The nature of mitochondrial respiration and discrimination between membrane and pump properties

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A new criterion is utilized for the interpretation of flow–force relationships in rat liver mitochondria. The criterion is based on the view that the nature of the relationship between the H^+/O ratio and the membrane potential can be inferred from the relationship between ohmic-uncoupler-induced extra respiration and the membrane potential. Thus a linear relationship between extra respiration and membrane potential indicates unequivocally the independence of the H^+/O ratio from the membrane potential and the leak nature of the resting respiration [Brand, Chien, and Diolez (1994) Biochem. J. **297**, 27–29]. On the other hand, a non-linear relationship indicates that the H^+/O ratio is dependent on the membrane potential. The experimental assessment of this relationship in the presence of an additional ohmic leak, however, is rendered difficult by both the uncoupler-induced depression of membrane potential and the limited range of dependence of the H^+/O ratio on the membrane potential. We have selected conditions, i.e. incubation of mitochondria at low temperatures, where the extent of non-linearity is markedly increased. It appears that the nature of the resting respiration of mitochondria *in vitro* is markedly dependent on the temperature: at low temperatures the percentage of resting respiration due to membrane leak decreases and that due to intrinsic uncoupling of the proton pumps increases.

INTRODUCTION

The nature of mitochondrial resting respiration, although fundamental, is an unsolved problem in bioenergetics. The debate arises from the interpretation of the pattern of the flow–force relationship between respiration and the protonmotive force (Δp), which is linear at low Δp and non-linear at high Δp. The non-linear relationship has been attributed either to membrane proton leak or to slip in the proton pumps, i.e. uncoupling not only as a membrane but also as a pump property [1–17]. More recently, the non-linear relationship has been attributed to a respiration-induced membrane perturbation causing a non-ohmic proton leak [18,19].

Recently, Brand et al. [20], starting from a study by Brown and Brand [12], have developed a new conceptual approach to discriminate between membrane leak and pump slip, and considered the membrane leak nature of resting respiration as conclusively proven under their experimental conditions. The approach is based not on the different behaviour of the various pumps [3,4] nor on a comparison between active and passive proton conductances [13,14,16], but on the interpretation of the relationship between uncoupler-induced extra respiration and Δp. Since the approach relies on the assessment of the flow–force relationships in the presence of ohmic uncouplers, and since these uncouplers depress Δp thereby also restricting the range of non-linearity, we have selected experimental conditions where the extent of non-linearity is enhanced, rendering easier the assessment of the flow–force relationships notwithstanding the depression of Δp. We find that mitochondria kept at low temperatures show a strongly biphasic relationship between respiration and Δp or between extra respiration and Δp. This indicates a marked dependence of the H^+/O ratio on Δp. Thus it appears that the new approach suggested by Brand et al. [20] reinforces the concept that resting respiration is largely due to slip at low temperature and to membrane proton leak at high temperature.

EXPERIMENTAL

Materials and methods

Rat liver mitochondria were prepared in a medium containing 0.25 M sucrose, 10 mM Tris and 0.1 mM EGTA according to standard procedures [21], and all the experiments were performed within 4 h of preparation. Mitochondrial protein was determined by the biuret method using BSA as a standard. The standard incubation medium contained 0.2 M sucrose, 30 mM Mops/Tris, 5 mM P_i/Tris, 5 mM succinate/Tris, 0.2 mM EGTA/Tris, 5 μM rotenone and 1 μg/mg oligomycin, pH 7.4. Rates of respiration and Δp were determined by measuring the rate of oxygen consumption with a Clark electrode (Yellow Springs) and the distribution of the lipophilic ion triphenylmethylphosphonium respectively, as described elsewhere [16]. The matrix volumes were determined as described in Pietrobon et al. [4]. The inner mitochondrial volume was about 1 μl/mg (between 0.9 and 1.0 μl/mg) and was constant under the prevailing experimental conditions. All experiments were conducted in the presence of 5 mM P_i/Tris and nigericin (14 pmol/mg), conditions which have been found to result in negligible pH changes. In the Figures the term $\Delta\psi$ (transmembrane electrical potential) is used instead of Δp solely to indicate that this was the parameter directly measured. It is, however, implicit that under the prevailing experimental conditions the two terms $\Delta\psi$ and Δp are interchangeable. Moreover, the membrane potential for mitochondria, chloroplasts, and bacteria is defined as $(\psi_{in} - \psi_{out})$, where 'in' represents the matrix phase. Thus the membrane potential values reported in the Figures should be seen as being representative of negative values. All the reagents were of

Abbreviations used: Δp, protonmotive force; $\Delta\psi$, transmembrane electrical potential gradient; J_o, rate of respiration; L_H, proton conductance; J_H^{leak}, rate of proton flux through leak; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; unc, uncoupler.

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maximal purity (commercial grade). Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was purchased from Sigma-Aldrich (Milano, Italy).

Theory

In a previous paper Brown and Brand [12] have shown that, during malonate titrations at 30 °C, (a) the relationship between respiration and Δp is non-linear whether in the presence or in the absence of BSA, and (b) after addition of BSA the relationship between the extra respiration (i.e. the differential respiration in the presence and absence of BSA) and Δp is also non-linear. The non-linear pattern of the latter relationship has been considered a feature of the Δp-dependent BSA-extractable leak pathway.

In a more recent paper, Brand et al. [20] have argued that, under conditions where the extra respiration is due to an ohmic uncoupler [carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)], if the relationship between this extra, CCCP-induced, respiration and Δp is linear, then the linearity indicates that the H^+/O ratio does not vary with Δp. The argument is intriguing because, although in the first instance it would appear that the properties of the resting respiration may not be inferred from those of the stimulated respiration, in reality the properties of the pumps may still be observed under conditions where there is an additional pump activity linked to an ohmic process.

Consider a steady state where the rate of proton extrusion by the redox pumps is equal to the ratio of proton influx via leak. In the presence of ohmic uncouplers there is an additional respiration (ΔJ_o) due to the extra proton influx; thus (cf. also [20]):

$$\Delta J_o = L_{unc} \times \Delta p/(H^+/O)_{\Delta p} \quad (1)$$

where L_{unc} is the coefficient of the proton conductance induced by the ohmic uncoupler (unc) and $(H^+/O)_{\Delta p}$ is the proton pump stoichiometry which may, in principle, be dependent on Δp. By dividing eqn. (1) by the uncoupler concentration, in order to normalize the plot, we have:

$$(\Delta J_o/[unc])_{\Delta p} = K \times \Delta p/(H^+/O)_{\Delta p} \quad (2)$$

where the coefficient K simply indicates that the uncoupler-induced proton conductance divided by the uncoupler concentration becomes a constant and is not affected by the membrane potential. According to eqn. (2) the H^+/O is described as Δp-dependent. The Δp-dependence of the H^+/O ratio gives a non-linear relationship between the uncoupler-stimulated respiration and Δp. On the other hand, if the H^+/O ratio is Δp-independent, eqn. (2) becomes:

$$(\Delta J_o/[unc])_{\Delta p} = K' \times \Delta p \quad (3)$$

According to eqn (3) the relationship between the extra respiration and Δp is linear. In summary, the comparison between eqns.(2) and (3) indicates that the relationship between uncoupler-induced extra respiration and Δp is linear or non-linear depending on the effect of Δp on the H^+/O ratio.

RESULTS

Figure 1 shows the relationships between the rate of respiration and $\Delta\psi$, in the absence or in the presence of low FCCP concentrations, as obtained during malonate titrations in mitochondria incubated at different temperatures. The relationship between resting respiration and $\Delta\psi$ was strongly biphasic at 15 °C (Figure 1a), almost biphasic at 25 °C (Figure 1b), and exponential at 37 °C (Figure 1c). Addition of FCCP resulted, as expected, in stimulation of respiration together with a depression of $\Delta\psi$. Uncoupler concentrations were selected to induce significant stimulation of respiration with only a slight depression of $\Delta\psi$. The higher FCCP concentrations were accompanied by greater decreases in $\Delta\psi$ and, consequently, by a shift in the relationship towards a $\Delta\psi$ range where the non-linearity is progressively diminished (see Figure 1a). The same results were obtained at low 2,4-dinitrophenol concentrations.

Figure 2 shows the relationship between the uncoupler-induced extra respiration (ΔJ_o), divided by the uncoupler concentration, and $\Delta\psi$, at three different temperatures. In order to render the experimental data more homogeneous and to facilitate the comparison, a normalization with respect to the potential was introduced, i.e. the values of ΔJ_o/[unc] were plotted versus the corresponding mean values of $\Delta\psi$ at each inhibitor concentration

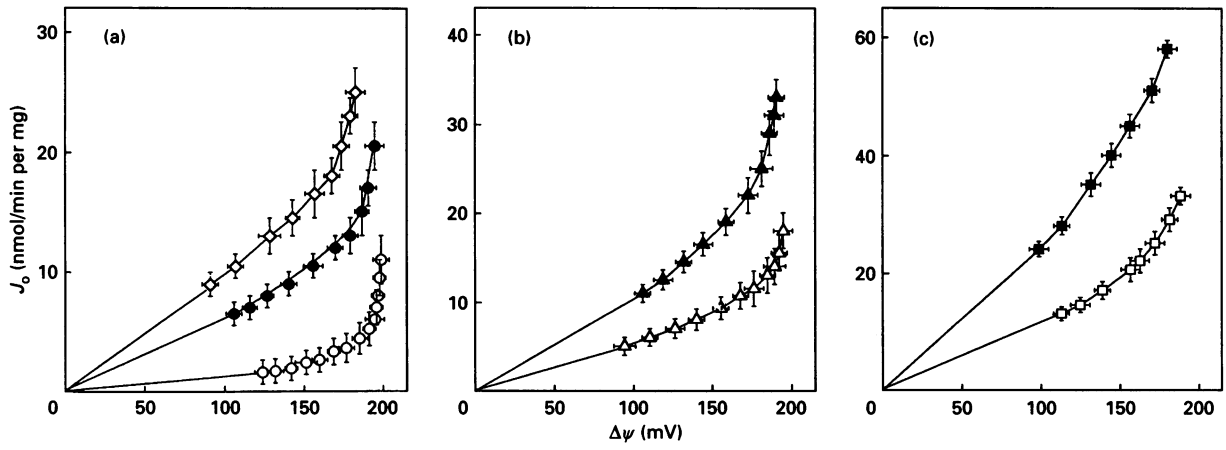

Figure 1 Effect of low uncoupler concentrations on the relationship between respiration and $\Delta\psi$ at various temperatures

Rat liver mitochondria (1 mg/ml) were incubated with the standard incubation medium at 15 °C (**a**), 25 °C (**b**) or 37 °C (**c**) in the absence (○, △, □) or in the presence of 5 (●, ▲, ■) or 10 (◇, **a**) pmol/mg FCCP. After 2 min of incubation, increasing amounts of malonate (0–5 mM) were added and the corresponding rate of respiration and membrane potential were measured. Values are means ± S.D. from a single repeat with seven mitochondrial preparations, except for the curve at 10 pmol/mg FCCP in (**a**), which shows the means ± S.D. from three mitochondrial preparations.

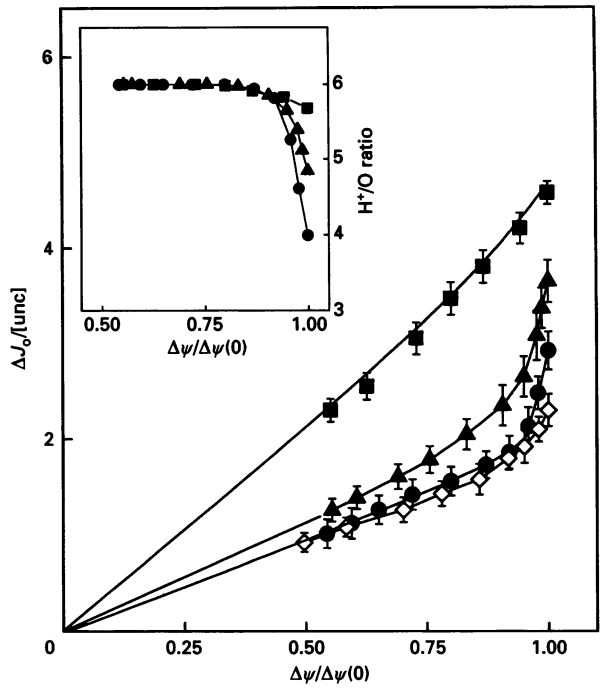

Figure 2 Relationship between differential respiration and $\Delta\psi$ at various temperatures

Data from Figure 1 were plotted as differential respiration, i.e. in the presence and absence of FCCP, divided by the concentration of FCCP as a function of the normalized membrane potential at 15 °C (●, 5; ◇, 10 pmol/mg FCCP), 25 °C (▲, 5 pmol/mg FCCP) and 37 °C (■, 5 pmol/mg FCCP). Inset: estimation of the dependence of the H^+/O ratio on the membrane potential at the three different temperatures. See the text for more details.

divided by the pertinent value without inhibitor, $\Delta\psi(0)$. Only deviations on the ordinate values were reported. The pattern of the ΔJ_o/[unc] versus $\Delta\psi$ relationship was markedly non-linear at low temperature (15 °C), showed mixed behaviour at intermediate temperature (25 °C) and was almost linear at high temperature (37 °C). At 15 °C the value of ΔJ_o/[unc] increased linearly from 1.01 to 1.86 in the wide 106–180 mV (0.54–0.92 normalized) potential range, and from 1.86 to 2.92 in the narrow 180–195 mV (0.92–1.00 normalized) range. At 25 °C the value of ΔJ_o/[unc] increased linearly from 1.15 to 2.35 in the 106–173 mV (0.55–0.90 normalized) potential range, and non-linearly from 2.35 to 3.55 in the 173–191 mV (0.90–1.00 normalized) range. Finally, at 37 °C, the value of ΔJ_o/[unc] increased almost linearly from 2.35 to 4.57 in the entire 100–181 mV (0.55–1.00 normalized) range. These data may be compared with the data reported by Brand et al. [20] who, at 37 °C, reported an increase in the value of ΔJ_o/[unc] from 0.5 to 0.8 in the 100–150 mV range. Figure 2 also shows the values of ΔJ_o/[unc] obtained by uncoupler-induced extra respiration at 15 °C using 10 pmol/mg FCCP.

The inset of Figure 2 shows a further elaboration of the experimental data, indicating the presumed change in the H^+/O stoichiometry with $\Delta\psi$ that is responsible for the observed relationship between ΔJ_o/[unc] and $\Delta\psi$. To perform this calculation, we have assumed a H^+/O pump stoichiometry of 6 at a low membrane potential, namely 100 mV. The value of the constant K was then calculated by inserting in eqn. (2) the values of ΔJ_o/[unc] at 100 mV and a pump stoichiometry equal to 6. This leads, by inverting the procedure (i.e. by starting from the calculated value of K and by inserting the experimental values of ΔJ_o/[unc] and of $\Delta\psi$), to values for the pump stoichiometry at each $\Delta\psi$ value. The data of the inset of Figure 2 show that there is a range of low $\Delta\psi$ values (0.5–0.9, normalized) where the H^+/O stoichiometry remains almost constant, while there is a range of higher values (0.9–1.0, normalized) where the stoichiometry decreases as $\Delta\psi$ increases. Thus it appears that the observed change in ΔJ_o/[unc] at high $\Delta\psi$ is accounted for by a decrease in the H^+/O stoichiometry from 6.0 to 4.0 at 15 °C, from 6.0 to 4.8 at 25 °C, and from 6.0 to 5.7 at 37 °C.

The results of Figure 2 suggest the following conclusions. First, the clear biphasicity of the ΔJ_o/[unc] versus $\Delta\psi$ relationship present at low temperatures tends to become less marked at higher temperatures. This explains the greater linearity reported by Brand et al. [20] at 37 °C. The temperature-dependent pattern is in accordance with the view that the increase in temperature is accompanied by an enhancement of the non-ohmic proton conductance (cf. ref. [16]). Secondly, the slope of the linear part of the relationship becomes steeper with an increase in temperature. This indicates that the increase in temperature is accompanied by increases in the values of the constants K and K' of eqns. (2) and (3), i.e. an increase in FCCP-catalysed proton translocation. Thirdly, the range of the non-linear respiration versus $\Delta\psi$ relationship is restricted by the increase in the FCCP concentration.

DISCUSSION

The 'slip' concept has gradually evolved from a tentative explanation for the nature of resting respiration [3] to represent a more general mechanism of uncoupling based essentially on alterations in pump properties rather than membrane properties. Examples of this evolution are the numerous studies in which a variety of uncoupling effects have been interpreted in terms of alterations of the pumps: the increase in respiration due to anaesthetics [14,15,22–24], or the decrease in H^+ extrusion due to heat treatment of the redox enzyme [25,26] or to addition of carbodi-imide reagents [27–29]. Uncoupling effects, similar to those reported for mammalian mitochondria and presumably due to alterations of pump mechanisms, have also been reported in a number of other instances [30–36]. In the present study we will use the slip concept to indicate all the alterations of the H^+/O ratio which cannot simply be explained by an increased circulation of protons across the membrane bilayer.

Brand and co-workers [37,38] have supported the view of a proton leak nature of resting respiration in mitochondria. In contrast, we have favoured the view of a partial slip nature of resting respiration, essentially on the basis of the low passive proton conductance [14–16]. The slip nature of the resting respiration in mitochondria is enhanced in the presence of BSA [15].

Recently, however, Brand et al. [20] have suggested that the relationship between uncoupler-induced extra respiration and Δp provides additional information. The argument is that, if the plot of uncoupler-induced extra respiration versus Δp is linear, or non-linear, then the H^+/O ratio for the resting respiration does not vary, or varies, with Δp. This is in principle correct. Intuitively, addition of an ohmic uncoupler (which increases the proton conductance in the membrane) should not change the dependence of the H^+/O ratio on Δp (which is an intrinsic pump property). However, the question is experimental, i.e. whether under conditions of diminished Δp the dependence of the H^+/O on Δp may still be identified. In fact, while the Δp-dependence of the H^+/O ratio is observed within a narrow range of high Δp values, the uncoupler depresses Δp and therefore shifts the respiration to a range of low Δp-dependence.

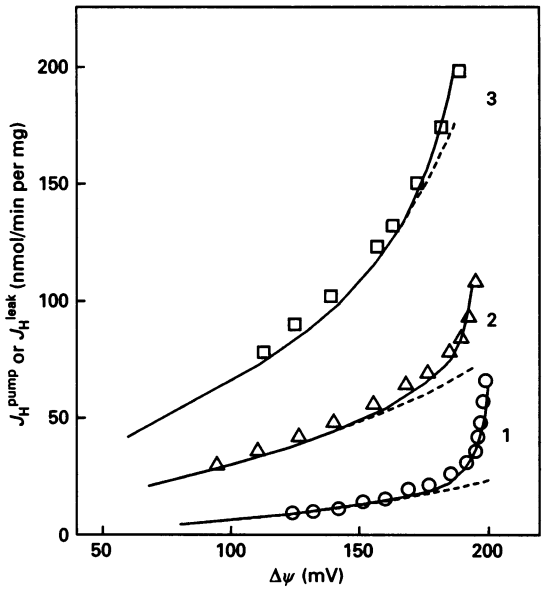

Figure 3 Computer simulations of the relationship between the rate of proton pumping or the rate of proton leak and membrane potential

The rate of proton pumping (solid lines) has been calculated by multiplying the simulated rate of respiration by the proton pump stoichiometry ($n = 6$). Broken lines represent the simulations of the corresponding relationships between the rate of proton leak and the membrane potential. The points represent the experimental data of the inhibitor titrations in the absence of FCCP, as reported in Figure 1. Simulation curves were obtained by assuming a certain degree of slip transition in the proton pump and by progressively decreasing the number of active proton pumps. In addition, increasing extents of non-ohmic leaks were considered. Curves 1: $L_H(0) = 0.07$ nmol/min per mg per mV, $\beta = 0.16$; curves 2: $L_H(0) = 0.18$, $\beta = 0.20$; curves 3: $L_H(0) = 0.45$, $\beta = 0.225$.

We have selected conditions where the extent of Δp-dependence of the H^+/O ratio is enhanced, i.e. mitochondria incubated at low temperatures. This allows us to perform the assay of uncoupler-induced respiration within a larger range of non-linearity with respect to Δp. We have found that, in the plots of $\Delta J_o/[unc]$ versus Δp, the biphasicity increases as the temperature decreases. The increased non-linearity of the plot of $\Delta J_o/[unc]$ versus Δp for mitochondria incubated at low temperature is a consequence of a marked decrease in the H^+/O ratio.

An attempt has been made to estimate the contributions of leak and slip to the resting respiration by applying computer simulations to the data of Figure 1. The simulation is based on a chemi-osmotic proton model constituted by two elements in parallel: (a) a proton pump model with a slip transition [39], and (b) a pathway of passive proton diffusion. The slip transition gives rise to non-linear relationships between the rate of electron transfer and the membrane potential [7,15,16,40].

The proton diffusion pathway was assumed to be exponentially dependent on the membrane potential:

$$J_H^{leak} = L_H(\Delta\psi) \times \Delta\psi \quad (4)$$

$$L_H(\Delta\psi) = L_H(0) \times \exp.(\beta F \Delta\psi / RT) \quad (5)$$

where J_H^{leak} is the rate of proton flux through leak and $L_H(\Delta\psi)$ represents the membrane-potential-dependent proton conductance. $L_H(0)$ is the proton conductance at zero membrane potential, while β is a parameter reflecting the exponential dependence of the passive proton flux on the membrane potential. β is correlated with the shape of the energy barriers to ion fluxes within the membrane [18,19]. A value of β in the range 0.16–0.49 is compatible with a proton leak localized at the level of the lipid membrane bilayer, while a value equal to 0.5 is indicative of integral protein-induced perturbations of the bilayer. The increase in passive proton diffusion may be simulated by assuming an increase either in the membrane conductance at zero membrane potential [$L_H(0)$] or in β (or both).

Figure 3 shows a comparison between the experimental data of Figure 1 in the absence of FCCP and the simulations of the relationship with respect to the membrane potential of either the rate of proton pumping (continuous line) or the rate of passive proton diffusion (broken line). The various curves have been obtained by introducing in the chemi-osmotic protonic circuit different extents of leaks and slips. In curves 1 the values of the parameters $L_H(0)$ and β were 0.07 nmol/min per mg per mV and 0.16 respectively. In curves 2 the extent of leak was increased: $L_H(0)$ to 0.18 and β to 0.20. Finally, in curves 3 the leak was further increased: $L_H(0)$ up to 0.45 and β to 0.225. The choice of increasing the extent of leak from curves 1 to 3 and of keeping constant that of slip implies that, in parallel, the percentage contribution of slip is progressively decreased. Comparison between the experimental respiration values and the simulations indicates the following features. First, at high membrane potential, the rate of leak accounts for less than 25% of the rate of respiration in curve 1, for about 50% in curve 2 and for more than 90% in curve 3. Secondly, at low membrane potential, the discrepancy between the rate of respiration and the amount of leak tends to disappear. Figure 3 thus shows that a discrepancy between the rate of proton pumping and that of proton leak becomes larger moving from a low to a high membrane potential. This is in accordance with the view that the higher the membrane potential the greater the extent of respiration not converted into proton pumping, i.e. there is increased energy dissipation due to a decrease in the pump stoichiometry at high membrane potential. Note that the continuous lines relative to curves 1, 2 and 3 reflect essentially the pattern obtained at 15 °C, at 25 °C and at 37 °C respectively.

The present data indicate that the contributions of leak and slip to resting respiration depend on the temperature of incubation. This is because of the marked temperature-dependence of the leak processes. Hence a decrease in the leak process at low temperatures is accompanied by a higher contribution of slip to the resting respiration, and vice versa with an increase in leak at high temperatures. The slip nature of the resting respiration does not necessarily reflect a kinetic transition of the type envisaged in the proton pump kinetic model of Pietrobon and Caplan [39], but may reflect a respiration-induced membrane perturbation that causes some conformational change in the respiratory enzyme, as suggested elsewhere [15,18].

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