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DICYCLOHEXYLCARBODIIMIDE INHIBITS PROTON TRANSLOCATION BY CYTOCHROME c OXIDASE

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SUMMARY

Mitochondrial cytochrome c oxidase has recently been shown to possess a proton-translocating activity. We report here that dicyclohexylcarbodiimide, a well-established inhibitor of ATP-linked proton pumps, inhibits proton translocation by cytochrome c oxidase whilst having only minor effects on its oxidative activity. The inhibition of the proton pump was time and concentration dependent, both in intact mitochondria and in reconstituted cytochrome c oxidase vesicles. This is the first observation of inhibition of a non ATP-linked proton pump by dicyclohexylcarbodiimide.

INTRODUCTION

Cytochrome c oxidase is the terminal electron transfer component of the mitochondrial respiratory chain. Until recently, it was considered that its sole function was to catalyse the vectorial transfer of electrons from ferrocytochrome c to molecular oxygen (1-3), leading to the consumption of protons from the mitochondrial interior (4). There is considerable evidence to indicate, however, that cytochrome c oxidase also carries out proton translocation, directed outwards in mitochondria, concomitantly with its redox changes. Initial observations of proton pumping by cytochrome c oxidase in mitochondrial systems by Wikström and co-workers (5,6) have been verified in other laboratories (7,8). Further strong evidence has been provided by demonstrations of ferrocytochrome c-induced proton translocation in reconstituted cytochrome c oxidase vesicles (6,9,10). These show that proton

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Abbreviations: DCCD, N,N'-Dicyclohexylcarbodiimide; Hepes, 2-(N-2-hydroxyethylpiperazin-N'-yl)ethane-sulphonic acid

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appearance at the vesicle exterior is the result of true proton translocation and that approximately one proton is translocated per ferrocytochrome c oxidised.

Dicyclohexylcarbodiimide (DCCD) inhibits a wide range of proton-translocating ATPases, including those of mitochondria (11), chloroplasts (12), Streptococcus faecalis (13) and chromaffin granules (14). There has been no demonstration up to now, however, of DCCD inhibition of a non ATP-linked H^+ pump. In this communication we report that the redox driven H^+ pump of cytochrome c oxidase is also strongly inhibited by DCCD. We propose that this inhibition may point to a similarity between the mechanisms of this and of ATP-linked, H^+-translocating systems.

EXPERIMENTAL

Cytochrome c oxidase was prepared from bovine heart, as described elsewhere (15).

Reconstituted cytochrome c oxidase vesicles were prepared at 4° C as follows. Cytochrome c oxidase (final concentration 7.5 μM) was added to a suspension of 40 mg/ml asolectin in 24.5 mM potassium cholate, 100 mM-Hepes, pH 7.2 which had been sonicated to clarity. The suspension was dialysed for 4 hours versus 100 volumes of 100 mM-Hepes, pH 7.2 then for a further 4 hours versus 200 volumes of 10 mM-Hepes, 27 mM-KCl, 73 mM-sucrose, pH 7.2 and finally for 12 hours versus 200 volumes of 1 mM-Hepes, 30 mM-KCl, 79 mM-sucrose, pH 7.2.

Rat heart mitochondria were prepared as described elsewhere (16), except that the final washing step was carried out in the experimental medium.

Ferrocytochrome c was prepared by adding a few grains of sodium dithionite to 40 mg/ml ferricytochrome c, 100 mM-Hepes, 40 mM-Tris, pH 7.4. Excess dithionite was removed using a column of Sephadex G-25 (coarse grade).

H^+ ejection was measured by monitoring the change in absorbance of phenol red at 556.6-504.5 nm with vesicles, or 560-580 nm with mitochondria, using an Aminco DW-2a spectrophotometer. Merck Titrisol grade hydrochloric acid was used for calibration.

Oxidative activity was measured by monitoring either the change in absorbance of cytochrome c at 550-540 nm or the consumption of O_2 using a Clark-type oxygen electrode.

Soy-bean phospholipids were once recrystallised and were obtained from Sigma Chemical Co. Ltd. as were cytochrome c, valinomycin, Hepes, rotenone, oligomycin and antimycin A. N-ethylmaleimide was obtained from Merck and Nagarse from Bacillus subtilis was from Serva. All other reagents were of analytical grade.

RESULTS

Inhibition of Ferrocyanide-Induced Proton Translocation in Mitochondria by Dicyclohexylcarbodiimide.

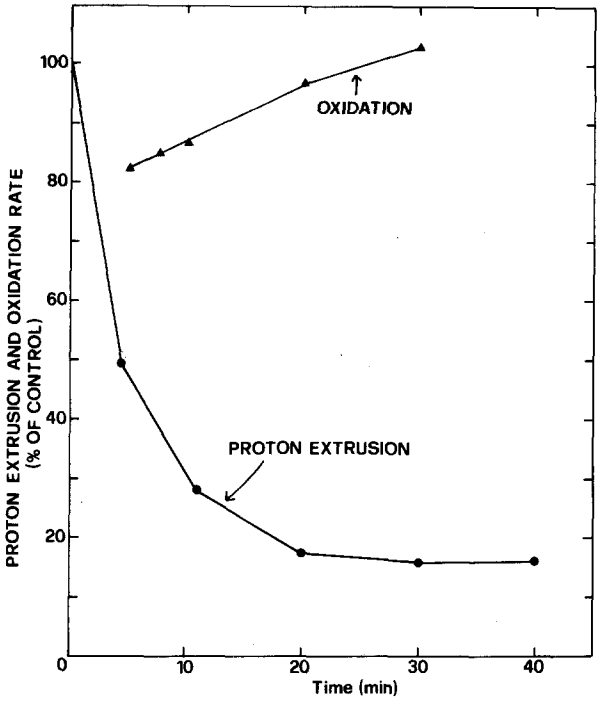

Figure 1 Time course of the effects of dicyclohexylcarbodiimide on H^+ translocation and oxidative activity in rat heart mitochondria.
Proton translocation: 0.5 ml of mitochondria (4.87 nmoles of cytochrome c oxidase) was added to 5.5 ml of 145 mM-choline chloride, 5 mM-KCl, 0.625 mM-ethylenediaminetetraacetate, 10 μM-phenol red, pH 7. Either 12.5 μl of an ethanolic solution of 0.1M-DCCD or 12.5 μl of ethanol was added and the suspension incubated at 13° C. At the times indicated the H^+ extrusion induced by 375 nmoles of potassium ferrocyanide to 1 ml of this was determined at 13° C as described by Wikström (5).
Oxidation: The procedure was as for the measurement of H^+ translocation with the following exceptions.0.3 ml of mitochondria (2.83 nmoles cytochrome c oxidase) was added to 2.15 ml of 150 mM-choline chloride, 0.15 mM-Hepes, 20 mM-sodium ascorbate, 20 mM-tetramethylphenylenediamine, pH 7.4 at 13° C. The volume of ethanol was 5 μl. At the indicated times 3 μl of 0.2M-sodium ascorbate, pH 7.4 was added and the O_2 consumption measured. The rate of O_2 consumption and the H^+ efflux in the samples containing DCCD are plotted as the percentage of those in the ethanol sample, incubated for the same time.

When potassium ferrocyanide was added to a suspension of rat heart mitochondria under conditions similar to those described by Wikström and co-workers (5,6), where cytochrome c oxidase should be the only operative component of the respiratory chain, H^+ ejection was observed. DCCD brought about a time-dependent inhibition of this H^+ efflux, and this reached 85 % under our experimental conditions (Figure 1). Of particularly interest are

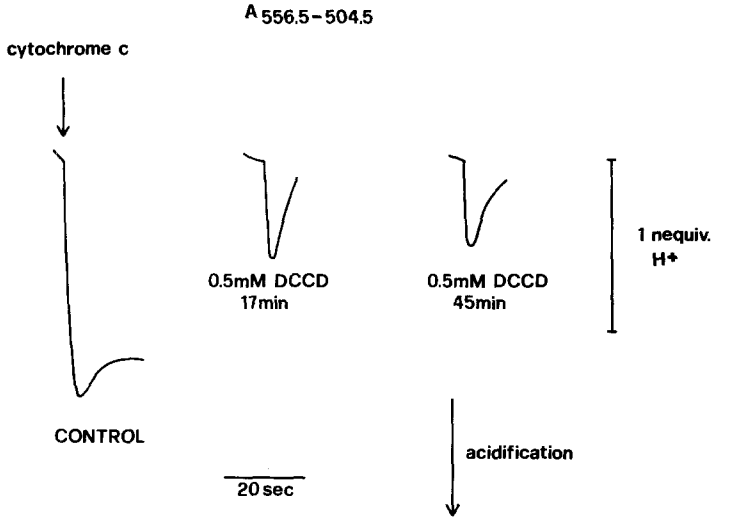

Figure 2 Dicyclohexylcarbodiimide inhibition of ferrocytochrome c-induced H^+ extrusion from cytochrome c oxidase vesicles.
50 µl of cytochrome c oxidase vesicles (0.3 nmoles cytochrome c oxidase) was added to 1 ml of 75 mM-choline chloride, 25 mM-KCl, pH 7.5 µl of 100 mM-DCCD or 5 µl of ethanol was then added and the suspension incubated at 13° C. The H^+ extrusion induced by 1.5 nmoles of ferrocytochrome c was then measured in the presence of 1 µM-valinomycin at the times indicated. For the ethanol control samples this was invariant during the 45 min. incubation.

the relatively minor effects on the oxidative activity which accompanied the potent inhibition of H^+ translocation. The inhibition of H^+ pumping shown in Figure 1 was obtained using 257 nmoles DCCD per nmole cytochrome c oxidase, though inhibition could also be observed at DCCD concentrations well below this (Figure 3).

Inhibition of Ferrocytochrome c-Induced Proton Translocation in Reconstituted Cytochrome c Oxidase Vesicles by Dicyclohexylcarbodiimide.

In order to confirm that the effects of DCCD seen in mitochondria were due to interaction with cytochrome c oxidase, similar experiments were carried out using reconstituted cytochrome c oxidase vesicles. Ferrocytochrome c induced the expulsion of protons from the vesicles in the presence of valinomycin as shown in Figure 2. Sufficient ferrocytochrome c was added for a single turnover of the oxidase as this provides a stable H^+ efflux with the optimal ratio of H^+ expelled to ferrocytochrome c oxidised (10). When

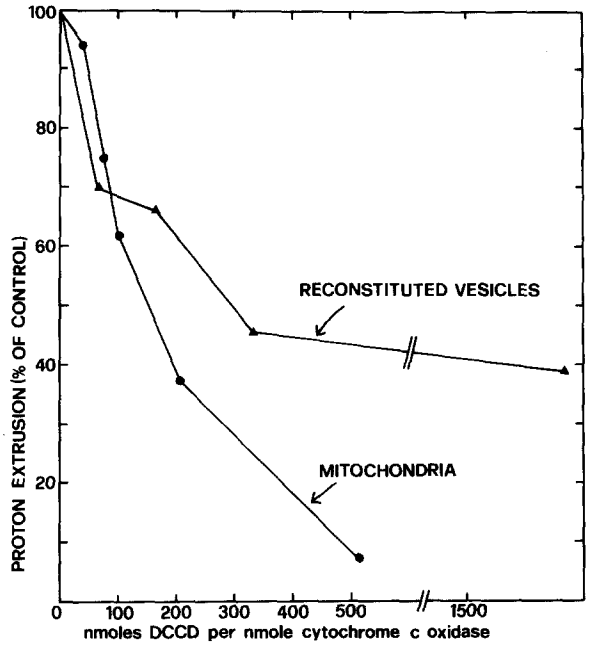

Figure 3 Concentration dependence of inhibition of H^+ translocation via cytochrome c oxidase by dicyclohexylcarbodiimide.
Cytochrome c oxidase vesicles: 50 µl of the vesicles (0.3 nmoles of cytochrome c oxidase) was added to 1.2 ml of 60 mM-choline chloride, 40 mM-KCl, 50 µM-phenol red, pH 7, followed by an ethanolic solution of DCCD to give the concentration indicated. The final volume of ethanol was always 10 µl. After 45 min. incubation at 13° C, the H^+ efflux caused by 5.45 nmoles of ferrocytochrome c was measured.
Mitochondria: The procedure was similar to that for the reconstituted vesicles except that 0.1 ml of mitochondria (0.97 nmoles of cytochrome c oxidase) was used and the medium was 145 mM-choline chloride, 5 mM-KCl, 10 µM-phenol red. At 15 min. the H^+ extrusion induced by 375 nmoles of potassium ferrocyanide was measured at 13° C as described by Wikström (5). The H^+ extrusions are plotted as the percentages of those in the samples with no DCCD, which were 2.57 and 4.04 nequiv. H^+ in vesicles and mitochondria respectively.

0.5 mM-DCCD was present, the H^+ efflux was inhibited by 58 % and 65 % after 17 and 45 minutes, respectively. The inhibition was calculated with respect to a control incubated for the same time in the presence of the same amount of ethanol. The time course of the DCCD inhibition (Figure 4) indicates that its onset was slower in vesicles than in mitochondria. In reconstituted vesicles, the strong inhibition of the proton pump was accompanied by only minor effects on oxidation, as in mitochondria. The concentration dependence

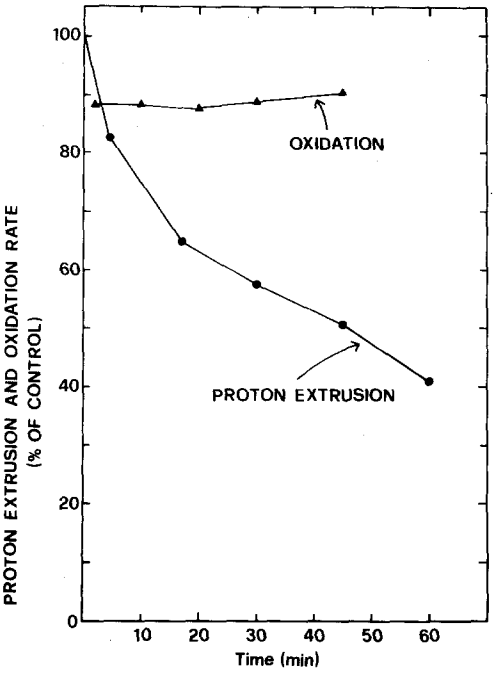

Figure 4 Time course of the effects of dicyclohexylcarbodiimide on H^+ translocation and oxidative activity in cytochrome c oxidase vesicles.
H^+ translocation: 0.3 ml of vesicles (1.8 nmoles cytochrome c oxidase) was added to 6 ml of 60 mM-choline chloride, 40 mM-KCl, 50 µM-phenol red, pH 7.4 followed by 6 µl of an ethanolic solution of 100 mM-DCCD or 6 µl of ethanol and the suspension incubated at 13° C. At the time indicated the H^+ efflux induced by 5.45 nmoles of ferrocytochrome c to 1 ml of this was determined at 13° C in the presence of 1 µM-valinomycin.
Oxidation: 50 µl of vesicles (0.3 nmoles cytochrome c oxidase) was added to 1 ml of 60 mM-choline chloride, 40 mM-KCl, 10 mM-Hepes, pH 7.2, followed by 10 µl of ethanol or 10 µl of 10 mM-DCCD. After incubation at 13° C for the time indicated, 50 µl of this was added to 1 ml of the same medium containing 1 µM-valinomycin and 4.36 µM-ferrocytochrome c. The rate of ferrocytochrome c disappearance and the H^+ efflux in the DCCD samples are shown as percentages of those in the ethanol controls incubated for the same times.

of the DCCD inhibition of H^+ pumping in vesicles (Figure 3) shows that, whilst still considerable, it was somewhat less than that in intact mitochondria.

DISCUSSION

DCCD clearly exerts a strong, time-dependent inhibition on H^+ extrusion by cytochrome c oxidase in both reconstituted vesicles and intact mitochondria and we believe that this is the first demonstration of in-

hibition of a non ATP-linked H^+ pump by this substance. The possibility that the inhibition was due to DCCD causing artefactual underestimation of the H^+ efflux (e.g. through interaction with phenol red or valinomycin, or by making the vesicular membranes H^+ permeable) was rigorously excluded by control experiments. The particularly potent inhibition in intact mitochondria is even more striking considering that other DCCD-binding proteins were present (17). The somewhat lower effects of DCCD in reconstituted vesicles than in mitochondria may reflect differences in the environments of cytochrome c oxidase in the two systems. The fairly slow kinetics of onset of the DCCD inhibition may indicate the formation of a covalent complex between the cytochrome c oxidase and DCCD as has been observed in the mitochondrial (17) and chloroplast (18) ATPases.

The minor effects on oxidation which accompanied the potent inhibition of the cytochrome c oxidase H^+ pump rule out the possibility that DCCD acts at the level of cytochrome c oxidation, thus blocking the energy input to the H^+ pump, as with inhibitors such as cyanide (9) and azide (19). They also distinguish the inhibition from the non-specific effects of DCCD reported by Carroll and Racker (20) which were strictly dependent on the presence of Tween 80. The DCCD inhibition we observe is thus a direct and specific effect at the level of the H^+-pumping mechanism of cytochrome c oxidase.

If the oxidative and H^+-translocating processes were strictly coupled, DCCD should exert effects of similar size on both (21). The inhibition of H^+-pumping observed here would thus be linked to a large stimulation of oxidative activity, through uncoupling at the molecular level, or to a strong inhibition of oxidation. The small effects on oxidation observed may indicate, therefore, that these processes are only loosely coupled.

We conclude that the inhibitory effects of DCCD may indicate a common mechanistic feature between the redox-linked H^+ pump of cytochrome c oxidase and the H^+-translocating ATPases. In addition, these effects should be

considered in the design and interpretation of experiments with intact mitochondria in which DCCD is used as a specific inhibitor of H^+-translocation by the ATPase.

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