Thermodynamic and steady-state-kinetic investigation of the effect of *NN'*-dicyclohexylcarbodi-imide on H^+ translocation by the mitochondrial cytochrome *bc*₁ complex

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1. Steady-state kinetic measurements showed that *NN'*-dicyclohexylcarbodi-imide decreased the observed $H^+/2e$ ratio of H^+ transport by mitochondria respiring on succinate, acting mainly at the cytochrome *bc*₁ complex. 2. Thermodynamic assessment of the $H^+/2e$ ratio by measuring the force ratio across the *bc*₁ complex showed that the inhibitor did not affect H^+ translocation. 3. Possible explanations of this disagreement between methods are examined; we conclude that the inhibitor does not alter the mechanistic stoichiometry of H^+ pumping by the *bc*₁ complex.

The protein-modifying reagent DCCD affects H^+ translocation by the mitochondrial ATPase (Beechey *et al.*, 1967; Kopecky *et al.*, 1983), by cytochrome oxidase (Casey *et al.*, 1980), by the mitochondrial transhydrogenase (Pennington & Fisher, 1981) and by other mitochondrial systems (Gauthier & Diwan, 1979; Jung *et al.*, 1980; Kolarov *et al.*, 1982; Warhurst *et al.*, 1982; Martin *et al.*, 1984). We have reported (Price & Brand, 1982, 1983) that DCCD decreases H^+ translocation by the cytochrome *bc*₁ complex in mitochondria without affecting electron transport, as have others (Lenaz *et al.*, 1982; Degli Esposti *et al.*, 1982). Similar results have been reported for the enzyme isolated and reconstituted into phospholipid vesicles (Beattie & Villalobo, 1982; Clejan & Beattie, 1983; Degli Esposti *et al.*, 1983; Lorusso *et al.*, 1983), although others (Nalecz *et al.*, 1983a,b) were unable to demonstrate this effect.

In the present paper we have investigated the effects of DCCD on H^+ translocation by the *bc*₁ complex in whole mitochondria using approaches different from those used before. We conclude that

the observed effects of DCCD do not represent a change in the mechanistic stoichiometry of H^+ ejection by the complex, but are caused by a process that interferes with kinetic, but not with thermodynamic, measurements of the $H^+/2e$ ratio.

Experimental

Mitochondria

These were isolated by conventional methods (Chappell & Hansford, 1972) in 250 mM-sucrose/5 mM-Tris-HCl/1 mM-EGTA (potassium salt), pH 7.4. Mitochondrial protein was assayed by a biuret method (Gornall *et al.*, 1949). Experiments on glycerol 3-phosphate oxidation were carried out using mitochondria from livers of rats pretreated with L-thyroxine (Alexandre *et al.*, 1980).

Steady-state kinetic determinations of $H^+/2e$

These were carried out as described by Al-Shawi & Brand (1981). The method involves measuring J_0 in a steady state, adding antimycin and measuring the initial rate of decay of the external pH using a pH electrode, then calculating $H^+/2e$ as the ratio of these rates.

Thermodynamic measurements

These were carried out as described by Brown & Brand (1985) in the medium described in Table 2 of the present paper. Mitochondrial volume, ΔpH and $\Delta\psi$ were measured with 3H_2O , [^{14}C]mannitol, [3H]acetate and [3H]methyltriphenylphosphonium. E_h' (Q-2) and E_h (cytochrome *c*) were measured by dual-wavelength spectrophotometry.

Abbreviations used: DCCD, *NN'*-dicyclohexylcarbodi-imide; J_0 , rate of oxygen consumption; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide; $\Delta\psi$, transmembrane difference in electrical potential; ΔpH , transmembrane difference in pH; Δp , protonmotive force; E_h , redox potential; ΔE_h , difference in redox potential between specified carriers; Q-2, ubiquinone-2; Q-10, ubiquinone-10; ΔG , Gibbs free-energy difference; F , Faraday constant.

Results and discussion

Fig. 1 shows the effect of DCCD on the H^+/O ratio for succinate measured by the steady-state kinetic method of Al-Shawi & Brand (1981). Just as in oxygen-pulse experiments (Price & Brand, 1982, 1983), the H^+/O ratio decreased from about 6 to about 4 with 80–100 nmol of DCCD/mg of protein. The time taken for maximum effect of DCCD was about 5 min at 30°C. The major effect of DCCD was an increase in J_0 , with little effect on the rate constant for H^+ re-entry after antimycin addition.

In Table 1 we show that DCCD had the same effect on H^+/O for glycerol 3-phosphate oxidation, and on K^+/O for succinate oxidation, showing that the effect of DCCD was not specific for succinate and was also observed on the charge/ O ratio. DCCD lowered the $H^+/2e$ ratio for the span succinate–ferricyanide, although not as much as for the whole span from succinate to oxygen. It had only a small effect on H^+/O for cytochrome oxidase under these conditions. Thus the steady-state kinetic method gave essentially the same results as the oxygen-pulse method (Price & Brand, 1982, 1983), appearing to show that DCCD decreased proton pumping by the bc_1 complex by about 1.2 to 1.9 $H^+/2e$.

A quite different approach to the measurement of the $H^+/2e$ stoichiometry of the bc_1 complex is to measure the force ratio $2\Delta E_h/\Delta p$, which approaches $H^+/2e$ as the complex approaches equilibrium. Thus if DCCD lowers the $H^+/2e$ ratio, either ΔE_h should decrease, or Δp should rise, or the reaction should move considerably further away from equilibrium. We found that under a variety of conditions the $H^+/2e$ ratio of the bc_1 complex measured in this way did not decrease in the presence of DCCD.

To show that this thermodynamic approach registered no decrease in $H^+/2e$ under precisely the conditions that the kinetic methods indicated a large decrease, we performed the experiment

Fig. 1. Effect of DCCD on H^+/O measured by the steady-state kinetic method

Mitochondria (10 mg of protein) were incubated anaerobically for 6 min in 5 ml of medium containing 200 mM-sucrose, 20 mM-KCl, 1 mM-EGTA (potassium salt), 5 mM-succinate (potassium salt), 5 μ M-rotenone, 40 nmol of *N*-ethylmaleimide/mg of protein, 500 ng of oligomycin/mg of protein, 350 pmol of carboxyatractylsides/mg of protein, 350 nmol of valinomycin/mg of protein, 3000 EC units of bovine catalase, and DCCD as indicated, at 30°C and pH 7.1. Respiration was initiated by addition of H_2O_2 solution. Steady state J_0 was measured with an oxygen electrode. Respiration was then inhibited by injection of antimycin (170 pmol/mg of protein) and the initial rate of change of extramitochondrial pH was recorded. Points represent means ($\pm$ S.E.M.) of three separate determinations.

Table 1. Steady-state kinetic measurements of $H^+/2e$

Experiments were carried out as described in Fig. 1. When glycerol 3-phosphate was used as substrate, succinate was replaced by 2 mM-glycerol 3-phosphate (sodium salt) (Gro-3-P) and 0.5 mM-malonate (sodium salt). K^+/O ratios were determined with a K^+ -sensitive electrode replacing the pH electrode in a similar medium, but with 20 mM-KCl replaced by 4 mM-KCl, and K^+ salts replaced by tetramethylammonium salts. For the span succinate–ferricyanide the medium included 1 mM- $K_3Fe(CN)_6$, 2 mM- $MgCl_2$ and 1 mM-KCN, and ferricyanide reduction was measured spectrophotometrically to give the steady-state rate of electron transport. For ascorbate/TMPD the succinate was replaced by 3 mM-ascorbate (potassium salt), 75 μ M-TMPD and 170 pmol of antimycin/mg of protein, and respiration was stopped by injection of 1.25 mM-KCN. Values are corrected for scalar H^+ changes. Results are expressed as means $\pm$ S.E.M. for the number of determinations shown in parentheses.

Substrate	Acceptor	$H^+/2e$	
		Control	+ DCCD (80 nmol/mg of protein)
Succinate	Oxygen	6.10 ± 0.20 (9)	4.23 ± 0.23 (4)
Gro-3-P	Oxygen	5.78 ± 0.25 (6)	4.35 ± 0.25 (4)
Succinate	Oxygen (K^+/O)	5.94 ± 0.55 (7)	3.88 ± 0.47 (7)
Succinate	Ferricyanide	4.51 ± 0.16 (4)	3.27 ± 0.18 (5)
Ascorbate/TMPD	Oxygen	2.12 ± 0.28 (4)	1.97 ± 0.28 (4)

Table 2. *Thermodynamic and kinetic determination of H⁺/2e*

Mitochondria (1 mg of protein/ml) were suspended in medium containing 200 mM-sucrose, 20 mM-tetramethylammonium chloride, 1 mM-EGTA (tetramethylammonium salt), 2 mM-KCl, 1 mM-mannitol, 100 μ M-potassium acetate, 50 μ M-ATP (sodium salt), 5 μ M-methyltriphenylphosphonium bromide, 5 μ M-rotenone, 30 nmol of *N*-ethylmaleimide/mg of protein, 500 ng of oligomycin/mg of protein, 10 μ M-Q-2, 3.3 μ M-cytochrome *c* (cyt. *c*), 330 pmol of valinomycin/mg of protein and either 0.5 μ l of ethanol/ml (control) or 100 nmol of DCCD/mg of protein. The final pH was 6.84 and the temperature 25°C. For measurement of mitochondrial volume the medium also contained ³H₂O (1 μ Ci/ml) and [¹⁴C]mannitol (0.14 μ Ci/ml), for Δ pH it contained [³H]acetate (0.8 μ Ci/ml) and [¹⁴C]mannitol and for $\Delta\psi$ it contained [³H]methyltriphenylphosphonium (0.05 μ Ci/ml). After 5 min incubation, 5 mM-succinate (tetramethylammonium salt) was added, then after a further 2 min measurements were made. For radioactive experiments, samples were centrifuged and supernatant and pellet were assayed for radioactivity, followed by calculation of volume, Δ pH, $\Delta\psi$ and Δ p. Determinations of E_h were carried out by dual-wavelength spectrophotometry (see Brown & Brand, 1985). H⁺ influx and J_0 were measured with electrodes as described by Al-Shawi & Brand (1981). Respiratory control was the ratio of J_0 after and before addition of 100 pmol of FCCP per mg of protein. Values are expressed as means $\pm$ S.E.M. for the number of determinations shown in parentheses.

	Control	+DCCD
Volume (μ l/mg of protein)	0.64 $\pm$ 0.08 (12)	0.79 $\pm$ 0.06 (12)
Δ pH (mV)	62.0 $\pm$ 2.5 (6)	48.9 $\pm$ 1.7 (6)
$\Delta\psi$ (mV)	108.5 $\pm$ 1.3 (6)	102.8 $\pm$ 1.6 (6)
Δ p (mV)	170.5	151.7
E_h (Q-2) (mV)	76.5 $\pm$ 10.3 (6)	99.8 $\pm$ 0.6 (6)
E_h (cyt. <i>c</i>) (mV)	275.9 $\pm$ 2.7 (6)	295.5 $\pm$ 2.1 (6)
ΔE_h (Q-2 - cyt. <i>c</i>) (mV)	199.4	195.7
$2\Delta E_h/\Delta p$	2.34	2.58
$\Delta E_h - \Delta p$ (mV)	28.9	44.0
H ⁺ influx rate (nmol of H ⁺ /s per mg of protein)	2.04 $\pm$ 0.16 (6)	2.19 $\pm$ 0.12 (6)
J_0 (nmol of O/s per mg of protein)	0.365 $\pm$ 0.012 (6)	0.780 $\pm$ 0.026 (6)
Respiratory-control ratio	3.17	1.39
Kinetic H ⁺ /2e (succinate-oxygen)	5.59 $\pm$ 0.28 (6)	2.80 $\pm$ 0.14 (6)

detailed in Table 2. We set up steady-state respiration in the presence of valinomycin and measured both the force ratio and the kinetic stoichiometry (by addition of antimycin) in exactly parallel determinations on the same preparation of mitochondria in the same medium

Control values of volume, Δp , ΔE_h and $2\Delta E_h/\Delta p$ were similar to those reported under similar conditions in more detail elsewhere (Brown & Brand, 1985). They indicate that the bc₁ complex pumps 2H⁺/2e (4H⁺/2e if the H⁺ translocated by reduced Q-10 diffusion from succinate dehydrogenase to the bc₁ complex is included) and is measurably displaced from equilibrium under these conditions (which were designed to give less than maximal Δp ; see Al-Shawi & Brand, 1981). Addition of DCCD caused a small decrease in Δ pH, $\Delta\psi$ and Δp (not a rise as predicted above) and had little effect on ΔE_h , although the E_h of both Q-2 and cytochrome *c* became more positive, $2\Delta E_h/\Delta p$ did not decrease, but rose slightly. The respiratory-control ratio decreased because of the increase in steady-state J_0 , but the mitochondria remained coupled. These results are consistent with a slight increase in proton permeability in the presence of DCCD, causing the reaction to move a little further from equilibrium. This is shown by the disequilibrium across the bc₁ complex ($\Delta E_h - \Delta p$; $-\Delta G/F$), which, assuming no stoichiometry

change, increased from 29 to 44 mV. The results are quite inconsistent with DCCD causing a decrease in the H⁺/2e ratio of the bc₁ complex in this experiment.

The exactly parallel determinations of H⁺/2e by the kinetic method are also reported in Table 2. The results were similar to those in Table 1: DCCD caused the measured H⁺/2e to decrease, in this particular case rather more than we usually find under slightly different conditions.

In a large number of experiments not done in parallel, under a variety of experimental conditions (e.g. no valinomycin, no Q-2, duroquinone replacing Q-2, no added cytochrome *c*, 20 mM-KCl present) we have consistently seen that the thermodynamic estimates of H⁺/2e show a small rise with DCCD, whereas the kinetic estimates show a large decrease.

A number of possible resolutions of the different conclusions about the effect of DCCD to be drawn from the thermodynamic and kinetic measurements can be suggested, but most of them do not stand up to scrutiny.

(1) The thermodynamic measurements fail to register the decrease in H⁺/2e with DCCD because the bc₁ complex moves very far out of equilibrium and the force ratio becomes a very poor indicator of H⁺/2e. We think this is untrue, because the mitochondria retain respiratory control (Table 2).

With sufficient FCCP present to give the same increase in J_0 as given by DCCD, the force ratio can still be measured (Brown & Brand, 1985). The complex does move further from equilibrium, but the force ratio does not increase very greatly. The change that is seen would not mask the very large decrease that is predicted from the kinetic measurements.

(2) The decrease in $H^+/2e$ from succinate to oxygen is in fact at cytochrome oxidase (which is known to pump less H^+ when DCCD is present under other conditions; Casey *et al.*, 1980) and is therefore not shown by the thermodynamic investigation of the bc_1 complex. Against this we can cite the data of Table 1 and of Price & Brand (1982, 1983), showing that under our relatively mild conditions there is little decrease in the $H^+/2e$ for cytochrome oxidase but a considerable decrease for the span succinate–ferricyanide.

(3) DCCD acts by increasing proton permeability, to which the thermodynamic analysis is relatively insensitive. It is clear that, at the concentrations we have used, DCCD does act in this way to a small extent (see Table 2 and Price & Brand, 1983). However, we have shown that addition of sufficient FCCP to give the same increase in J_0 as given by DCCD has no effect on the measured $H^+/2e$ ratio in either oxygen-pulse (Price & Brand, 1983) or steady-state kinetic determinations (Al-Shawi & Brand, 1981).

(4) A sub-population of the mitochondria burst or become very H^+ -permeable on addition of DCCD, so that up to half J_0 is not coupled to H^+ ejection, but the remaining mitochondria are essentially unaffected. This would cause kinetic methods to underestimate $H^+/2e$ by overestimating the relevant J_0 . It would not necessarily affect the thermodynamic measurements, since Δp is measured only in the non-burst population and measured ΔE_h might not be greatly affected. We have not rigorously excluded the possibility of proton-specific leaks, but have shown bursting and non-specific leakage to be unlikely in several ways. These include measurements of the following: the release of glutamate dehydrogenase (Price & Brand, 1983) and malate dehydrogenase; release of K^+ ; the latency of succinate dehydrogenase in the presence of phenyl malonate; and the intramitochondrial volume, using 3H_2O and $[^{14}C]$ sucrose, and by monitoring light-scattering changes (M. D. Brand, M. K. Al-Shawi, G. C. Brown & B. D. Price; unpublished work). There do, however, appear to be damaging effects of DCCD in respiring mitochondria measured by many of these tests at DCCD concentrations of over 100–200 nmol/mg of protein, values rather higher than the ones we have used.

(5) DCCD induces rapid K^+/H^+ exchange or H^+

uniport in energized mitochondria so that measured $H^+/2e$ and $K^+/2e$ ratios are decreased when measured kinetically, but affected little when measured thermodynamically. The exchange would have to shut off rapidly when the oxygen was all consumed in oxygen-pulse experiments or when antimycin was added in steady-state kinetic experiments, otherwise these methods would successfully correct for the increased H^+ back-flow. We have found this possibility difficult to disprove or substantiate. There are reports, however, of effects of DCCD on both K^+ uniport and K^+/H^+ antiport (Jung *et al.*, 1980; Martin *et al.*, 1984).

Other experiments lend support to the suggestion that the effect of DCCD on the measured $H^+/2e$ of the bc_1 complex is not a change in the mechanistic stoichiometry. In experiments with purified mitochondrial bc_1 complex and bacterial reaction centres inlaid into phospholipid vesicles we have been unable to demonstrate any effects of DCCD on $H^+/2e$ other than those due to uncoupling (M. D. Brand & P. R. Rich, unpublished work; Rich, 1984). Similar results have been described by Nalecz *et al.* (1983a,b), despite reports by others that DCCD does decrease the mechanistic $H^+/2e$ ratio in such systems (see the introduction).

Our results lead us to abandon our earlier conclusion that DCCD lowers the mechanistic stoichiometry of the bc_1 complex (Price & Brand, 1982, 1983), although we cannot establish the precise nature of the effect of DCCD. We suggest that other reports in the literature showing effects of DCCD on H^+ translocation by bc_1 complex in intact mitochondria (Lenaz *et al.*, 1982; Degli Esposti *et al.*, 1982) should be treated with caution, as should similar reports dealing with reconstituted bc_1 complex (Beattie & Villalobo, 1982; Clejan & Beattie, 1983; Degli Esposti *et al.*, 1983; Lorusso *et al.*, 1983).

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