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DCCD INHIBITS PROTON TRANSLOCATION AND ELECTRON FLOW AT THE SECOND SITE OF THE MITOCHONDRIAL RESPIRATORY CHAIN

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DCCD inhibits formation of a succinate-driven transmembrane pH gradient in submitochondrial particles, as shown by inhibition of fluorescence quenching of 9-aminoacridine, without concomitant inhibition of succinate oxidation. On the other hand ubiquinol-cytochrome c reductase activity is inhibited by DCCD. Half-inhibition of both fluorescence quenching and ubiquinol-cytochrome c reductase occur at 35 μM DCCD. The results suggest that DCCD inhibits proton pumping activity coupled to electron flow through the bc_1 complex.

INTRODUCTION

Dicyclohexylcarbodiimide (DCCD) is a well known inhibitor of proton translocating ATPases, such as those involved in oxidative phosphorylation (1) and in photophosphorylation (2). Its mode of action concerns specific covalent binding to one hydrophobic subunit of the enzymes (3,4), namely to a buried glutamic acid residue (5), thus hindering proton translocation.

More recently DCCD has been found to act on non-ATP linked H^+-translocating systems, such as mitochondrial cytochrome c oxidase (6,7) and photosynthetic electron flow at the level of plastoquinone (8). The effects of DCCD in cytochrome oxidase were considered further evidence for the association of a proton pump to the complex (9).

The bc_1 complex (Complex III) of the mitochondrial respiratory chain is associated with vectorial proton translocation (10), although it is not solved whether the proton pumping activity takes place through direct H^+ transport by ubiquinone (11) or through specific channels in the polypeptides of the complex (12).

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In this communication we present evidence that DCCD inhibits proton translocation and oxidative activity in the ubiquinone--cytochrome c span of the chain;this finding represents additional evidence for the association of a proton channel to electron transfer at the second coupling site.

METHODS

Submitochondrial particles ETP were prepared by sonic oscillation (13).

Ubiquinol cytochrome c reductase was assayed at room temperature as described by Rieske (14);the reduction of cytochrome c (10 μM) was monitored at 550 nm in a Cary 15 spectrophotometer using the extinction coefficient of 18.7 $mM^{-1}cm^{-1}$.In order to follow the rapid initial phase of cytochrome c reduction,the sample cuvette was supplied with an automatic device allowing a mixing time of about 200 msec.The antimycin insensitive reaction never accounted for more than 10% of the overall reaction rate. Succinate ubiquinone reductase activity was assayed as described previously (15).Succinate oxidase was assayed polarographically with a Clark electrode at room temperature in a medium containing:sucrose,0.5M;glycylglycine,pH 7.8,40 mM;$MgCl_2$,6 mM;KCl,100 mM;EDTA,1 mM;valinomycin,1 μM;rotenone,0.1 μg/mg protein;and ETP, 0.5 mg,in a total volume of 2 ml;after 5-min preincubation the reaction was started by addition of 6 mM succinate.

The determination of the succinate-driven transmembrane pH difference was obtained indirectly by measuring energy-dependent quenching of fluorescence of 9-aminoacridine (9-AA) in presence of valinomycin to collapse the $\Delta\psi$ component of the protonmotive force (16).The quenching was determined in a Perkin-Elmer MPF4 spectrofluorimeter,with an excitation wavelength of 400 nm and an emission wavelength of 460 nm.The assay medium was identical to that used for succinate oxidase,except that 1.5 μM 9-AA was also added to the medium.The quenching obtained by addition of 6 mM succinate was calibrated with a control containing 0.05% Triton-X-100. In other experiments the quenching associated with the activity of cytochrome oxidase was determined in presence of antymicin A (0.5 ug/ml) with 0.5 mM ascorbate and 21 μg/ml of TMPD (4,4,4',4'-tetramethylphenylendiamine).

DCCD from Sigma was kept in ethanol at -20°C at 10 mM.

RESULTS

Addition of succinate to ETP induces a rapid quenching of 9-AA fluorescence which decays slowly until the system becomes anaerobic (Fig. 1). The difference in pH between the inner and outer compartments (ΔpH_{i-o}) is related to the quenching of the amine by the following relation (16):

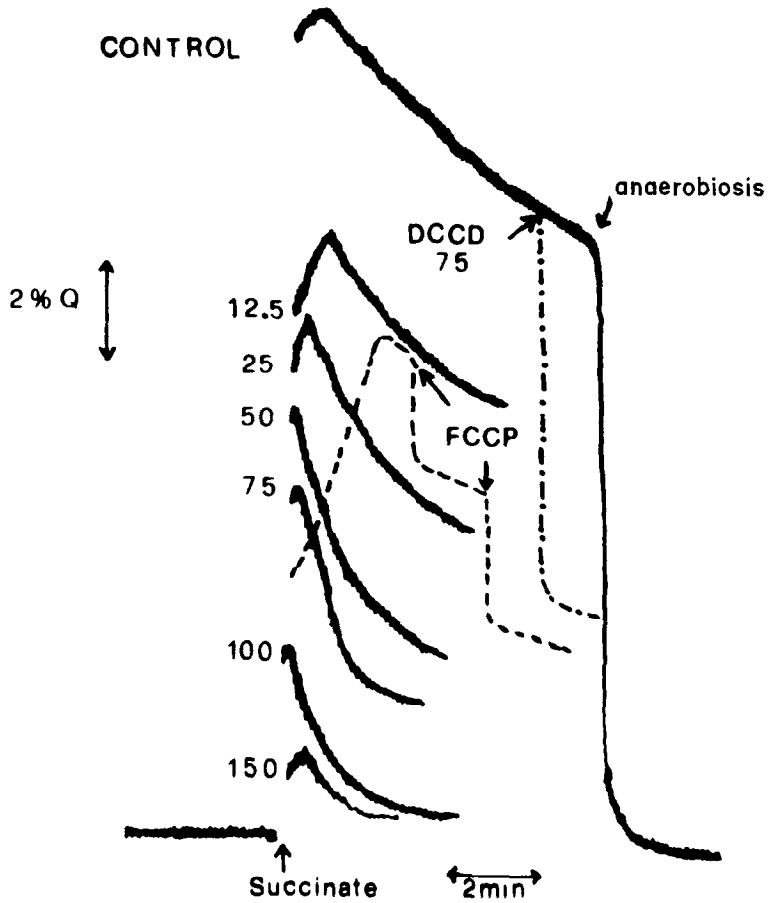

Fig.1. Effect of DCCD on the quenching of 9-AA fluorescence upon energization of ETP with succinate.The dashed line refers to a sample with 10^{-8} M FCCP;the arrows indicate successive additions of 10^{-8} M FCCP.The numbers refer to DCCD concentration in μM.

$$\log \frac{Q}{100-Q} = -\Delta pH_{i-o} - \log V_o + \log V_i$$

where Q is the percentage fraction of the total fluorescence quenched upon energization,and V_o and V_i are the volumes of the external and internal compartments respectively.Assuming a V_i of ETP of 0.75 μg/mg protein at high osmolarity of the medium (17) the quenching observed corresponds to a ΔpH of about 3.15.

Incubation of DCCD with the system for 5-min before the addition of the respiratory substrate induces a progressive decrease of quenching with almost complete inhibition at 150 μM. The rate of decay of the quenched fluorescence,negligible in the control,grows progressively higher,becoming over 20-fold with

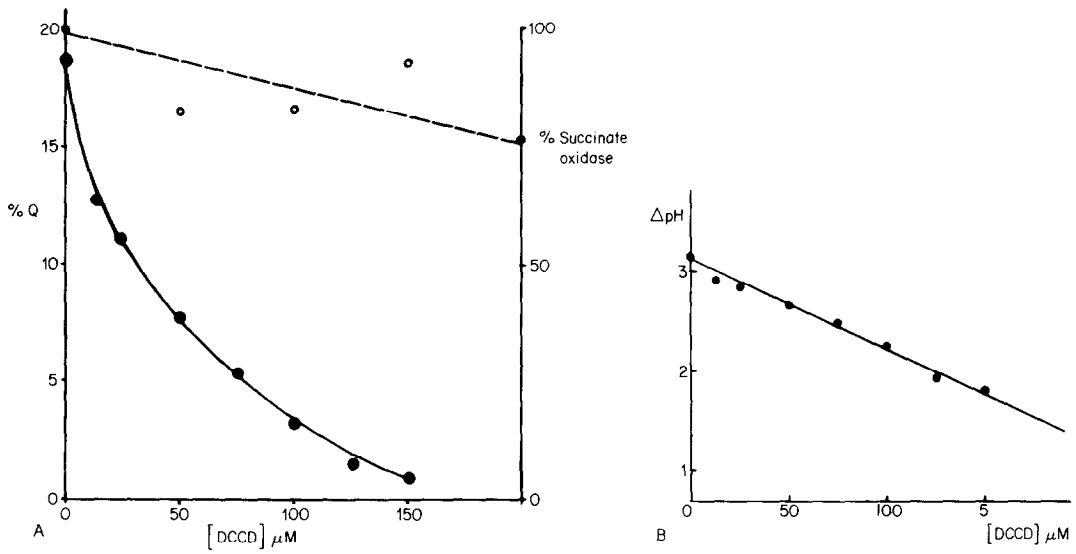

Fig.2. A. Effect of DCCD on the quenching of 9-AA fluorescence upon energization of ETP with succinate and on succinate oxidation. ●---●,quenching;o---o,succinoxidase activity. B. Effect of DCCD on the ΔpH.

respect to the control at 75 μM DCCD.The inhibition is not time-dependent and can be observed also by adding DCCD after energization by succinate.The effect of 10^{-8}M FCCP is also shown for comparison. The quenching associated with cytochrome oxidase (driven by ascorbate plus TMPD in presence of antimycin,not shown in the Figure) is inhibited only 14% by 75 μM DCCD whereas the succinate-driven quenching is inhibited over 60%.

The effect of DCCD on succinate-driven quenching of 9-AA in comparison with the effect on succinate oxidase is shown in Fig. 2A. The extent of quenching undergoes a hyperbolic decrease in presence of increasing DCCD concentrations;the inhibition of the calculated ΔpH is linear (Fig. 2B).From Fig.2A,half inhibition of quenching can be calculated at 35 μM DCCD.Under the same conditions the rate of succinate oxidation is only marginally decreased.

On the other hand,the rate of ubiquinol-1 cytochrome c reductase shown in Fig.3 is dramatically decreased by DCCD,with half inhibition occurring at 35 μM.The rate of succinate ubiquinone-1 reductase (not shown) is not inhibited by DCCD at concentrations up to 300 μM.

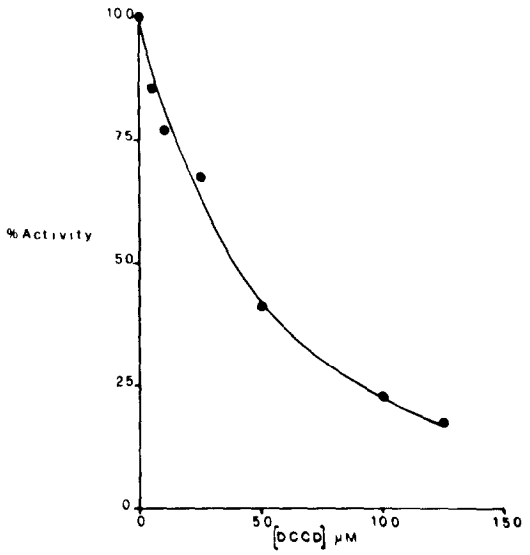

Fig.3. Effect of DCCD on the rates of ubiquinol-1 cytochrome c reductase in ETP.

DISCUSSION

In this study we demonstrate that DCCD concentrations of the order of those inhibiting H^+ translocation in cytochrome oxidase (6) also strongly inhibit H^+ translocation associated with succinate oxidation in submitochondrial particles,as shown by inhibition of the formation of a pH gradient investigated by quenching of 9-AA fluorescence.

The inhibition of quenching is almost complete at 150 μM DCCD,indicating that besides H^+ translocation associated with Complex IV (6,7),also H^+ extrusion by Complex III must be inhibited.It has not been possible to investigate the pH gradient associated with electron transfer from ubiquinol to cytochrome c, since absorbance changes associated with ubiquinol oxidation and cytochrome c reduction interfere with the amine fluorescence.We have however observed that quenching associated with cytochrome oxidase is only marginally inhibited by 75 μM DCCD under conditions (short preincubation) where the succinate-driven quenching is inhibited over 60%.This finding agrees with the observation (6,7) that a long preincubation is necessary to show inhibition of H^+ translocation in cytochrome oxidase.

We have excluded by rigorous controls that the inhibition is an artifact due either to interaction of DCCD with 9-AA or to ethanol;it is also unlikely that DCCD either enhances proton permeability across the membrane or prevents the collapse of the membrane potential induced by valinomycin and K^+,thus decreasing the ΔpH component of the protonmotive force,since DCCD does not inhibit at Site 3 under our conditions;furthermore DCCD had no effect on the valinomycin plus K^+ induced H^+ permeability in cytochrome oxidase vesicles (7).Moreover the inhibition of the pH gradient by DCCD is not due to decreased electron transport,with block of energy input to the H^+ pump as with genuine electron transfer inhibitors (18);in fact succinoxidase activity under identical conditions is only marginally affected.An apparent contradiction to this observation is the potent inhibition exerted by DCCD on ubiquinol cytochrome c reductase in ETP.This striking difference can be easily explained by the fact that both succinate dehydrogenase and cytochrome oxidase represent rate-limiting steps in the transfer of electrons from succinate to oxygen (19), with the oxidation of ubiquinol by cytochrome c proceeding several times faster than the oxidation of succinate by ubiquinone. Under such conditions a decrease of electron transfer through Complex III cannot be detected by studying succinate oxidation.

If the oxidative and H^+-translocating processes were strictly coupled,as are the H^+ pump and ATP-hydrolytic activity of membranous ATPase,DCCD should exert similar effects on both.This is indeed the case when we examine the effect of the carbodiimide on the establishment of ΔpH through Complexes III plus IV and on ubiquinol oxidation by cytochrome c.In the case of cytochrome oxidase reconstituted in liposomes (6) no inhibition of oxidation was observed,suggesting a loosely coupled system.

Following the reasoning of Sane et al (8) for chloroplasts, also in mitochondria the oxidation of ubiquinol appears to be associated with H^+ translocation involving a proton conducting channel (12);DCCD is postulated to block proton conduction through the channel,thus inhibiting electron flow.From our study,DCCD is a new addition to the list of inhibitors of respiration at en-

ergy-coupling site 2 in mitochondria (20);the inhibition of electron flow at the level of Complex III is however apparent only if no rate-limiting steps are present in the oxidation rate.

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