Biochem. J. (1991) 275, 75–80 (Printed in Great Britain)

Effect of protonmotive force on the relative proton stoichiometries of the mitochondrial proton pumps

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The rate of phosphorylation of ADP by isolated mitochondria respiring on succinate was set by addition of ATP, ADP or ADP plus malonate. We measured the rates of phosphorylation and respiration and the protonmotive force under each of these conditions. We measured the oxygen consumption required to drive the proton leak at the protonmotive force reached under each condition and subtracted it from the respiration rate during phosphorylation to determine the oxygen consumption driving phosphorylation. By dividing the rate of phosphorylation by the rate of respiration driving phosphorylation we calculated the mechanistic P/O ratio (number of molecules of ADP phosphorylated per oxygen atom reduced). This ratio was the same at high, intermediate and low values of protonmotive force, indicating that the relative stoichiometries of the mitochondrial protonmotive-force-producing and protonmotive-force-consuming pumps (i.e. $H^+/O:H^+/ATP$) are independent of the protonmotive force. This greatly weakens the case for a decrease in stoichiometry, or 'slip', in the mitochondrial proton pumps at high protonmotive force.

INTRODUCTION

The observation that the relationship between mitochondrial respiration rate and the protonmotive force (Δp) is non-linear has been made by a number of groups (Nicholls, 1974; Pietrobon *et al.*, 1981, 1983; Krishnamoorthy & Hinkle, 1984; O'Shea *et al.*, 1984; Duszynski & Wojtzak, 1985; Brown & Brand, 1986; see Brand, 1990). There are two main interpretations of this finding (for review see Garlid *et al.*, 1989; Murphy, 1989). The first, initially suggested by Nicholls (1974), is that the mitochondrial proton conductance (or leak) increases at high values of Δp. The second is that the stoichiometries of the mitochondrial proton pumps decrease (i.e. the pumps 'slip') at high Δp (Pietrobon *et al.*, 1981, 1983; Zoratti *et al.*, 1986).

Direct measurement of the mitochondrial proton conductance has confirmed that it does indeed increase at high Δp (Krishnamoorthy & Hinkle, 1984; O'Shea *et al.*, 1984; Brown & Brand, 1986; Murphy & Brand, 1987, 1988*a*,*b*; Zolkiewska *et al.*, 1989), but whether the increase in proton conductance is sufficient to quantitatively account for the relationship between mitochondrial respiration rate and Δp remains a matter of contention. The initial evidence for slip in the mitochondrial proton pumps came from observations that the relationship between respiration rate and Δp depended on the proton pump used to generate Δp (after correction for the mechanistic stoichiometries of the various proton pumps used) (Pietrobon *et al.*, 1981, 1983). This should not be the case if the non-linearity of the relationship between respiration rate and Δp is due to the proton conductance properties of the membrane. Other workers have, however, reported that the relationship between respiration rate and Δp does not depend on the proton pump used to generate Δp (Nicholls, 1974; Murphy *et al.*, 1986; Brown, 1989; Zolkiewska *et al.*, 1989). Further evidence that the increase in proton conductance at high Δp is not sufficient to account for the relationship between respiration rate and Δp was presented by Zoratti *et al.* (1986), who measured the mitochondrial proton conductance in the presence of valinomycin-induced diffusion potentials and compared this with the respiration rate at the same potential. These workers concluded that the proton conductance at any value of $\Delta\Psi$ (the transmembrane difference in electric potential) was not sufficient to account for the respiration rate at the same $\Delta\Psi$. However, the opposite conclusion was reached from similar experiments by Brown & Brand (1986).

In an effort to resolve this controversy, Murphy & Brand (1987, 1988*a*,*b*) measured steady-state H^+/O ratios as a function of $\Delta\Psi$ and concluded that there was indeed a decrease in the H^+/O ratio at high values of $\Delta\Psi$. However, subsequent identification by Brown (1989) of a possible source of error in those experiments has reduced our confidence in their conclusion. Zolkiewska *et al.* (1989) applied a method similar to that developed in Murphy & Brand (1987,1988*a*), and concluded that the proton conductance of the mitochondrial inner membrane was quantitatively more important than changes in the stoichiometry of the mitochondrial proton pumps in accounting for the relationship between mitochondrial respiration rate and Δp.

In the present paper we test the hypothesis that the mitchondrial proton pumps slip differently at high Δp by measuring the relative proton stoichiometries of the respiratory chain and ATP-synthesizing reactions (as the mechanistic P/O ratio, i.e. the number of molecules of ADP phosphorylated per oxygen atom reduced) across a range of values of Δp. We demonstrate that the P/O ratio, and thus the relative 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.

EXPERIMENTAL

Mitochondria were isolated from female Wistar rats (200–300 g) according to standard procedures (Chappell & Hansford, 1972), in a medium containing 250 mM-sucrose, 5 mM-Tris and 1 mM-EGTA, pH 7.4. Mitochondrial protein was assayed by the biuret method (Gornall *et al.*, 1949).

Rates of mitochondrial phosphorylation (using the pH change associated with the scalar reaction monitored with a pH electrode) and oxygen consumption (using an oxygen electrode) were

Abbreviations used: P/O, number of molecules of ADP phosphorylated per oxygen atom reduced; Δp, protonmotive force; ΔpH, transmembrane difference in pH; z, factor to convert from pH units to mV ($2.3\,RT/F$); $\Delta\Psi$, transmembrane difference in electric potential; Ph_3MeP^+, methyltriphenylphosphonium cation; state 3, condition with excess ADP and excess substrate present; state 4, condition with ATP and excess substrate present; e, electron.

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measured simultaneously with mitochondrial membrane potential [using an electrode sensitive to the lipophilic cation methyltriphenylphosphonium (Ph_3MeP^+)] in a 3 ml magnetically stirred Perspex chamber. The chamber contained a Clarke-type oxygen electrode and was sealed with a Perspex lid through which a pH-sensitive combination glass electrode and a Ph_3MeP^+-sensitive electrode (Brown & Brand, 1985) were inserted. The outputs of the pH- and Ph_3MeP^+-sensitive electrodes were fed to separate digital voltmeters whose reference sockets were connected together. The output of the oxygen electrode and the output of the voltmeter connected to the pH electrode were fed to a double-channel chart recorder. The output of the voltmeter connected to the Ph_3MeP^+-sensitive electrode was fed to a separate chart recorder. Addition of Ph_3MeP^+ had no effect on the pH signal. Addition of small amounts of acid equivalent to the amounts used here had no effect on the signal of the Ph_3MeP^+-sensitive electrode.

The mitochondrial P/O ratio was determined at 25 °C in 3 ml of medium containing 2 mg of mitochondrial protein/ml, 120 mM-KCl, 5 mM-P_i, 2 mM-$MgCl_2$, 1 mM-EGTA and 100 μM-acetate (made pH 7.4 with KOH) supplemented with 50 μM-bis-adenosyl pentaphosphate (to inhibit adenylate kinase), 5 μM-rotenone and 0.2 μg of nigericin/mg of protein (to clamp ΔpH, i.e. the transmembrane difference in pH, close to zero). The mitochondria were incubated in this medium for 5 min, during which time the Ph_3MeP^+-sensitive electrode was calibrated by 1 μM additions of Ph_3MePBr to a final concentration of 5 μM; then 2.5 mM-succinate (made pH 7.4 with KOH) was added. P/O ratios were measured under three conditions. (*a*) In state 3 the medium was supplemented with 2.5 mM-ADP [di(monocyclohexylammonium)salt], after which the pH was re-adjusted to just below 7.4. (*b*) In 'state 3 + malonate', 0.25 mM-malonate (made pH 7.4 with KOH) was also added. (*c*) In state 4, 1 mM-ATP [di(monoethanolammonium) salt, pH 6.8] was added after the succinate and the pH was re-adjusted to just below 7.4.

For each of the three conditions, P/O ratios were measured as follows. The rates of mitochondrial respiration and phosphorylation and the extramitochondrial Ph_3MeP^+ concentration were measured during the steady state initiated by addition of succinate. When the pH reached 7.4, 9.6 μM-carboxyatractyloside was added to inhibit the adenine nucleotide translocator, and the extramitochondrial ATPase activity was assayed from the rate of change in pH. Carboxyatractyloside was used rather than oligomycin so that any ATP hydrolysis by broken mitochondria was fully accounted for. The pH electrode was then calibrated by addition of standard 0.1 M-HCl. Absolute rates of phosphorylation were determined by adding the forward rates of ATP synthesis and the background rates of ATP hydrolysis after carboxyatractyloside inhibition, assuming 0.8 H^+ consumed per ATP synthesized (Alberty, 1969). (Note that in state 4 the background ATP hydrolysis rate is equal to the entire rate of ATP synthesis since, by definition, there is no net synthesis of ATP in state 4.) Oligomycin (1 μg/mg of mitochondrial protein) was then added to inhibit any residual ATP synthesis and the Ph_3MeP^+ signal was titrated to its steady-state value (before carboxyatractyloside) by small additions of 250 mM-malonate (made pH 7.4 with KOH). The new oxygen consumption rate (required to drive the proton leak at this potential) was recorded. The membrane potential was calculated from the Ph_3MeP^+ signal using a Ph_3MeP^+-binding correction of 0.54 (Hafner *et al.*, 1990*a*). Air-saturated medium was assumed to contain 475 nmol of O/ml at 25 °C (Reynafarje *et al.*, 1985). From this experiment we calculated the net rate of ATP synthesis in the steady state, the absolute rate of extramitochondrial ATP hydrolysis (after inhibition with carboxyatractyloside) and, by addition, the absolute rate of ATP synthesis in the steady state. The oxygen consumption required to drive this ATP synthesis was calculated as the rate of oxygen consumption in the steady state minus the rate of oxygen uptake at the same potential with oligomycin present, i.e. in the absence of ATP synthesis (see the Theory section).

ΔpH and mitochondrial volume were determined from the distribution of [^{14}C]sucrose and [^{3}H]acetate or ^3H_2O respectively. In some experiments ΔΨ was measured from the distribution of [^{3}H]Ph_3MeP^+. These measurements were as described in Brown & Brand (1985). ATP [di(monethanolammonium) salt] was from Sigma Chemical Co. (Poole, Dorset, U.K.). Other materials were from the sources indicated in Hafner & Brand (1988) and Hafner *et al.* (1988).

THEORY

At any value of Δp, the mitochondrial respiratory chain will pump protons out with an H^+/O stoichiometry of n_1. We assume that if slip in the proton pumps does occur, then it is dependent only on Δp, so that n_1 is a constant at defined Δp. There will be an opposing leak of protons back across the mitochondrial inner membrane. The rate of this proton leak can be measured as the oxygen consumption rate in the presence of oligomycin (J_L) at that potential multiplied by n_1. The rate at which protons are used to drive phosphorylation can be determined by taking the oxygen consumption rate driving phosphorylation and multiplying by n_1. The oxygen consumption rate driving phosphorylation can be determined as the total oxygen consumption rate during phosphorylation ($J_{tot.}$) minus the oxygen consumption rate driving the proton leak at the same value of Δp (J_L) (see Hafner *et al.*, 1990*b*). Thus the rate of proton pumping by the respiratory chain at any given Δp to drive ATP synthesis is equal to $n_1 (J_{tot.} - J_L)$.

During phosphorylation, the Δp-consuming reactions (i.e. the ATP synthase, adenine nucleotide translocator and phosphate transporter) use protons with an H^+/ATP stoichiometry of n_2 to synthesize ATP. Again we assume that if the ATPase does slip, n_2 is a function only of Δp. Thus the rate at which protons are being used to drive ATP synthesis at any given Δp equals $n_2 \cdot J_p$.

In any steady state, the rate of proton pumping to drive ATP synthesis must equal the rate of proton use by the ATP-synthesizing reactions. Thus

$$n_1(J_{tot.} - J_L) = n_2 \cdot J_p$$

By simple rearrangement we arrive at:

$$J_p/(J_{tot.} - J_L) = n_1/n_2$$

Thus the expression is equal to the relative proton stoichiometry of the mitochondrial respiratory chain and ATP synthesis reactions at the value of Δp at which the measurements are made. It is, of course, also the mitochondrial mechanistic P/O ratio. Thus by measuring the rates of oxygen consumption and phosphorylation as well as the rate of oxygen consumption driving the mitochondrial proton leak at the same Δp, the relative stoichiometries of the mitochondrial Δp-producing and -consuming pumps can be determined. By performing the experiment under different conditions we can determine if this ratio changes with Δp and thus test the hypothesis that the mitochondrial proton pumps slip differently at high Δp.

RESULTS

Δp-dependence of the P/O ratio

Fig. 1 shows the dependence of the mitochondrial P/O ratio on ΔΨ under conditions where ΔpH is clamped at zero by the addition of nigericin so that ΔΨ is approximately equal to Δp.

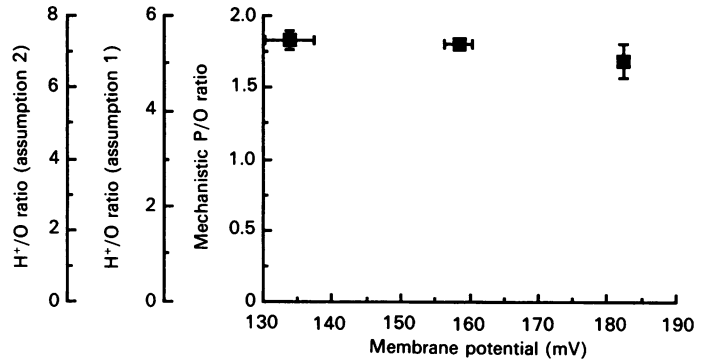

Fig. 1. Effect of $\Delta\Psi$ on the mechanistic P/O ratio of mitochondria

P/O ratios were measured as the steady-state net rate of ATP synthesis plus the rate of ATP hydrolysis after addition of carboxyatractyloside, divided by the steady-state rate of oxygen consumption minus the rate of oxygen consumption at the same Δp in the presence of oligomycin (as described in the text) in state 4, state 3 and state 3+0.25 mM-malonate. Data are from three independent experiments; error bars represent S.E.M. P/O ratios under the three different conditions were not significantly different from each other ($P > 0.2$ using Student's t test). H^+/O ratios for the electron transport from succinate to oxygen were calculated from the same data, assuming a constant H^+/ATP ratio for ATP synthesis of 3 (assumption 1) or 4 (assumption 2), both including 1 H^+ for the combined movements of phosphate and adenine nucleotides.

Fig. 1 shows that the P/O ratio is roughly constant across a range of values of $\Delta\Psi$. None of the P/O ratios was significantly different from any of the others ($P > 0.2$ using Student's t test). This demonstrates that the ratio between the proton stoichiometries of the respiratory chain and the ATP synthesis reactions does not change with Δp. The simplest interpretation is that neither changes its stoichiometry with Δp. The absolute value of the P/O ratio is close to the value of 1.75 proposed by Beavis & Lehninger (1986), who also corrected for the mitochondrial proton leak. The most likely source of error in the absolute value observed here is the assumption of 0.8 H^+/ATP consumed during the scalar reaction of ATP synthesis; errors in this assumption will not affect the conclusion that the ratio does not change with Δp.

Δp-dependence of the H^+/O and H^+/ATP ratios

At present the values of the mitochondrial H^+/O and H^+/ATP ratios are uncertain. Fig. 1 also shows the dependence of the H^+/O ratio for electron transport from succinate to oxygen on $\Delta\Psi$ calculated from the P/O ratios, assuming constant H^+/ATP ratios of 3 and 4 (including 1 for transport of ADP, ATP and P_i). Fig. 1 shows that the mitochondrial H^+/O ratio for the span succinate to oxygen is roughly constant, and that any change in the H^+/O ratio is less than approx. 0.6. Thus changes in the H^+/O ratio of cytochrome oxidase from 4 to 2 going from low to high Δp (Murphy & Brand, 1988*b*) are not apparent. The dependence of the H^+/ATP ratio (including 1 for transport of ADP, ATP and P_i) was assessed in the same way, assuming constant H^+/O ratios of 6, 7 and 8 (results not shown). Any change in the H^+/ATP ratio was less than approx. 0.4.

Validation of the method used to measure P/O ratios

For the method to work, it is necessary that the oxygen consumption driving the proton leak during phosphorylation is accurately determined after inhibition of phosphorylation with carboxyatractyloside and oligomycin. Several control experiments were performed to eliminate artefacts that would result in the oxygen consumption driving the leak at the same $\Delta\Psi$ after inhibition of phosphorylation under- or over-estimating the actual oxygen consumption that was driving the leak during phosphorylation.

(*a*) The method measures the rate of oxygen consumption used to drive the proton leak at the same $\Delta\Psi$ as in the phosphorylating steady state. However, it is Δp that drives the proton leak (Brown & Brand, 1986), and thus if ΔpH was different under phosphorylating and non-phosphorylating conditions, the determination of the oxygen consumption driving the leak would be wrong. To test this, we measured ΔpH under phosphorylating and non-phosphorylating conditions. Fig. 2 shows that ΔpH was neither consistently nor significantly different under non-phosphorylating and phosphorylating conditions. Under each condition the two ΔpH values were not significantly different ($P > 0.2$ using Student's t test). Even if the small apparent differences in mean ΔpH under non-phosphorylating and phosphorylating conditions in state 4 and state 3+malonate are real, this would not change the conclusions drawn from Fig. 1. Assuming that $\Delta\Psi$ and ΔpH have the same effect on mitochondrial proton conductance per mV (Brown & Brand, 1986), we calculate that the P/O ratios would be 1.78 ± 0.13 in state 4, 1.79 ± 0.04 in state 3 and 1.87 ± 0.08 in state 3+malonate. None of these values is significantly different from the others ($P > 0.2$ using Student's t test).

(*b*) If the binding of Ph_3MeP^+ to the mitochondria was altered by addition of carboxyatractyloside or oligomycin, then measuring the oxygen consumption required to balance the proton leak at the same external Ph_3MeP^+ concentration would be inappropriate. To test this, we investigated whether carboxyatractyloside or oligomycin altered the measured membrane potential under conditions where we would predict that they should have no effect. Thus we investigated the effect of adding oligomycin to mitochondria inhibited with carboxyatractyloside. $\Delta\Psi$ (measured from the uptake of $[^3H]Ph_3MeP^+$) in the presence of carboxyatractyloside alone was 170.4 ± 0.4 mV; in the presence of carboxyatractyloside and oligomycin it was 169.6 ± 0.6 mV (means $\pm$ S.D. for three determinations on one preparation). Thus oligomycin appears to have no effect on the binding of Ph_3MeP^+. To investigate if carboxyatractyloside changes the binding of Ph_3MeP^+, we supplemented the medium with 2% bovine serum albumin to remove any unbound non-esterified fatty acids and so prevent the possibility that carboxyatractyloside might change $\Delta\Psi$ by preventing uncoupling by non-esterified fatty acids as reported by Andreyev *et al.* (1988). $\Delta\Psi$ was measured from the uptake of $[^3H]Ph_3MeP^+$, assuming that 10% of the Ph_3MeP^+ binds to albumin (Nobes *et al.*, 1990). In the presence of oligomycin alone $\Delta\Psi$ was 177.3 ± 0.4 mV (mean $\pm$ S.E.M., $n = 3$); in the presence of oligomycin and carboxyatractyloside it was 177.7 ± 0.8 mV ($n = 3$). Thus carboxyatractyloside also appears to have no effect on the binding of Ph_3MeP^+.

(*c*) The oxygen consumption required to balance the proton leak at a given Δp would not be the same after inhibition with carboxyatractyloside and oligomycin if they change the kinetics of the proton leak. Carboxyatractyloside is reported to inhibit fatty acid uncoupling of mitochondrial respiration (Andreyev *et al.*, 1988). Thus, if there were significant amounts of unbound non-esterified fatty acids in the mitochondria, then addition of carboxyatractyloside might change the proton leak. To test this we investigated the effect of adding carboxyatractyloside in the presence of oligomycin (and the absence of bovine serum albumin). Mitochondria were incubated in state 4, state 3 and state 3+malonate. Oligomycin was added, the external Ph_3MeP^+ concentration was titrated back to the steady-state phosphorylating level with malonate and the respiration rate was

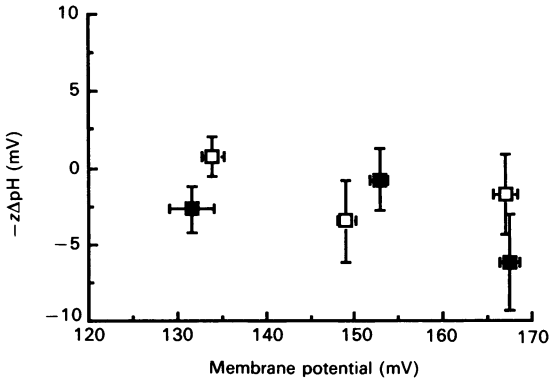

Fig. 2. Comparison of $-z\Delta pH$ under phosphorylating and non-phosphorylating conditions

$-z\Delta pH$ ($z = 2.3\ RT/F$) was measured in state 3, state 3+malonate and state 4 (□) and in the three conditions plus 9.6 μM-carboxyatractyloside, 1 μg of oligomycin/mg of mitochondrial protein and enough malonate to bring $\Delta\Psi$ back to roughly the phosphorylating value (■). $\Delta\Psi$ was determined from the distribution of [^{3}H]Ph_3MeP^+. Error bars represent S.E.M. for three independent experiments. None of the pairs of $-z\Delta pH$ values were significantly different ($P > 0.2$ using Student's t test).

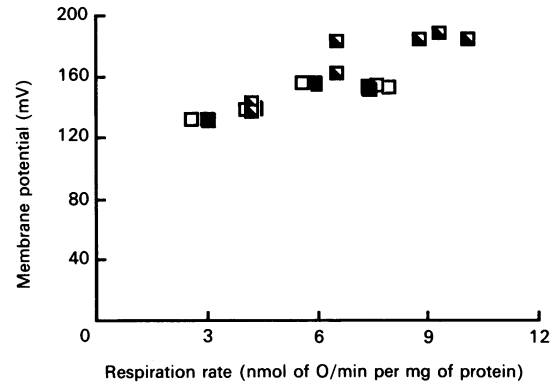

Fig. 3. Effect of carboxyatractyloside on mitochondrial proton leak

Mitochondria were incubated under state 3, state 3+malonate or state 4 conditions and 1 μg of oligomycin/mg of mitochondrial protein was added. The Ph_3MeP^+ signal was then titrated back to the phosphorylating value with potassium malonate (pH 7.4) and the respiration rate was recorded (□). Carboxyatractyloside (9.6 μM) was then added and the Ph_3MeP^+ signal was titrated back to the original value (if necessary) and the new respiration rate was recorded (■). The data are from two independent experiments.

measured; 9.6 μM-carboxyatractyloside was then added and (if necessary) the external Ph_3MeP^+ concentration was once more titrated back to the original value. The new respiration rate was recorded. Fig. 3 shows that addition of carboxyatractyloside had no significant effect on the oxygen consumption required to balance the proton leak. The lack of effect of carboxyatractyloside on the oxygen consumption required to balance the proton leak is presumably a reflection of low contamination of the mitochondria by unbound non-esterified fatty acids, the relative insensitivity of non-esterified fatty acid uncoupling of rat liver mitochondria to carboxyatractyloside (Schönfeld, 1990) and the ability of adenine nucleotides (Andreyev *et al.*, 1988) and KCl (results not shown) to inhibit carboxyatractyloside-sensitive uncoupling by non-esterified fatty acids. A similar experiment was carried out with oligomycin: Fig. 4 shows that oligomycin

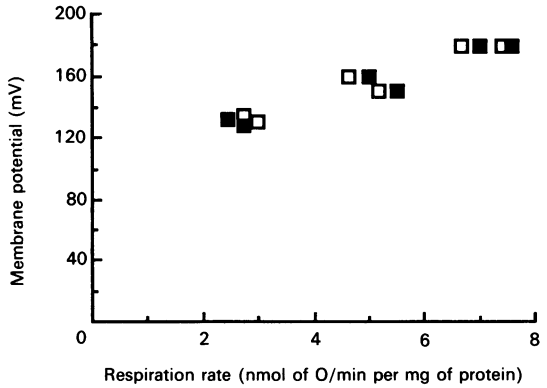

Fig. 4. Effect of oligomycin on mitochondrial proton leak

Mitochondria were incubated in state 3, state 3+malonate or state 4, and 9.6 μM-carboxyatractyloside was added. The Ph_3MeP^+ signal was then titrated back to the phosphorylating value with potassium malonate (pH 7.4) and the respiration rate was recorded (□). Oligomycin (1 μg/mg of mitochondrial protein) was then added and the Ph_3MeP^+ signal was titrated back to the original value (if necessary), and the new respiration rate was recorded (■). The data are from a single experiment.

had no effect on the mitochondrial proton leak. Thus there is no reason to suspect that the kinetics of the proton leak are changed by these inhibitors.

If there is a significant amount of intramitochondrial ATP hydrolysis, this would continue after addition of carboxyatractyloside, and would introduce an error in our measurement of the rate of ATP hydrolysis. If this were the case, adding oligomycin should cause a decrease in the apparent mitochondrial proton leak in the presence of carboxyatractyloside. However, Fig. 4 shows that this did not happen.

DISCUSSION

There have been previous investigations of the effect on P/O ratios of altering the respiration rate (e.g. Ernster & Nordenbrand, 1974). Such studies made no correction for the leak of protons, so the overall P/O ratios that were measured were found to vary as the contribution of the proton leak to oxygen consumption varied. In the present paper we have subtracted all oxygen consumption used to drive the proton leak and so report only the mechanistic P/O ratio. Our results show that the P/O ratio (and thus the relative proton stoichiometries of the respiratory chain and ATP-synthesizing reactions) is constant across a range of values of Δp.

The conclusion that the relative proton stoichiometries are constant is similar to that reached by Brown (1989), who compared inhibitor titrations of proton pumping rates against Δp for various proton pumps. Like Brown (1989), we are unable to rule out the possibility that the respiratory chain and ATP-synthesizing reactions change their stoichiometries in proportion over the range of Δp studied. Thus if the H^+/e (e = electron) stoichiometries of the proton pumps in the respiratory chain all change in the same way with changes in Δp, so does the H^+/ATP ratio of the ATP synthase. However, in conjunction with the results of Brown (1989), we are able to rule out the possibility that the ATP synthase slips, in the sense that it pumps less protons at high Δp during ATP hydrolysis but requires more protons at high Δp during ATP synthesis. This is because Brown (1989) showed that the H^+/ATP ratio during hydrolysis of ATP is constant relative to the H^+/O ratio, and in the present work we have shown that the H^+/ATP ratio during synthesis of ATP is also constant relative to the H^+/O ratio.

The lack of change in the relative stoichiometries of the mitochondrial Δp-producing and Δp-consuming pumps is contrary to the findings of Pietrobon *et al.* (1981, 1983), but in agreement with a number of other workers (Nicholls, 1974; Murphy *et al.*, 1986; Brown, 1989; Zolkiewska *et al.*, 1989). Potential reasons why such a discrepancy should arise are discussed in Brown (1989), who stressed the critical importance of using exactly the same conditions for assaying the different pumps. Our method is unique in this respect, as we compare the stoichiometry of the Δp-producing and Δp-consuming pumps in the same set of mitochondria at the same time. Note that if the Ph_3MeP^+-binding correction used in Pietrobon *et al.* (1981) is used to calculate $\Delta\Psi$ in our experiments, our values of Δp under state 4 conditions are as high as or higher than the maximum values of Δp reached during ATP hydrolysis by Pietrobon *et al.* (1981), demonstrating that the discrepancy between our findings and those of Pietrobon *et al.* (1981) are not due to failure to reach high enough values of Δp in the present study.

What can we say about the possibility that the mitochondrial Δp-consuming and Δp-producing pumps all change stoichiometry in the same ratio as Δp varies? In the light of the present results, evidence that any one of the pumps changes stoichiometry would require that all the pumps change stoichiometry. Such evidence comes from two experiments that have attempted to measure the proton permeability properties of the mitochondrial inner membrane at high values of Δp (Zoratti *et al.*, 1986; Murphy & Brand, 1987, 1988*a*,*b*). Both of these studies reported that the passive permeabilities of the mitochondrial inner membrane could not account for the relationship between respiration rate and Δp. Opposing evidence comes from Brown & Brand (1986) and Zolkiewska *et al.* (1989), who reported that the proton leak did account for the relationship.

Brown (1989) has pointed out that in the experiments of Murphy & Brand (1987, 1988*a*,*b*) the magnitude of the reversed pH gradient set up during the measurements of proton leak rate may have been underestimated. Since the driving force on protons is not $\Delta\Psi$ but Δp (Brown & Brand, 1986), this would cause underestimation of the proton conductance, so making the conclusions of Murphy & Brand (1987, 1988*a*,*b*) less reliable.

Could ΔpH also have affected the experiments of Zoratti *et al.* (1986)? These workers measured the respiration rate and Δp of respiring mitochondria. The respiration rate was inhibited and after 10 s a valinomycin-induced diffusion potential was created and the rate of proton entry was measured. Zoratti *et al.* (1986) reported that the passive proton permeability measured at different values of $\Delta\Psi$ could not account for the relationship between respiration rate and Δp. They recognized that changes in ΔpH could introduce uncertainties in the driving force for proton re-entry, but argued that variation in ΔpH after inhibition of respiration was negligible, since there was negligible K^+ efflux after inhibition by antimycin, apparently indicating that there was also negligible H^+ influx. However, negligible observed K^+ efflux does not indicate negligible H^+ influx at this point, because there was no valinomycin present and so the rate of K^+ efflux would not be equal to the rate of H^+ influx if other ions were to move. From Fig. 1 of Zoratti *et al.* (1986) we can calculate that at the highest value of Δp, the rate of proton re-entry, nJ_e, was about 96 nmol of H^+/min per mg of protein. A period of 10 s was allowed between inhibition of respiration rate by antimycin and addition of valinomycin, so approx. 16 nmol of H^+ entered in this interval. Assuming an internal volume of 1 μl/mg of protein, and allowing the 2 mM-phosphate to remain at equilibrium, this means some 14 nmol of H^+/mg of protein was buffered by the mitochondria. The internal buffering capacity of mitochondria has been estimated as 16 nmol of H^+/mg of protein per pH unit at pH 7.4 (Mitchell & Moyle, 1969). Thus the internal pH would have dropped by up to 0.88 pH unit. This is equivalent to a change in $-z\Delta$pH of 51.5 mV, which is more than enough to superimpose the respiration-driven and diffusion-potential-driven proton permeability curves in Fig. 3 of Zoratti *et al.* (1986). Of course the changes in $-z\Delta$pH calculated here will be an overestimate, because as Δp drops, the rate of proton re-entry will slow, but a change in $-z\Delta$pH of 30 mV (which seems entirely reasonable) would still be enough to superimpose the curves in Fig. 3 of Zoratti *et al.* (1986). It follows that if ΔpH is a driving force for proton leak across the mitochondrial inner membrane, the results of Zoratti *et al.* (1986) are not good evidence for slip.

Thus there is no reason to suppose that the passive proton permeability of the membrane cannot completely account for the relationship between respiration rate and Δp, and there is no good evidence for changes in the stoichiometry of any of the mitochondrial proton pumps in intact mitochondria.

Conclusion

The relative stoichiometries of the mitochondrial proton pumps are constant across a range of values of Δp. There is no good evidence to suggest that any of the mitochondrial proton pumps slip at high Δp, and so it seems that a matched stoichiometry change at high Δp in the mitochondrial proton pumps is an unlikely explanation of the constant relative stoichiometries of the mitochondrial Δp-producing and Δp-consuming pumps. Rather, it seems much more likely that the relationship between respiration rate and Δp can be explained entirely by the passive proton permeability properties of the mitochondrial inner membrane.

We are grateful to Mark Leach and Mary George for assistance. R.P.H. is grateful to Girton College, Cambridge, for the award of a research fellowship. This work was supported by a grant from the Science and Engineering Research Council to M.D.B.

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Received 31 July 1990/16 October 1990; accepted 15 November 1990