The inhibition of proton translocation in the mitochondrial bc_1 region by dicyclohexylcarbodiimide

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1. INTRODUCTION

N',N'-Dicyclohexylcarbodiimide (DCCD), a hydrophobic reagent of protein carboxyl groups, inhibits the formation of the transmembrane pH difference driven by succinate in submitochondrial particles, without significant inhibition of succinate oxidation [1,2]. A complete collapse of the succinate-driven membrane potential is also induced by DCCD at similar concentrations (unpublished results with Dr M.C. Sorgato). Moreover, DCCD inhibits succinate and ubiquinol–cytochrome *c* reductase activities in mitochondria [3], but at concentrations at least 1 order of magnitude higher than those affecting proton translocation. Analogous effects have been found in chloroplasts at the level of plastoquinol oxidation [14].

Although DCCD is an unspecific protein reagent, it is able to inhibit a number of proton-translocating membrane-bound enzymes besides H^+-ATPase [5], like NADH–$NADP^+$-transhydrogenase [6] and cytochrome oxidase [7,8], by stoichiometrically binding to the enzymes. The elucidation of the effects of this powerful inhibitor in the bc_1 region constitutes an important clue for discriminating among different possible mechanisms for the proton–electron transfer in this span of the respiratory chain. Here we show that DCCD inhibits proton translocation by the bc_1 complex either 'in situ' in rat liver mitochondria, or in the isolated enzyme reconstituted in lipid vesicles, without significant changes of the proton conductance of the membranes and without inhibition of the electron transfer activity of the complex, indicating that a H^+-pump mechanism can be primarily uncoupled by the reagent.

2. MATERIALS AND METHODS

Rat liver mitochondria (RLM) were prepared as in [9] and used immediately after preparation or after treatment with hypotonic–isotonic KCl to remove the outer membrane and endogenous cytochrome *c* [10] (KCl-RLM); protein was measured by a biuret method [11]. Isolated bc_1 complex was purified from beef heart mitochondria as in [12] and contained 2.6 nmol/mg protein of cytochrome c_1. With ubiquinol-2 as substrate it exhibited maximal activities at ~ 100 μmol · min^{-1} · mg^{-1}. The quinol was prepared from ubiquinone-2 (a kind gift from Eisai Co., Tokyo) as in [12] and used immediately after preparation. The reconstitution of the bc_1 complex into liposomes was accomplished essentially as in [13].

The final dialysis step was carried out against 230 vol. of 0.2 M sucrose, 30 mM KCl, 0.5 mM EDTA and 1 mM K-Hepes (pH 7.5) for ~ 10 h.

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Abbreviations: CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; DCCD, *N', N'*-dicyclohexylcarbodiimide; EDTA, ethylene diaminotetraacetate; Q_2H_2, ubiquinol-2; RLM, rat liver mitochondria

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After incorporation, the redox activity of the enzyme was usually at least 5-fold stimulated by uncouplers like CCCP.

The proton-translocating activities were measured at room temperature using the indicator phenol red (20 μM). The medium used for mitochondrial suspensions was 0.2 M sucrose, 30 mM KCl, 0.5 mM EDTA, 1 mM K-Hepes with or without 2 mM KCN (pH 7.5) containing 1 μg/ml each of valinomycin and rotenone and 2 μg/ml of oligomycin. With intact RLM, the mitochondrial concentration was 2.5–5 mg/ml and the signals were monitored in the dual wavelength mode at 566–582 nm in a Sigma Biochem. ZWS11 spectrophotometer; at this wavelength couple we have observed the minimal interferences by the redox changes of endogenous cytochromes on the absorbance of the indicator under our experimental conditions. In the succinate–ferricyanide assay the concentration of the electron acceptor was usually 125 μM. With KCl-RLM the mitochondrial concentration was kept at 1 mg/ml and 20 μM cytochrome *c* (type VI Sigma) plus 100 μg ferricyanide were added to the assay mixture. Due to the spectral interferences of cytochrome *c* reduction at the above wavelength couple, we have monitored the absorbance changes of phenol red at 556.5 nm (an isosbestic point of cytochrome *c*) either in the Sigma Biochem. spectrophotometer or in a Cary-15 spectrophotometer equipped with a rapid mixing apparatus [3].

With bc_1 reconstituted in lipid vesicles the assay medium was 0.2 M sucrose, 30 mM KCl, 0.5 mM EDTA and 0.5 mM K-Hepes (pH 7.6) containing 0.5 μg/ml of valinomycin; cytochrome *c* and ferricyanide concentrations were 11 and 55 μM, respectively, and that of the enzyme 0.2–0.4 mg/ml. In all assays the signals were calibrated with N/100 HCl Titrisol (Merck) and were corrected by the absorbance changes found in the absence of phenol red. The reaction was always started by the electron donor, either 5 mM K-succinate or 25–30 μM ubiquinol-2.

Succinate oxidation in RLM was measured with an oxygen electrode without valinomycin in the medium, whereas NADH oxidase either polarographically or spectrophotometrically at 340 nm. Other electron transfer activities were measured in the Cary-15 spectrophotometer as in [3] under the same assay conditions used for the proton-translocating assays. Ferricyanide and cytochrome *c* reduction were monitored at 420 and 550 nm, respectively.

DCCD from Sigma was dissolved in ethanol at 0.05–0.1 M and stored at −20°C. When added to the assay mixture, it was usually pre-incubated for 10 min at room temperature.

3. RESULTS AND DISCUSSION

Fig.1 shows the effect of DCCD on proton translocation associated with ubiquinol-2–cytochrome *c*–ferricyanide reductase in KCl-RLM. The acidification of the external medium is completely inhibited by antimycin A (trace a in fig.1A), and only a portion of the decrease in absorbance of phenol red is insensitive to uncouplers (trace b) due to scalar protons produced by ubiquinol oxidation. DCCD completely inhibits the uncoupler-sensitive portion of the acidification at ~170 nmol/mg protein, with half-inhibition at 82 nmol/mg protein. Under the same experimental conditions the rate of electron transfer from ubiquinol-2 to cytochrome *c* is not affected by the inhibitor, remaining antimycin-sensitive as in the controls (fig.1B).

Very similar results have been obtained in intact

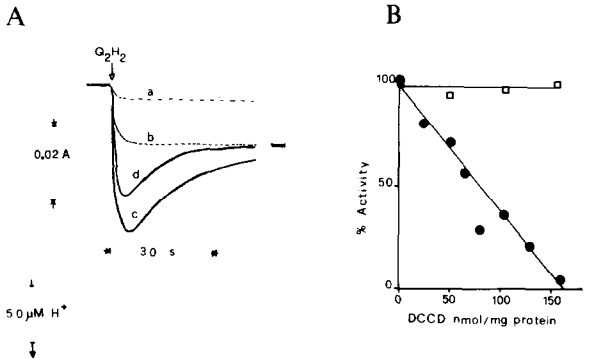

Fig.1. Inhibition by DCCD on proton translocation of KCl-RLM associated with ubiquinol-2 (30 μM)–cytochrome *c*–ferricyanide reductase. The absorbance changes were monitored at 556.5 nm in the Sigma Biochem. spectrophotometer. (A) Experimental traces: (a) + antimycin A 2 μg/ml; (b) + 10 μM CCCP; (c) control; (d) + DCCD 65 nmol/mg protein. (B) Effect of increasing DCCD concentrations on proton translocation (●—●) and on ubiquinol-2–cytochrome *c* reductase under the same assay conditions (○—○).

Table 1

Effect of DCCD (100 nmol/mg protein) on various activities of the succinate–ferricyanide assay in RLM (4 mg/ml)

Activity	Control	+ DCCD	%Control
Succinate–ferricyanide reductase (μM/min)	164	146	89
Extent of CCCP-sensitive external acidification ($\Delta A_{566-582}$)	0.0070	0.0027	38
First-order rate constant of H^+-backflow (s^{-1})	0.082	0.078	95

mitochondria using either ubiquinol-2 or succinate as the driving substrate to oxygen, and also in the succinate–ferricyanide reductase assay in the presence of KCN. In all assays the half-inhibitions occur at 70–90 nmol/mg protein, and the vectorial acidification can be completely abolished at ~200 nmol/mg protein. Under the same assay conditions the rates of electron transfer activities are only slightly decreased by DCCD.

Several lines of evidence demonstrate that DCCD does not enhance the permeability of the membrane to protons. In submitochondrial particles we found that the rate of H^+-backflow under anaerobiosis is not enhanced by the diimide, contrary to the effect of genuine uncouplers [2]. Analysis of the traces in the experiments quoted here also shows that the re-alkalinization of the medium upon exhaustion of the oxidant or of the reductant is only slightly affected by DCCD (table 1).

Further observations on the effects of the diimide come from the oxygen electrode experiments. Succinate oxidation in oligomycin-inhibited and malonate-activated RLM is stimulated >5-fold by CCCP; DCCD concentrations leading to progressive inhibition of H^+-translocation enhance state-4 respiration in a progressive fashion, as expected for an agent preventing the formation of a transmembrane electrochemical gradient which limits the electron flow. On the other hand, the CCCP-stimulated respiration is only marginally decreased by DCCD. Under the same assay conditions DCCD does not significantly alter the rate of proton backflow upon oxygen exhaustion as monitored by the phenol red indicator (fig.2). With 200 nmol/mg of the inhibitor the rate of proton re-entry is increased only 20%, when the external acidification is inhibited by 85%. As a comparison, a limited amount (1 μM) of CCCP leading to a 63% inhibition of the acidification, increases by 100% the rate of proton backflow. This demonstrates that the uncoupler-like effect of DCCD on state-4 respiration is not due to an enhanced proton conductance of the mitochondrial membrane.

However, other experimental evidence could partially account for the increased rate of succinate oxidation in RLM; the succinate–ubiquinone re-

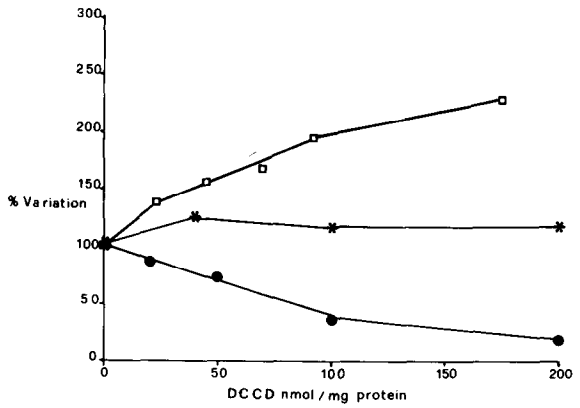

Fig.2. Effect of DCCD on state-4 succinate oxidase (□—□), on proton translocation monitored at 566–582 nm with phenol red in the presence of valinomycin (●—●), and on the rate constants of proton backflow upon attainal of anaerobiosis (*—*) in RLM (2.5 mg/ml) pre-activated by 1 mM malonate.

ductase activity is stimulated up to 2-fold by DCCD at < 300 nmol/mg protein, similarly to that reported in beef heart mitochondria [3]. The significance of this observation is under investigation.

An alternative explanation for the effects of DCCD could be that the reagent is capable of inducing an all-or-none disruption of a progressive number of mitochondria, leaving the remaining mitochondria completely unaffected. We have excluded such a possibility, however, on the basis of a number of functional criteria assessing the integrity of the mitochondrial suspensions. For instance, the rate of NADH oxidase in RLM, which is cryptic in intact mitochondria, is not enhanced by DCCD up to 200 nmol/mg protein, and the same for the rate of NADH–ferricyanide reductase in the presence of KCN, indicating that the inhibitor does not destroy the membrane integrity of mitochondria. This is also confirmed by the fact that DCCD is not able to stimulate ubiquinol–cytochrome *c* reductase activity in KCl-RLM or in beef heart mitochondria [3] or submitochondrial particles [1], differing from detergents [14].

DCCD can also inhibit the proton-translocating activity of the purified bc_1 complex inlayed in phospholipid vesicles (fig.3). Under experimental conditions very close to those employed in KCl-RLM (cf.fig.1) we have observed half-inhibition of the CCCP-sensitive external acidification at ~400–450 nmol DCCD/nmol cytochrome c_1. Considering the content of cytochrome c_1 in RLM (= 0.12 nmol/mg protein) the half-inhibitions found in mitochondria assume values even higher, ~650 nmol DCCD/nmol cytochrome c_1. This indicates that such a rather high ratio of the diimide to the enzyme is required for the binding to the complex capable of blocking its proton-translocating activity. The rate of ubiquinol-2–cytochrome *c* reductase under the same conditions is not affected by the inhibitor, as also indicated by the rates of scalar proton production by a second pulse of ubiquinol in the presence of CCCP (cf.fig.3). In the absence of valinomycin or other uncouplers, however, DCCD induces a stimulation (up to 65%) of the reductase rate in parallel to the inhibition of the proton extrusion. This effect is not due to an alteration of the proton permeability of the proteoliposomes induced by the diimide, as checked by the rate of H^+-backflow after exhaustion of the quinol, in agreement with [7].

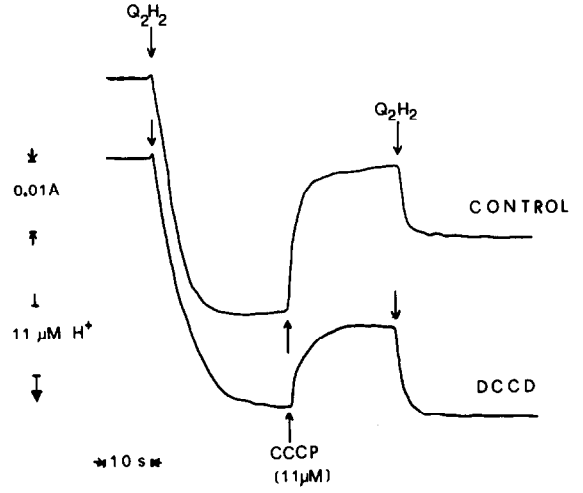

Fig.3. Effect of DCCD (344 nmol/nmol cytochrome c_1) on the CCCP-sensitive external acidification produced by isolated bc_1 complex incorporated in proteoliposomes (0.35 mg/ml). Ubiquinol-2 was 27 μM at each pulse. The traces were obtained at 556.5 nm in the Cary 15 spectrophotometer. The rates of scalar acidification of the second ubiquinol pulse were in the range of 5 μM $H^+ \cdot s^{-1}$ with or without DCCD.

Here, our findings suggest that DCCD primarily inhibits H^+-translocation of the bc_1 complex without affecting electron transfer and, differing from uncouplers, without enhancing the membrane permeability to protons. The loose coupling between H^+-translocation and electron transfer is not unexpected if protons are ejected via a channel rather than a redox loop mechanism [15], since protons not entering the channel may be released from the bc_1 complex by a different route. Inhibition of the redox activities of the bc_1 complex at higher concentrations [3] is probably due to secondary effects independent of the primary effect on the proton channel, by analogy to that found in cytochrome oxidase [8].

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