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RELATIONSHIP BETWEEN THE ENERGY COST OF ATP TRANSPORT AND ATP SYNTHESIS IN MITOCHONDRIA *

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Liver mitochondria from rats kept on a high-protein diet exhibit an increased rate of respiration upon addition of ornithine in the presence of HCO_3^- and NH_4^+. This is the manifestation of intramitochondrial utilization of ATP for the synthesis of citrulline. State 3 respiration of these mitochondria could be adjusted to the same rate as that produced by ornithine by either using limiting amounts of hexokinase or titration with atractyloside. Under such conditions, in both systems the proton-motive force, the intramitochondrial ATP/ADP ratio and the redox state of the respiratory chain were the same. In contrast to this, the ATP/O ratio (equal to 2 × citrulline/O ratio) in mitochondria synthesizing citrulline was higher than the glucose 6-phosphate/O ratio in the system where ATP was trapped extramitochondrially. The ratio of these two ratios was close to 1.5 with both glutamate and succinate as respiratory substrates. From these results it can be concluded that the translocation of ATP against ADP and phosphate utilizes an amount of the chemiosmotic proton gradient equal to half of that needed for the synthesis of ATP in the inner compartment.

Introduction

Isolated mitochondria are able to phosphorylate external ADP against a high phosphorylation potential [1,2]. The energy required for this process can be calculated from the relationship:

$$\Delta G = \Delta G'_o + 2.3RT \log \frac{[ATP]}{[ADP] \cdot [P_i]} \quad (1)$$

where $\Delta G'_o$ is the standard free energy of ATP synthesis, R the gas constant and T the absolute temperature. It is well established that ΔG for the extramitochondrial compartment is higher than that for the matrix compartment, since, in the resting state, the phosphorylation potential $[ATP]/[ADP] \cdot [P_i]$ outside mitochondria is much higher than inside [1–4]. The difference represents the energy required for the inward transport of ADP and P_i and the outward transport of ATP [3]. This energy cost has been evaluated as 2–4.5 kcal/mol [1,2,5].

It has been shown [6] that the translocation of ATP against ADP in the inner mitochondrial membrane is fully electrogenic and, therefore, the combined action of the adenine nucleotide carrier and the phosphate carrier in mitochondria phosphorylating external ADP is associated with the inward transport of one vectorial proton per molecule of ATP exported. It can be thus expected that the efficiency of oxidative phosphorylation should be higher when the formation of internal ATP is taken into account

* A preliminary report of this study has already appeared [9]. Abbreviations: TPMP, triphenylmethylphosphonium bromide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

as compared to the situation when ATP synthesized intramitochondrially is subsequently transported to the external compartment. In the latter case, the translocation utilizes a portion of the electrochemical proton gradient which could otherwise be used for ATP formation.

To verify experimentally this assumption we compared the efficiency of oxidative phosphorylation in mitochondria with extra- and intramitochondrial ADP-regenerating systems. The latter was represented by mitochondria synthesizing citrulline, the process occurring in the matrix compartment and utilizing two molecules of ATP for each molecule of citrulline formed. Citrulline production is enhanced in animals kept on a high-protein diet [7] and, indeed, it has been found [8] that only in mitochondria from such rats is the respiration rate significantly increased upon addition of ornithine in the presence of HCO_3^- and NH_4^+. As the extramitochondrial ATP-utilizing system the glucose-hexokinase trap was used. Comparison of the phosphorylation efficiency in these two systems may provide a novel approach to the evaluation of the energy cost of ATP-ADP translocation and of the relationship between ATP synthesis and proton transfer in coupled mitochondria.

Materials and Methods

Rat liver mitochondria were prepared [10] from animals fed with a high-protein diet (75 g casein, 25 g starch, 2.5 g sunflower oil and 1.5 g NaCl) ad libitum. Mitochondria were suspended in 300 mM mannitol. Protein was determined by the biuret method using bovine serum albumin as standard. Citrulline was assayed colorimetrically [11]. Adenine nucleotides were determined by standard enzymatic assays in neutralized $HClO_4$ extracts [12]. Mitochondrial respiration was measured with a Clark-type electrode. All incubations were carried out at 25°C. Mitochondria were incubated in the following standard medium: 50 mM KCl, 20 mM $KHCO_3$, 5 mM KH_2PO_4, 10 mM glutamate or succinate, 15 mM glucose, 0.5 mM EGTA, 2 mM $MgCl_2$, 10 mM NH_4Cl and 25 mM Tris-HCl (pH 7.2) which was gassed with a mixture of 95% O_2 and 5% CO_2. Intra- and extramitochondrial adenine nucleotide profiles were determined in whole extracts without separation of the mitochondria from the medium as described previously [4]. The transmembrane potential ($\Delta\psi$) and the pH gradient between the matrix and the external medium (ΔpH) were calculated from the distribution of [^{3}H]TPMP [13] and [^{14}C]acetate [14], respectively. The matrix space was calculated from the difference between the ^3H_2O space and the [^{14}C]sucrose space.

[*methyl*-^{3}H]TPMP was obtained from New England Nuclear (Boston, MA., U.S.A.), other radiochemicals were from The Radiochemical Centre (Amersham, U.K.) and the Institute of Nuclear Research (Swierk, Poland). Chemicals and biochemicals were of the highest purity commercially available.

Results

Fig. 1 shows that, in the medium containing HCO_3^- and NH_4^+, the respiration of mitochondria isolated from rats kept on a high-protein diet can be markedly stimulated not only by ADP but also by ornithine. The rate of respiration obtained with ornithine usually amounted to 50–60% of that produced by excess of glucose plus hexokinase. The rate of mitochondrial respiration in the glucose-hexokinase system could be easily adjusted to any of the intermediate levels between the active and the resting states by titration with either hexokinase or the inhibitor of adenine

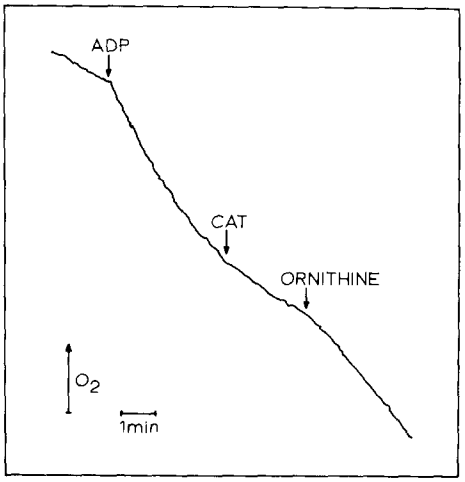

Fig. 1. Stimulation of the respiration rate by ADP and ornithine. Oxygraphic trace. Mitochondria (2 mg protein) were suspended in 1.0 ml of the standard medium with glutamate as substrate. Additions: 0.23 mM ADP, 5 μM carboxyatractyloside (CAT) and 10 mM ornithine.

TABLE I

MEMBRANE ENERGY STATE OF MITOCHONDRIA RESPIRING AT VARIOUS ENERGY DRAINS

Mitochondria (8.5 mg protein) were suspended in 1.1 ml of the standard medium with glutamate as substrate and containing 0.15 mM ADP, 0.1 mM [^{14}C]acetate and 0.15 nM [^{3}H]TPMP. Where indicated, 10 mM ornithine or hexokinase was added. After 3 min at 25°C samples were layered on silicone oil and centrifuged. Radioactivity was measured in the bottom $HClO_4$ extracts and the upper supernatants. Sucrose and water spaces were measured in parallel runs; they amounted to 2.2 and 2.9 μl/mg, respectively. n.d., not determined.

Conditions	Respiration rate (ngatom O/min per mg protein)	$TPMP_{in}/TPMP_{out}$	$\Delta\Psi$ (mV)	$Acetate_{in}/acetate_{out}$	ΔpH (mV)
State 4	8.9	667	166	7.5	52
Ornithine	25.0	381	152	7.4	51
Limiting hexokinase	24.2	361	151	7.8	53
Excess of hexokinase (state 3)	45.2	287	145	n.d.	n.d.

TABLE II

INTERNAL ADENINE NUCLEOTIDES IN MITOCHONDRIA RESPIRING AT THE SAME RATE IN THE ORNITHINE AND THE GLUCOSE-HEXOKINASE SYSTEMS

Mitochondria were suspended in the standard medium containing 10 mM glutamate, 0.15 mM ATP and either 10 mM ornithine or limiting amounts of hexokinase as indicated. Adenine nucleotides (expressed in nmol/mg protein) were assayed after 5 min incubation at 25°C.

Additions	Respiration rate (ngatom O/min per mg protein)	ATP	ADP	AMP	ATP/ADP
Ornithine	25.0	10.2	3.1	0.4	3.3
Hexokinase	24.2	10.6	3.1	0.4	3.4

nucleotide translocase, atractyloside. This enabled us to compare mitochondrial respiring at the same rate in the ornithine and the glucose-hexokinase systems.

Table I shows the accumulation of [^{3}H]TPMP and [^{14}C]acetate in mitochondria respiring in these two systems. Although there are some doubts whether the accumulation of TPMP is a good measure of the absolute value of the transmembrane potential [15] because of possible binding of the compound to mitochondrial membranes, it remains a good tool for comparison of values in different metabolic states of the same mitochondrial preparation. As can be seen in Table I, in mitochondria respiring at equal rates, the proton-motive force, i.e., the sum of $\Delta\psi$ and ΔpH, is identical in both systems. Table II shows that in mitochondria with internal and external ADP-regenerating systems respiring at the same rate the intramitochondrial ATP/ADP ratios are also the same. Redox changes of nicotinamide nucleotides and cytochrome *c* produced by addition of ornithine and ADP were similar as well (Fig. 2). All these data indicate that the redox pressure and the 'back energy pressure' on the respiratory chain are the same in mitochondria in the ornithine and the glucose-hexokinase systems at equal rates of respiration.

In contrast to this, P/O ratios in both systems were different at the same respiration rate. This was demonstrated in experiments in which mitochondrial respiration in the presence of glucose and hexokinase was adjusted with atractyloside to a rate identical with that in the presence of ornithine. In an experiment illustrated in Fig. 3, P/O ratios with glutamate as substrate were 1.78 and 1.20 with ornithine and hexokinase, respectively, and the ratio of these two P/O ratios amounted to 1.48. In seven similar experiments the mean value of the ratio of P/O ratios with orni-

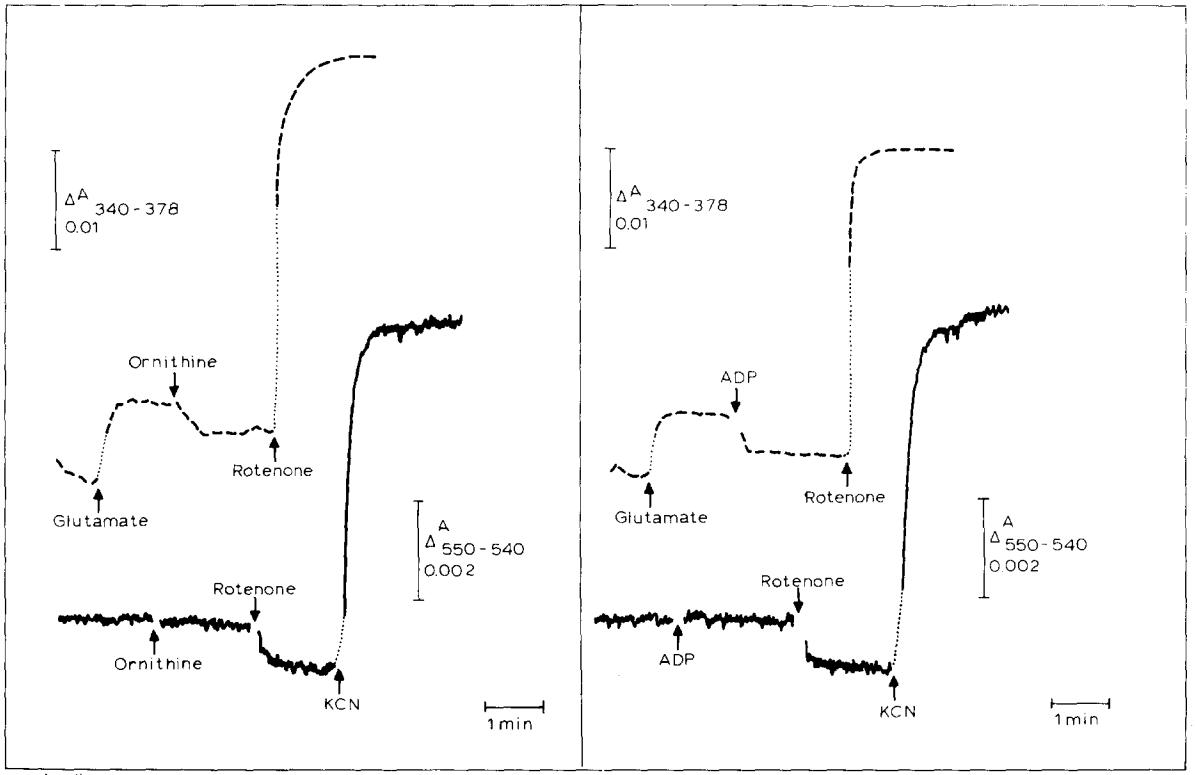

Fig. 2. Oxidation of nicotinamide nucleotides and cytochrome *c* upon addition of ADP and ornithine. Differential absorbance traces at 340–378 nm (dashed lines) and 550–540 nm (solid lines). Mitochondria (3.1 mg protein) were suspended in 2.0 ml of the standard medium. Measurements were made with continuous stirring in the cuvette. Additions: 10 mM glutamate, 10 mM ornithine, 2 mM ADP, 2 μM rotenone and 1 mM KCN. Mitochondria used in this experiment exhibited almost the same rate of respiration with ADP and ornithine.

thine and hexokinase was 1.58 ± 0.05 (±S.E.). With succinate as substrate the respiration in the glucose-hexokinase system was adjusted by limiting amounts of hexokinase. In the experiment shown in Table III mean values of the P/O ratio amounted to 1.09 and 0.73 with ornithine and glucose plus hexokinase, respectively. Thus, in all cases and with both respiratory substrates used the ratio between P/O ratios in the two systems was very close to 1.5.

P/O ratios obtained in this investigation were lower than those usually reported in the literature. The likely explanation of this fact is a submaximal rate of respiration, since (i) HCO_3^- produces a slight suppression of the respiration and (ii) in our hands the respiration in the presence of ornithine only seldom exceeded 60% of the full state 3 respiration. The dependence of the P/O ratio on the rate of respiration is a result of the competition between the flux of protons through the ATP-synthesizing system (ATPase and translocase) and the proton leak. This is verified by titrating mitochondria respiring at submaximal rates with an uncoupler (Fig. 4). Although the rate of respiration increased, the P/O ratio decreased because the proton leak was increased by the uncoupler.

Discussion

The results presented here clearly show that the net production of ATP per oxygen atom consumed is much higher when ATP is utilized inside the mitochondrion than when it is exported to the external compartment. To evaluate the relationship between the energy required for the synthesis of ATP and for its transport we shall discuss the problem in terms of 'chemiosmotic protons' rather than in usual energy

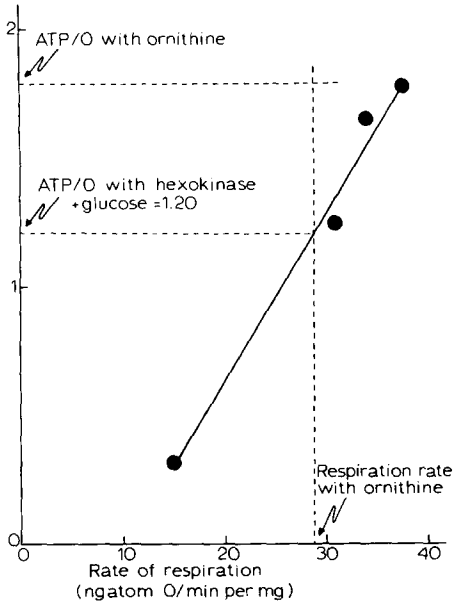

Fig. 3. Phosphorylation efficiency in the glucose-hexokinase and the ornithine systems. The standard medium contained 2.2 mg mitochondrial protein/ml and either 25 μM ADP, an excess of hexokinase and varying amounts of atractyloside (•), or 10 mM ornithine and 10 μM carboxyatractyloside. The rate of respiration with ornithine was 28 ngatom O/min per mg and the ATP/O ratio was 1.78. The ATP/O ratio for the glucose-hexokinase system corresponding to the same rate of respiration can be interpolated from the graph as 1.20. Ordinate: P/O.

units. Because of the electrogenic character of the ATP-ADP transfer [6], the concerted action of the adenine nucleotide translocase and the phosphate carrier results in a net uptake of one proton per molecule of ATP exported. In contrast to this, no energy in chemiosmotic terms is lost for the transport of metabolites in the citrulline system, since the ornithine$^+$-H$^+$ antiport [16] as well as the transfer of citrulline [16], CO_2 and NH_3 are electroneutral.

The proton fluxes in the two systems are schematically represented in Fig. 5, where p and p' denote the rate of proton extrusion, a and a' are proton fluxes through the ATPase, t designates the proton flux through the translocase plus the phosphate carrier and l and l' indicate proton leaks. For the glucose-hexokinase system we have the following relationship:

$$p = a + t + l \quad (2)$$

where for the ornithine system this is simplified to:

$$p' = a' + l' \quad (3)$$

At equal rates of oxygen uptake p and p' are equal. Therefore:

$$a + t + l = a' + l' \quad (4)$$

Furthermore, it seems feasible that the proton leak, whatever it represents, depends on the proton-motive force and the energy state of the mitochondrial membrane. Because the proton-motive force as well as the ATP/ADP ratio and the redox state of the respiratory chain appear to be identical in mitochondria respiring at the same rate, independently of the site where ATP is drained (Tables I and II and Fig. 2), it can be assumed that the leak is also identical in both systems ($l \approx l'$). Thus:

$$a + t = a' \quad (5)$$

TABLE III

P/O RATIOS IN MITOCHONDRIA RESPIRING AT THE SAME RATE IN THE ORNITHINE AND THE GLUCOSE-HEXOKINASE SYSTEMS

Mitochondria (3.1 mg protein) were suspended in 3.0 ml of the standard medium containing 10 mM succinate, 0.15 mM ATP and 1 μM rotenone. After addition of 10 mM ornithine or an appropriate amount of hexokinase aliquots were withdrawn to measure the rate of citrulline synthesis and glucose 6-phosphate formation.

	System	
	Ornithine	Glucose-hexokinase
Respiration rate (ngatom O/min per mg protein)	80.6	79.4
Citrulline formation (nmol/min per mg protein)	44.1	
Citrulline/O	0.547 [a]	
Glucose 6-phosphate formation (nmol/min per mg protein)		58.2
Glucose 6-phosphate/O		0.73

[a] Corresponds to P/O = 2 × 0.547 = 1.09.

or

$$\frac{t}{a} = \frac{a'}{a} - 1 \qquad (6)$$

Assuming that the synthesis of ATP in both systems is connected with the transfer of the same amount of proteins through the ATPase, the ratio a'/a can be substituted by the ratio of ATP/O in the ornithine system to ATP/O in the glucose-hexokinase system:

$$\frac{a'}{a} = \frac{(\text{ATP/O})_{\text{ornithine}}}{(\text{ATP/O})_{\text{glucose-hexokinase}}} = \frac{2 \times \text{Citrulline/O}}{\text{Glucose 6-phosphate/O}} \qquad (7)$$

It has been observed in this investigation that this ratio is very close to 1.5, e.g., it amounted to 1.48, in Fig. 3 and 1.49 in Table III. Thus, the t/a ratio is 0.5. This means that the combined transport of adenine nucleotides and phosphate in mitochondria phosphorylating external ADP produces an additional discharge of the proton gradient which is equal to half of the proton flux through the ATPase. Since the transfer of one molecule of ATP against one molecule of ADP and phosphate is associated with the flux of one proton, it follows that the synthesis of one molecule of ATP inside the mitochondrion utilizes two protons, i.e., the ratio H^+/ATP_{in} is 2. This is in good agreement with the H^+/ATP stoichiometry found for submitochondrial particles hydrolyzing ATP [17,18]. Therefore, it can be concluded that the energy cost of the export of ATP synthesized intramitochondrially is equal to half of the energy needed for the synthesis of ATP in the inner compartment. This is in agreement with estimations based on the difference of the phosphorylation potential between the matrix and the external compartment [19–21]. Furthermore, it follows that the H^+/ATP_{out} ratio is 3. This is an important conclusion in connection with the H^+/O ratio in the respiratory chain which has been very much disputed recently [22–26].

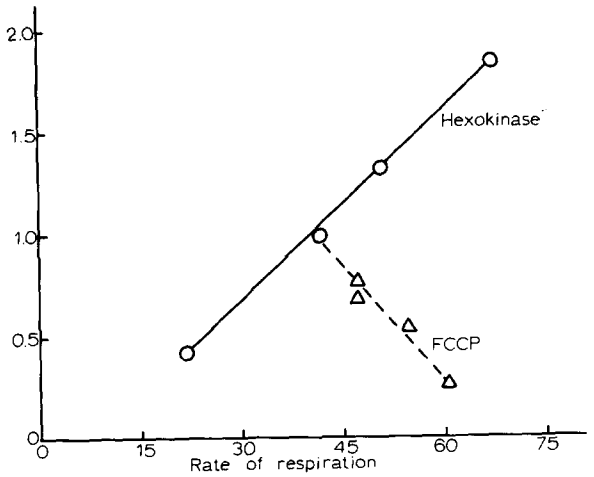

Fig. 4. Dependence of the phosphorylation efficiency on the rate of respiration at different states of energy coupling. Liver mitochondria (2.7 mg protein) were obtained for this experiment from rats fed with normal laboratory chow and were suspended in 1.1 ml of the standard medium containing 10 mM glutamate, 5 mM malate and 0.15 mM ADP. Mitochondrial respiration was stimulated either with increasing amounts of hexokinase (○) or with a limited amount of hexokinase plus increasing amounts of the uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (△). Ordinate: P/O.

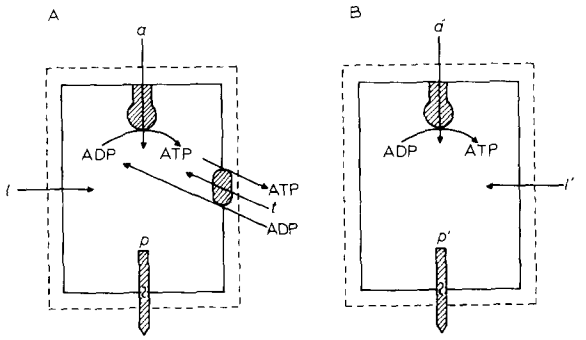

Fig. 5. Scheme of proton fluxes in mitochondria phosphorylating external and internal ADP.

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