The Rate of ATP Synthesis by Submitochondrial Particles Can Be Independent of the Magnitude of the Protonmotive Force

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The problem of whether the rate of ATP synthesis is proportional to the magnitude of the protonmotive force has been studied in submitochondrial particles. It was found that the rate of ATP synthesis can decrease at constant protonmotive force and is more closely related to the rate of substrate oxidation.

The chemiosmotic mechanism (Mitchell, 1966, 1976) of energy coupling requires that there should be a defined relationship between the magnitude of a transmembrane electrochemical proton gradient (protonmotive force, Δp) and the extent to which processes driven by Δp (e.g. ATP synthesis) are displaced from equilibrium. In contrast, the statement of the chemiosmotic hypothesis (Mitchell, 1966, 1976) makes no prediction as to the relationships between changes in the rates of either energy-yielding or energy-utilizing processes and the magnitude of Δp , yet knowledge of these relationships is important both for an overall understanding of oxidative phosphorylation (and other related processes) as well as for applying the principles of irreversible thermodynamics to bioenergetics (van Dam & Westerhoff, 1977; Rottenberg, 1978, 1979; Stücki, 1978).

In earlier work we and others have found that a decrease of the rate of respiration of mitochondria or submitochondrial particles does not cause a proportional drop in Δp ; a significant drop in Δp is only observed at very high extents of inhibition (Nicholls, 1974; Sorgato *et al.*, 1978; Sorgato & Ferguson, 1979). Inhibition of respiration will, however, decrease the rate of ATP synthesis, which therefore raises the important question as to whether the rate of ATP synthesis decreases whilst the accepted immediate driving force, Δp , remains constant. The objective of the present paper is to report on investigations with submitochondrial particles that show that there is not necessarily a close relationship between the size of Δp and the rate of ATP synthesis, a finding that is not readily accommodated within a simple chemiosmotic scheme (cf. Casadio *et al.*, 1978).

Abbreviation used: Δp , protonmotive force (electrochemical proton gradient expressed in mV).

Materials and Methods

Bovine heart submitochondrial particles were prepared as described by Ferguson *et al.* (1977) and Δp was determined by using the flow-dialysis procedure (Sorgato & Ferguson, 1979). The rate of ATP synthesis catalysed by the particles in the flow dialysis cell was determined in a parallel experiment in which the potassium thio[^{14}C]cyanate or [^{14}C]methylamine used for determination of Δp was omitted from the cell and $^{32}\text{P}_i$ was added to an otherwise identical reaction mixture. The reaction was started by the addition of ADP, and aliquots of the reaction mixture were withdrawn at 3, 5 and 8 min from the flow dialysis cell for analysis of the amount of ATP synthesized by measuring the incorporation of $^{32}\text{P}_i$ into organic material (Sugino & Miyoshi, 1964). The respiration rate in the flow dialysis cell was estimated by transferring 0.1 or 0.2 ml of the contents of the cell to a closed reaction chamber fitted with a Clark-type oxygen electrode and containing the reaction medium used in the flow dialysis experiment to give a final volume of 2.5 ml. The temperature was 25°C.

ATP and ADP were obtained from Boehringer; lyophilized ammonium-free hexokinase was purchased from Sigma. Sources of other reagents and details of additional procedures are given by Sorgato & Ferguson (1979).

Results and Discussion

Table 1 shows the results of experiments in which the rate of succinate oxidation, the rate of ATP synthesis and Δp were each measured for four preparations of submitochondrial particles in the presence and absence of malonate. It is evident in each case that, although malonate has its expected

Table 1. *Effect of inhibition of succinate oxidation by malonate on the rate of ATP synthesis and the size of the protonmotive force generated by submitochondrial particles*

The flow dialysis cell contained in a total volume of 1 ml: 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid; sodium salt], 200 mM-sucrose, 2 mM-MgCl₂, 50 mM-KCl, 10 mM-KH₂PO₄, 20 mM-glucose, 0.17 mM-ADP, submitochondrial particles (approx. 9 mg of protein), and 25 U of salt-free hexokinase. The initial concentration of sodium succinate was 20 mM and where added the sodium malonate concentration was 0.95 mM. For determination of Δp , potassium thio[¹⁴C]cyanate (6 μ M) or [¹⁴C]methylamine (15 μ M) were additionally added, and for measurement of the rate of ATP synthesis 2 μ Ci of ³²P_i was present. The rate of ATP synthesis was linear over the 8 min period tested in Expts. 3 and 4, whereas in Expts. 1 and 2 a slight initial lag was observed, and thus the averaged rate over 8 min is given. The temperature was 25°C and the pH was 7.5. The submitochondrial particles were preincubated with 5 mM-sodium succinate for at least 10 min at 25°C to activate succinate dehydrogenase (Singer, 1968).

Expt.	Malonate	Respiration rate (ng-atom of O · min ⁻¹ · mg ⁻¹)	Inhibition (%)	ATP synthesis rate (nmol · min ⁻¹ · mg ⁻¹)	Inhibition (%)	Δp (mV)
1	—	166	—	83	—	185
	+	93	44	41	50	185
2	—	161	—	78	—	175
	+	87	46	36	54	180
3	—	225	—	51	—	195
	+	115	55	23	57	185
4	—	150	—	93	—	175
	+	60	58	28	70	188

effect of decreasing the rates of both succinate oxidation and ATP synthesis, the magnitude of Δp is virtually insensitive to the presence of malonate. The titre of malonate was chosen so as to inhibit the succinate oxidation rate by approx. 50%. Thus conditions were obtained in which it had previously been shown that the passive proton conductance of the particles varies so as to maintain a constant Δp despite the inhibition of respiration-driven proton translocation (Sorgato & Ferguson, 1979).

The conclusion from Table 1 is that the rate of ATP synthesis declines in close relation to the extent of inhibition of respiration, yet the magnitude of Δp , which it should be emphasized was measured under conditions in which the submitochondrial particles were synthesizing ATP, shows no corresponding decrease. It is difficult to reconcile this result with the standard chemiosmotic principle, as the ATP synthase enzyme should be insensitive to the extent to which the respiratory chain is inhibited, and is expected to be responsive only to the magnitude of the bulk phase Δp , provided that ADP and P_i are not rate limiting. In view of the widespread evidence for chemiosmotic mechanisms it is therefore appropriate to consider some alternative explanations of our data that might remove the apparent inconsistencies with the chemiosmotic principle.

The possibility that the insensitivity of Δp to the addition of malonate is a consequence of the experimental method having an upper limit of detection, leading to an underestimate of the real Δp by an amount that would decrease as respiration is inhibited, has been ruled out by previous experi-

ments (Sorgato & Ferguson, 1979). Extra evidence against this possibility was provided by the observation that the Δp developed by the particles used for Expts. 3 and 4 (Table 1) was increased to 225 mV when ATP synthesis was inhibited by the addition of oligomycin (1 μ g/mg of particle protein) to the reaction mixture described in Table 1. This latter value is the same as that reported previously (Sorgato & Ferguson, 1979) for submitochondrial particles under non-phosphorylating but otherwise similar conditions, and also suggests that ATP synthesis places a demand on Δp .

Another interpretation of the data in Table 1 requires (cf. Casadio *et al.*, 1978) that only a limited proportion of the submitochondrial particles are active in ATP synthesis. Thus a drop in Δp upon addition of malonate might only occur in this population of the particles, so that, overall, little or no change in Δp would be seen. Several arguments militate against this possibility. First, the suggested proportion of phosphorylating particles cannot be very limited, as when the particles are actively driving ATP synthesis a decrease in Δp can be observed (see above). The logarithmic response of the methods used for determining Δp means that the value obtained would be biased towards the putative population of phosphorylating particles that might reasonably be expected to generate a higher Δp . The same logarithmic response also means that the methods are quite sensitive to relatively small changes when Δp is high, as is the case in the present work. Second, we have found that 50% inhibition of respiration with malonate is accompanied by only a

very small (approx. 3%; M. C. Sorgato, D. Branca & S. J. Ferguson, unpublished work) decrease in the maximum value of the phosphorylation potential that can be attained, whereas a model in which Δp decreases greatly in the supposed phosphorylating fraction of submitochondrial particles predicts that the phosphorylation potential should also show a considerable decrease. The rates of ATP synthesis given in Table 1 are lower than some other values reported [e.g. $160 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (Thayer & Hinkle, 1975)] with succinate as substrate, but this cannot be taken as evidence for a relatively small fraction of phosphorylating particles. It should be noted that KCl, which ensures that both components of Δp are readily detectable but can inhibit somewhat the rate of ATP synthesis (Thayer & Hinkle, 1975, and references therein), is present as a component of a standard reaction mixture also used in previous work (Sorgato & Ferguson, 1979).

A final point to consider is that ATP synthesis was insensitive to added carboxyatractyloside (3 nmol/mg of particle protein), showing that the reaction was catalysed by submitochondrial particles rather than by contaminating mitochondria. This observation eliminates the possibility that malonate might be preferentially inhibiting the respiration and ATP synthesis of contaminating mitochondria rather than acting on submitochondrial particles.

It is concluded from the above arguments that any heterogeneity in the submitochondrial particle preparations does not mask a decrease of Δp in parallel with the inhibition by malonate of respiration and ATP synthesis. Consequently the implications of a straightforward interpretation of the data in Table 1 require consideration. It is difficult to account for the variation of the ATP synthesis rate being independent of Δp without requiring an interaction between the respiratory chain and the ATP synthase enzyme. Thus, changes in the respiration rate would be reflected in the ATP synthesis rate even when the magnitude of the accepted intermediate in oxidative phosphorylation, Δp , is not itself perturbed. Essentially the same conclusion has been drawn from studies on bacterial chromatophores (Baccarini Melandri *et al.*, 1977; Casadio *et al.*, 1978; Melandri *et al.*, 1978) although Δp was measured by procedures, carotenoid band shift and 9-aminoacridine fluorescence, different from those used in the present work.

The results of the present work suggest a role for relatively localized interactions between the ATP synthase and electron transport chains. Similar indications have come from related experiments with bacterial chromatophores (Baccarini Melandri *et al.*, 1977; Casadio *et al.*, 1978; Melandri *et al.*, 1978; Petty & Jackson, 1979) and vesicles (Kell *et al.*, 1978). Furthermore, direct coupling between oxi-

dation and phosphorylation has been suggested on the basis of the observation that ATP synthesis or partial uncoupling can stimulate mitochondrial respiration to equal extents although having very different effects on the protonmotive force (Rottenberg, 1978, 1979). Nevertheless it is difficult to envisage the physical basis of an interaction between the respiratory chain and ATP synthase unless it is really a reflection of a restricted proton circuit between these two components.

There is only one other study of the relation between the magnitude of Δp and the rate of ATP synthesis in submitochondrial particles (Thayer & Hinkle, 1975). In that work it was found that there was a correlation between the rate of ATP synthesis and the estimated size of a Δp which was imposed on the particles by rapid changes in the external pH and development of potassium diffusion potentials. A crucial difference between that work and the present investigation is that the artificially induced Δp decays with time so that, in contrast with the present work, true steady-state conditions were not achieved. Thus there is not necessarily a conflict with the results of the present study.

Finally it is instructive to assess our results against the framework of irreversible thermodynamics that has been advocated for oxidative phosphorylation (van Dam & Westerhoff, 1977; Rottenberg, 1978, 1979; Stücker, 1978). In some of these treatments (van Dam & Westerhoff, 1977; Rottenberg, 1978) it is suggested that the rate of ATP synthesis might be a composite function both of a linear dependence on Δp and of a linear dependence on the phosphorylation potential with the assumption, in line with chemiosmotic theory, that there is no direct coupling between oxidation and phosphorylation. To determine whether our data (Table 1) fit this relationship a value for the phosphorylation potential is thus required. It is estimated, assuming $\Delta G^{\circ} = 4.6 \text{ kcal} \cdot \text{mol}^{-1}$ ($19.2 \text{ kJ} \cdot \text{mol}^{-1}$) (Robbins & Boyer, 1957) and equilibrium for the hexokinase reaction, that the phosphorylation potential under the conditions used for the experiments shown in Table 1 reached on average $4.4 \text{ kcal} \cdot \text{mol}^{-1}$ ($18.4 \text{ kJ} \cdot \text{mol}^{-1}$) in the presence of malonate and $5.5 \text{ kcal} \cdot \text{mol}^{-1}$ ($23.0 \text{ kJ} \cdot \text{mol}^{-1}$) in its absence after a reaction time of 8 min in the flow dialysis cell. This corresponds closely to the time at which Δp is determined in the flow dialysis experiments. Thus the difference in phosphorylation potential cannot account for the large decrease in the rate of ATP synthesis that occurs upon adding malonate. On the contrary, given that Δp is not altered by malonate, the lower phosphorylation potential estimated in the presence of this inhibitor suggests that the rate of ATP synthesis should be higher, as, in the linear expression for the rate of ATP synthesis, the phosphorylation potential will

contribute as a negative term (van Dam & Westerhoff, 1977; Rottenberg, 1978).

A further problem is that, strictly, the application of linear phenomenological equations is restricted to conditions where a system is close to equilibrium. It is evident that the presence of glucose plus hexokinase prevents the magnitude of the phosphorylation potential from reaching equilibrium with Δp , so that our experiments have been conducted under conditions where the ATP synthase reaction is far from equilibrium. Hence, despite suggestions (Rottenberg, 1978, 1979) that irreversible thermodynamics may be applied when a system is far from equilibrium, one possible conclusion from our experiments is that a linear analysis is not necessarily applicable under such conditions. Alternatively it might be that the expression for the rate of ATP synthesis should include a linear term dependent on the redox potential and on a coefficient reflecting the activity of the respiratory chain. The question remains, however, as to how to write a formal mechanistic description to account for the apparent independence of the rate of ATP synthesis from the size of Δp under the conditions (Table 1) tested in the present work.

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