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MEASUREMENT OF THE INTRAMITOCHONDRIAL P/O RATIO

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SUMMARY

Measurements of the initial rate of ATP synthesis and the initial rate of oxygen consumption in mitochondria in which transport of ADP, Pi and ATP were inhibited were used to obtain a value for the intramitochondrial P/O ratio. With succinate as substrate this method yielded a P/O ratio of 2.8 for the phosphorylation of intramitochondrial ADP.

INTRODUCTION

The question of whether the entry of phosphate and ADP into the mitochondrial matrix and exit of ATP from the matrix during oxidative phosphorylation constitute an obligatory energy-requiring system has been considered by a number of authors, notably Mitchell (1), and Klingenberg (2). More recently Brand and Lehninger (3), Alexandre et al. (4), and Hinkle and Yu (5) have presented models in which the inward translocation on the phosphate and adenine nucleotide carriers of $1H^+$ per ATP synthesized is an obligatory reaction during oxidative phosphorylation of cytoplasmic ADP. This obligatory H^+ transport arises because of the electroneutral entry of $1H^+$ with phosphate on the phosphate carrier (6) and the net outward movement of one negative charge associated with exchange of ADP^{3-} for ATP^{4-} on the adenine nucleotide carrier (7,8).

As emphasized by Klingenberg and associates (2,7,8), this input of energy into translocation reactions is responsible for the observed difference in phosphorylation potential between the

Abbreviation: EGTA, ethyleneglycolbis(aminoethyl)tetraacetate

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mitochondrial matrix and the cytosol, and means that a substantial proportion of the energy passed to the cytosolic ATP pool during oxidative phosphorylation comes not from the synthesis of ATP per se but from coupled phosphate and adenine nucleotide transport. This process may constitute 1/3 (3,5) or 1/4 (4) of the total energy input, depending upon whether translocation of 3 or 4 H^+ is required during synthesis of one ATP.

A simple prediction of these models is that if oxidative phosphorylation is allowed to proceed without concomitant transport reactions, more of the H^+ flux will be directed through the ATPase than would otherwise be possible, and thus a greater amount of ATP should be formed per oxygen consumed. Thus in the absence of transport of substrates and products of the ATPase reaction the P/O ratio should be increased. Assuming a stoichiometry of $2H^+$ imported by the ATPase per ATP synthesized (3,9,10), and an H^+/O ratio of 6 for succinate (11,12) the P/O ratio for succinate should rise from 2.0 to 3.0. With an ATPase stoichiometry of $3H^+/ATP$ (4) and an H^+/O ratio of 8 for succinate (13-15) the P/O ratio for succinate should rise from 2.0 to 2.67 for synthesis of ATP without import and export of ADP, Pi and ATP.

Transport reactions may be dissociated from oxidative phosphorylation either by working with inverted submitochondrial particles, in which case the ATPase is directly accessible to added ADP and phosphate, or by working with intact mitochondria, measuring the reaction of intramitochondrial ADP and phosphate to form intramitochondrial ATP. In both cases inhibitors of adenine nucleotide and phosphate transport need to be added. I have adopted this second approach, and present results indicating that the intramitochondrial P/O ratio for succinate is indeed greater than 2.0, apparently reaching values of about 2.8. These results have been presented briefly (16) and are supported by those of Duszyński et al. (17), who found that the intramitochondrial P/O ratio was higher than the extramitochondrial P/O ratio using an indirect method.

MATERIALS AND METHODS

Rat liver mitochondria with a respiratory control ratio of 4 to 5 with succinate as substrate were prepared by conventional methods in 250mM sucrose/1mM EGTA/5mM Tris-HCl, pH 7.4, from rats which had been starved overnight. Protein was measured according to (18).

ATP production was measured from experiments carried out in the barrel of a 10ml RePette dispensing syringe containing a magnetic stirring bar which gave efficient mixing (< 0.2 sec). The reaction was initiated by addition of succinate with a microsyringe through the tip of the dispensing syringe, 0.5ml samples were then ejected into 75µl 1.6M perchloric acid at 1 second intervals timed manually. After centrifugation, neutralization and recentrifugation, ATP was assayed using the luciferase-luciferin system in a DuPont 760 Luminescence Biometer.

Oxygen consumption was measured in parallel incubations carried out in an oxygen electrode cell (Rank Bros., Bottisham, Cambridge, England). The electrode was fitted with a thin teflon membrane to decrease the response time, and was calibrated as described by Robinson and Cooper (19).

RESULTS

The basis of the method used was to preincubate rotenone-treated mitochondria with phosphate to ensure an adequate intramitochondrial phosphate pool, then to inhibit the phosphate carrier with mersalyl and the adenine nucleotide carrier with atractyloside to prevent any transport of these compounds during the subsequent reaction. The addition of a low concentration of succinate then initiated electron transport with concomitant phosphorylation of the intramitochondrial ADP pool. Dissipation of the electrochemical H^+ gradient ($\Delta\mu H^+$) by calcium cycling was prevented by the presence of EGTA. Dissipation by H^+ leakage was minimized by using a low succinate concentration which, together with any slight inhibition of the dicarboxylate carrier by the relatively low amounts of mersalyl added, ensured that H^+ ejection by the respiratory chain was rate-limiting. Thus $\Delta\mu H^+$ presumably did not reach a very large value, and the H^+ leak, which is a function of $\Delta\mu H^+$, was also small. That the oxidation of succinate was rate limiting was shown by the lack of stimulation of oxygen consumption following addition of uncoupler. The P/O ratio was estimated following rapid sampling and assay of total ATP and monitoring of oxygen consumption in exactly parallel experiments.

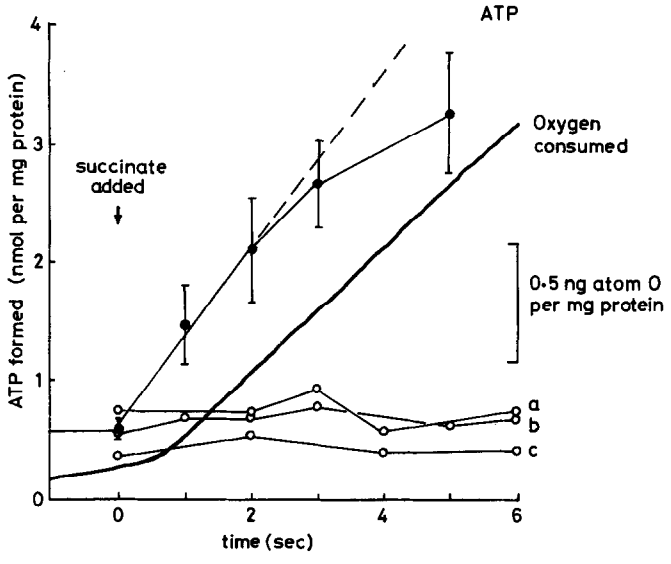

Figure 1. Oxygen consumption and phosphorylation of intramitochondrial ADP.
Mitochondria (17.5 mg protein) were suspended in 5 ml of medium containing 120mM KCl/3mM Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid)/ 2mM potassium phosphate/1mM EGTA/40μM potassium atractylate/10μM rotenone at pH 7.2, 23°. After 3 min 15 nmol mersalyl/mg protein was added. After a further 1 min a 500μl sample was taken and quenched in perchloric acid and then 200μM potassium succinate was added and further 500μl samples were taken at 1 second intervals. Samples were assayed for ATP as described in the experimental section. Oxygen consumption was measured in exactly parallel experiments. Where indicated (a) 1μg oligomycin/mg protein, (b) 1μg antimycin/mg protein or (c) 2.5mM malonate were added at the start. Error bars represent S.D. of 5 separate experiments. The rate of ATP synthesis was 45 nmol/min/mg protein, the rate of oxygen consumption was 16.2 ng atom/min/mg protein, giving an apparent P/O ratio of $\frac{45}{16.2}$ = 2.78.

The result of one such experiment is shown in Figure 1. Because of the small pool of ADP within the matrix the rate of phosphorylation was linear for only a few seconds before it decreased. The line shown is a least-squares plot using the first three data points, giving a rate of ATP synthesis of 45 nmol/min/mg protein. The corresponding rate of oxygen consumption from 4 experiments was 16.2 $\pm$ 0.4(S.D.) ng atom O/min/mg protein with no corrections applied. The measured intramitochondrial P/O ratio for succinate was therefore 2.78. Analysis of a number of such experiments gave a value of 2.8 $\pm$ 0.2(S.D.). The controls show that there was negligible ATP synthesis in the presence of antimycin, malonate, or oligomycin.

Similar experiments were carried out with the mersalyl omitted so that phosphate transport could occur. These gave a value of 1.55 $\pm$ 0.2(S.D.) for the intramitochondrial P/O ratio for succinate. In other experiments both mersalyl and atractyloside were omitted, and phosphorylation of extramitochondrial ADP (100 nmol/mg protein) was measured, giving a P/O value of about 1.7.

The intramitochondrial P/O ratio was also measured by coupling matrix ATP formation to carbamoyl phosphate synthase and ornithine carbamoyltransferase in mitochondria from rats in which these enzymes had been maximally induced by a high-protein diet, so that a steady-state turnover of ATP led to a continuous synthesis of citrulline from added ornithine, NH_3 and CO_2. Comparison of the rate of citrulline synthesis with that of oxygen consumption over a 2 min period in the presence of mersalyl, atractyoside and EGTA led to an intramitochondrial P/O ratio for succinate of 1.4 $\pm$ 0.2(S.D.). The reason for this low value is possibly an inadequate rate of ATP turnover by the citrulline synthesis system with consequent diversion of H^+ to pathways other than ATP synthesis.

DISCUSSION

The results presented here indicate an intramitochondrial P/O ratio for succinate of 2.8, in contrast to the P/O value of less than 2 obtained when transport reactions were not prevented.

There are, however, several potential sources of error in the approach used in this paper. It is possible that the relatively slow response time of the oxygen electrode may cause an underestimation of the initial rate of oxygen consumption leading to an overestimate of the P/O ratio. However, since oxidation of added succinate was rate-limiting it does not seem likely that the true initial rate of oxygen consumption was greater than the rate a few seconds later. The oxygen uptake rates were linear for 10 - 20 sec after the initial lag of 0.5 - 1.0 sec. The possibility that the true oxygen consumption rate lagged significantly behind the rate

of electron flow through the proton-translocating regions of the respiratory chain due to initial changes in the redox state of chain components cannot be entirely ruled out. The small size and rapid consumption of the intramitochondrial ADP pool made it necessary to take the ATP synthesis rate from only a few time points and may also have led to inaccuracies.

A potential theoretical problem during net ATP synthesis in any small compartment (which might also affect measurement of P/O ratios in other systems, e.g. in bacteria (20)), is that ATP synthesis is accompanied by scalar alkalinization of that compartment. If the pH gradient set up in this way could be used for further ATP synthesis this would cause a substantial overestimate of the true P/O ratio (by up to about 50%). However, in the absence of movements of a counter-ion to charge-balance the required H^+ influx this effect may be expected to have been small in the experiments described here.

I therefore conclude that the intramitochondrial P/O ratio for succinate is greater than 2, and probably lies in the range 2.6 to 3.0. The method used is not sufficiently accurate to distinguish between predicted values of 2.67 (4) and 3.0 (3). These high P/O ratios for reaction of intramitochondrial ADP and phosphate to form intramitochondrial ATP lend support to the suggestion that translocation of phosphate and adenine nucleotides is a normal energy-requiring step during oxidative phosphorylation in intact mitochondria.

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