ON THE RELATIONSHIPS BETWEEN THE STOICHIOMETRY OF OXIDATIVE PHOSPHORYLATION AND THE PHOSPHORYLATION POTENTIAL OF RAT LIVER MITOCHONDRIA AS FUNCTIONS OF RESPIRATORY STATE

E. Jack DAVIS, Lawrence LUMENG and Donald BOTTOMS
Departments of Biochemistry and Medicine, Indiana University School of Medicine
and
V.A. Hospital Indianapolis, Indiana 46202 U.S.A.

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1. Introduction

The characteristics of the mitochondrial adenine nucleotide exchange carrier have been studied in detail (see e.g. ref. [1]). Exchange of ADP and ATP across the mitochondrial inner membrane, mediated by this carrier, appears to be the rate-limiting step for oxidative phosphorylation under conditions which obtain in vivo [1–3].The asymmetry in the specificity of the transport of adenine nucleotide results in an asymmetric distribution of ADP and ATP on the two sides of the membrane, the ATP/ADP ratio being greater outside the mitochondria than inside [1, 4, 5]. As predicted by Klingenberg and co-workers [4] the formation and maintenance of this gradient of phosphorylation potential (ATP/ADP × P_i) would be expected, on thermodynamic grounds, to consume metabolic energy.

The present communication reports the results of experiments carried out with liver mitochondria under conditions in which respiration is controlled by a constant and limiting supply of ADP, in such a manner that in vivo conditions are closely simulated: i.e., (a) a high extramitochondrial phosphorylation potential is maintained, and is constant throughout an incubation; and (b) the concentrations of inorganic phosphate and of extramitochondrial adenine nucleotides are held constant and within the physiological range of concentration. The data are interpreted as being a direct estimation of energy requirement for maintenance of a high cytosolic phosphorylation potential. The results further indicate that 'state 4' respiration is mainly accounted for by the energy requirement for maintenance of a high extramitochondrial phosphorylation potential.

2. Experimental

Rat liver mitochondria were prepared according to Johnson and Lardy [6], and highly purified mitochondrial ATPase from bovine heart mitochondria was isolated by a modification (Dr. J. Fessenden-Raden, unpublished) of the method described by Penefsky [7]. The activity of different preparations of this ATPase was 40–80 enzyme units/mg of protein when assayed at 25°C. Respiration studies were carried out in a Gilson 'oxygraph'. When mitochondria were to be separated from the medium, incubations were carried out under oxygen. However, identical results were obtained when incubations were in an air atmosphere. Rapid separation of mitochondria was attained by rapid centrifugation through Silicone oil into 2 M $HClO_4$, essentially as previously reported [8], except that an Eppendorf microcentrifuge, modified with a light-weight swing-out head was used. Intra- and extramitochondrial adenine nucleotides were separated and counted essentially as described by Heldt et al. [4]. Extra-matrix water was determined from the amount of radioactivity in the mitochondrial pellet after incubation with [^{14}C] sucrose. Matrix water was taken as 1.2 μl/mg of protein [4]. In-

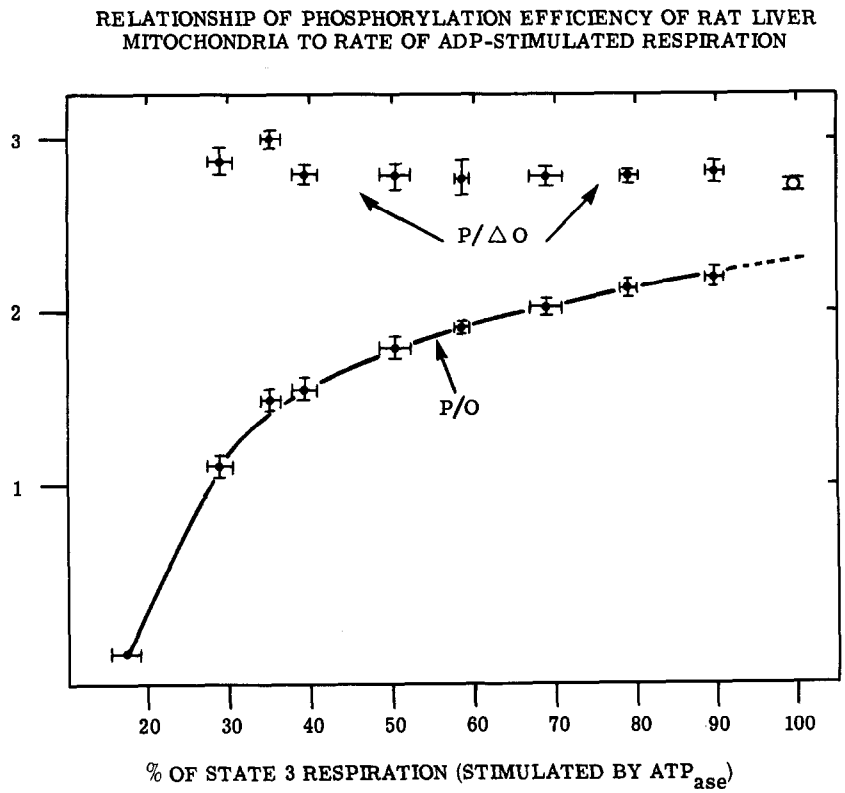

Fig. 1. Correlation of the yield of oxidative phosphorylation of mitochondria as a function of respiratory state. Rat liver mitochondria (2 mg protein/ml) were incubated at 30°C in a medium containing 66 mM KCl, 33 mM Tris–HCl, 2 mM potassium phosphate, 2 mM metal ion-free ATP, 6.6 mM $MgCl_2$, 10 mM K-glutamate and 2 mM K-malate to a final volume of 2.0 ml and a pH of 7.4, in a Gilson 'oxygraph'. After a period of 2 min, varying amounts of purified ATPase were added in order to obtain a desired degree of respiratory stimulation. P/O ratios were calculated from the amounts of ATPase added and the rates of respiration after addition of ATPase. P/ΔO ratios represent the ratios of ATPase activity to the increments of respiration resulting from ATPase activity. The individual points represent the means of 4 to 6 individual incubations, with S.E.M. (vertical lines) and the ranges of rates of respiration effected by a given amount of ATPase (horizontal lines). The P/O obtained on addition of 960 nmoles of ADP (corrected for AMP) is shown by the small circle ± S.E.M. from 18 determinations.

organic phosphate was determined colorimetrically according to Baginski et al. [9]. The activity of purified ATPase was assayed by following the oxidation of NADH in the presence of phospho*enol*pyruvate, pyruvate kinase and lactic dehydrogenase. Since it was important to know the ATPase activity with great accuracy for the calculations of phosphorylation efficiencies, the ATPase assays were carried out in media which were identical to those used for mitochondrial incubations. The results obtained were identical, whether the assays were carried out in the absence of mitochondria, or with mitochondria plus rotenone and atractyloside (2 μg/mg mitochondrial protein).

ADP was standardized spectrophotometrically using phosphopyruvate, pyruvate kinase and lactate dehydrogenase (ADP content) followed by adenylate kinase (AMP content). The terminology for mitochondrial respiratory states of Chance and Williams [10] is used.

3. Results and conclusions

Fig. 1 shows the results of a series of experiments in which the respiration of liver mitochondria oxidizing glutamate plus malate was stimulated under

Table 1
Calculated gradients in phosphorylation potentials inside and outside the mitochondrial matrix as a function of respiratory state.

Resp. state (% of state 3)	ATP/ADP	$[P_i]$ mM (outside)	ΔG_c (kcal/mole)	ATP/ADP	$[P_i]$ mM (inside)	ΔG_c (kcal/mole)	ΔG_c (out)$-\Delta G_c$ (in) (kcal/mole)
20 (State 4)	82	2	6.27	3.7	17	3.18	3.09
44	64	2	6.12	3.9	16	3.18	2.94
66	30	2	5.68	3.8	14	3.30	2.38
79	26	2	5.59	3.8	13	3.34	2.25

Incubation conditions were identical to the Experiments in fig. 1, except that they were carried out in a shaking water bath under 100% oxygen. Reactions were initiated by addition of various amounts of ATPase, and were terminated after 3 and 6 min in parallel incubations either by direct addition of $HClO_4$, or by rapid centrifugation of the mitochondria through silicone into $HClO_4$. Since the ATP/ADP ratios, as well as phosphate concentrations, were essentially identical at the two time periods, the numbers in the table represent the mean of the two values.

standard conditions by the addition of increasing amounts of ATPase. The P/O ratio (bottom curve) was calculated from the known activity of ATPase, and the rate of respiration attained after addition of a given amount of ATPase. As seen from this curve, the P/O ratio increases from zero (state 4) to a value (extrapolated) of about 2.33 at state 3. However, the phosphorylation associated with ATPase-stimulated respiration ($P/\Delta O$) was, within experimental error, a constant at all increments of respiration between state 4 and state 3 and, in all cases, the $P/\Delta O$ ratio was near 3.0. In contrast, when a standard amount of ADP was added in a series of incubations (small circle), the mean observed ADP/O ratio was 2.79 ± 0.03 (n = 18 ± S.E.M.). In another set of experiments carried out as in fig. 1 (not shown), in which 10 mM succinate was substrate (in presence of rotenone), the extrapolated P/O value was 1.46, the calculated $P/\Delta O$ for five ATPase-stimulated states of respiration between state 4 and 88% of state 3 were all near 2.0 (range, 1.82–2.14), and the P/O determined with 660 nmoles of added ADP was 1.77 ± 0.05 (n = 12).

Table 1 summarizes the results of a typical experiment in which the difference in phosphorylation potentials outside and inside the mitochondrial matrix space are calculated, assuming that the standard free energy of hydrolysis of ATP ($\Delta G_0'$) is the same in both compartments, and that the measured concentrations are equivalent to activities. Thus, if the above assumptions are correct, the difference (out *minus* in) in the concentration terms (ΔG_c) represents the difference in phosphorylation potentials of the two compartments, where $\Delta G_P = \Delta G_0' + 1.36 \log [ATP]/[ADP][P_i]$. As shown in this table the calculated ΔG_c inside the mitochondria in state 4 is about 3 kcal/mole less than that in the suspending medium. This value is slightly higher than the value calculated by Klingenberg and co-workers [1, 4], and slightly lower than the value found by Slater et al. [5], owing presumably to slightly different experimental conditions. As respiration is increased toward state 3, the gradient in phosphorylation potential is largely maintained, with the gradient in ΔG_c dropping by only about 0.8 kcal/mole between state 4 and 79% of state 3. It is also interesting to note that the measured mitochondrial ATP/ADP ratio does not change as a function of the respiratory state. The possibility that this constancy is an artifact due to limitation in the rapidity of stopping the reactions has been excluded, since the ratio of extractable NADH and NAD^+ varies as a function of respiratory state, whether the reaction is stopped immediately with acid, or if the mitochondria are centrifuged through silicone (L. Lumeng and E.J. Davis, in preparation).

The following important conclusions can be drawn from these results: 1) So long as the cellular consumption of ATP does not exceed the capacity of the mitochondria for oxidative phosphorylation, the cytosolic phosphorylation potential is maintained at a value substantially higher than that of the mitochondria; 2) The non-phosphorylating respiration observed under state 4 conditions persists at higher rates of respiration.

This is accounted for at least in part by the energy required to maintain a high external phosphorylation potential, as has been suggested [4, 11]. This conclusion is supported by a recent communication by Ogata et al. [12], who reported that there is a basal, time-independent increment of non-phosphorylating respiration of mitochondria during state 3 respiration imposed by varying amounts of added ADP. It is suggested by these authors that this 'extra' respiration is used for mitochondrial transitions from state 3 to state 4; and [3]. Since the mitochondrial ATP/ADP ratio appears to be independent of the respiratory state per se, it is suggested that the *extra*-mitochondrial phosphorylation potential (dictated in part by the mitochondria) and the *intra*-mitochondrial reduction potential ($NADH/NAD^+$) are both reflections of the energetic state of the mitochondria.

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