The relationship between the rate of respiration and the protonmotive force

The role of proton conductivity

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1. It is shown by titrating a suspension of rat liver mitochondria with either ADP or an uncoupler that a specific rate of respiration may not have a unique associated value of the protonmotive force. Alternatively, a specific protonmotive force may not be associated with a unique rate of respiration. 2. It seems that the rate of respiration and the protonmotive force are more sensitive to the agents used for the titrations than to each other. 3. Such observations are not easily explained by the chemiosmotic hypothesis. It is, however, possible provided that the proton conductivities, i.e. the rates of dissipation of the protonmotive force, are considered to be different for each of the agents used to titrate the rate of respiration at the same protonmotive force, or vice versa.

The free energy made available by the redox reactions catalysed by the membranous respiratory electron-transport chains is conserved by an electrochemical protonic potential difference (Δp) across the membrane. The Δp generated in this way is then used to promote the reversible synthesis of ATP. The Δp therefore may be both generated and consumed by the electron-transporting devices and the ATPases that act as reversible proton pumps. The conceptual development of this mechanism is largely attributed to the theoretical and experimental breakthrough of Mitchell's chemiosmotic hypothesis (Mitchell, 1961, 1968; Nicholls, 1982).

The relationship between the rate of electron transport and the magnitude of the Δp is, however, disputed. Thus the mechanism of 'respiratory control' is also disputed. Mitchell (1979) has suggested that the Δp is de-localized over the entire organelle or bacterium, although it is also acknowledged that the protonic current is probably kinetically localized. The chemiosmotic hypothesis thus predicts that some relationship exists between the rate of respiration and the magnitude of the Δp . Although Mitchell (1968) has never stated that this relationship should be a linear function, many research workers assume that it is (see, e.g., Wilson & For-

man, 1982) and are perplexed when complex relationships are found. Such relationships, however, have been found to exist between the Δp and the rate of respiration in all systems studied (see for a review McCarthy & Ferguson, 1982). Recent and comprehensive studies have been made on the relationship between the rate of respiration and the Δp in the mitochondrial system by Wilson & Forman (1982) and Zoratti *et al.* (1983). These authors have concluded that, as their observations do not correspond to their interpretation of the chemiosmotic hypothesis, then the hypothesis cannot be the best description of oxidative phosphorylation. Clearly either the hypothesis itself is inadequate or the specific interpretation of it must be in error.

A closer examination of the predictions of the chemiosmotic hypothesis may resolve the apparently paradoxical observations made by Wilson & Forman (1982) and Zoratti *et al.* (1983). We have thus attempted to illuminate the relationship between the rate of respiration and the Δp in terms of the chemiosmotic hypothesis.

In the present paper we endeavour to show that, as long as different proton conductivities are considered, the chemiosmotic hypothesis is quite competent to explain the results of the above apparent idiosyncracies. By following these considerations we hope that it will become clearer that the mechanism of respiratory control is as Mitchell (1968) first described, although a finer subtlety is also apparent.

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Materials and methods

All reagents used during this work were of the highest purity available. Biochemical reagents were obtained generally from Sigma Chemical Co. (Poole, Dorset, U.K.). Radioisotopically labelled compounds were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Bovine serum albumin was kindly given by Dr. J. D. McGivan. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, valinomycin and rotenone were made up to 1.0 mM with 96% (v/v) ethanol.

Preparation of mitochondria was carried out by standard methods. Mitochondrial protein was determined by the biuret method (Gornall *et al.*, 1949). Mitochondrial preparations typically contained between 60 and 100 mg of protein/ml. The Δp was determined by using the isotope-distribution technique (Nicholls, 1974). $^{86}\text{Rb}^+$ (and valinomycin) were used to measure the mitochondrial membrane potentials. 5,5-Dimethyl[2- ^{14}C]oxazolidine-2,4-dione was used to measure the mitochondrial pH gradients. $^3\text{H}_2\text{O}$ and [^{14}C]sucrose were used to measure the mitochondrial volumes.

A thermostatically controlled glass reaction vessel equipped with a magnetic stirring bar was used to treat the mitochondrial suspension with the various reagents. The reaction media contained 250 mM-sucrose, 3 mM-choline/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 3 mM-succinate, 2 mM- MgCl_2 , 2 mM inorganic phosphate, 0.7 μM -valinomycin and 1.0 μM -rotenone, and were supplemented with the following concentrations of labelled compounds for Δp determination: 10 μM - $^{86}\text{RbCl}$ and 6 μM -5,5-dimethyl[2- ^{14}C]oxazolidine-2,4-dione.

All calculations of the mitochondrial volumes and Δp were performed as described by Nicholls (1974). For each determination of the Δp the mitochondrial volume was measured and not assumed to be constant. Depending on the circumstances, the volume was found to vary from 0.4 μl /mg of protein to 1.2 μl /mg of protein.

The rates of respiration of the mitochondrial suspensions were assessed by using a Clark-type oxygen electrode suspended in the glass reaction vessel, both constructed by the Science Faculty Workshop at the University of Bristol.

Results and discussion

Tables 1 and 2 indicate a series of experiments that suggest that the relationship between the rate of respiration and the magnitude of the Δp is not a simple linear function. At the same rate of respiration the Δp may have a non-unique value, or alternatively at the same Δp the rate of respiration is also not unique. It seems that the rate of respiration

Table 1. Measured Δp of a suspension of rat liver mitochondria may not have a unique associated rate of respiration

The determinations of the Δp and the rate of respiration were carried out as indicated in the Materials and methods section. The Δp was titrated to the indicated values with 0.1 M-ADP or 1 mM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The rate of respiration was then determined. The ADP-treated mitochondria were sampled for Δp determination 20 s after the addition of ADP, at which time the rate of respiration was constant. The experiments were carried out at pH 7.2 and at 25°C. The measured values of Δp and rates of respiration are the means $\pm$ S.E.M. for six replicates.

Δp (mV)	Agent used to titrate Δp	Rate of respiration (nmol of O_2 /min per mg of protein)
190 $\pm$ 5	No addition	11.6 $\pm$ 0.3
180 $\pm$ 5	ADP	89.0 $\pm$ 0.8
180 $\pm$ 5	FCCP	12.4 $\pm$ 0.9

Table 2. Dependence of the Δp on the nature of the stimulation of the rate of respiration of rat liver mitochondria

The determinations of the rate of respiration and the Δp were carried out as indicated in the Materials and methods section. The rate of respiration was titrated to the indicated values with 0.1 M-ADP or 1 mM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The ADP-treated mitochondria were sampled for Δp determination 20 s after the addition of ADP, at which time the rate of respiration was constant. The experiments were carried out at pH 7.2 and at 25°C. The measured values of rates of respiration and Δp are the means $\pm$ S.E.M. for six replicates.

Rate of respiration (nmol of O_2 /min per mg of protein)	Agent used to stimulate respiration	Δp (mV)
12 $\pm$ 0.7	No addition	190 $\pm$ 5
100 $\pm$ 0.7	ADP	150 $\pm$ 5
100 $\pm$ 0.8	FCCP	0

and the magnitude of the Δp are more sensitive to the agents used for the titration than to each other. The important question that must be addressed, therefore, is what instructs the respiratory chain to limit its turnover rate. Is it the Δp , which is the parameter used in the classical explanation for the rate limitation? Or is there some other more complex coupling mechanism of which we only have a limited understanding, the result of which makes the above relationships obscure (see, e.g., Kell, 1979)? Or have we merely misunderstood the more subtle predictions of the chemiosmotic hypothesis?

To clarify the issue, let us consider the factors that generate and maintain the Δp , together with the factors that dissipate the Δp . Eqn. (1) indicates that at a steady-state Δp the rate of generation of Δp ($J_{\text{gen.}}$) is given by the rate of electron transport ($J_{\text{ox.}}$) multiplied by the number of protons (n) translocated or abstracted from the intramitochondrial space per oxygen atom consumed. The rate of dissipation ($J_{\text{dis.}}$) of the Δp is given by the proton leak through the membrane (L), which is a function of the Δp itself, i.e.

$$J_{\text{ox.}} \cdot n = L \cdot \Delta p \quad (1)$$

If we again consider the results shown in Tables 1 and 2, it is clear that we (and of course Wilson & Forman, 1982, and others) are comparing only two variables when in fact there are actually four possibilities.

We consider the n factor to be constant (see below), although Pietrobon *et al.* (1982) suggest that this may also vary. The remaining variable, therefore, is the proton conductivity. Now, which parameters constitute the proton-leakage term? Firstly, there is the natural leak of the phospholipid membrane, but this must be added together with any other devices that may catalyse the leakage of protons through the membrane. In the case of the experiments described in Tables 1 and 2, the latter devices are the reversible ATPases under phosphorylating conditions and the proton ionophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, with which the mitochondria have been treated. We should ask ourselves, therefore, is it likely that the ATPase and the ionophore would be responsible for exactly the same rate of proton leakage at the same Δp ? The results given in the Tables indicate that this possibility is not the case.

An explanation of the observations indicated by Tables 1 and 2, together with the results given in the cited work of Wilson & Forman (1982) and Zoratti *et al.* (1983), would be now appropriate, and is as follows.

With eqn. (1) in mind, let us consider the possibility that the rate of proton conductance across the mitochondrial membrane is not the same if it is catalysed by the reversible ATPases or the proton ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. One would then predict that the observed relationships between the various rates of respiration and the associated Δp would not be simple functions of each other.

Under conditions of a change from the controlled state of mitochondria to conditions of rapid phosphorylation, the proton conductance of the ATPase becomes relatively very high, i.e. the L term is very high. Eqn. (1) therefore predicts either that the rate of respiration should increase or that the steady-state Δp decreases. In fact both occur at

once (see, for a review, Ferguson & Sorgato, 1982). If, however, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and ADP are used to titrate suspensions of coupled mitochondria, as in Table 2, to the same higher rate of respiration, and then sampled for Δp determination, then the steady-state Δp is found to be much larger in the case of the ADP-increased rate. The reason, we suggest, is: under the conditions of complete uncoupling by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, the proton conductance catalysed by the uncoupler is higher than that catalysed by the ATPases, and thus the steady-state Δp is much smaller. Alternatively, under conditions whereby the Δp is titrated with the two reagents but the Δp is maintained at a relatively high value, as in Table 1, the proton conductance due to the ATPase is much higher than that due to the uncoupling with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Thus the ADP-stimulated rate of respiration is also much higher than that of the uncoupler-stimulated mitochondria.

It would seem that an important condition is fulfilled for the efficient operation of oxidative phosphorylation by the highly conducting ATPase. If the ATPase under phosphorylating conditions were not the most able dissipator of the Δp , i.e. with respect to other dissipative processes, then oxidative phosphorylation would not operate with any semblance of efficiency.

The consequence of such phenomena are, therefore, apparently arbitrary relationships between the rate of respiration and the Δp . For only if the same proton conductance were induced fortuitously at the same Δp would the same rate of respiration result.

It is now worthwhile to reconsider the question put at the beginning of this exercise: 'what instructs the respiratory chain to limit its rate of turnover?'

The latter conclusions described above lead us to the apparently paradoxical situation of suggesting that, in addition to the Δp , the proton conductance also has some influence on the rate of respiration. The proton conductance and the Δp may thus be varied either by the presence of an uncoupler or by the turnover of the ATPase itself. The Δp is therefore a macroscopic phenomenon that may limit respiration if the rate of its generation is greater than the rate of its dissipation (eqn. 1). If, however, the rate of generation of the Δp is the same as the rate of dissipation, then at a constant Δp the leak rate will be the only factor that determines the rate of respiration. The consequence, therefore, would be, under some circumstances, a direct relationship between the rate of respiration and the rate of phosphorylation. The magnitude of the Δp would simply reflect the relationship between $J_{\text{gen.}}$ and

$J_{\text{dis.}}$. Such relationships have often been shown (Mandolino *et al.*, 1983), and have been used to suggest that the chemiosmotic hypothesis is not a complete enough description of oxidative phosphorylation (Wilson & Forman, 1982). Clearly, such conclusions are now no longer valid.

Two more points are relevant to our discussion. The first concerns the stoichiometric value, n . We have considered that this parameter does not vary. It is possible, however, to vary the n value for the whole respiratory chain merely by inhibiting segments of the chain with rotenone and/or antimycin A and then supplying reducing equivalents as succinate and ascorbate + ferrocyanide respectively. Now, the controlled rate of respiration is progressively higher as more of the segments of the respiratory chain are inhibited. Under these circumstances two conditions prevail: the Δp is about the same (Nicholls, 1982) and the natural leak through the membrane is about the same for the various inhibited states. The remaining variable thus is the n value. Eqn. (1) predicts that to maintain the same Δp and the same L value at a lower n value the rate of respiration must increase, which of course is the observation. This is precisely the argument that we have applied, but advocating a variable L value at constant n value.

The second point concerns the mechanism by which the ATPase may vary its conductivity or rate of consumption of protons. It has been suggested by Schonfeld & Neumann (1977) that the current-voltage relationship of the ATPase in question behaved like a semiconductor diode. These authors suggested this description simply to describe the current-voltage relationship of the ATPase. We must point out, however, that this provides an excellent electrical analogy to our preceding discussion. One of the main uses of a diode in electrical circuits is to provide a constant voltage across a load for a variation in the load impedance. Thus in biological terms the ATPase diode would attempt to regulate the conduction of protons while allowing the Δp to remain about the same. The rate of respiration will maintain and is limited by the Δp , but the rate of synthesis of ATP will also influence the rate of respiration. The synthesis of

ATP therefore takes place with as large a driving force as possible.

Finally, we suggest that further research in this interesting field might be directed towards the mechanisms of the control of the ATPase conductance. The elegant work of Jackson and co-workers (Clark *et al.*, 1983) is of particular interest.

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