The effect of chloroform on mitochondrial energy transduction

Lee-Feng CHIEN and Martin D. BRAND*

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

The effect of chloroform on mitochondrial respiration with succinate was investigated by applying the method of Brand, Chien and Diolez [(1994) *Biochem. J.* **297**, 27–29] to examine whether chloroform causes redox slip (fewer protons pumped per electron transferred) during mitochondrial electron transport. *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine (TMPD), which lowers H^+/O (the number of protons pumped to the external medium by the electron transport complexes per oxygen atom consumed) by altering the electron flow pathway, was investigated for comparison. Non-phosphorylating mitochondria that had been treated with 350 μ M TMPD or 30 mM chloroform were titrated with malonate in the presence of submaximal concentrations of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Linear relations between CCCP-induced extra respiration and protonmotive force were obtained. These results showed that there was no measurable

protonmotive force-dependent or rate-dependent slip in mitochondria treated with either TMPD or chloroform. However, both TMPD and chloroform seemed to decrease H^+/O in a manner independent of protonmotive force and rate. The relationship between non-phosphorylating respiration and protonmotive force was simulated in mitochondria of which 25 % of the total population were assumed to have been broken. The simulation showed that the apparent decrease in H^+/O on the addition of TMPD or chloroform to mitochondria could be in principle accounted for by breakage. Assays of mitochondrial breakage (ATP hydrolysis in the presence of atractyloside and oxidation of exogenous NADH) showed that chloroform broke mitochondria but TMPD did not. We conclude that chloroform changes the measured H^+/O as an artifact by causing mitochondrial breakage and does not cause measurable redox slip, whereas TMPD genuinely lowers H^+/O .

INTRODUCTION

Nicholls [1,2] was the first to demonstrate a nonlinear relation between respiration rate (J_o) and protonmotive force (Δp) when non-phosphorylating mitochondria were titrated with a respiratory inhibitor. This nonlinearity might be due to an increase in proton leak conductance of the inner membrane at high Δp while the number of protons pumped by the respiratory chain per oxygen consumed (H^+/O) remains constant [2,3]. Alternatively, it might be caused by increased redox slip (fewer protons pumped per electron transferred) at high Δp , with constant proton leak conductance [4]. In this case H^+/O would decrease at high Δp . There is considerable controversy over which interpretation is correct [5–8].

Rottenberg [9,10] addressed the mechanism of how chloroform uncouples succinate respiration from ATP synthesis and hydrolysis in mitochondria. Rottenberg [11] suggested that the collapse of Δp is not the cause of uncoupling by chloroform. He postulated that the 'decoupling' is due to interference with intramembrane processes that mediate direct energy transfer between the electron transport complexes and the ATPase. In addition, Luvisetto et al. [12] reported that chloroform affects the plot of conductance against membrane potential ($\Delta\psi$) in the non-ohmic region. They suggested that the apparent conductance reflects a property of a pump (or the pumps) rather than a property of the membrane. Subsequently, Luvisetto et al. [13] suggested that there is an effect of chloroform at the level of the cytochrome oxidase proton pump, with chloroform acting as a slip inducer.

The validity of the technique for directly measuring proton conductance used by Luvisetto et al. [12,13], and also by Murphy and Brand [14], has been questioned and shown to be unreliable

[5,15]. As discussed by Zoratti et al. [16], the passive proton permeability can be measured from the rate of K^+ efflux in respiration-inhibited mitochondria. The approach depends on the assumption that the initial rate of K^+ efflux after the addition of respiratory inhibitor is equal to the rate at which protons diffuse into the matrix just before and just after inhibition. Zoratti et al. used the rate of K^+ efflux after addition of valinomycin to mitochondria in low- K^+ media to estimate the proton permeability. The K^+ diffusion potential was calibrated against the steady-state distribution of triphenylmethylphosphonium (TPMP⁺). However, the rapid build-up of a reverse ΔpH makes it difficult if not impossible to be confident that the true initial rate of K^+ efflux is measured. Unfortunately, Luvisetto et al. [12,13] investigated the effects of chloroform on H^+ permeability using this method. In addition, Luvisetto et al. apparently measured the rate of K^+ efflux at different times and under different conditions in experiments to measure direct proton permeability and in experiments to measure Δp and J_o , during the incubations with agents such as chloroform (Figure 3c in [12] and Figure 8 in [13]).

Hafner and Brand [17] demonstrated that P/O (the maximum number of molecules of ADP phosphorylated to ATP per oxygen atom consumed), and thus the relative proton pumping stoichiometries of the Δp -producing and Δp -consuming reactions, is constant as Δp varies, greatly weakening the case for Δp -dependent slip in the mitochondrial proton pumps. In addition Zolkiewska et al. [18] suggested that the proton conductance of the mitochondrial inner membrane is quantitatively more important than changes in the stoichiometry of the mitochondrial proton pumps in accounting for the relationship between mitochondrial respiration rate and Δp . Brown [15] showed that the relative H^+/O ratios of the different respiratory chain pumps

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Δp , protonmotive force; $\Delta\psi$, membrane potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; H^+/O , number of protons pumped to the external medium by the electron transport complexes per oxygen atom consumed; J_o , rate of oxygen consumption; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; TPMP⁺, triphenylmethylphosphonium.

* To whom correspondence should be addressed.

remain constant as Δp is varied. Thus the results obtained and the conclusion made by Luvisetto et al. that chloroform causes slip as well as increasing the conductance to protons are perhaps misinterpreted.

An experiment was therefore devised to discriminate between redox slip and proton leak in mitochondria and in hepatocytes [7,19–21]. The method distinguishes between four possible models. Model 1 postulates increased proton leak conductance of the mitochondrial inner membrane at high Δp , with H^+/O remaining constant. Model 2 proposes increased redox slip at high Δp with constant proton leak conductance; i.e. an increase in Δp decreases H^+/O . Model 3 proposes increased proton leak conductance at high electron-transfer rates. The inner membrane becomes more permeable to protons perhaps because active respiratory complexes seal the membrane less effectively. Model 4 proposes increased redox slip at high turnover rates of the respiratory complexes. The results described previously [7,8,21,22] demonstrate that redox slip does not happen in isolated mitochondria and in hepatocytes under the experimental conditions used, at physiological temperature; i.e. only model 1 is correct.

It should be possible to apply this method to find out whether putative slip-inducers such as chloroform do indeed cause Δp -dependent or rate-dependent redox slip, by repeating the experiments in the presence of the agent. The slopes of the graphs generated by the method are proportional to H^+/O , so by comparing these slopes in the presence and in the absence of the putative slip-inducers it should also be possible to detect changes caused by the agents in the underlying H^+/O . In the present paper the method of Brand et al. [7] is applied to examine whether chloroform causes redox slip [13,23] during mitochondrial electron transport at physiological temperature. *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine (TMPD), which lowers H^+/O by bypassing the cytochrome *bc*₁ complex, has been described as a redox slip inducer [24], and is used as the comparative control. Our results show that neither chloroform nor TMPD causes Δp -dependent or rate-dependent redox slip. They both lower the apparent H^+/O ; this effect of chloroform is an artifact caused by mitochondrial breakage, whereas the effect of TMPD is genuine.

THEORY

The theory of the method is given in Brand et al. [7]. Briefly, because the proton conductance caused by the classical uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) is independent of Δp [25], it is possible to derive the relationship between extra J_o caused by addition of a small amount of CCCP (ΔJ_o) and Δp . It turns out that:

$$\frac{\Delta J_o}{[CCCP]} = \frac{\Delta p \times C^{CCCP}}{(H^+/O) \times [CCCP]} = \frac{\Delta p \times \text{constant}}{H^+/O} \quad (1)$$

where C^{CCCP} is the proton conductance of CCCP. The same equation holds in the presence of a putative slip-inducer: any proton leak caused by the agent is subtracted during calculation of ΔJ_o , but the induced change in H^+/O still affects the new ΔJ_o .

If $\Delta J_o/[CCCP]$ is plotted against Δp the line will pass through the origin and will have a slope inversely proportional to H^+/O [eqn. (1)]. If in the presence of an added agent the plot of $\Delta J_o/[CCCP]$ against Δp is linear and can be superimposed on the plot for control mitochondria, the added agent does not cause Δp -dependent slip (as in model 1), and any effect that it has on the system must be through the proton leak or the rate of the electron transport chain. In contrast, any extra nonlinearity in

the graph with added agent present compared with control graphs indicates that the agent induces Δp -dependent redox slip (as in model 2). If the agent lowers H^+/O in a way that depends on turnover rate, i.e. causes rate-dependent slip, there would be different curves in the plot of $\Delta J_o/[CCCP]$ against Δp at different [CCCP] (as in model 3). If the agent causes rate-dependent leak, there would also be different curves at different [CCCP] (as in model 4).

The slope of the plot of $\Delta J_o/[CCCP]$ against Δp depends on $C^{CCCP}/[CCCP]$ and on H^+/O [eqn. (1)]. If a putative slip-inducer changes H^+/O to a new constant value but does not change the proton conductance of CCCP, then it will change the slope of the line. The relative slopes in the presence and absence of the agent will give the relative change in H^+/O . However, other effects such as mitochondrial breakage can also change the slope of these plots (see below) so such changes must be interpreted cautiously.

EXPERIMENTAL

Measurement of respiration and $\Delta\psi$ with time

Each rat liver mitochondrial preparation was made by the method of Chappell and Hansford [26] from one 200–300 g Wistar rat. The rate of oxygen consumption, measured with a Clark-type oxygen electrode, was calculated by assuming that air-saturated medium at 37 °C contains 406 nmol/ml O [27]. Mitochondria (1 mg/ml protein) were incubated with 4 mM succinate at 37 °C in medium containing 100 mM NaCl, 3 mM Hepes, 5 mM Na₃PO₄ and 1 mM EGTA, adjusted to pH 7.0 with NaOH, plus 5 μ M rotenone. Respiratory control ratios were approx. 4–6, using the rates during phosphorylation of ADP and non-phosphorylation (without oligomycin), by the method of Chance and Williams [28]. The mitochondrial $\Delta\psi$ was measured with a TPMP⁺-sensitive electrode as described by Brown and Brand [29]. Electrode measurements of $\Delta\psi$ were corrected for TPMP⁺ binding [29]. Mitochondria were incubated with or without 15 mM chloroform.

Measurement of respiration and Δp

Each rat liver mitochondrial preparation was isolated from three or four Wistar rats. Δp was measured by the method of Brand [30]. Mitochondria (2 mg/ml protein) were incubated at 37 °C in the medium described above plus 100 pmol of valinomycin/mg of protein and 1 μ g of oligomycin/mg of protein. $\Delta\psi$ was measured with 0.04 μ Ci/ml ⁸⁶Rb, and mitochondrial volume was measured with 1 μ Ci/ml ³H₂O and 0.1 μ Ci/ml [¹⁴C]sucrose. Mitochondria were titrated with sodium malonate in the absence of CCCP and in the presence of 20 or 40 nM CCCP. For TMPD experiments, mitochondria were incubated with or without 350 μ M TMPD for 2 min. For chloroform experiments, mitochondria were incubated with or without 30 mM chloroform for 3 min. Membrane potentials and respiration rates were measured simultaneously in parallel incubations. All values of Δp were calculated from the measured $\Delta\psi$ using the relationship $\Delta p = 1.1\Delta\psi - 11.9$ as described in Brand et al. [7].

Swelling experiments and enzyme assays

The rate of rapid mitochondrial swelling was determined by measuring the increase of transmittance at 520 nm [31,32] with a thermostatically controlled Uvikon 930 spectrophotometer (Kontron Instruments). The assay for ATP hydrolysis [15,33,34] and stimulation of mitochondrial respiration by externally added NADH [35,36] are described in the legends to the tables.

Statistics

Errors were calculated from n independent mitochondrial preparations. Student's t test was used for comparisons of two values. The COMPREG program [37] was used for comparisons of lines fitted by linear regression. A P value of less than 0.05 was considered statistically significant.

RESULTS

We have previously shown that the effects of chloroform on isolated mitochondria are complex [38]. Chloroform inhibits the activity of the substrate oxidation reactions and stimulates the proton leak or redox slip reactions but has little direct effect on the reactions of ATP synthesis and export. In addition we found that the proton leak (or slip reactions) increases with time in the presence of chloroform, as shown in Figure 1 (top panel). The

Figure 1 Mitochondrial respiration rate (top panel) and $\Delta\psi$ (bottom panel) as a function of incubation time in the presence of chloroform

Mitochondria (1 mg/ml protein) were incubated at 37 °C. Respiration rate and $\Delta\psi$ were measured as described in the Experimental section. Upper panel: for measurement of non-phosphorylating rate (●) and uncoupled rate (■), mitochondria were incubated in 2 ml of reaction medium with 200 μ M ATP and 4 mM succinate. After 1 min, 15 mM chloroform was added to initiate the experiment. At the times shown the non-phosphorylating rate was measured, then 400 pmol/mg carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added and the uncoupled rate was measured. If the rate of phosphorylating respiration (△) was measured, 200 μ M ADP was added at the times shown. Points are means $\pm$ S.E.M. for three independent experiments. Lower panel: mitochondria (1 mg/ml protein) were incubated in 3.5 ml of incubation medium containing in addition 100 pmol of nigericin/mg of protein. Five successive aliquots (1 μ M) of TPMP⁺ were added to the medium up to 5 μ M to calibrate the TPMP⁺ electrode. Then 4 mM succinate was added to generate the membrane potential. After incubation for 1 min, 15 mM chloroform was added (■) or not (□), and $\Delta\psi$ was measured at each successive minute in the non-phosphorylating state. Points are means $\pm$ range for two independent experiments.

Figure 2 Malonate titrations of non-phosphorylating respiration and Δp with submaximal amounts of CCCP in control (A) and TMPD-treated (B) mitochondria

Mitochondria (2 mg protein/ml) were incubated with 4 mM succinate at 37 °C. Δp and J_o were measured as described in the Experimental section. (A) Titrations were performed by adding 0–25 mM sodium malonate with 0 (□), 20 (○) or 40 (△) nM CCCP. (B) Titrations were repeated with 0 (■), 20 (●) or 40 (▲) nM CCCP in the presence of 350 μ M TMPD. The lowest point on each curve contained 25 mM malonate and 1 μ M CCCP to inhibit respiration fully and bring $\Delta\psi$ to zero. Points are means $\pm$ S.E.M. for three independent experiments.

non-phosphorylating respiration, which is a measure of the proton leak, was stimulated by chloroform but varied with time. The inhibition of phosphorylating and uncoupled rate by chloroform was obviously enhanced with time. Figure 1 (bottom panel) shows that the mitochondrial $\Delta\psi$ decreases with time in the presence of chloroform. El-shenawy and Abdel-Rahman [39] also observed that the effect of chloroform on hepatocytes is time-dependent. The effect of TMPD on mitochondria is time-dependent but complex and we have not included it here. Therefore all measurements were made at an exact time after the addition of TMPD or chloroform. We found that 30 mM chloroform was needed at 2 mg/ml mitochondrial protein to give significant respiratory stimulation, and used these concentrations throughout (except for Figure 6).

TPMD causes no measurable Δp -dependent slip or rate-dependent slip or leak at physiological temperature

The experiments were performed by titrating non-phosphorylating mitochondria with sodium malonate in the presence of 0, 20 or 40 nM CCCP at 37 °C, in the absence or presence of TMPD. Lower CCCP concentrations were used here than in Brand et al. [7] because the uncoupling effect of a slip-

Figure 3 $\Delta J_0/[CCCP]$ against Δp for control (A) and TMPD-treated (B) mitochondria

ΔJ_0 is the difference between the measured value of J_0 with CCCP present and the interpolated value at the same Δp with CCCP absent. Points are means $\pm$ S.E.M. derived from the data of Figure 2. (A) The continuous line is a linear regression calculated from all the points with 20 (●) or 40 (▲) nM CCCP present. The broken line (□) shows a predicted curve for the Δp -dependent slip if the nonlinearity of the non-phosphorylating respiration titrations were caused entirely by slip superimposed on a small ohmic proton leak. This line of theoretical slip is generated to represent zero endogenous leak. A CCCP concentration of 4.1 nM is calculated as that which would have to be added to mitochondria to cause the lowest three points, lying on the linear region of the non-phosphorylating titration in Figure 2(A), to fall on the experimental curve in this figure. (B) As in (A) but with the addition of 350 μ M TMPD. The continuous line is a linear regression calculated from all the points with 20 (■) or 40 (◆) nM CCCP present. The broken line (○) shows the theoretical Δp -dependent slip superimposed on a small ohmic proton leak equivalent to 6.1 nM CCCP.

inducer would also increase J_0 . TMPD is a redox reagent that can accept electrons from ascorbate or from succinate via ubiquinone and donate them to cytochrome *c*. TMPD would therefore cause a change in H^+/O by shuttling electrons from ubiquinone directly to cytochrome *c*, bypassing proton translocation by the intervening cytochrome *bc_1* complex.

Figure 2(A) shows the control malonate titrations of non-phosphorylating respiration and Δp in the absence of CCCP and with two different concentrations of added CCCP. The curves show the usual nonlinear behaviour. As the amount of CCCP is increased, the non-phosphorylating respiration increases while Δp decreases and the nonlinearity becomes less pronounced, as described in Brand et al. [7]. Figure 2(B) shows the same experiment in the presence of 350 μ M TMPD. The curves are again nonlinear. The J_0 values are approximately doubled by TMPD but there is little effect on Δp .

Figure 3(A, filled symbols) shows that the plot of $\Delta J_0/[CCCP]$ against Δp is linear and passes close to the origin. The points for

20 nM CCCP fit well to a straight line by linear regression. This linearity of the plot indicates that H^+/O does not decrease as Δp increases; i.e. no Δp -dependent slip occurs in the normal mitochondria. The points for 40 nM CCCP are also a good fit to a straight line. Furthermore the lines for the two different CCCP concentrations were not significantly different from each other ($P < 0.05$) when the data were analysed by COMPREG [37]. The continuous line is a linear regression calculated from all these points with different amounts of CCCP present. One single line can fit both sets of data. That is, H^+/O does not decrease as the turnover rate of the proton pumps increases. Therefore no rate-dependent slip or leak occurs in normal mitochondria. This confirms the conclusion of Brand et al. [7,20] that no Δp -dependent slip and no rate-dependent slip or leak occurs in isolated mitochondria under these conditions at physiological temperature. As a comparison a predicted curve is generated for the Δp -dependent slip (Figure 3A, open symbols) if the nonlinearity of the non-phosphorylating respiration titration is caused entirely by slip superimposed on a small ohmic proton leak. CCCP at 4.1 nM would have to be added to mitochondria that theoretically had zero endogenous leak to cause the lowest three points of the non-phosphorylating titration in Figure 2(A) to fall on the curve in Figure 3(A). It is clear that the experimental points (filled symbols) show no tendency to follow the line predicted for slip (open symbols). The data are not consistent with the hypothesis of slip in the proton pumps that increases at higher values of Δp . This result concurs with Brand et al. [7,20] that all of the nonlinearity 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. The lowest point lying off the origin on this curve, representing conditions where ΔJ_0 and Δp should be zero, merely reflects residual experimental error at low respiration rate and Δp .

Figure 3(B, filled symbols) shows the derived graph of $\Delta J_0/[CCCP]$ against Δp in the presence of TMPD. The plot is also linear and passes close to the origin. The experimental points for 20 or 40 nM CCCP fit to a straight line by linear regression. This linearity of the plot indicates that H^+/O does not decrease as Δp increases, showing that no Δp -dependent slip occurs in TMPD-treated mitochondria. The lines for the two different CCCP concentrations are not significantly different as analysed by COMPREG. One single line can fit both sets of data. Thus H^+/O does not decrease as the turnover rate of the proton pumps increases; i.e. no rate-dependent slip or leak occurs in the TMPD-treated mitochondria. In the presence of TMPD the ohmic endogenous leak is calculated to be equivalent to 6.1 nM added CCCP to generate the line predicted for redox slip. This is greater than the equivalent CCCP concentration in the absence of TMPD, probably indicating that TMPD has a small uncoupling effect in the present experiments.

Chloroform causes no measurable Δp -dependent slip or rate-dependent slip or leak at physiological temperature

Figure 4(A) shows the result of malonate titrations of non-phosphorylating respiration and Δp as the paired control for the chloroform experiments confirming Figure 2(A). Figure 4(B) shows the same experiment in the presence of 30 mM chloroform in the absence of CCCP, and with two different concentrations of added CCCP. The relationship between non-phosphorylating respiration and Δp in the presence of chloroform is again nonlinear. J_0 is increased approx. 1.5-fold by chloroform with CCCP absent, and Δp is decreased by approx. 10 mV compared with the control.

The derived graph of $\Delta J_0/[CCCP]$ against Δp is plotted for the

Figure 4 Malonate titrations of non-phosphorylating respiration and Δp with submaximal amounts of CCCP in control (A) and chloroform-treated (B) mitochondria

Mitochondria (2 mg/ml protein) were incubated at 37 °C. Δp and J_o were measured as described in the Experimental section. (A) Titrations were performed by adding 0–25 mM sodium malonate with 0 ($\square$), 20 ($\circ$) or 40 ($\triangle$) nM CCCP. (B) Titrations were repeated with 0 ($\blacksquare$), 20 ($\bullet$) or 40 ($\blacktriangle$) nM CCCP in the presence of 30 mM chloroform. Points are means $\pm$ S.E.M. for three independent experiments.

control experiment in Figure 5(A, filled symbols). The plot is linear and passes close to the origin in the same way as the control line in Figure 3(A). CCCP at 6.9 nM mimics endogenous leak. Figure 5(B, filled symbols) shows the derived graph of $\Delta J_o/[CCCP]$ against Δp in the presence of chloroform. The plot is linear and passes close to the origin. The points for 20 or 40 nM CCCP are both well fitted by a straight line. The graph reveals that there is no detectable Δp -dependent slip in chloroform-treated mitochondria. The lines for the different CCCP concentrations are not significantly different from each other. A single line can fit both data sets, indicating that there is no rate-dependent slip or leak in chloroform-treated mitochondria. The broken line (open symbols) shown in Figure 5(B) is generated to predict theoretical slip in the presence of chloroform; the ohmic endogenous leak is equal to 6.2 nM added CCCP. As the experimental points show no tendency to follow the line predicted for slip, the data are not consistent with the hypothesis of slip. The result shows that there is no measurable Δp -dependent slip in chloroform-treated mitochondria.

The analysis in Figures 3(B) and 5(B) tests the possibility that in the presence of the added agents the whole of the nonlinearity in Figures 2(B) and 4(B) is caused by redox slip. To test whether any of the extra nonlinearity caused by the agents [i.e. Figure 2(B) minus Figure 2(A); Figure 4(B) minus Figure 4(A)] was due

Figure 5 $\Delta J_o/[CCCP]$ against Δp for control (A) and chloroform-treated (B) mitochondria

Points are means $\pm$ S.E.M. derived from the data of Figure 4 as described in the Experimental section. (A) The continuous line is a linear regression calculated from all the points with 20 nM ($\bullet$) or 40 ($\blacktriangle$) nM CCCP present. The broken line ($\square$) represents predicted Δp -dependent slip superimposed on a small ohmic proton leak equivalent to 6.9 nM CCCP. (B) As in (A) but with the addition of 30 mM chloroform. The continuous line is a linear regression calculated from all the points with 20 ($\blacksquare$) or 40 ($\blacklozenge$) nM CCCP present. The broken line ($\circ$) shows the theoretical slip superimposed on a small ohmic leak equivalent to 6.2 nM CCCP.

to slip, we plotted graphs like those in Figures 3 and 5 for this extra component (results not shown). In each case the data could be fitted by straight lines, providing no evidence that the agents caused Δp -dependent slip to be superimposed on the endogenous Δp -dependent leak.

The change in the slope of the plot of $\Delta J_o/[CCCP]$ against Δp in the presence of chloroform or TMPD is not due to changes in the conductance of CCCP

Figure 3 shows that the replots of $\Delta J_o/[CCCP]$ against Δp in the absence and presence of TMPD are two significantly different lines, analysed by COMPREG, with different slopes. TMPD increases the gradient of the plot approx. 1.8-fold from 0.0080 to 0.0142 nmol O/min per mg of protein per nM per mV. Figure 5 shows that the replots of $\Delta J_o/[CCCP]$ against Δp in the absence and presence of chloroform are two significantly different lines, analysed by COMPREG, with different slopes. Chloroform changes the gradient of the plot 1.6-fold from 0.0085 to 0.0137 nmol O/min per mg of protein per nM per mV. The increase in the slope of the plot of $\Delta J_o/[CCCP]$ against Δp in the presence of chloroform is similar to that in the presence of TMPD.

Figure 6 Mitochondrial swelling in ammonium thiocyanate as a function of CCCP concentration in the absence and in the presence of TMPD (A) or chloroform (B)

(A) Mitochondria (0.3 mg/ml protein) were incubated in 3 ml of medium containing 120 mM ammonium thiocyanate, 5 μ M rotenone, 1 mM EGTA, 3 mM Hepes (made to pH 7.0 with NH_4OH), 2 mM KCN (made to pH 7.0 with HCl) and 2 mM ascorbate at 37 $^\circ\text{C}$ in the absence ($\square$) or presence ($\blacksquare$) of 350 μ M TMPD. The rate of mitochondrial swelling was determined by measuring the increase of transmittance at 520 nm. Points are means $\pm$ range for two independent experiments. (B) Mitochondria were incubated as above in the absence ($\square$) or presence ($\blacksquare$) of 15 mM chloroform except that no ascorbate was added. Points are means $\pm$ S.E.M. for four independent experiments.

Why are the slopes different in the absence and in the presence of TMPD (Figure 3) or chloroform (Figure 5)? According to eqn. (1) there are two factors that could change the slope: one is C^{CCCP} and the other is H^+/O . Do TMPD or chloroform lower H^+/O or change C^{CCCP} ?

The conductance of CCCP was examined by measuring the initial rate of mitochondrial swelling monitored at 520 nm in isotonic ammonium thiocyanate [31,32]. In this medium the rate of swelling is limited by the proton permeability of the mitochondrial membrane. The experiment was performed in the absence and in the presence of TMPD. However, oxidized TMPD absorbs strongly at 520 nm and the absorbance gradually increases as TMPD becomes more oxidized. Thus ascorbic acid was added to reduce TMPD and potassium cyanide was added to inhibit cytochrome oxidase to avoid TMPD oxidation. Oxidized and reduced TMPD were assumed to have the same effects on the conductance of CCCP. Figure 6(A) shows the mitochondrial swelling rate as a function of CCCP concentration in the absence and in the presence of TMPD. The two lines are not significantly different, indicating that the conductance of CCCP is not changed by TMPD.

We conclude that the increase in the slope of the plot of $\Delta J_o/[\text{CCCP}]$ against Δp in the presence of TMPD is caused by a decrease in H^+/O . As shown in Figure 3, TMPD increases the gradient of the plot of $\Delta J_o/[\text{CCCP}]$ against Δp approx. 1.8-fold compared with the control. If an H^+/O value of 6 is used for the oxidation of succinate [40], then TMPD decreases H^+/O to 3.3, compared with the predicted H^+/O value of 4 if all of the electron flow proceeds from ubiquinone via TMPD. Thus TMPD causes no Δp -dependent redox slip, rate-dependent redox slip or proton leak, but decreases H^+/O by a fixed amount.

To determine whether the changed slope of the plot of $\Delta J_o/[\text{CCCP}]$ against Δp caused by chloroform is due to a change in the conductance of CCCP, the conductance of CCCP in the absence and in the presence of chloroform was also examined by measuring mitochondrial swelling in ammonium thiocyanate. The two lines in Figure 6(B) are not significantly different, i.e. the conductance of CCCP is not changed by chloroform. This seems to suggest that the increase in the slope of the replot of $\Delta J_o/[\text{CCCP}]$ against Δp in the presence of chloroform could be a consequence of a decreased H^+/O caused by the addition of chloroform. If this interpretation is correct, chloroform would have to change H^+/O from 6 to 3.7, but this conclusion will be shown below to be incorrect.

The effect of mitochondrial breakage: a simulation

The method of Brand et al. [7] was not originally designed to compare two different conditions, and some complications arise if it is used in this way. One of the main complications is the effect of mitochondrial breakage. The calculation of ΔJ_o as J_o (plus CCCP) $- J_o$ (minus CCCP) at each experimental value of Δp (plus CCCP) assumes that all mitochondria in a population respond to Δp as it is changed by titration with malonate. However, any fully uncoupled mitochondria will respond to malonate but not to Δp . As shown in Figure 7(A), less malonate is needed to give a particular Δp when CCCP is present. In principle, to calculate ΔJ_o on addition of CCCP one should subtract the rate of the coupled control population at the same Δp but the rate of the uncoupled control population at the same malonate concentration. For a well-coupled preparation this is a small correction that can safely be ignored, but if a substantial proportion of the mitochondrial population is fully uncoupled the required correction becomes significant.

Hence the relationship between respiration rate and Δp was simulated for a mitochondrial population that was assumed to be 25% broken, to model the effect of any agent-induced mitochondrial breakage.

To simplify the model we assumed that the data in Figure 7(A) came from a fully coupled homogeneous population of mitochondria with a respiratory control ratio of 5.0, that 25% of this population was then completely broken with no effect on the remaining 75%, and that the sensitivity of respiration to malonate was the same as in the control curve in Figure 7(A) under all conditions. More complex assumptions could have been made, but this would not change the conclusion reached below that breakage could be the cause of the chloroform-induced change in slope in Figure 5. For each point in Figure 7(A) the new J_o of mitochondria with 25% breakage was calculated. The new rate, $J_{o(25\%,y,z)}$, was calculated as:

$$J_{o(25\%,y,z)} = 0.75 \times J_{o,y} + 5 \times 0.25 \times J_{o,z}$$

where $J_{o(25\%,y,z)}$ represents the new respiration rate of mitochondria with 25% breakage generated from any point of Figure 7(A) at y value of Δp and z concentration of malonate, $J_{o,y}$

Figure 7 Malonate titrations of J_o and Δp with submaximal amounts of CCCP in intact mitochondria (A), simulation result of malonate titrations of non-phosphorylating respiration and Δp in mitochondria assuming 25% breakage (B), and $\Delta J_o/[CCCP]$ against Δp for intact and broken mitochondria (C)

(A) J_o and Δp were obtained at different concentrations of sodium malonate in the presence of 0 ($\square$), 20 ($\circ$) or 40 ($\triangle$) nM CCCP. Points are means $\pm$ S.E.M. for nine independent experiments (including data from Figures 2 and 4). The numbers next to the points represent the concentrations of malonate in mM. (B) J_o and Δp for mitochondria assuming 25% breakage were calculated from (A) as described in the text. Points represent malonate titrations in the presence of 0 ($\blacksquare$), 20 ($\bullet$) or 40 ($\blacktriangle$) nM CCCP. (C) The data were derived from (A) and (B) as described in the text. The lines are linear regressions calculated from all the points for intact mitochondria with 20 ($\square$) or 40 ($\circ$) nM CCCP present, and points for 25% broken mitochondria with 20 ($\blacksquare$) or 40 ($\bullet$) nM CCCP present respectively.

represents the respiration rate of intact mitochondria at y value of Δp , and $J_{o,z}$ represents the respiration rate of intact mitochondria in the absence of CCCP at z concentration of malonate.

Table 1 ATPase assay

Mitochondria (2 mg/ml protein) were incubated at 37 °C in medium containing 100 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 3 mM Hepes (made to pH 7.4 with NaOH), 3 μ M rotenone, 200 pmol of FCCP/mg of protein and 0.2 nmol of antimycin/mg of protein. ATP (0.2 mmol) (pH 7.4 with NaOH) was added after 6 min incubation, and the scalar H^+ production rate was measured using a pH electrode. ATPase rates were measured immediately after the addition of ATP. Where indicated, 18 nmol of atractyloside/mg of protein or 2 μ g of oligomycin/mg of protein was added at the start and 30 mM chloroform or 350 μ M TMPD was added after 3 min. At the end of the experiment 2 μ l of 0.1 M HCl was added as calibration. Abbreviations: atract., atractyloside; olig., oligomycin. Values are means $\pm$ S.E.M. for three independent experiments.

	Total activity including non-mitochondrial ATPase activity (nmol H^+ /min per mg)	Corrected activity excluding non-mitochondrial ATPase activity (nmol H^+ /min per mg)	Activity with atractyloside (% of relevant control activity)
Control	38 $\pm$ 5	26 $\pm$ 6	100
Control + atract.	16 $\pm$ 5	4 $\pm$ 6	15
Control + atract. + olig.	12 $\pm$ 3	0	0
TMPD	31 $\pm$ 6	24 $\pm$ 6	100
TMPD + atract.	11 $\pm$ 4	4 $\pm$ 4	17
TMPD + atract. + olig.	7 $\pm$ 2	0	0
Chloroform	75 $\pm$ 12	39 $\pm$ 15	100
Chloroform + atract.	63 $\pm$ 20	27 $\pm$ 22	70
Chloroform + atract. + olig.	36 $\pm$ 9	0	0

Note that breakage will not alter Δp because the broken mitochondria have neither ^{86}Rb uptake nor matrix volume.

Figure 7(B) shows the results of the simulation. The J_o values of mitochondria with 25% breakage are increased approx. 1.5–2-fold compared with those of intact ones. The graph of $\Delta J_o/[CCCP]$ against Δp for the mitochondria with 25% breakage is plotted in Figure 7(C). The plot is linear and passes close to the origin. The points for 20 nM CCCP fit well to a straight line with a slope of 0.132 nmol O/min per mg of protein per nM per mV. The points for 40 nM CCCP fit a straight line with a slope of 0.0082 by linear regression but it is not a good fit; this may be due to the errors for the first two points at high potential. If only the plot with a good fit is considered, the slope of the plot with 20 nM CCCP present for the broken mitochondria is 1.5 times the control (0.0088 nmol O/min per mg of protein per nM per mV, derived from Figure 7A).

The slope of the plot of $\Delta J_o/[CCCP]$ against Δp from the simulated result for mitochondria with 25% breakage is similar to those from the experimental data for TMPD-treated or chloroform-treated mitochondria shown in Figure 3B and Figure 5B. This implies that the apparent decrease of H^+/O ratio in the presence of TMPD or chloroform could be caused by about 25% breakage of mitochondria by these two reagents.

Mitochondrial breakage by TMPD and chloroform

The following experiments were performed to test whether mitochondria are broken in the presence of TMPD or chloroform.

Measurement of ATP hydrolysis

Disruption of the mitochondrial inner membranes was assayed by measuring net scalar proton production during ATP hydrolysis [15]. In the presence of atractyloside to inhibit ADP/ATP

Table 2 NADH permeability assay

Mitochondria (2 mg/ml protein) were incubated in medium of 100 mM NaCl, 5 mM Na_2PO_4 , 4 mM succinate, 1 mM EGTA, 3 mM Hepes (made to pH 7.0 with HCl), 5 μM cytochrome *c* and 200 μM ADP at 37 °C; 3 min after the addition of Triton X-100 or chloroform, 0.6 mM NADH was added and the oxygen consumption rate was measured. Values are means $\pm$ S.E.M. for three independent experiments. The numbers in parentheses are the breakage expressed as a percentage of the value with chloroform plus Triton X-100.

	Respiration rate (nmol O/min per mg protein)	Breakage (%)
Control	21 $\pm$ 1	21
30 mM chloroform	36 $\pm$ 13	36 (48)
0.1 % Triton X-100	101 $\pm$ 27	100
30 mM chloroform + 0.1 % Triton X-100	77 $\pm$ 29	76 (100)

exchange [34], ATP hydrolysis can only occur in broken mitochondria or by non-mitochondrial hydrolysis. Non-mitochondrial ATP hydrolysis can be estimated after the addition of oligomycin, which inhibits the mitochondrial ATPase.

Table 1 shows the ATPase activity in the presence of TMPD or chloroform. TMPD slightly decreased ATP hydrolysis. After correction for non-mitochondrial ATPase, the breakage in TMPD-treated mitochondria was similar to that in the control mitochondria. Chloroform increased ATP hydrolysis approx. 2-fold compared with the control. The results represent a high percentage breakage of mitochondria in the presence of chloroform compared with the control; i.e. there is about 55 % mitochondrial breakage by chloroform as determined by measuring ATP hydrolysis. This indicates that TMPD does not break mitochondria but chloroform does break a large proportion of mitochondria.

Measurement of NADH permeability

NADH is oxidized by complex I on the matrix face of the membrane. Intact mitochondria are unable to use externally added NADH, because NADH cannot cross the mitochondrial inner membrane. Experiments were performed to measure the oxygen consumption rate during NADH oxidation [35,36] with the addition of ADP and cytochrome *c*. The results are summarized in Table 2. A maximal respiration rate of 100 % was measured when 0.1 % Triton X-100 was added to break the mitochondria completely. The respiration rate of normal mitochondria is approx. 21 % of the maximal rate. This result suggests that mitochondria are somehow damaged during preparation and agrees with approx. 15 % of breakage in normal mitochondria by measuring ATP hydrolysis (Table 1). The respiration rate of chloroform-treated mitochondria is approx. 36 % of the maximum. However, the respiration rate was inhibited to approx. 76 % when Triton X-100 plus chloroform were present. Therefore when the rate in the presence of Triton X-100 plus chloroform is considered as 100 %, chloroform would increase the rate to approx. 48 % of maximum. Thus chloroform increases NADH oxidation by approx. 27 % over the control, i.e. there is 27 % mitochondrial breakage by chloroform determined by measuring NADH oxidation.

DISCUSSION

In this paper we have applied the method of Brand et al. [7] to distinguish proton leak from redox slip during mitochondrial

electron transport with added chloroform. The results show that there is no Δp -dependent slip, no rate-dependent slip and no rate-dependent proton leak in the presence of chloroform under our conditions. Chloroform causes an apparent decrease in H^+/O , calculated from an increase in the slope of a plot of $\Delta J_o/[\text{CCCP}]$ against Δp . Simulation of the experiment showed that this effect can be explained by mitochondrial breakage. Two different assays of mitochondrial breakage demonstrate that chloroform does break 27–55 % of the mitochondria, suggesting that the apparent increase in H^+/O caused by chloroform is an artifact. TMPD also increases the slope of the plot of $\Delta J_o/[\text{CCCP}]$ against Δp . Unlike chloroform, TMPD does not break the mitochondria, so it does cause a decrease in H^+/O in these experiments.

Recently, Luvisetto et al. [23] have applied the method of Brand et al. [7] in mitochondria supplemented with chloroform at 25 °C and reported that the extent of the nonlinearity of the relationship between the ohmic uncoupler-induced extra respiration and $\Delta\psi$ was enhanced by chloroform. They suggested that the increase in respiration caused by chloroform cannot simply be explained by an increased circulation of protons across the membrane bilayer. However, their results showed no effect of chloroform over most of the experimental range; the conclusion of redox slip depended on a single point, making their conclusion weak. In addition their analysis was performed at 25 °C rather than 37 °C as in our work. Canton et al. [8] reported that at low temperatures the percentage of resting respiration due to membrane leak decreases and that due to slip of proton pumps increases, so it may be that chloroform can induce some redox slip at unphysiologically low temperatures.

We conclude that there is no evidence that chloroform causes redox slip under our conditions at 37 °C.

We thank Mrs Julie Buckingham for technical assistance. This work was supported in part by a grant from the Medical Research Council.

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Received 2 May 1996/21 August 1996; accepted 29 August 1996