THE ATPase AS AN IRREVERSIBLE COMPONENT IN ELECTRON TRANSPORT LINKED ATP SYNTHESIS

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1. Introduction

The reversibility of the ATPase that participates in oxidative phosphorylation is implicit within the idea that the phosphate potential (energy stored in ATP) comes into equilibrium with the respiratory chain [1]. This is the basis of an explanation of respiratory control in which ADP stimulated respiration (state 3) continues until this equilibrium is reached, whereupon respiration decreases to a controlled rate (state 4). Our purpose in this paper is to enquire whether this equilibrium view is always correct by considering the properties of the oxidative phosphorylation apparatus of 'inside out' phosphorylating membrane vesicles from *Paracoccus denitrificans* which exhibit respiratory control [2,3,4].

2. Results and discussion

The vesicles of *P. denitrificans* have very little ATPase activity [5] compared with the rate at which they are able to synthesise ATP. Typically the rate of ATP synthesis is one hundred times greater than the ATP hydrolysis rate observed when ATP is added to unenergised vesicles. The very low ATPase activity does not cause an enhancement of 1-anilino-1-naphthalene-8-sulphonate fluorescence [6], although succinate oxidation can support this process [6]. Therefore if the ATPase of these vesicles is to function reversibly, it is necessary to postulate that it is triggered during oxidative phosphorylation into a state which can function reversibly. The following experiment was carried out to test this possibility. Vesicles of *P. denitrificans* were incubated under a variety of conditions with [γ-^{32}P]ATP, and the $^{32}P_i$ released was analysed. The $^{32}P_i$ may result from both ATPase activity and ATP $\rightleftharpoons$ P_i exchange. This procedure did not detect any stimulation of P_i release by concurrent electron transport (state 4), or oxidative phosphorylation (state 3) (table 1). The amount of ADP added in the experiment in which the effect of oxidative phosphorylation was measured, was such that net ATP synthesis should have been complete after 4 min. The rate of ATP synthesis was 65 times faster (table 1) than the rate of $^{32}P_i$ release from [γ-^{32}P]ATP. Hence there is no evidence from the experiments in table 1 that the ATPase acts reversibly during concurrent electron transport or oxidative phosphorylation.

Table 2 shows that the ADP/O ratio remains constant as the rate of NADH oxidation is decreased progressively down to 17% of the original rate when the vesicles are titrated with rotenone. This implies that the amount of ADP phosphorylated is constant, and that the phosphate potential generated is essentially independent of the NADH oxidation rate. An alternative interpretation of the constant ADP/O ratio is that a decrease in the amount of ADP phosphorylated is paralleled by a decrease in the P/O ratio, such that the ADP/O ratio is only apparently constant as the respiratory rate is reduced. We consider this explanation unlikely; indeed a constant P/O ratio has been demonstrated for mitochondria over a wide range of succinate oxidation rates [7]. In order to maintain a constant phosphate potential, an equilibrium mechanism of ATP synthesis requires that any intermediate between the respiratory chain and the ATPase must be able to reach a high enough concentration to maintain this potential, even when the rate

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Table 1
Release of $^{32}P_i$ from [γ-^{32}P]ATP by membrane vesicles of *P. denitrificans*

Reaction conditions	c.p.m. in isobutanol layer	Rate of release of $^{32}P_i$ from [γ-^{32}P]ATP (nmoles·min^{-1}·mg^{-1})
No NADH oxidation	10 800	34
State 4 NADH oxidation	9 000	27
State 3 NADH oxidation	7 200	22

For comparison, the rate of ATP synthesis under these conditions was 1.4 μmoles min^{-1}·mg^{-1}, and typical ATPase activity of these vesicles is 20 to 30 nmoles min^{-1}·mg^{-1} [5]. Vesicles were prepared from cells grown on succinate and nitrate as described previously [15] except that 10 mM ATP was added to the 100 mM Tris-acetate in which the lysozyme treated cells were resuspended. Vesicles (0.1 mg protein) were added to a basic medium containing Tris-phosphate (10 mM in phosphate) pH 7.3 and 5 mM magnesium acetate. For experiments in which the effect of state 4 respiration was examined, 0.1 mg yeast alcohol dehydrogenase (Sigma), 0.6 mM NAD and 30 μl ethanol were present, to which 0.2 mM ADP was added for the experiment in which state 3 respiration occurred. The total volume was 3 ml. 1 mM [γ-^{32}P]ATP (8.8×10^6 c.p.m. μmole^{-1}) was added to start the reaction, and the samples incubated at 30°C. After 10 min 1 ml of 15% trichloroacetic acid was added, and the samples put on ice for five minutes. Precipitated protein was removed by centrifugation, and 0.05 ml of the supernatant taken for the total counts. 1 ml of the supernatant was added to 1 ml of 2.5% ammonium molybdate in 1 M sulphuric acid. After allowing five minutes for the formation of phosphomolybdate, 2.5 ml of isobutanol–benzene (1:1, v:v) was added with thorough mixing. The isobutanol layer was allowed to separate and 1 ml withdrawn for counting. The counts shown in the table have been corrected for the counts (12 000 c.p.m.) which were found in the isobutanol layer when 1 mM [γ-^{32}P]ATP was incubated alone in the basic medium.

Table 2
The ADP/O ratio of *P. denitrificans* vesicles at different rates of NADH oxidation

Respiration rate (μg atoms O_2·min^{-1}·mg protein^{-1})	Rotenone concentration (μM)	ADP/O ratio
0.72	0	1.8
0.60	0.66	1.9
0.24	1.66	2.1
0.18	3.3	2.1
0.06	13.2	2.1

0.5 mg vesicles were added to a medium containing in a total volume of 3 ml: Tris-phosphate (10 mM in phosphate), pH 7.3, 5 mM magnesium acetate, 0.2 mg yeast alcohol dehydrogenase (from Sigma), and 30 μl ethanol. The reaction was started by the addition of NAD^+ (0.6 mM) and subsequently ADP (0.2 mM). The appropriate amount of rotenone (Sigma) was added two minutes before the NAD^+. Oxygen uptake was measured at 30°C with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England). ADP/O ratios were calculated by the procedure of Chance and Williams [16].

of energy input is reduced. For instance, in terms of the chemiosmotic hypothesis [8], a proton motive force would have to be maintained constant as the oxidation rate is drastically reduced. However, if the ATPase were to work irreversibly, then the maximum phosphate potential could be achieved irrespective of the oxidation rate; it would just take longer to reach the final phosphate potential when the respiratory rate is slower.

We must now consider the implications if the ATPase in *P. denitrificans* is not fully reversible. First, poising the phosphate potential against a property of the membrane, such as a proton motive force, is inappropriate. Secondly, the nature of respiratory control must be kinetic rather than thermodynamic, relying on the availability of ADP and phosphate rather than the value of the ratio $[ATP]/[ADP][P_i]$.

It is appropriate to assess whether the conclusions drawn here from studies on one species of bacterium may be applied to other electron transport-linked phosphorylating systems. The problem posed by the absence of ATP hydrolysis by the ATPase in the thylakoid membrane of chloroplasts has been recognised before [9], and it was suggested that the ATPase is triggered into a reversible form by electron transport [9]. Avron and Jagendorf [10] showed that the release of $^{32}P_i$ from $[\gamma^{32}P]ATP$ was not stimulated by electron transport, although Shavit et al. [11] did detect a small electron transport dependent $ATP \rightleftharpoons P_i$ exchange in chloroplasts which was very much slower than the rate of ATP synthesis. Thus it appears that the ATPase of the thylakoid membrane, like that of *P. denitrificans*, is not fully reversible. The irreversibility associated with the ATPase of the thylakoid membrane can account for the observation that in chloroplasts the size of the phosphate potential does not influence either the rate of ATP synthesis at any level of a transmembrane pH gradient, or the threshold level of this pH gradient that is required for ATP synthesis [12].

The mitochondrial ATPase appears to behave reversibly in that ATP can be hydrolysed and reversed electron transport driven, but an element of irreversibility may be introduced to mitochondrial oxidative phosphorylation by the energy dependent anisotropy of the adenine nucleotide translocator [13]. Respiratory control in mitochondria may operate through an equilibrium mechanism in contrast to the kinetic mechanism that is proposed in this paper for *P. denitrificans*. Therefore although the inner mitochondrial membrane and the plasma membrane of *P. denitrificans* have many features in common [14], they may differ in their respiratory control mechanisms.

It seems to us that there are good reasons for believing that, at least in chloroplasts and in *P. denitrificans*, ATP synthesis is not a fully reversible process because the ATPase functions irreversibly. Although the mechanism of this irreversibility may be related to the presence of an ATPase inhibitor protein, or to another feature of the ATPase complex, we believe that consideration should now be given to the view that equilibration between the phosphate potential and the electron transport chain may not occur.

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