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A Clarification of the Effects of DCCD on the Electron Transfer and Antimycin Binding of the Mitochondrial bc_1 Complex

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Abstract

We have studied in detail the effects of dicyclohexylcarbodiimide (DCCD) on the redox activity of the mitochondrial bc_1 complex, and on the binding of its most specific inhibitor antimycin. An inhibitory action of the reagent has been found only at high concentration of the diimide and/or at prolonged times of incubation. Under these conditions, DCCD also displaced antimycin from its specific binding site in the bc_1 complex, but did not apparently change the antimycin sensitivity of the ubiquinol–cytochrome *c* reductase activity. On the other hand, using lower DCCD concentrations and/or short times of incubation, i.e., conditions which usually lead to the specific inhibition of the proton-translocating activity of the bc_1 complex, no inhibitory effect of DCCD could be detected in the ubiquinol–cytochrome *c* reductase activity. However, a clear stimulation of the rate of cytochrome *b* reduction in parallel to an inhibition of cytochrome *b* oxidation has been found under these conditions. On the basis of the present work and of previous reports in the literature about the effects of DCCD on the bc_1 complex, we propose a clarification of the various effects of the reagent depending on the experimental conditions employed.

Key Words: bc_1 complex; DCCD; ubiquinol–cytochrome *c* reductase; antimycin; pre-steady-state kinetics; cytochrome *b*; beef heart mitochondria.

Introduction

Dicyclohexylcarbodiimide (DCCD),[3] a well established carboxyl reagent (Solioz, 1984), has been shown to block the proton-translocating activity of the mitochondrial bc_1 complex without changing the proton conductance of the membrane (Lenaz *et al.*, 1982; Degli Esposti *et al.*, 1982, 1983; Price and

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[3] Abbreviations: DCCD, *N'*,*N'*-dicyclohexylcarbodiimide; Q_1H_2, ubiquinol-1; Q_2H_2, ubiquinol-2.

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Brand, 1982a, b; Beattie and Villalobo, 1982b; Lorusso *et al.*, 1983; Nalecz *et al.*, 1983). Most of the published reports outline a specific inhibition of proton translocation with no relevant effects on the redox activity of the bc_1 complex (Degli Esposti *et al.*, 1982, 1983; Price and Brand, 1983; Beattie and Villalobo, 1982b; Lorusso *et al.*, 1983), but some other reports indicate that the electron transfer activity of the enzyme is also affected by DCCD (Lenaz *et al.*, 1982; Nalecz *et al.*, 1983; Degli Esposti *et al.*, 1981). However, these results were usually obtained only at high ratios of the diimide to the bc_1 complex, and at times of incubation longer than 30 min. If the reaction of the DCCD with the enzyme is accomplished at relatively low concentrations, and within 10 min of incubation, only the proton translocating activity is selectively inhibited, whereas the redox function is even stimulated, particularly under coupled conditions (Degli Esposti *et al.*, 1983; Price and Brand, (1983).

Therefore, depending on the binding conditions employed, one may discriminate between the primary action on the proton pumping activity and the secondary, less specific inhibition of the electron transfer activity of the bc_1 complex (Degli Esposti *et al.*, 1983). It is important to stress and support this point experimentally, since it could be meaningful for the clarification of the mechanism of action of the bc_1 complex. A redox-linked Bohr mechanism (Lorusso *et al.*, 1983) or a proton pumping system (Degli Esposti *et al.*, 1983; Beattie and Villalobo, 1982b) could be in accordance with such "decoupling" effect of DCCD, particularly if the redox pathway within the complex has not been altered by the reagent.

Clejan and Beattie (1983) have recently shown that the treatment of yeast bc_1 complex with DCCD induces a displacement of the antimycin binding to the enzyme, and a lower sensitivity of the ubiquinol–cytochrome *c* reductase to the antibiotic. Such findings may raise doubts on the above interpretation, as they could indicate that the electron transfer catalyzed by the bc_1 complex is modified by DCCD and is less affected by the inhibitory action of the most powerful inhibitor of the enzyme, namely antimycin. This indication may be more in accordance with a ligand-conducting mechanism for proton translocation in the bc_1 complex (Mitchell, 1976), which can explain the "decoupling" effect of DCCD as a deviation of the redox pathway induced by the reagent in some critical steps, normally tightly coupled to proton exchange reactions. The apparently unaltered redox activity of the bc_1 complex would no longer be physiological, and therefore would be less sensitive to the specific inhibitor antimycin.

The purpose of this paper is to analyze the experimental conditions leading to the inhibition by DCCD of the redox activity of the bc_1 complex and the displacement of the antimycin binding, in order to better understand the mechanism of action of the reagent.

Materials and Methods

Beef heart mitochondria were a generous gift from the late Prof. D. E. Green, Madison, Wisconsin. Sonic submitochondrial particles were prepared by the method of Hansen and Smith (1964). Fraction S_1 and isolated bc_1 complex were prepared as described by Rieske (1967).

Ubiquinols were reduced by the method of Rieske (1967). Reconstitution of the isolated bc_1 complex into asolectin vesicles was accomplished by the cholate-dialysis procedure, as previously described (Degli Esposti *et al.*, 1983).

Ubiquinol–cytochrome *c* reductase was assayed as previously described (Degli Esposti *et al.*, 1983). The rapid reduction of cytochrome *b* was followed either at 430–410 or at 562–575 nm by means of a stopped-flow apparatus in a dual-wavelength Sigma Biochem spectrophotometer (Degli Esposti *et al.*, 1983). The transmembrane pH gradient built up by succinoxidase activity was monitored by the quenching of 9-aminoacridine (Lenaz *et al.*, 1982).

The red shift produced by antimycin binding on the reduced cytochrome *b* spectrum was monitored either in the α-band, at 564–558 nm, or in the Soret region, at 433–424 nm (Von Jagow *et al.*, 1982). Spectra of cytochromes were obtained in a Perkin-Elmer 559 split-beam spectrophotometer. Cytochrome c_1 was evaluated after dithionite reduction at 554–541 nm with an extinction coefficient of $28\,mM^{-1}$, higher than the coefficient reported for the purified cytochrome (Rieske, 1967) to account for the overlapping of reduced cytochrome *b* at the above wavelength couple. Such determination was found to adequately correspond to that obtained after the specific reduction of cytochrome c_1 by ascorbate.

DCCD from Sigma was prepared as a 0.1 M solution in ethanol immediately before the experiments.

Results

Effects of DCCD on the Ubiquinol-Cytochrome c Reductase Activity in the Isolated bc_1 Complex

We have previously reported that the ubiquinol–cytochrome *c* reductase activity of intact mitochondrial particles is clearly inhibited by relatively high concentrations of DCCD (Degli Esposti *et al.*, 1981; Lenaz *et al.*, 1982). This action is less evident in the detergent-solubilized bc_1 complex extracted from beef heart mitochondria, displaying a remarkable positive cooperativity (Fig. 1A). Titers as high as 5–10,000 mol of DCCD per mole of cytochrome c_1 are

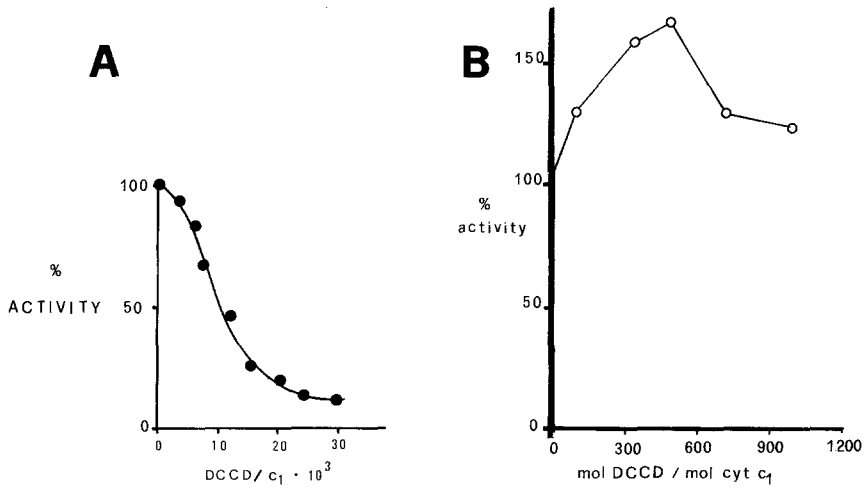

Fig. 1. (A) Inhibition of the Q_1H_2 (55 μM)–cytochrome *c* (10 μM) reductase activity in the isolated bc_1 complex (to a final concentration of 4 nM). DCCD was added directly to the assay mixture and incubated 2 min with the enzyme. If the reagent were incubated with the concentrated solution of the complex, a somewhat lower titer was obtained. (B) Effect of DCCD (incubated for 5 min at room temperature with the concentrated enzyme suspension) on the Q_2H_2 (27 μM)–cytochrome *c* (11 μM) reductase activity of bc_1 proteoliposomes retaining a respiratory control ratio of 5. The final concentration of the enzyme was approximately 5 nM.

required for half-inhibition of the redox activity of the isolated enzyme at times of incubation shorter than 10 min (room temperature). When the time of incubation is increased, the effect of the diimide increases, with a half-time of about 2 h at 4°C. For instance, with an overnight incubation with 50 mol of DCCD per mole of cytochrome c_1 at 4°C, we observed an exent of inhibition around 30% of the Q_1H_2–cytochrome *c* reductase activity.

However, once reconstituted into proteoliposomes, the isolated bc_1 complex becomes less sensitive in its redox activity to such inhibitory action of DCCD. This could be partly due to the dilution of the reagent in the lipid bilayer, and to the direct reaction with some phospholipids (Degli Esposti *et al.*, 1983). Actually, under coupled conditions (i.e., in the absence of protonophores or valinomycin plus KCl), the treatment with DCCD stimulates the redox activity of bc_1-proteoliposomes (Fig. 1B). At diimide concentrations higher than 500–1000 mol per mole of cytochrome c_1, or with times of incubation longer than 10 min, an inhibitory action is seen as in the detergent-solubilized enzyme (Degli Esposti *et al.*, 1983). The reductase rates in the presence of the diimide remain antimycin-sensitive as in the controls. The stimulation of the redox activity under coupled conditions could not be ascribed to an uncoupling-like effect of DCCD in the proton permeability of the lipid vesicles (Degli Esposti *et al.*, 1983).

Thus, the effects of DCCD on the redox activity of the bc_1 complex are easily distinguishable from the more specific inhibition on the proton-translocating activity, which has a very rapid time course (Price and Brand, 1982b; Degli Esposti *et al.*, 1983) and usually occurs at diimide concentrations lower than 200 mol per mole of cytochrome c_1 (Degli Esposti *et al.*, 1983; Lorusso *et al.*, 1983).

Effects of DCCD on the Redox Changes of Cytochrome b

Under conditions leading to the "decoupling" effect of DCCD, the rate of cytochrome *b* reduction and reoxidation are significantly altered (Degli Esposti *et al.*, 1983). In coupled submitochondrial particles 192 mol DCCD per mole of cytochrome c_1 induce a 50% decrease of the transmembrane pH gradient driven by aerobic succinate oxidation (without affecting the succinoxidase activity), and a clear stimulation of cytochrome *b* reduction by succinate (Fig. 2). Similar data have been obtained in the presence of KCN,

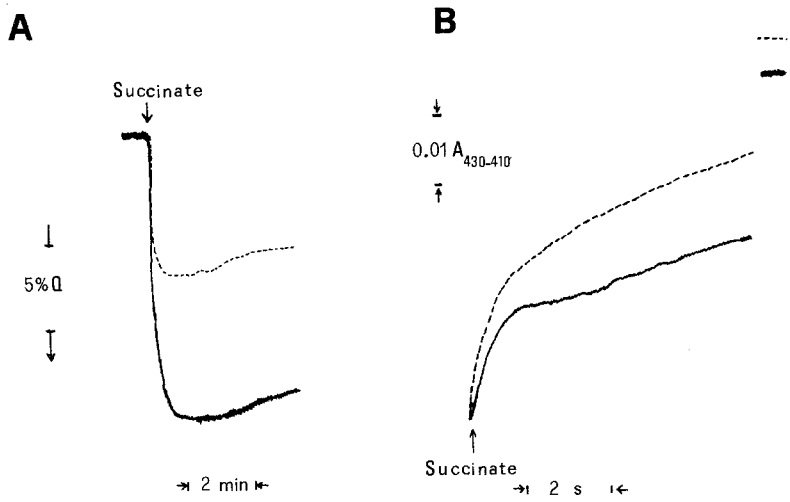

Fig. 2. (A) Quenching of 9-aminoacridine during aerobic succinate oxidation with submitochondrial particles to a final concentration of about 80 nM cytochrome c_1. The assay conditions were those previously described (Lenaz *et al.*, 1982). The dashed line represents the trace obtained in the presence of 192 mol of DCCD per mole of cytochrome c_1 incubated for 10 min at room temperature directly in the assay mixture. Under such conditions, no change in the rate of both succinoxidase and ubiquinol–cytochrome *c* reductase activity was detected. (B) Stopped-flow traces of the reduction of cytochrome *b* by succinate in submitochondrial particles (to about 1 μM cytochrome c_1 at the final concentration) previously oxidized with 3 μM ferricyanide in the absence (solid line) and in the presence (dashed line) of 192 mol of DCCD per mole of cytochrome c_1. The experimental conditions were identical to those in A. The upper short lines represent the final extents of cytochrome *b* reduction. Succinate was 5 mM in both A and B.

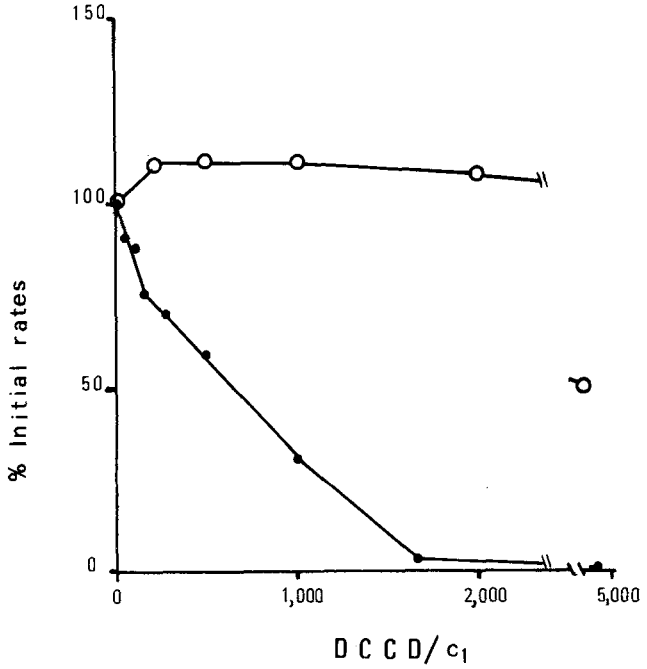

Fig. 3. (●) DCCD titration of the rapid oxidation of cytochrome *b* by 330 μM ferricyanide in fraction S_1 (to a final concentration of 0.2 μM cytochrome c_1) previously reduced by 5 mM succinate and 0.2 mM NADH. The traces were obtained in the stopped-flow apparatus, and were over 80% sensitive to antimycin (Degli Esposti *et al.*, 1983). DCCD was incubated with the concentrated enzymatic solution for 10 min at room temperature. (○) Succinate (5 mM)–cytochrome *c* reductase (5 μM) activity of 0.02 μM cyt c_1 of fraction S_1 under the same conditions, and effect of DCCD. As before, the reagent was incubated with the concentrated solution of the enzyme. The experiments were carried out in the 0.67 M sucrose, 50 mM Tris-Cl buffer, pH 8.0.

and also in intact mitochondria. Increasing the DCCD concentrations to values leading to an inhibition of also the ubiquinol–cytochrome *c* reductase activity results in a progressive loss of the stimulating effect on cytochrome *b* reaction.

An interesting observation is that the modification of the reduction pattern of cytochrome *b* by substrates is much clearer when the preparation is partially prereduced. Since it is quite difficult to maintain the redox state of mitochondrial particles, or even of mitochondrial subfractions, at a carefully controlled oxidized state in the stopped-flow apparatus, we were not able to obtain a reliable correlation between such effect of DCCD and the inhibition on the proton-translocating activity of the same preparation.

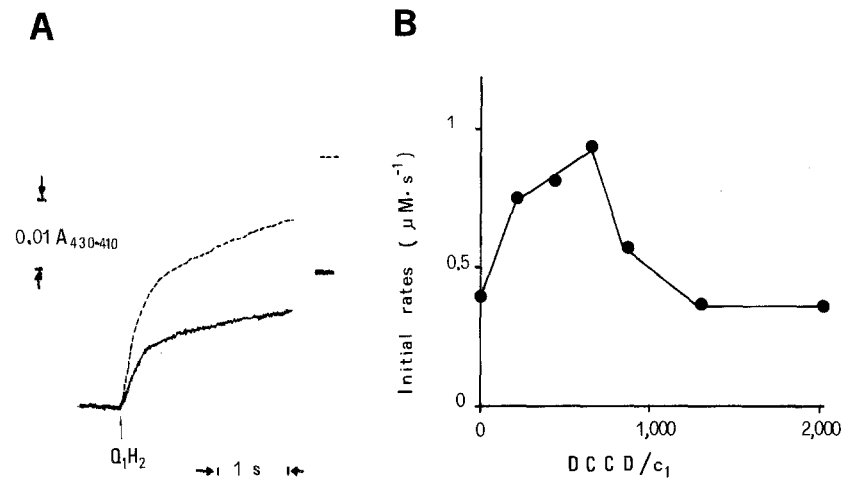

Fig. 4. (A) Rapid reduction of cytochrome *b* by ubiquinol-1 (6 μM) in fraction S_1 to about 60 nM cytochrome c_1. The dashed line represents the trace obtained in the presence of 217 mol of DCCD per mole of cytochrome c_1 preincubated 2 min directly in the assay mixture. (B) DCCD titration of the rapid reduction of cytochrome *b* by ubiquinol-1 under the same conditions as in A. The experiments were performed in the 0.1 M K-phosphate, 1 mM EDTA buffer, pH 7.4. The extinction coefficient used for cytochrome *b* was 160 mM^{-1}.

A more meaningful correlation has been found comparing the latter effect of the diimide with its inhibition of the reoxidation of reduced cytochrome *b* by ferricyanide, as shown in Fig. 3 (cf. Degli Esposti *et al.*, 1983). In fact, it is much simpler to maintain the mitochondrial fraction prereduced than completely oxidized during the stopped-flow experiments.

It has been reported that DCCD also modifies the redox responses of cytochrome *b* in the isolated bc_1 complex reconstituted in proteoliposomes, abolishing electrogenic changes due to reverse electron flow in the complex (Beattie and Villalobo, 1982b). We have found that the rate of cytochrome *b* reduction or oxidation can be affected by the reagent even in detergent-solubilized fractions enriched in the bc_1 complex, such as fraction S_1 (cf. Figs. 3 and 4). It is interesting to note the similarity between the DCCD stimulation of cytochrome *b* reduction by ubiquinol (cf. Fig. 4) and the analogous stimulation of the ubiquinol–cytochrome *c* reductase in coupled proteoliposomes (cf. Fig. 1B).

DCCD Modification of the Antimycin Binding to the bc_1 Complex

In intact cytochrome *c*-depleted mitochondria we have observed that DCCD is able to abolish the red shift induced by antimycin in the reduced spectrum of cytochrome *b* (from 562 to 564 nm), as shown in Fig. 5. This

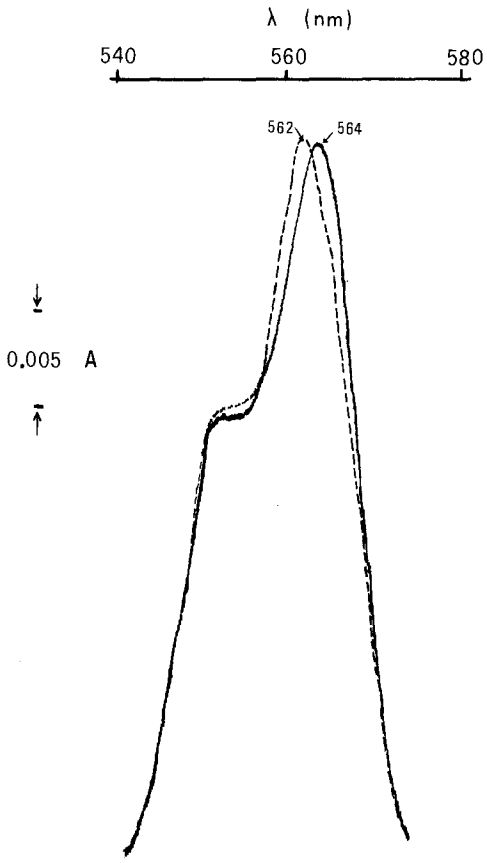

Fig. 5. Dithionite-reduced spectrum of cytochrome *c*-depleted mitochondria, obtained with the method described by MacLennan *et al.* (1966), at about 1.3 μM cytochrome c_1 with 10 μM antimycin in the 0.1 M K-phosphate buffer, pH. 7.4. The dashed line refers to the spectrum obtained in the presence of 1900 mol DCCD per mole of cytochrome c_1 incubated for about 1 h at room temperature, directly in the cuvette. Note the disappearance of the red shift of the cytochrome *b* maximum toward 564 nm.

action, which occurs only at concentrations higher than 1000 mol per mole of cytochrome c_1 and with long incubation times, is accompanied by alterations of the oxidant-induced *b* extrareduction by ferricyanide, and by a higher level of *b* reduction by substrates (cf. Figs. 2 and 4). In the absence of antimycin, no modification of the spectrum of the bc_1 complex completely reduced by dithionite has been observed (Degli Esposti *et al.*, 1981). These

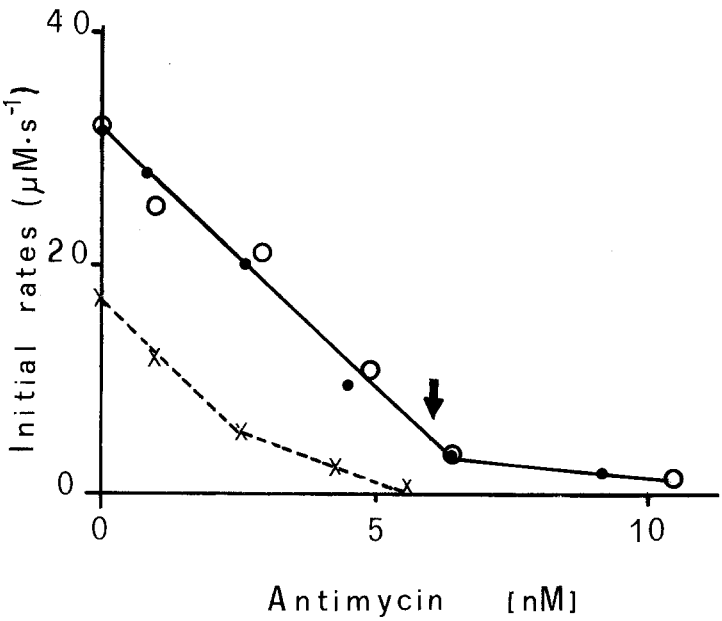

Fig. 6. Antimycin titration of the Q_2H_2–cytochrome *c* reductase activity in the isolated bc_1 complex at 6 nM. Q_2H_2 and cytochrome *c* concentrations were 90 and 20 μM, respectively. (○) Control without DCCD; (●), with 600 mol of DCCD per mole of cytochrome c_1 incubated at 0°C with the concentrated solution of the enzyme for about 5 min; (X), with 1200 mol of DCCD per mole of cytochrome c_1 incubated for 60 min at 0°C with the concentrated solution of the enzyme. The arrow indicates the stoichiometric equivalence between antimycin and cytochrome c_1.

data appear in close agreement with those reported in yeast mitochondria (Clejan and Beattie, 1983), and suggest that DCCD is able to displace antimycin from its binding site in the bc_1 complex, leading to the red shift in the reduced cytochrome *b* spectrum. However, it is to be noted that the conditions required to observe such effects correspond to those inducing the less specific action of DCCD on the redox activity of the bc_1 complex (cf. Fig. 1).

In fact, a clear decrease of the ubiquinol–cytochrome *c* reductase activity follows the modification of the antimycin titer in the isolated bc_1 complex from beef heart, as shown in Fig. 6. It appears that only the molecules of the enzyme not modified by the reagent maintain a regular capability to bind antimycin, which is always able to inhibit over 95% the rate of cytochrome *c* reduction. At short times of incubation or at lower DCCD concentrations, i.e., under conditions where the diimide specifically blocks the proton-pumping activity of the complex without affecting its redox activity (Degli Esposti

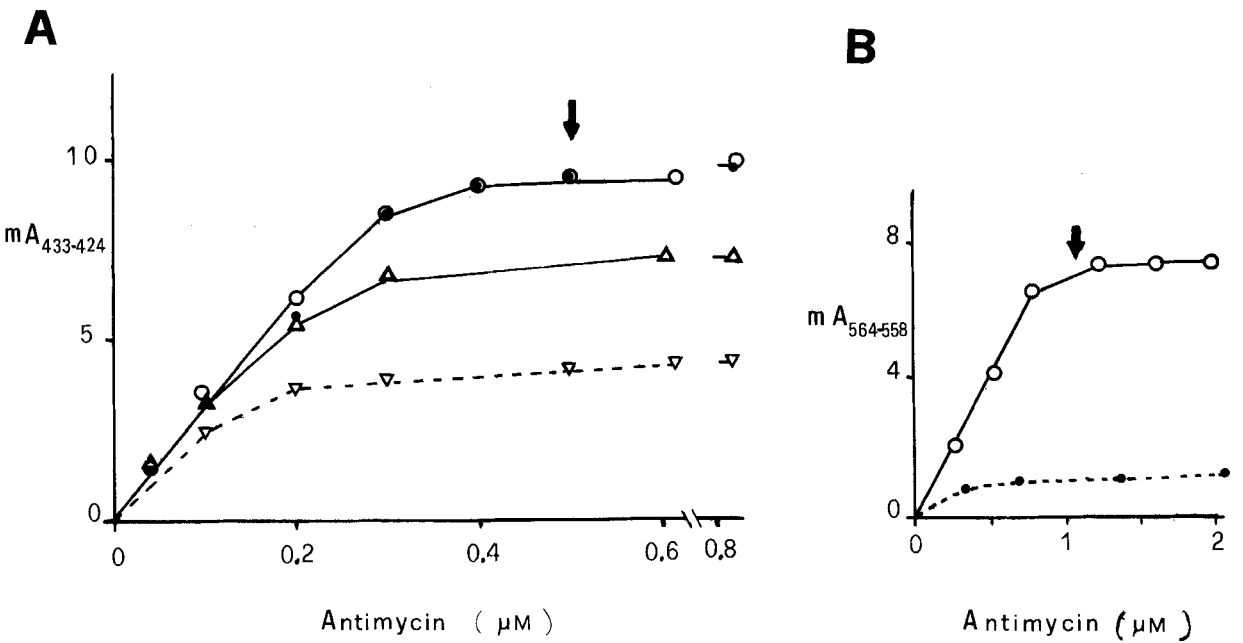

Fig. 7. (A) Antimycin titration of the red shift of the reduced spectrum of cytochrome *b* in the isolated bc_1 complex incubated in the same 0.2 M sucrose, 30 mM KCl, 0.5 mM K-Hepes buffer, pH 7.2, used in the proton translocating assays of the bc_1-proteoliposomes (Degli Esposti *et al.*, 1983), with 217 mol of DCCD per mole of cytochrome c_1 at room temperature in the concentrated solution of the enzyme. (○) Control without DCCD; (●) with DCCD, after 10 min of incubation; (△) after 80 min of incubation. The dashed line (▽) represents the titer obtained with 2200 mol of DCCD per mole of cytochrome c_1 incubated overnight at 0°C with the complex. Under the same conditions no change in the antimycin titration was seen in the controls. The final cytochrome c_1 concentration was 0.5 μM. (B) Same as (A), with 1.2 μM cytochrome c_1 in the 0.1 M K-phosphate buffer, pH 7.4, in the presence of 1% of K-cholate to better clarify the suspension. DCCD was 1600 mol per mole of cytochrome c_1 (●), and was incubated for about 80 min at 0°C. In both (A) and (B) the bc_1 complex was reduced with 35 mM succinate plus solid dithionite.

et al., 1983), the antimycin titer is not affected by DCCD, as also shown in Fig. 6.

In order to investigate the interference on the antimycin binding to the bc_1 complex by DCCD, we have studied the red shift induced by the antibiotic in the dithionite-reduced cytochrome *b* (cf. Fig. 5). Such parameter, stoichiometrically linked to the binding and the inhibitory action of antimycin (Von Jagow *et al.*, 1982), is more accurate than the antimycin-fluorescence quenching method, which is complicated by the light-scattering produced by DCCD in aqueous solution (Degli Esposti *et al.*, 1981; Clejan and Beattie, 1983).

The binding of antimycin is not significantly altered by 217 mol of DCCD per mole of cytochrome c_1 (a concentration usually inhibiting over 70% of the proton-translocating activity of the bc_1 complex, cf. Degli Esposti *et al.*, 1983) up to 1 h of incubation at room temperature (Fig. 7A). Only at longer times is a certain decrease of the maximal red shift observed, concomitantly with a slight decrease of the redox activity of the enzyme. For a relevant modification of the antimycin titration of the red shift, an overnight incubation with one order of magnitude higher DCCD concentration is required (cf. Fig. 7A). These results have been obtained without adding detergents to clarify the suspension of the bc_1 complex; if 1% cholate were added, a more rapid and potent effect of DCCD is observed, as shown in Fig. 7B. Such evidence indicates that the detergent has accelerated the accessibility of the DCCD-sensitive groups in the enzyme whose modification alters the antimycin binding.

Discussion

As previously discussed (Degli Esposti *et al.*, 1983), the effects of DCCD in the bc_1 complex are varied, involving different parameters of the enzyme depending on the experimental conditions used. The reason for this may be the nature of the DCCD modification itself, which is a quite nonspecific complexation with carboxyl (or even hydroxyl) groups in a hydrophobic environment, with possible cross-linking reactions (Solioz, 1984). However, due to the rapid time course of inhibition of the proton-translocating activity of the bc_1 complex (Price and Brand, 1983; Degli Esposti *et al.*, 1983), this effect can be considered rather specific, or primary. Also the stimulation of cytochrome *b* reduction by substrates, and the inhibition of its reoxidation by ferricyanide, seem to be a consequence of the primary action of the diimide, or at least they are concomitant of it (cf. Figs. 2–4). Other effects induced by DCCD occur only after a prolonged incubation and/or with higher concentrations of the reagent (Degli Esposti *et al.*, 1983). They include an

Table I. Review of the Various Effects Induced by DCCD on Functional and Structural Parameters of the Mitochondrial bc_1 Complex

Parameter	DCCD effects	
	Primary action	Secondary action ($\geq$ 1000 mol/mol c_1 and/or for more than 30 min)
Ubiquinol–cytochrome *c* reductase activity	None or partial stimulation under coupled conditions (1–4)[a]	Inhibition (1,2,3,5)
Proton translocation	Inhibition (1,5,7)	Inhibition (1–7)
Antimycin-sensitivity of redox activity	None (1–3)	Decreased sensitivity (5) None (1)
Antimycin binding	None (1,3)	Decreased binding (1,5)
Reduction of cytochrome *b* by substrates	Stimulation (1,3,5)	None or partial inhibition (1,3,5)
Reoxidation of cytochrome *b* by ferricyanide	Inhibition (1,3)	Inhibition (1,3)
Reverse electron flow	Inhibition (7)	Inhibition (7)
[^{14}C]DCCD binding	Mainly to subunit VIII (3,4)	Broad labelling of many subunits (3,4); large binding to cytochrome *b* (6,8)
Subunits structure and interaction	Modification of the migration of Band VIII (3,4); some cross-linking between bands V and VIII (3,4,6)	Large cross-linking between bands V, VII, and VIII (3,4,6)

[a] References: (1) Present work; (2) Degli Esposti *et al.*, 1982; (3) Degli Esposti *et al.*, 1983; (4) Lorusso *et al.*, 1983; (5) Clejan and Beattie, 1983; (6) Nalecz *et al.*, 1983; (7) Beattie and Villalobo, 1982b; (8) Beattie and Clejan (1982a).

inhibition of the ubiquinol–cytochrome *c* reductase activity (cf. Figs. 1 and 6) and a decreased binding of antimycin to the complex (cf. Figs. 5 and 7), without a clear modification of the antimycin sensitivity of the ubiquinol–cytochrome *c* reductase activity (cf. Fig. 6), and no significant alteration of the redox changes of cytochrome *b* (cf. Fig. 4). Such effects can be collectively ascribed to a less specific, or secondary, action of DCCD on the bc_1 complex. In fact, they are accompanied by a broad labeling of almost all the subunits of the enzyme, as indicated by binding experiments with radioactive DCCD (Degli Esposti *et al.*, 1983; Nalecz *et al.*, 1983; Lorusso *et al.*, 1983).

After analysis of the data in the literature on this subject, we can ascribe most of the observed effects of DCCD to the primary or secondary action, depending just upon the experimental conditions employed. The picture of the experimental evidence thus obtained is presented in Table I. Major discrepancies to such rationalization may be due to structural variations

between bc_1 complexes prepared with different methods or from different sources.

The present data show that the primary action of DCCD on the proton translocating activity of the bc_1 complex cannot be the result of a modified electron transfer pathway, with consequent loss of antimycin sensitivity. If the experimental conditions are not carefully controlled, it is easy to observe effects of the diimide which are related to its secondary action.

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