18), and exhibited activities up to 100 μmol/min per mg protein with ubiquinol-2.

Ubiquinols were prepared as described in Ref. 18. Reconstitution of the purified b-c_1 complex into crude soybean phospholipid vesicles was accomplished as previously described [9], according to the procedure in Ref. 19. The proteoliposomes exhibited respiratory control ratios usually ranging between 5 and 9.

Proton-translocation assays. All the assays were performed at room temperature (22 ± 2°C). The transmembrane pH gradient driven by succinate oxidation in submitochondrial particles was measured by the fluorescence quenching of 9-aminoacridine [8]. The external acidification associated with succinate oxidation by either oxygen or ferricyanide in mitochondria was monitored using the pH indicator phenol red as described in Ref. 9. The assay mixture was 0.2 M sucrose, 30 mM KCl, 0.5 mM EDTA, buffered at pH 7.4–7.8 with 1 mM K^+-Hepes or 2 mM Tris-HCl, containing 1 μg/ml of both rotenone and valinomycin and 2 μg/ml of oligomycin, with a mitochondrial concentration of 2–5 mg/ml. The proton extrusion associated with quinol oxidase activity was monitored with phenol red at 556.5 nm or with the indicator bromocresol purple [20] at 585 or 590 nm with 0.5–1 mg/ml of mitochondrial protein. The assay mixture was the same as before, except that the medium was buffered with 0.5 mM K^+-Hepes to pH 7.5 with phenol red, or to 7.0 with bromocresol purple. The reaction was initiated by ubiquinol-2 at concentrations usually between 20 and 30 μM. In some experiments 5 μM cytochrome *c* (Sigma, type III) was added to enhance the extent of proton translocation.

The proton-translocating activity of the b-c_1 complex reconstituted into phospholipid vesicles was assayed with the indicator bromocresol purple under identical conditions in the presence of 0.18–0.2 mg/ml of proteoliposomes and 11 μM cytochrome *c* plus 50–100 μM ferricyanide [9].

The absorbance changes were monitored either in a Sigma Biochem dual-wavelength spectrophotometer or in a Cary 15 spectrophotometer, and were routinely calibrated with HCl N/100 Titrisol Merck; the signals gave linear responses with the H^+ concentration, and were corrected, when necessary, for the spectral interferences found in the absence of indicators.

Electron-transfer activities. The redox activities were assayed under the same experimental conditions employed in the proton-translocating assays. Succinate-ubiquinone-dichlorophenolindophenol reductase was assayed as described in Ref. 21 by starting the reaction with 15–20 μM DCIP. Succinate- and ubiquinol-cytochrome *c* reductase activities were measured as previously described [22]. The respiratory control ratio of b-c_1 complex proteoliposomes was estimated from the stimulation of the ubiquinol-2-cytochrome *c* reductase rate by 10 μM CCCP plus 1 μg/ml valinomycin.

Rapid reduction and reoxidation of cytochrome *b* were followed either at 430–410 nm or at 562–575 nm in the Sigma Biochem Spectrophotometer equipped with a stopped-flow apparatus [22]. The reduction of cytochrome $c + c_1$ was followed at 552–540 nm.

[^{14}C]DCCD-binding studies and SDS gel electrophoresis. [^{14}C]DCCD from CEA, France, was preincubated at different times with 1–2 mg/ml of purified *b*-c_1 complex or proteoliposomes, usually in the same buffer used for the proton-translocation assay. The unbound reagent was removed by two washings of 96% cold acetone followed by 5 min centrifugation at 4000 rpm, and the protein pellets were dissolved in the sample lysing buffer containing 2.5% SDS, 5% mercaptoethanol, 10% glycerol, 63 mM Tris-HCl, pH 6.8, at 2 mg/ml [23]. The samples were denatured for 5 min at 100°C, or for 2 h at room temperature (no significant change in the gel pattern was detected in the two cases), and then run in 15% acrylamide slab gels according to Ref. 23 at 10 mA for 10 h. The running gels of about 9 cm length were stained and destained as described in Ref. 24 or directly sliced in 2-mm thick slices. The gel slices were digested in 15% H_2O_2 at 55°C overnight, and counted in 4.5 ml of Insta-Gel Hewlett-Packard scintillation fluid using a Packard Tri-carb scintillation counter.

We have also performed SDS-polyacrylamide gel electrophoresis using 12–20% linear gradients of acrylamide [25] in collaboration with Drs. C. Montecucco and H. Gutweniger from the Institute of Pathology, University of Padua. In these systems the gels were sliced and counted as described in Ref. 25.

Miscellanea. Protein was measured by a biuret method [28]. The quinol concentration was evaluated at 290 nm using an extinction coefficient of 4 mM^{-1}. DCCD from Sigma was dissolved in ethanol at 0.1–0.05 M and stored at −20°C. Only freshly prepared solutions were added to the assay mixtures to final concentrations always below 10^{-3} M; the inhibitor was usually preincubated 5–10 min at room temperature, since the half-time of inhibition was found to range between 1 and 3 min.

Soybean phospholipids were purchased from Associated Concentrates, NY, U.S.A., and used as such without purification. Ubiquinones were a kind gift from Eisai Co., Tokyo, Japan. All other reagents were the purest commercially available.

Results

Effects of DCCD on the span between succinate and oxygen

In bovine heart submitochondrial particles the ΔpH promoted by succinate oxidase activity is completely inhibited by DCCD at concentrations around 200 nmol per mg protein, corresponding to 800–1000 mol per mol cytochrome c_1, without any decrease of the electron transfer to oxygen [8,27]. Also, the transmembrane potential driven by succinate oxidase activity is completely abolished by DCCD in tightly coupled submitochondrial particles, as found in collaboration with Dr. C. Sorgato from the Institute of Biological Chemistry of the University of Padua, using a flow-dialysis technique [28] (Fig. 1).

In bovine heart mitochondria the inhibitor titer for external acidification is close to that found for ΔpH in submitochondrial particles, whereas in rat liver mitochondria is clearly higher with respect to cytochrome c_1 concentration (Table I), and almost coincident with the values obtained in the succinate-ferricyanide assay or in the ubiquinol-2-cytochrome *c*-ferricyanide assay in the same system [9]. In both mitochondria and submitochondrial

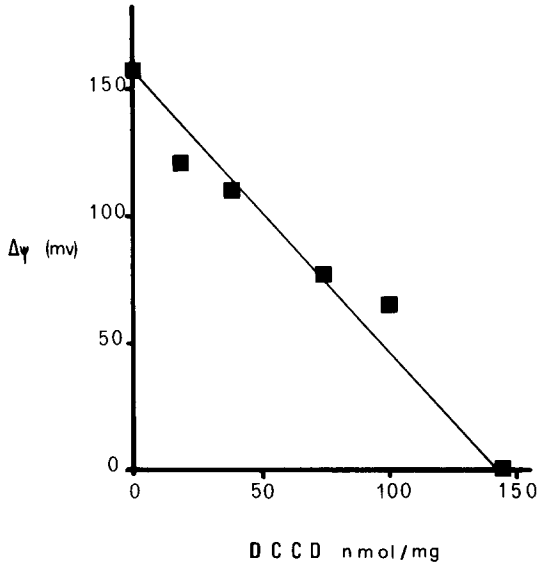

Fig. 1. Inhibition by DCCD of the transmembrane electrical potential built up during succinate oxidation in bovine heart submitochondrial particles; the experimental conditions were those described in Ref. 28.

TABLE I

DCCD TITER FOR HALF-INHIBITION OF THE PROTON-TRANSLOCATING ACTIVITY OF DIFFERENT MITOCHONDRIAL PREPARATIONS WITH 5 mM SUCCINATE AT pH 7.8

All the assays were performed during aerobic succinate oxidation except the succinate-ferricyanide assay.

Mitochondrial preparation	pH indicator	Apparent half-inhibition (mol DCCD per mol cytochrome c_1)
Bovine heart submitochondrial particles	9-aminoacridine	200–270
Cytochrome *c*-depleted bovine heart mitochondria	phenol red	280
Rat liver mitochondria	phenol red	650
Rat liver mitochondria [a]	phenol red	670

[a] Succinate-ferricyanide (125 μM) assay in the presence of 2 mM KCN.

particles DCCD is not able to enhance the proton permeability of the membrane at concentrations blocking proton translocation (cf. Table I of Ref. 9 and Fig. 6 of Ref. 27, and also Ref. 10); moreover, DCCD can affect the rate of succinate oxidase or succinate-cytochrome *c* reductase only at much higher concentrations, with apparent half-inhibition at greater than or equal to 6000 mol per mol cytochrome c_1 [29]. Thus, at diimide concentrations below 1000 mol per mol b-c_1 complex, the proton extrusion coupled to succinate oxidation is highly inhibited without any relevant decrease of the steady-state redox activity of ubiquinol-cytochrome *c* reductase [9]. We have found, however, that under such conditions the redox responses of endogenous cytochromes display some alterations [30]. The reduction of cytochrome *b* upon anaerobiosis is clearly accelerated by DCCD, with a modification of the reduction pattern from biphasic to monophasic (Fig. 2). On the other hand, the reduction of the *c*-type cytochromes upon anaerobiosis appears to be only slightly enhanced under the same conditions. The acceleration of cytochrome *b* reduction induced by DCCD is evident also by stopped-flow techniques even in the isolated b-c_1 complex.

Once reduced by substrates, cytochrome *b* is

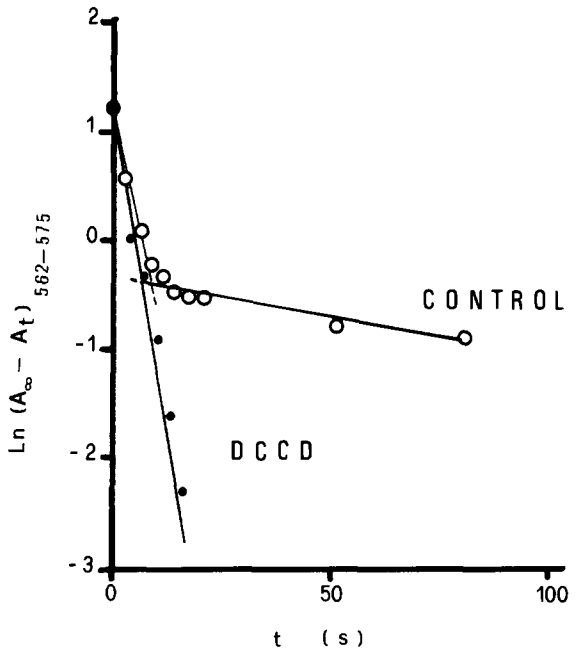

Fig. 2. Semilogarithmic plots of the reduction of endogenous cytochrome *b* upon anaerobiosis in submitochondrial particles (2 mg/ml) after a pulse of 5 mM succinate. The experimental conditions were those described in Ref. 8, and DCCD was 400 mol per mol cytochrome c_1.

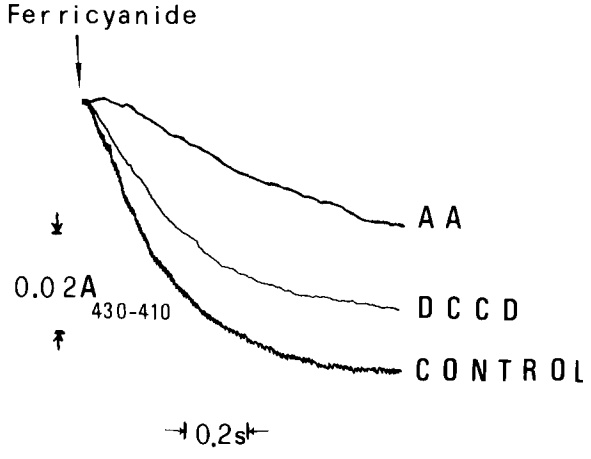

Fig. 3. Effect of DCCD (500 mol per mol cytochrome c_1) on the rapid reoxidation of cytochrome *b* by 330 μM ferricyanide in a crude succinate-cytochrome *c* reductase preparation suspended in 0.66 M sucrose, 50 mM Tris-HCl, 1 mM histidine, pH 8.0, at a final concentration of about 0.4 μM cytochrome *b*. The enzyme was previously reduced with 0.25 mM NADH plus 10 mM succinate for 5 min. AA, plus 2 μM antimycin A.

reoxidized by excess ferricyanide at lower rates when intact mitochondria or mitochondrial subfractions like crude succinate-cytochrome *c* reductase [22] are pretreated with low concentrations of DCCD (Fig. 3). Cytochrome *b* rapid oxidation by ferricyanide is more than 80% antimycin sensitive under the conditions employed in the experiment of Fig. 3, and the antimycin-sensitive rates are clearly inhibited by the diimide, with a resulting titer about one order of magnitude lower than that of the overall succinate-cytochrome *c* reductase activity of the preparation.

Effects of DCCD on the span between succinate and ubiquinone

DCCD is not able to inhibit the succinate-ubiquinone reductase activity in mitochondria up to concentrations of 8000–10000 mol per mol cytochrome c_1. In contrast, in the concentration range blocking proton translocation associated with succinate oxidation, the succinate-ubiquinone reductase rates are accelerated up to 2-fold (Fig. 4) (cf. Refs. 9 and 29). The rate of DCIP reduction stimulated by DCCD appears less sensitive to thenoyltrifluoroacetone (a specific inhibitor of Complex II [31]), and is decreased by addition of purified superoxide dismutase (bovine blood, Sigma).

We are going to investigate further these effects.

Effect of DCCD on the span between ubiquinol and cytochrome c

The proton-translocating function of the b-c_1 complex has been investigated by a rapid pulse of

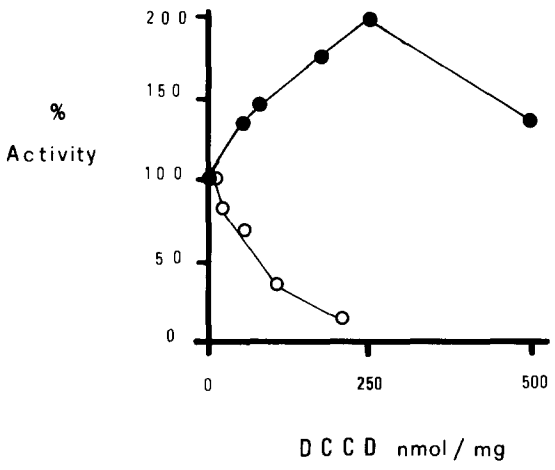

Fig. 4. Stimulation by DCCD of the succinate-Q_1-DCIP reductase activity in rat liver mitochondria (1 mg/ml) with 5 mM succinate, 75 μM Q_1 and 17 μM DCIP (●———●). For comparison, the inhibition of the vectorial acidification monitored by the phenol red indicator with aerobic 5 mM succinate and 2.5 mg/ml of mitochondria is shown (○———○).

ubiquinol-2 either in mitochondria or in the isolated b-c_1 complex reconstituted in proteoliposomes. Due to the very low K_m of 0.6 μM for this quinol (Degli Esposti, M., unpublished result) and the high enzymatic rates driven [18], after a rapid pulse of ubiquinol-2 the complex can undergo multiple turnovers at steady state in the presence of enough oxidants like oxygen or exogenous cytochrome *c* plus excess ferricyanide. In intact mitochondria the aerobic acidification obtained with excess cytochrome *c* can be an indication of the proton-translocating function of the b-c_1 com-

TABLE II

EFFECT OF DCCD ON THE INITIAL RATES OF AEROBIC PROTON TRANSLOCATION AND CYTOCHROME *c* REDUCTION

Experiments were performed on coupled rat heart mitochondria (0.47 mg/ml) with 30 μM Q_2H_2, in the presence of 5 μM exogenous cytochrome *c*. The pH indicator was phenol red, and the absorbance changes were monitored at 556.5 nm as in Fig. 4. The pH of the medium was 7.4.

Addition	Rates of external acidification (μM/s)	Rates of H^+ back-flow (μM/s)	Rates of cytochrome *c* reduction (μM/s)
None	5.7	0.43	5.6
53 nmol/mg DCCD	2.8	0.37	7.1
266 nmol/mg DCCD	0	–	9.2

plex; in fact, under such conditions, cytochrome *c* is rapidly reduced, reaches a brief steady-state level (concomitant with the maximal extent of acidification), and is then reoxidized at a somewhat lower rate, so that the vectorial proton extrusion is predominantly driven by the ubiquinol-cytochrome *c* reductase activity.

In coupled rat liver mitochondria DCCD inhibits the vectorial acidification associated with quinol oxidase activity (Fig. 5), with half-inhibition values of about 700 mol per mol cytochrome c_1, i.e., almost coincident with that found in the ubiquinol-cytochrome *c* span assayed in the presence of KCN (cf. Fig. 1 in Ref. 9).

We have observed that under certain conditions, particularly at high concentrations, the inhibitor produces a release of excess scalar, CCCP-insensitive protons during quinol oxidation (result not shown). As shown in Table II in the case of rat heart mitochondria, but observed in all preparations, DCCD clearly decreases also the initial rates of acidification coupled to quinol oxidation, whereas the initial rates of cytochrome *c* reduction are generally enhanced, without any increase of the rate of proton back-flow. This stimulation of cytochrome *c* reduction may be due to many reasons: firstly, the decrease of the residual electrochemical potential induced by the inhibitor (cf. Fig. 1) should enhance the electron-transfer activity, which is normally inhibited by $\Delta\Psi$; secondly, the DCCD concentration used can inhibit the redox activity of cytochrome *c* oxidase [5], so that the production of excess scalar protons could be promoted by faster cytochrome *c* reduction not compensated by oxygen consumption. Another factor for increased production of scalar protons in the DCCD-treated particles could be the complexation of the diimide with phospholipids [32] interfering with protonation-deprotonation equilibria during cytochrome *c* reduction, which has been found to be accompanied by H^+ uptake [33].

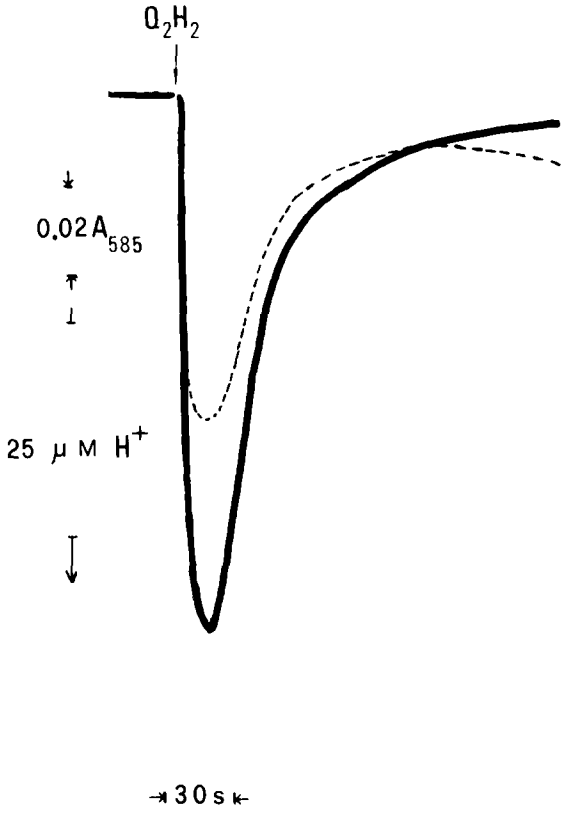

Fig. 5. Vectorial proton extrusion associated with ubiquinol-2 (27 μM) oxidation in aerobic rat liver mitochondria (1.03 mg/ml) and effect of 100 nmol DCCD/mg protein; the traces were obtained at pH 7.0 with the indicator bromocresol purple, and also 5 μM cytochrome *c* was added to the assay mixture. The dashed line is that found in the presence of the inhibitor.

In the isolated *b*-c_1 complex we have preferred to monitor the proton-translocating activity of *b*-c_1 complex proteoliposomes by means of the pH indicator bromocresol purple, since its absorbance changes at 585–590 nm do not suffer from spectral interference from cytochrome *b* reduction as for phenol red at 556.5 nm. However, with both indicators, we have found that in DCCD-treated proteoliposomes the sensitivity of the dyes to the external proton concentration is higher than in untreated proteoliposomes. This may be due to a decreased buffering capacity of the assay mixture induced by the diimide. In fact, DCCD lowers the passive membrane permeability in proteoliposomes with *b*-c_1 complex isolated from yeast mitochondria [11], hence limiting the exit of the internal buffer of the vesicles; DCCD can also complex some ionizable groups in the proteoliposomes that contribute to the overall buffering capacity of the assay system [32]. For this reason we have calibrated the indicator responses in the presence and absence of DCCD, correcting the observed absorbance changes for the actual sensitivity of the dye to acidification. For instance, in the experiment presented in Fig. 6A, the ab-

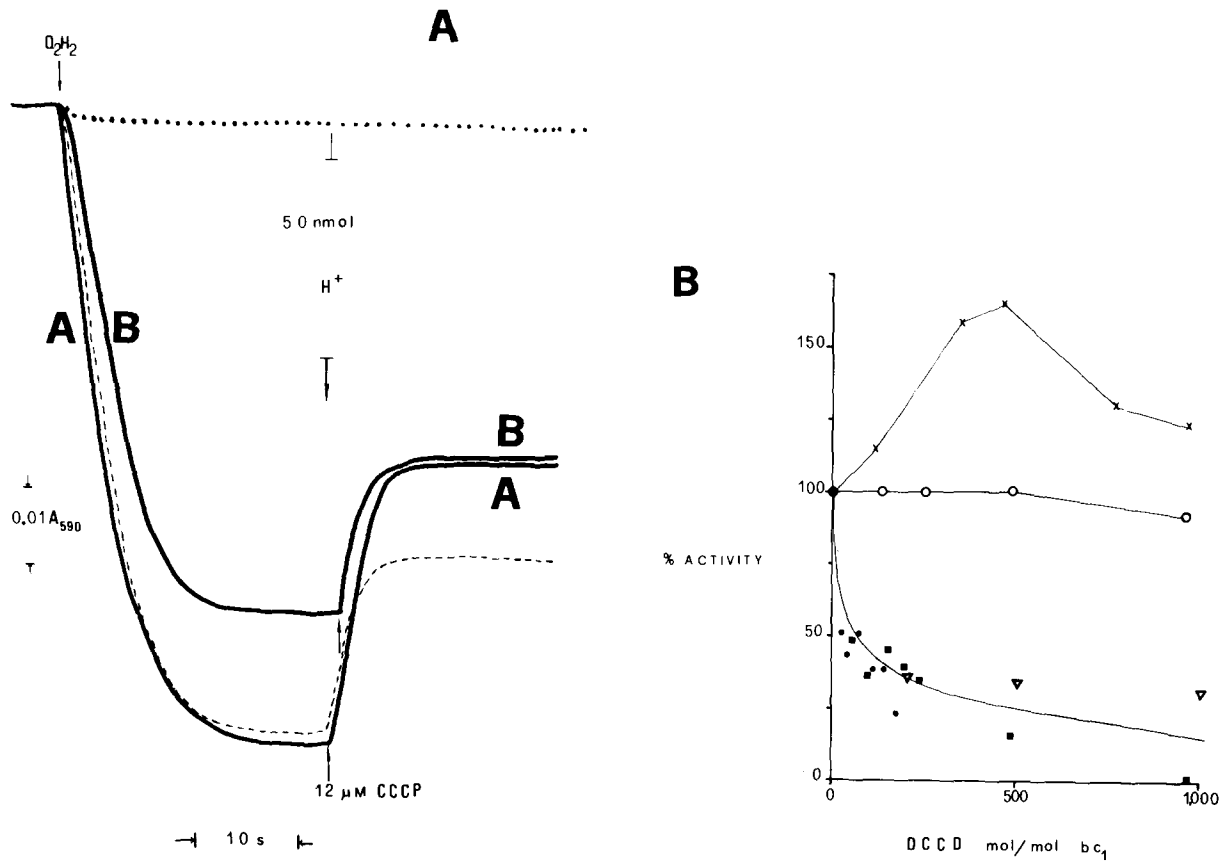

Fig. 6. (A) Proton translocation of b-c_1 complex proteoliposomes (0.18 mg/ml) exhibiting a respiratory control ratio of 9, followed at 590 nm in a Cary-15 spectrophotometer equipped with a rapid-mixing apparatus [29]. (Trace A) Control trace with 45 nmol of Q_2H_2; the dotted line refers to the trace obtained in the presence of 2 μM antimycin; (trace B) trace in the presence of 68 mol DCCD per mol cytochrome c_1, corrected for the different calibration for H^+ (20% higher than in the control, see text). The dashed line represents the experimental trace obtained with DCCD which was not corrected for the different sensitivity of bromocresol purple. The stoichiometry of the reaction was $3.8H^+/2e^+$ in the control, of which $1.6H^+/2e^-$ were CCCP sensitivie, and $2.8H^+/2e^+$ in the presence of DCCD, of which $0.8H^+/2e^-$ were CCCP sensitive. (B) Titration of the effect of DCCD on the CCCP-sensitive external acidification (the different symbols (▽, ■, ●) refer to different experiments), and on the rate of Q_2H_2-cytochrome c reductase under the same conditions (○——○), and in the absence of valinomycin (×——×) in b-c_1 complex proteoliposomes under the same experimental conditions as in A.

sorbance change of bromocresol purple was found to be 20% higher in the presence of the diimide, so that, after correction, the decrease of proton translocation induced by the inhibitor is more evident, and the stoichiometry of scalar protons approaches $2H^+/2e^-$, as expected. Using bromocresol purple as indicator at pH 7.0, we have found a stoichiometry of the overall proton extrusion coupled to ubiquinol-2-cytochrome c reductase of $3.7H^+/2e^-$ in b-c_1 complex proteoliposomes (mean of four experiments). About one-half of the acidification (mean value = $1.8H^+/2e^-$) is reversed by uncouplers like CCCP, and hence is due to vectorial proton extrusion (Fig. 6A). These findings agree with the previously reported stoichiometries approaching $4H^+/2e^-$ obtained by direct measurements of pH [11,19].

The inhibition of DCCD of the vectorial proton extrusion exhibits biphasic behavior (Fig. 6B), and the apparent half-inhibition values range between 60 and 100 mol DCCD per mol cytochrome c_1. Under the same experimental conditions the rates of ubiquinol-2-cytochrome c reductase are not altered up to 1000 mol per mol cytochrome c_1,

whereas a partial stimulation of the redox activity is observed in the absence of valinomycin (Fig. 6B), as expected by the decreased transmembrane proton pressure elicited in the presence of the diimide. As already pointed out for mitochondria, this effect is not due to an increased permeability of the membrane vesicles to protons. In fact, the proteoliposomes retain good respiratory control ratios throughout the DCCD concentration range inhibiting proton translocation, and the rate of proton back-flow is not accelerated, but rather decreased by the inhibitor. As an example, under conditions identical to those of Fig. 6, 200 mol DCCD per mol cytochrome c_1 decreased the rate of proton back-flow from 0.80 to 0.44 min^{-1}. This evidence suggests that DCCD is not able to 'uncouple' the lipid vesicles with reconstituted b-c_1 complex, in agreement with results obtained for other enzymes (cf. Refs. 4, 5 and 7) and the same enzyme [9–11].

The antimycin titer of the ubiquinol-cytochrome c reductase is not modified by treatment with DCCD (at least below 1000 mol per mol cytochrome c_1), thus making it unlikely that the inhibition of the proton translocation may be due to a bypass of the normal electron pathway owing to a relevant modification of the enzymatic mechanism.

The redox activity of the detergent-solubilized b-c_1 complex is also inhibited by DCCD, but at much higher concentrations, with apparent half-inhibition values ranging between 5000 and 10000 mol per mol cytochrome c_1 (cf. Ref. 29). The inhibition pattern is usually sigmoidal, with a strong positive cooperativity.

Binding of radiolabelled DCCD to the b-c$_1$ complex

[^{14}C]DCCD binds to the detergent-solubilized b-c_1 complex with a half-time of saturation of about 22 min at room temperature, and of 40 min at 0°C.

We experimented with different systems for washing away the unbound reagent from the enzyme. The most effective system was that using precipitation with 96% acetone, followed by one or more acetone washings, which lead to the removal of the majority of unbound reagent without changing the relative staining intensities of the different bands of the b-c_1 complex in the SDS gel electro-

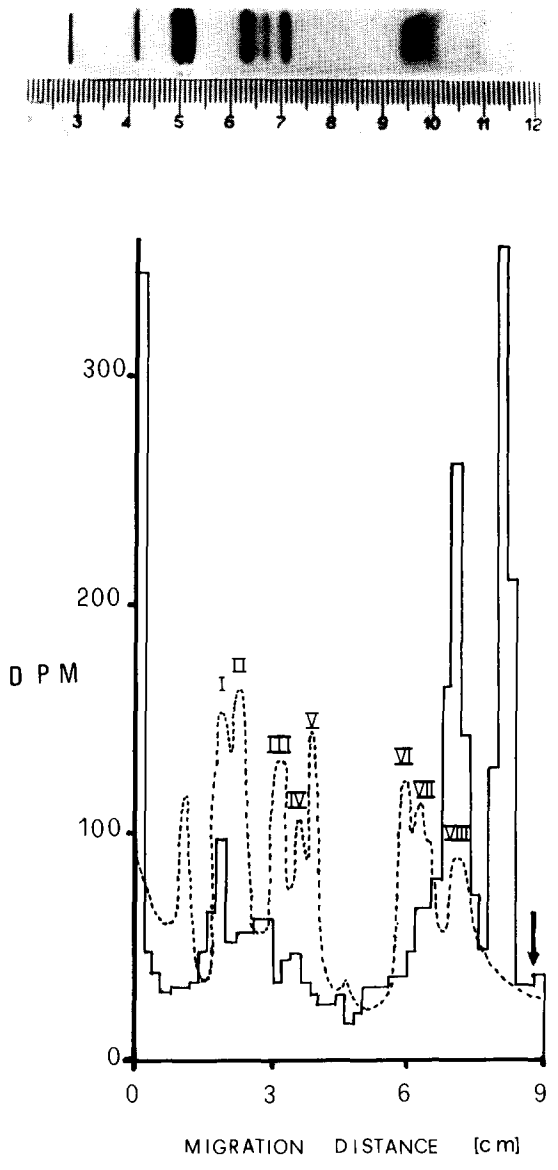

Fig. 7. Gel slices pattern of detergent-solubilized b-c_1 complex preincubated 10 min at room temperature under the same experimental conditions as in Fig. 6 with 100 nmol radiolabelled DCCD per mg protein (i.e., 35 mol per mol cytochrome c_1). 100 μg of acetone-washed samples were applied to a 15% acrylamide slab gel and run in parallel columns; one-half of the gel was stained and destained as in Ref. 24, and the other half was sliced, digested and counted without staining. The dashed line represents the densitometric trace at 560 nm of the stained gel, where the protein bands of the b-c_1 complex are indicated with the correspondent Roman number according to Ref. 23. The arrow corresponds to the tracking dye migration. The gel pattern of b-c_1 complex polypeptides was found to be identical in the absence of DCCD. A photograph of the stained gel is shown in the inset of the figure.

phoresis profile, whereas washings performed by 90% acetone or chloroform/methanol partly remove some low molecular weight components. However, most phospholipids associated with the isolated b-c_1 complex are coprecipitated by 96% acetone with the enzyme. In order to evaluate the extent of DCCD complexed by phospholipids, the acetone-washed pellets were subsequently extracted with chloroform/methanol (2 : 1, v/v). Less than 50% of the radioactivity was detected in the chloroform/methanol extract, of which a relevant portion was still unbound DCCD, and the remaining DCCD associated to phosphatidylserine [32], and possibly to cardiolipin and to the proteolipid fraction, as judged by thin-layer chromatography (Timoneda, J. and Barber, T., unpublished results; see also Ref. 34).

By Scatchard analysis we have found that the maximal binding of DCCD to the b-c_1 complex is about 0.8 mol per mol cytochrome c_1 with 4 h of incubation at room temperature and pH 7.0, and with chloroform/methanol extraction of the samples in order to minimize unspecific binding to lipids and the extent of unbound reagent.

However, it is not easy to establish a reliable quantitative correlation between the binding of radiolabelled DCCD to one or more specific polypeptides of the enzyme and the inhibition of its proton-translocating activity. One main reason is that the binding of the diimide may involve cross-linking reactions, occurring either within one single polypeptide, or between different subunits [1]. We have, in fact, observed that at relatively high DCCD concentrations (greater than or equal to 200 mol per mol cytochrome c_1) a new band appears with a molecular weight of about 43 000, with a concomitant disappearance of most of Band VIII and of Band V (the iron-sulfur protein of the b-c_1 complex [23]). Moreover, a significant amount of radioactivity remains in the stacking gel and in the top of the running gel when DCCD is allowed to react with the b-c_1 complex, possibly due to the formation of highly aggregated cross-linked products which cannot enter the running gel.

An important factor which appears to affect qualitatively the binding of DCCD to the b-c_1 complex is the time of incubation with the reagent. With approx. 10 min of preincubation at room temperature the most labelled polypeptide is a protein band of about 9500 Da, namely, Band VIII in gel systems resolving eight bands of the enzyme [23] (Fig. 7). Under such conditions, only one other protein with a molecular mass of about 50 000 Da is also labelled, although to a much lower extent. This polypeptide can be identified with Band I (namely, Core protein I [23]) of the b-c_1 complex, but may also correspond, at least in part, to a contamination by the β-subunit of F_1-ATPase, which is commonly seen in the gel pattern just ahead of Band I [23], and has been proven to bind DCCD as well [35]. The peak of radioactivity comigrating with the gel front is constituted by DCCD both unbound and complexed to phospholipids (cf. Fig. 7). In fact, after a chloroform/methanol extraction, this peak is much lower, whereas the binding profile to the polypeptides, and particularly to Band VIII, is unchanged. This result excludes that the labelling of Band VIII might be due to contamination by the DCCD-binding protein of F_0-ATPase, which has a molecular weight close to that of Band VIII [3], since this protein should be extracted with chloroform/methanol [3]. Such results appear in partial accordance with previous findings [36].

However, when the time of incubation is greater than 10–20 min, the resulting binding profile is more complex, as many other proteins start to be labelled. Some of them appear contaminations from other complexes of the respiratory chain (particularly, a few bands with molecular mass greater than 40 000 Da, and some between 20 000 and 14 000 Da), but may also be due to cross-linked products induced by prolonged reaction with DCCD. Other peaks of radioactivity also correspond to some of the eight subunits of the b-c_1 complex, particularly to Bands I, IV (cytochrome c_1 [23]), VI and VII. At 4 h of incubation, almost all the protein bands visible in the gel pattern appear to be labelled to different extents by DCCD, but the most heavily labelled one remains Band VIII. This unspecific protein binding with long preincubation is accompanied by a certain loss of the redox activity of the enzyme. On the other hand, the saturation of the binding to Band VIII is much faster than that to all other protein bands, with a half-time between 2 and 3 min, i.e., quite close to the half-time for the inhibition of the proton-translocating activity of the b-c_1 complex

proteoliposomes, which is about 1 min, in accordance with what found in intact coupled mitochondria [37].

In preliminary experiments performed in collaboration with Dr. C. Montecucco of the Institute of Pathology, University of Padua, using a high-resolution SDS gel electrophoresis system [25], we have essentially confirmed the above findings.

Similar results have been also obtained with b-c_1 complex reconstituted in lipid vesicles, but the presence of high concentrations of phospholipids interferes with the normal migration of the proteins, particularly in the low molecular mass range, leading to less clear data.

Discussion

DCCD induces a complex series of effects on the succinate-cytochrome c span of the mitochondrial respiratory chain. We can, however, distinguish two main actions: a primary action, occurring at less than or equal to 1000 mol DCCD per mol cytochrome c_1, which involves inhibition of the proton-translocating activity of the b-c_1 complex concomitant with an alteration of the redox responses of cytochrome b, but without any apparent decrease of the steady-state electron-transfer activity of the enzyme; and a secondary action, occurring at much higher concentrations, which leads to an inactivation of the reductase activity of the complex.

From the labelling experiments we can make a tentative correlation between the primary action and the binding to Band VIII of the complex (cf. Fig. 7). The almost specific labelling of this subunit under reagent concentrations and mode of incubation (e.g., 10 min at room temperature) leading to block of proton translocation, but not of the maximal redox activity of the enzyme (cf. Fig. 6B), clearly supports this correlation. Band VIII, constituted by three small polypeptides [25], does not carry any prosthetic group, but appears to be directly involved in the binding of antimycin [38]; the function of these polypeptides, however, is at present unknown. Since with preincubation longer than 10–20 min, and/or at DCCD concentrations higher than 50 mol per mol cytochrome c_1, other protein bands appear to be labelled by the reagent, any clear and unequivocal conclusion cannot be reached about the exact correspondence between the structural modification and the primary action of the inhibitor. Moreover, the evidence for cross-linking reactions between various subunits of the b-c_1 complex may indicate that, at least partially, the inhibition of proton translocation could be due to condensation reactions between different polypeptide chains induced by DCCD. Such cross-linking effects may be more relevant in the secondary action of the diimide, occurring at higher concentrations, since they directly involve an essential redox group of the enzyme as the iron-sulfur protein. The cooperative inhibition of the reductase activity in the isolated enzyme is also in accordance with this view.

Contrary to a recent report by Beattie and Clejan [39], no clear labelling of cytochrome b (Band III) has been observed in comparison to that of other protein bands, even after prolonged preincubation.

In view of the implications on the mechanism of the proton-electron transfer at the level of the second coupling site, the primary action of DCCD needs to be further discussed here. How does the binding of this carboxyl reagent to the b-c_1 complex block proton translocation and affect the redox responses of cytochrome b? As suggested by the DDCD binding at times corresponding to the time course of inhibition of proton translocation, we could speculate that at least one (if not all) polypeptide making up Band VIII may be involved in the proton-extrusion device of the b-c_1 complex. These proteins, in fact, contribute to the membrane sector of the enzyme, as indicated by hydrophobic labelling [25]. The binding of DCCD to one or more of these polypeptides could alter the redox responses of cytochrome b, which is also deeply buried in the membrane core of the complex [25,40], through modifications of the protein-protein or protein-lipid interactions inside the structure of the membranous portion of the enzyme. If this interpretation, which appears only tentative at the present stage, were correct, it should mean that the redox centers of the b-c_1 complex and the site of proton translocation are physically separated in different subunits, and functionally connected through its quaternary structure in the membrane.

Other explanations are possible. Since during the catalytic function of the b-c_1 complex a species of 'bound' ubisemiquinone is formed, whose stabilization is quite sensitive to the conformation of the enzyme [41], one possibility could be that the DCCD binding alters such stabilization of the radical. Some of the effects of the diimide found in the succinate-ubiquinone reductase (cf. Fig. 4) may be explained by a similar action, which should lead to enhanced disproportionation of semiquinones to molecular oxygen. Such a mechanism should result also in a lower sensitivity of cytochrome c reduction to antimycin A, since it could be promoted by superoxide anions, with a consequent 'uncoupling' of a Q-cycle mechanism [42], and direct equilibration of the proton gradient. Although we were able to detect a slight overproduction of oxygen radicals during the succinate-cytochrome c reductase activity (Rugolo, M. and Degli Esposti, M., unpublished results), no significant alteration of the sensitivity to antimycin of the ubiquinol-cytochrome c reductase activity has been observed, indicating that no major disruption of the electron flow is induced by DCCD in the b-c_1 complex.

Therefore, the present findings suggest the existence of a DCCD-sensitive proton pump in the b-c_1 complex responsible for the proton translocation at the second coupling site of the mitochondrial respiratory chain.

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