Proton translocation by the mitochondrial cytochrome *b*–*c*₁ complex is inhibited by *NN'*-dicyclohexylcarbodi-imide

Brendan D. PRICE and Martin D. BRAND

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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NN'-Dicyclohexylcarbodi-imide at low concentrations decreases the $H^+/2e$ ratio for rat liver mitochondria over the span succinate to oxygen from 5.9 ± 0.3 (mean $\pm$ S.E.M.) to 4.0 ± 0.1 and for the cytochrome *b*–*c*₁ complex from 3.8 ± 0.2 to 1.9 ± 0.1 , but has little effect on the $H^+/2e$ ratio of cytochrome oxidase. The decrease in stoichiometry is due, not to uncoupling or inhibition of electron transport, but to inhibition of proton translocation. *NN'*-Dicyclohexylcarbodi-imide thus 'decouples' proton translocation in the cytochrome *b*–*c*₁ complex.

DCCD has been shown to inhibit proton translocation by the F_0 component of the mitochondrial ATPase (Beechey *et al.*, 1966), by the mitochondrial transhydrogenase (Pennington & Fisher, 1981) and by cytochrome oxidase (Casey *et al.*, 1979). Prochaska *et al.* (1981) have demonstrated that DCCD binds to a glutamate residue within both cytochrome oxidase and the ATPase. This acidic residue may play a role in proton translocation via proton channels.

We have previously found the $H^+/2e$ ratio for the span succinate to oxygen to be 6 (Brand *et al.*, 1976; Al-Shawi & Brand, 1981). Other studies have obtained values of 4 (Moyle & Mitchell, 1978) and 8 (Alexandre & Lehninger, 1979). The value of the $H^+/2e$ stoichiometry is important in understanding the molecular mechanism of proton translocation. In the present paper we report that DCCD changes $H^+/2e$ ratios by inhibiting proton translocation by the cytochrome *b*–*c*₁ complex; this effect is not due to uncoupling or to inhibition of electron transport. A preliminary report of this work has already been published (Price & Brand, 1982).

Materials and methods

Rat liver mitochondria were prepared by the method of Chappell & Hansford (1972) and suspended at 65 mg of protein/ml (Gornall *et al.*, 1949) in 0.25 M-sucrose / 1 mM-EGTA / 5 mM-Tris / HCl buffer, pH 7.2.

Abbreviation used: DCCD, *NN'*-dicyclohexylcarbodi-imide.

Oxygen-pulse-type experiments (Mitchell & Moyle, 1967; Brand *et al.*, 1976) were performed with either oxygen (17 nmol of O) or $K_3Fe(CN)_6$ (60 nmol) as electron acceptor as follows: mitochondria (10 mg) were suspended anaerobically in 5 ml of 120 mM-KCl / 1 mM-EGTA, pH 7.0, containing succinate (3 mM), rotenone (3 μ M), *N*-ethylmaleimide (30 nmol/mg of protein) and oligomycin (0.6 μ g/mg of protein). For the $H^+/2e$ ratio for succinate to oxygen, air-saturated KCl was used to initiate the reaction. For the cytochrome *b*–*c*₁ complex, KCN (2 mM) was added and $K_3Fe(CN)_6$ was used as electron acceptor. For cytochrome oxidase, the cytochrome *b*–*c*₁ complex was inhibited by antimycin A (0.2 nmol/mg), and ascorbate (3 mM) / tetramethyl-*p*-phenylenediamine (60 μ M) was used as electron donor. Oxygen was the electron acceptor. Either DCCD or ethanol (control) was then added and the mitochondria were incubated anaerobically for 15 min at 30°C in a Clark Oxygen Electrode (Rank Bros., Bottisham, Cambridge, U.K.). Valinomycin (40 μ M) was then injected, the pH (measured with a Pye–Unicam Ingold pH electrode connected to a Pye PW 9004 pH-meter and a Bryans 28000 chart recorder) was adjusted to 7.0 with KOH or HCl, and the electron acceptor was rapidly injected.

$H^+/2e$ ratios were calculated by plotting $\log \Delta[H^+]$ against time, and the total $\Delta[H^+]$ was calculated by back-extrapolation of the semi-logarithmic plot obtained (Mitchell & Moyle, 1967; Brand *et al.*, 1976). Net scalar proton changes were subtracted. The slope of the semi-logarithmic plot is the first-order rate constant for proton re-entry into the mitochondria and will increase if uncoupling occurs.

This rate constant was therefore used as a measure of uncoupling.

Uncoupled electron transport was measured in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (150 pmol/mg of protein). Oxygen consumption was determined in aerobic oxygen-pulse medium with and without DCCD. The reaction was started with 3 mM-succinate. The rate of electron transport from succinate to ferricyanide was measured by calculating the initial uncoupled rate of scalar proton production from mitochondria under the conditions used to measure the coupled $H^+/2e$ value for the cytochrome *b-c*₁ complex.

All reagents were of analytical grade. DCCD was obtained from BDH Chemicals, Poole, Dorset, U.K. Oligomycin, valinomycin and antimycin were obtained from Boehringer, Lewes, East Sussex, U.K. All other reagents were obtained from BDH Chemicals or Sigma Chemical Co., Poole, Dorset, U.K.

Results and discussion

Table 1 summarizes the effects of DCCD on the combined $H^+/2e$ ratio for the cytochrome *b-c*₁ complex and cytochrome oxidase and the individual $H^+/2e$ ratios for these complexes. The span succinate to oxygen shows a decrease of 2 in the $H^+/2e$ ratio; dissection of the chain shows that there is only a small effect on cytochrome oxidase, but that the cytochrome *b-c*₁ complex registers a decrease of 2 in the $H^+/2e$ stoichiometry.

Fig. 1 shows the DCCD-dependence of the $H^+/2e$ ratio for the span succinate to oxygen and for the cytochrome *b-c*₁ complex. In both cases there is a loss of 2 translocated protons per $2e$. In each case the effect plateaus at about 130 nmol of DCCD/mg of protein.

There are three possible causes of this effect. (i) Partial uncoupling by DCCD; this would increase the rate of proton backflow during an oxygen pulse and might cause underestimation of the number of

protons ejected, leading to an apparent decrease in $H^+/2e$. (ii) Inhibition of electron transport, causing a decrease in the rate of proton ejection. Backflow would now become relatively more significant, leading to underestimation of $H^+/2e$. (iii) Inhibition of proton translocation by DCCD within the cytochrome *b-c*₁ complex.

To eliminate (i), the experiment shown in Fig. 2 was performed. This shows that there was no change in the rate constant for proton re-entry into the mitochondria in the presence of DCCD when the pH gradient was established when the span succinate to oxygen was studied. With succinate to ferricyanide, the rate constant increased by less than 10% (results not shown). To eliminate (ii), uncoupled rates of electron transport from succinate to oxygen were measured. These were unchanged [264 ± 13 nmol of e^- /min per mg of protein ($n = 10$) without DCCD and 248 ± 16 nmol of e^- /min per mg of protein ($n = 8$) with DCCD], as were uncoupled rates of scalar proton production by the span succinate to ferricyanide [88 ± 5 ($n = 10$) to 78 ± 10 nmol of H^+ /min per mg of protein ($n = 10$)]. Thus DCCD neither increases permeability towards H^+ nor inhibits electron transport.

Fig. 1. Titration of the effect of DCCD on the $H^+/2e$ ratio for rat liver mitochondria

Conditions were as described in the Materials and methods section. ●, Succinate-oxygen span; ○, succinate-ferricyanide span. Bars indicate S.E.M. Results are averages of ten determinations.

Table 1. Effects of DCCD on the $H^+/2e$ ratio for rat liver mitochondria

DCCD was at 130 nmol/mg of protein; other incubation conditions were as described in the Materials and methods section. Values are means $\pm$ S.E.M., with the numbers of observations in parentheses. Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

Respiratory span	$H^+/2e$	
	-DCCD	+DCCD
Succinate-oxygen	5.9 ± 0.3 (20)	4.0 ± 0.1 (21)
Ascorbate/TMPD-oxygen	2.5 ± 0.1 (15)	2.2 ± 0.1 (14)
Succinate-ferricyanide	3.8 ± 0.2 (10)	1.9 ± 0.1 (10)

Fig. 2. Effect of DCCD on permeability towards H^+ and State-4 respiratory rate of rat liver mitochondria. The rate constant for H^+ re-entry (O) is equal to the slope of a plot of $\log \Delta[H^+]$ against time after an oxygen pulse. Coupled (State-4) rates of electron transport (●) were measured in an oxygen electrode under aerobic conditions in the medium used for oxygen pulses for the span succinate to oxygen. Results are averages of ten determinations. Bars indicate s.e.m.

Measurement of State-4 rates of electron transport through the span succinate to oxygen (Fig. 2) revealed that coupled rates approximately doubled over the range in which the $H^+/2e$ ratio falls (see Fig. 1). An increase in the rate of State-4 electron transport is to be expected, since only 4 protons are being ejected from the mitochondria per O atom consumed instead of 6. This means that, in order to generate the same electrochemical gradient of H^+ , the mitochondria must respire at a higher rate to extrude the required number of protons.

These results indicate that DCCD inhibits proton translocation by the cytochrome $b-c_1$ complex and that DCCD does not act by uncoupling or inhibiting electron transport. Rather, DCCD acts by 'decoupling' two of the protons normally translocated by the cytochrome $b-c_1$ complex.

In these experiments, we have not seen the abolition by DCCD of the proton-pumping capacity of cytochrome oxidase reported by Casey *et al.* (1979) (see Table 1). The reason seems to be that those workers used much higher concentrations of DCCD and longer incubation times. Under our mild conditions, we see only a small effect on cytochrome oxidase at concentrations where the cytochrome $b-c_1$ complex is affected.

In summary, we present direct evidence for the inhibition of proton pumping by DCCD in the cytochrome $b-c_1$ region with no uncoupling or inhibition of electron transport. In a recent paper Lenaz *et al.* (1982) came to a similar but non-quantitative conclusion after using a method substantially different from ours. Since we find that only two protons are lost as a result of the action of DCCD, the inference is that there are two separate mechanisms of proton translocation by the cytochrome $b-c_1$ complex, only one of which is decoupled by DCCD.

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