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Partial uncoupling, or inhibition of electron transport rate, have equivalent effects on the relationship between the rate of ATP synthesis and proton-motive force in submitochondrial particles

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The rates of electron transport and of ATP synthesis have been measured in bovine heart Mg-ATP submitochondrial particles oxidising succinate under conditions of partial attenuation of the proton-motive force by malonate or FCCP. This paper reports evidence that the relationship between the rate of ATP synthesis and the magnitude of the proton motive force is independent of the mode by which the decrease of the proton motive force is achieved.

ATP-synthesis rate *Submitochondrial particle* *Proton-motive force* *Localised proton coupling* *Energy transduction*

1. INTRODUCTION

There are at least two conflicting views as to the nature of the relationship between the rate of ATP synthesis and the magnitude of the proton-motive force across an energy transducing membrane.

In the first of these it is supposed, in line with the original chemiosmotic theory [1], that the rate of ATP synthesis would be solely a function of the magnitude of Δp [2,3]. Although such an expectation has been confirmed in experiments with thylakoids from chloroplasts [4], results that are inconsistent with the first hypothesis have been found in mitochondria [5,6] and chromatophores from photosynthetic bacteria [7]. These experiments have shown that equal attenuation of the rate of ATP synthesis with either uncouplers or electron-transport inhibitors are accompanied by different reductions of the magnitude of Δp [5–7]. Such experiments have been particularly prominent in casting doubt on whether the chemiosmotic theory of energy coupling provides a full description of the linkage between electron transport and ATP synthesis [8]. This conclusion is of such importance that further experiments of this kind need to be performed with a variety of systems and experimental methods.

The present paper reports a study of the relationship between the rate of ATP synthesis and the magnitude of Δp in bovine heart Mg-ATP submitochondrial particles under conditions of controlled reduction of Δp with either a protonophore or an inhibitor of electron transport.

Abbreviations: Δp, proton-motive force: $\Delta\bar{\mu}_H/F = \Delta\psi + RT2.303/F \cdot \Delta pH$, where $\Delta\psi$ is the electrical component and ΔpH the chemical component of the electrochemical gradient of protons ($\Delta\bar{\mu}_H$) existing across an energy-transducing membrane. F is the Faraday constant; Ap_5A, P^1,P^5-di(adenosine-5′-)pentaphosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SMP, submitochondrial particles

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2. MATERIALS AND METHODS

Type II bovine heart Mg-ATP SMP were prepared according to [9] and suspended in 10 mM P_i/21 mM Tris (pH 7.3), 5 mM $Mg(CH_3COO)_2$. Before all experiments reported here, the stock suspension of particles was incubated for 30 min at 37°C with 2.5 mM sodium malonate in order to maximally stimulate succinate dehydrogenase activity [10].

The rate of ATP synthesis and the magnitude of Δp measurements were made under the same conditions and in the same experimental set-up. More specifically, the upper chamber of a glass flow-dialysis cell used for the determination of Δp [11] was fitted with a Clark-type oxygen electrode and a thermostatically controlled water jacket. The rate of ATP synthesis was determined in the following way: 3.3 ml of 10 mM P_i/21 mM Tris (pH 7.3), 5 mM $Mg(CH_3COO)_2$ in the upper chamber of the dialysis cell contained 6.6 mg protein of Mg-ATP SMP, 2 mM potassium phosphate (plus 1 μCi $^{32}P_i$), 20 mM glucose, 90 μM Ap_5A and 50 mM sodium succinate. Two 0.1 ml samples were withdrawn to assay the percentage of precipitation of $^{32}P_i$ by the method of Sugino and Miyoshi [12]. Routinely this was found to be $\geqslant$99.8%. After 5 min a water-saturated stream of oxygen was blown over the suspension with an intensity sufficient to saturate the suspension, as indicated by the electrode; 25 units/ml of salt-free yeast hexokinase and 190 μM ADP were then added to the mixture in such a volume that the final volume of the suspension was increased 1%; this was taken as zero time. For determining the rate of ATP synthesis, three 0.1-ml samples were collected within the experimental time of 3–4 min. Before withdrawal of a sample for measurement of the rate of ATP synthesis [12], the oxygen stream was interrupted to allow the collection of the sample. The stream of oxygen was then immediately restored over the suspension where it remained until the next sample was withdrawn.

FCCP and sodium malonate, when present, were added at the beginning of the incubation period. ATP, as glucose 6-[^{32}P]phosphate, was separated from $^{32}P_i$ according to the method of Sugino and Miyoshi [12]. Plots of esterified ^{32}P against time were linear. ^{32}P was counted by the Cerenkov method.

The reaction mixture in which the particles were suspended was such that only the electrical component of Δp needed be measured [13]. Thus, under these conditions, $\Delta\psi = \Delta p$. The determination of the magnitude of the membrane potential was made from measurement of the uptake into the particles of $S^{14}CN^-$ [11]. The conditions for determining $\Delta\psi$ were the same as those used for determining the rate of ATP synthesis except that buffer was pumped through the lower chamber of the flow-dialysis cell, starting at the time when ADP and hexokinase were added. Also, the stream of water-saturated oxygen was blown over the sample continuously throughout $\Delta\psi$ determination. $KS^{14}CN$ was 3.5 μM and CCCP, added to collapse the membrane potential [11] between 3 min 15 s and 3 min 20 s following the addition of ADP and hexokinase, was 26 μM. The internal volume used for calculating the magnitude of $\Delta\psi$ was 1.3 μl/mg protein of SMP [13]. Temperature in all experiments was 25°C.

Protein was determined according to [14]. FCCP was from Boehringer (Mannheim, FRG) and dissolved at 27 μM in ethanol. CCCP was from Boehringer and dissolved at 12.5 mM in ethanol. Ap_5A (as lithium salt) was purchased from Sigma. $^{32}P_i$ and $KS^{14}CN$ were purchased from the Radiochemical Centre (Amersham, Bucks, England).

3. RESULTS AND DISCUSSION

Fig.1 gives the results of experiments in which the rate of ATP synthesis catalysed by submitochondrial particles oxidising succinate was measured at a range of different values of Δp. Two methods were used to lower the steady-state value of Δp below its maximum; either the electron transport rate was reduced by adding selected concentrations of malonate or, alternatively, low concentrations of the protonophore FCCP were added. Attenuations of Δp by either procedure had equivalent effects on the rate of ATP synthesis (fig.1). As explained in section 2, particular care was taken to ensure that the rate of ATP synthesis and Δp determinations were both measured under identical conditions. Moreover, precautions were taken to ensure that the action of the ATP-synthase inhibitor protein did not affect the results. This was done in the following way. When

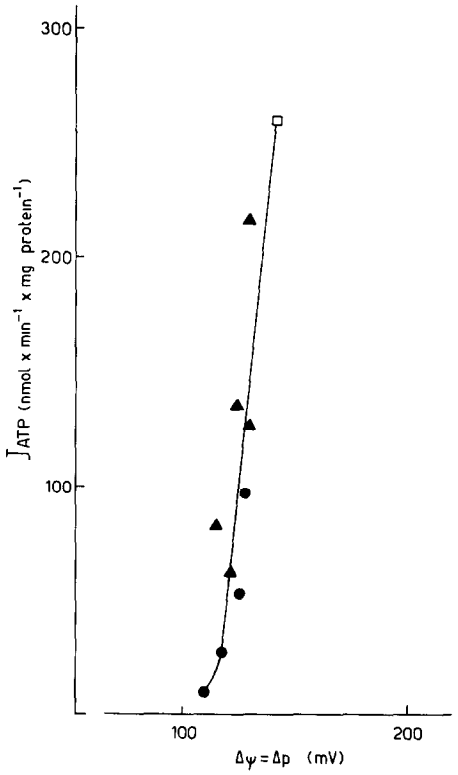

Fig.1. Relationship between the rate of ATP synthesis (J_{ATP}) and the magnitude of the membrane potential in Mg-ATP SMP oxidising succinate, in the absence and presence of increasing concentrations of malonate (▲) or FCCP (●). The rate of ATP synthesis was measured by trapping the ATP ^{32}P produced with glucose and hexokinase and $\Delta\psi$ from the extent of $S^{14}CN^-$ uptake into the particles [11]. Malonate concentrations (▲) used to lower $\Delta\psi$ ranged from 0.72 to 3.63 mM, while FCCP concentrations (●) ranged from 0.14 to 0.41 μM. For experimental details see section 2 and the text. (□) Indicates that no malonate or FCCP was added to the particles. Under identical experimental conditions, but for the absence of both ADP and malonate or FCCP, the membrane potential value developed by the particles was 155 mV.

the particles were added to the flow-dialysis cell in the presence of succinate, the reaction medium remained aerobic for between 25 and 150 s depending on the extent of inhibition by malonate, i.e., the time necessary to exhaust the dissolved oxygen. This period was sufficient for maximal activation of the ATP-synthase, which is assumed to be related to dissociation of the ATPase inhibitor protein from the ATP-synthase upon development of $\Delta\psi$ [15], because extending the aerobic period, by supplying a water-saturated stream of oxygen, did not further increase the subsequent rate of ATP synthesis. Abolition of the aerobic period before the addition of ADP and hexokinase (see section 2), i.e., by adding succinate, ADP and hexokinase simultaneously to the particles, resulted in a lower initial rate of ATP synthesis that increased with time. Hence rates of ATP synthesis shown in fig.1 were linear through the period examined as not influenced by an increased dissociation of the inhibitor protein during the experiment.

The data shown in fig.1 are consistent with a scheme in which the rate of ATP synthesis can be related solely to the magnitude of the protonmotive force. This conclusion is in agreement with the findings made in experiments with thylakoids by Portis and McCarty [4], but differs from the results reported from experiments with chromatophores from photosynthetic bacteria [7] and rat liver [5] or plant [6] mitochondria. The latter studies indicated that a particular rate of ATP synthesis was not associated with a unique value of Δp. Thus it was reported that a given reduction in the rate of ATP synthesis was associated with a larger drop in Δp when an uncoupler was present compared with an inhibitor of electron transport. Comparison of experiments in which such findings were made [5–7] with the data of Portis and McCarty [4] and the present paper, shows that discrepancy principally arises in the measurements of Δp and the rate of ATP synthesis in the presence of an uncoupler. In fact in all published works it is reported that reductions in the rate of electron transport are accompanied by small decreases in Δp. It has been argued that this is a physiological necessity that prevents decreases in the ATP content, ion and substrate gradients and other reactions in cells where electron transport is operating at a submaximal rate [2,3]. Reasons why Δp should have been underestimated in the presence of an uncoupler are not easy to envisage. Possibly, in the case of experiments in which Δp was determined using rapid centrifugation of mitochondria [5], an underestimate for Δp arose owing to concentration of the uncoupler within the sedimenting mitochondria, acceleration of the rate of anaerobiosis and efflux of the probes used for the estimation of Δp.

The reports of correlation of a given rate of

ATP synthesis with different values of Δp depending on the experimental conditions have led to suggestions of the existence of intramembrane proton circuits between the electron transport chain and the ATP–synthase complexes [8], or, alternatively, that, even though Δp is the intermediate between electron transport and ATP synthesis, other factors, including perhaps a direct regulatory effect of the electron transport chain on the ATP-synthase, might regulate the rate of ATP synthesis [16,17]. The results here do not require the postulate of any factor additional to Δp to determine the rate of ATP synthesis.

In a previous paper from this laboratory [18], it was reported that inhibition by approx. 50% of succinate oxidation with malonate in SMP caused a proportional inhibition of the rate of ATP synthesis without any detectable decrease in the magnitude of Δp. On the contrary, we now find that 50% inhibition of the rate of ATP synthesis is associated with a small diminution of Δp (by approx. 10 mV). Indeed the very small changes in Δp, associated with substantial inhibition of ATP synthesis, can make difficult the task of correlating properly Δp with rates of ATP synthesis. In addition, another factor to consider is that in [18] a high ionic strength medium containing sucrose was used, in which both $\Delta\psi$ and ΔpH components of Δp were measured. Consequently a small change in Δp would be reflected in smaller changes in each of the $\Delta\psi$ and ΔpH values. Although reinspection of our earlier data gives no grounds for altering the estimates of $\Delta\psi$ and ΔpH, we have to suggest that a decrease in Δp of around 10 mV at 50% inhibition of ATP synthesis (fig.1 of this paper) might have escaped detection when divided between $\Delta\psi$ and ΔpH components [18]. An extra consideration is that there is now evidence [19] that $\Delta\psi$ and ΔpH may be both overestimated in the sucrose medium owing to binding of the probes for $\Delta\psi$ and ΔpH to the membranes. Any such overestimation would also have contributed to mask small changes in Δp [18].

Fig.1 also shows that the rate of ATP synthesis tended towards zero, but was not abolished, at a value of $\Delta\psi$ of approximately 110 mV. The trend of the relation between Δp and the rate of ATP synthesis, as shown in fig.1, is similar to that found in *Paracoccus denitrificans* membrane vesicles [20]. This type of behaviour warrants consideration as to whether a kinetic threshold value, below which ATP synthesis is abolished, truly exists for ATP-synthase in conditions under which there is no thermodynamic constraint from the magnitude of the phosphate potential. In this respect it is of interest that the lowest value of Δp tested in the experiments reported in fig.1, at which ATP synthesis still occurs, is lower than that found in some other comparable studies with energy transducing membranes [5–7,21], and also lower than the values reported for experiments in which a voltage pulse [22] or artificially imposed Δp [23] are imposed in submitochondrial particles.

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