

## RESEARCH COMMUNICATION

## Experimental discrimination between proton leak and redox slip during mitochondrial electron transport

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By measuring the relationship between protonmotive force and the increment in oxygen consumption by mitochondria treated with submaximal amounts of uncoupler, we have experimentally tested four different models of imperfect coupling of oxidative phosphorylation. The results show that the increased rate of oxygen consumption at high protonmotive force is explained entirely by the dependence on protonmotive force of the passive

proton leak conductance of the mitochondrial inner membrane. There is no measurable contribution from redox-slip reactions in the proton pumps caused by high protonmotive force. Neither is there any contribution from increased proton conductance of the membrane or increased redox slip in the respiratory chain caused by high turnover rates of the complexes.

## INTRODUCTION

When non-phosphorylating mitochondria are titrated with respiratory inhibitor, there is a non-linear relationship between respiration rates and protonmotive force ( $\Delta p$ ) [1]. There are currently four main models to explain this non-linearity, with radically different implications for the mechanism of proton translocation. The first model [1] postulates increased proton leak conductance of the inner membrane at high  $\Delta p$ , while the number of protons pumped by the respiratory chain per oxygen consumed ( $H^+/O$ ) remains constant. The second model [2] proposes increased redox slip at high  $\Delta p$  with constant proton leak conductance. Redox slip is electron flow without proton pumping to the external medium; it decreases  $H^+/O$ . The third model [3,4] proposes increased proton leak conductance at high electron-transfer rates, perhaps because active respiratory complexes seal the membrane less effectively, and the fourth model [5] proposes increased redox slip at high turnover rates of the complexes. Despite much work on this important question [1–28], the validity of each of these models is uncertain. We present here an experiment, developed from preliminary work described in [29], that shows that only the first model is correct.

## METHODS

Measurement of oxygen consumption and  $\Delta p$ 

Rat liver mitochondria (2 mg of protein/ml) were incubated for 2 min in medium containing 100 mM NaCl, 3 mM Hepes, 5 mM  $Na_3PO_4$ , 4 mM succinate, 1 mM EGTA, 5  $\mu M$  rotenone, 100 pmol of valinomycin/mg of protein and 1  $\mu g$  of oligomycin/mg of protein at 37 °C and adjusted to pH 7.0 with NaOH. Oxygen consumption was measured in duplicate with two oxygen electrodes. The respiratory control ratio [rate with 1  $\mu M$  carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)/rate with oligomycin] was greater than 9. To avoid complications from possible variations in the binding of lipid-soluble organic probes such as methyltriphenylphosphonium ion, membrane

potential was instead measured in parallel in this sodium-based medium using  $^{86}Rb$  in the presence of valinomycin [30]. Mitochondrial volume was measured with  $^3H_2O$  and [ $^{14}C$ ]sucrose [30] and was constant during the titrations. In control experiments  $\Delta pH$  was measured with [ $^3H$ ]acetate and [ $^{14}C$ ]sucrose; it varied linearly from +5 mV to –10 mV.  $\Delta p$  under the conditions used here was found to be related to membrane potential by the equation  $\Delta p = (1.1 \times \text{membrane potential}) - 11.9$ . All values of  $\Delta p$  were calculated from the measured membrane potential using this relationship. This explains the difference between the  $\Delta p$  values calculated here and those given in our preliminary report [29], in which  $\Delta pH$  was wrongly estimated to be about –20 mV at all potentials because of binding of [ $^{14}C$ ]methylamine.

## Theory

The logic of the experiment is as follows. The proton conductance ( $C^{CCCP}$ ) catalysed by the classical uncoupler CCCP is known [8] to be independent of  $\Delta p$ . In other words, the proton flux catalysed by CCCP depends linearly on potential; it is ohmic. We can measure the relationship between respiration rate,  $J_o$ , and  $\Delta p$  in isolated mitochondria prevented from making ATP, and then add a low concentration of CCCP and repeat the measurement. If endogenous leak or slip depends only on  $\Delta p$  (models 1 and 2), then all of the extra respiration rate in the presence of CCCP ( $\Delta J_o$ ) at each value of  $\Delta p$  must be used to drive protons through the uncoupler. If we find that the relationship between this extra respiration rate and  $\Delta p$  is linear, then  $H^+/O$  does not vary with  $\Delta p$ . The non-linearity in the original relationship between  $J_o$  and  $\Delta p$  when the natural conductance operates must then be due to the  $\Delta p$ -dependence of the natural proton leak (model 1). If we find that the relationship between  $\Delta J_o$  and  $\Delta p$  is non-linear, then  $H^+/O$  must decrease at higher  $\Delta p$  and the non-linearity in the original relationship between  $J_o$  and  $\Delta p$  must be due to slip (model 2).

The extra proton influx will be given by  $C^{CCCP}$  multiplied by the value of  $\Delta p$ , and will be equal to the extra proton efflux in the

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone;  $\Delta p$ , protonmotive force;  $J_o$ , rate of oxygen consumption;  $H^+/O$ , number of protons pumped to the external medium by the electron-transport complexes per oxygen atom consumed;  $C^{CCCP}$ , proton conductance catalysed by CCCP.

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steady state. This in turn will be given by the extra respiration rate ( $\Delta J_0$ ) multiplied by  $H^+/O$ . Therefore:

$$\Delta J_0 = \frac{\Delta p \cdot C^{CCCP}}{H^+/O} \quad (1)$$

If we vary the concentration of CCCP we add, we can also test for proton leak and redox slip that depend on electron-transfer rate (models 3 and 4), as described below. The easiest way to display results using several different concentrations of CCCP is to normalize the plot with respect to CCCP by dividing both sides of eqn. (1) by the concentration of added CCCP. Thus:

$$\frac{\Delta J_0}{CCCP} = \frac{\Delta p \cdot C^{CCCP}}{H^+/O \cdot CCCP} = \frac{\Delta p \cdot \text{constant}}{H^+/O} \quad (2)$$

A graph of  $\Delta J_0/CCCP$  against  $\Delta p$  will pass through the origin and will have slope inversely proportional to  $H^+/O$ . If  $H^+/O$  remains constant as  $\Delta p$  is varied (in other words, if the original non-linear titrations of  $\Delta p$  and  $J_0$  are explained entirely by  $\Delta p$ -dependent changes in proton conductance; model 1), then this derived plot will give a straight line through the origin. If, however, slip occurs to any significant extent (model 2),  $H^+/O$  will vary with  $\Delta p$  and the derived plot will give a curve, with slope rising as  $\Delta p$  rises.

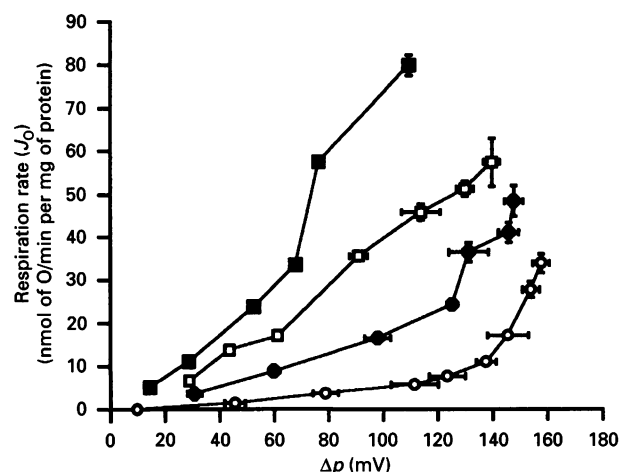
We can use the same experiment to test whether endogenous proton leak is a function of  $J_0$  (model 3) and whether the proton pumps slip as a function of turnover rate (model 4). In other words, we can test the assumption made above that endogenous leak or slip depends only on  $\Delta p$  and is independent of the turnover of the pumps. We can do this by using different CCCP concentrations to increase  $J_0$ . If leak depends on  $J_0$  (model 3), then the total proton conductance will be higher than anticipated when more CCCP is present and the graphs of  $\Delta J_0/CCCP$  against  $\Delta p$  representing higher concentrations of CCCP will be steeper than those representing lower uncoupler concentrations. If slip depends on  $J_0$  (model 4), then  $H^+/O$  will be lower at higher CCCP concentrations, and again the graphs will be steeper. On the other hand, if models 3 and 4 are wrong and electron-transport rate does not influence the endogenous proton leak rate or  $H^+/O$ , then the lines with different CCCP concentrations will superimpose.

The same experiment also tests for any direct effect of added CCCP; if CCCP itself changes the endogenous leak or causes slip, then once again higher CCCP concentrations will give steeper lines.

## RESULTS AND DISCUSSION

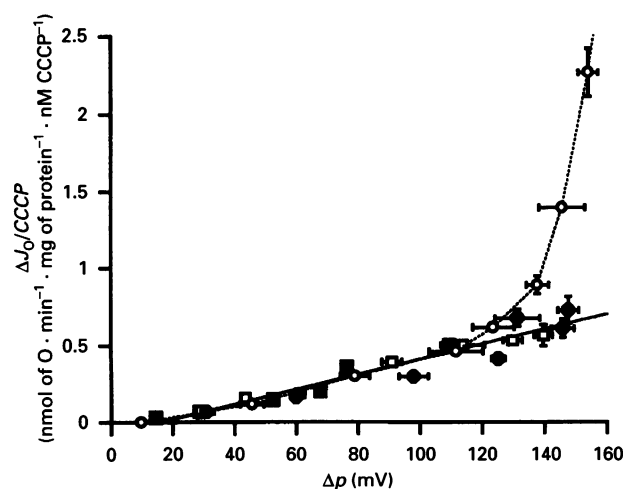
The Figures show the results of the experiment. Figure 1 shows a malonate titration of respiration and  $\Delta p$  of rat liver mitochondria respiring on succinate in the absence of CCCP, and three titrations with submaximal concentrations of added CCCP. In the absence of CCCP there is the expected non-linear relationship between  $J_0$  and  $\Delta p$ ; as the amount of CCCP is increased, the non-linearity becomes less pronounced.

Figure 2 shows the derived secondary plot of  $\Delta J_0/CCCP$  against  $\Delta p$ . The plot is linear (continuous line) and passes close to the origin, as predicted if all of the non-linearity of the titration in the absence of CCCP is caused by the endogenous non-ohmic proton leak and none is due to redox slip in the proton pumps (model 1). As a comparison, the curve expected if all of the non-linearity of the titration in Figure 1 were due to slip



**Figure 1** Malonate titrations of State-4 respiration of rat liver mitochondria in the presence of different submaximal amounts of CCCP

Mitochondria were incubated with 4 mM succinate at 37 °C and  $\Delta p$  and  $J_0$  were measured as described in the Methods section. Titrations with malonate were carried out essentially as previously described [30] by including different concentrations of tetramethylammonium malonate in the presence of 0 nM (○), 40 nM CCCP (●), 80 nM CCCP (□) and 150 nM CCCP (■). Points are means  $\pm$  S.E.M. from four or five repeats on two or three rat liver mitochondrial preparations, except for the curve with 150 nM CCCP, which represents the mean  $\pm$  range for two repeats on a single mitochondrial preparation. The lowest point on the control curve contained 20 mM malonate and 1  $\mu$ M CCCP to fully inhibit respiration and bring  $\Delta p$  to zero.



**Figure 2**  $\Delta J_0/CCCP$  versus  $\Delta p$

$\Delta J_0$  is the difference between the measured value of  $J_0$  with CCCP present and the interpolated value at the same  $\Delta p$  with CCCP absent, from Figure 1. ●, 40 nM CCCP; □, 80 nM CCCP; ■, 150 nM CCCP. The continuous line is a linear regression calculated using all these points with CCCP present; lines for the different CCCP concentrations were not significantly different from each other, disproving models 3 and 4. The broken line (○) shows the result expected for these curves with CCCP present if the non-linearity of the State-4 titration is caused entirely by  $\Delta p$ -dependent slip (model 2) superimposed on a small ohmic proton leak equivalent to 12.1 nM CCCP. To generate this line we calculated the amount of CCCP that would have to be added to mitochondria that theoretically had zero endogenous leak to cause the lowest four points of the State-4 titration in Figure 1 to fall on the curve in Figure 2 for the titrations in the presence of CCCP. This was 12.1 nM CCCP; the rest of the broken line was generated assuming zero endogenous leak, but this concentration of CCCP. The broken line is clearly different from the continuous line, disproving model 2.

is shown by open circles (broken line). Since the lowest point on this curve represents conditions where  $\Delta p$  should be zero, the small intercept on the  $\Delta p$  axis reflects residual consistent experimental error at low  $\Delta p$ . It is clear that the experimental points show no tendency to follow the line predicted for slip; the data is not consistent with the hypothesis of slip in the proton pumps that increases at higher values of  $\Delta p$  (model 2).

The points for the three titrations with different amounts of CCCP superimpose completely, showing that there is no increase in the endogenous proton conductance as the turnover rate of the proton pumps increases; model 3 is incorrect. There is also no change in  $H^+/O$  ratio dependent on the rate of electron transport, as this too would cause the lines representing the higher uncoupler concentrations to be steeper. Model 4 is therefore also incorrect. Note that the line at the highest concentration of CCCP does not contribute to the conclusion that there is no slip, since the potential reached is not great enough for non-linearity to be marked. However, this line is important for the subconclusion that  $H^+/O$  is independent of rate, since this CCCP concentration gives the highest rates.

The linearity seen in this experiment also confirms an earlier report [8] that the conductance catalysed by CCCP is ohmic. Non-linearity of CCCP conductance would be expected to show up as a steeper slope in Figure 2 at high  $\Delta p$ , and this was not observed. Krishnamoorthy and Hinkle [8] showed that 21 nM CCCP linearized the relationship between proton flux and membrane potential up to 200 mV in soya-bean lipid vesicles (again with a 20–30 mV intercept, probably due to small consistent experimental errors in membrane potential at low values). These experiments measured proton fluxes directly with a pH electrode and so do not depend on assumptions about the existence or otherwise of slip reactions; they provide independent evidence that CCCP catalyses an ohmic conductance.

Krishnamoorthy and Hinkle [8] also showed that the relationship between proton flux and membrane potential in mitochondria was linear in the presence of 4.2 nM CCCP. If we subtract the intrinsic oxygen consumption in the absence of CCCP and replot the mitochondrial data in Figure 3 of reference [8] as  $\Delta J_o$  against  $\Delta p$ , then it gives a near-linear graph, confirming our conclusion that there is no increase in  $\Delta J_o$  at higher potential, i.e. that there is no significant slip reaction in the redox proton pumps. The anomalous dependence of proton flux on  $\Delta pH$  seen in [8] at  $\Delta pH$  values of more than 1 pH unit (60 mV) is not relevant to our experiments, where  $\Delta pH$  was less than 10 mV in all cases.

The superimposability of the lines in our Figure 2 with different CCCP concentrations shows that CCCP itself does not increase the endogenous proton conductance or cause redox slip, disproving proposals that classical uncouplers like carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone ('FCCP') and CCCP are also slip-inducers [12,13].

We conclude that the State-4 titration in Figure 1 is completely explained by the non-ohmic nature of the endogenous leak of

protons across the mitochondrial inner membrane, and that slip reactions are insignificant. Neither the endogenous proton conductance of the inner membrane nor the  $H^+/O$  ratio is affected by the turnover rate of the redox proton pumps. Using the method introduced here with mitochondria or with artificial vesicles containing redox proton pumps, it should now be possible to test claims that particular compounds such as fatty acids, carbodi-imides, thyroid hormones and local anaesthetics are able to induce slip in the redox proton pumps [19]. The method should also work in intact cells.

This work was supported by grants from the Agricultural and Food Research Council (to M. D. B.) and the Royal Society/Centre National de la Recherche Scientifique (to P. D.).

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