DETERMINATION OF THE FREE-ENERGY DIFFERENCE OF THE ADENINE NUCLEOTIDE TRANSLOCATOR REACTION IN RAT-LIVER MITOCHONDRIA USING INTRA- AND EXTRAMITOCHONDRIAL ATP-UTILIZING REACTIONS

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1. Introduction

During mitochondrial oxidative phosphorylation oxidative free energy is transduced for the synthesis of ATP from ADP and phosphate. The rate of oxidative phosphorylation and consequently of oxygen consumption must be geared to the ATP requirements of the cell. Extensive studies have been carried out on the control of respiration in isolated mitochondria. Chance and Williams [1] proposed that the only factor controlling the rate of respiration is the availability of ADP, i.e., that kinetic regulation occurs. Klingenberg [2,3], however, proposed that the rate of respiration is thermodynamically controlled by the extramitochondrial phosphate potential. Slater et al. [4], Davis and coworkers [5–7] and Kunz and coworkers [8,9] concluded that the rate of mitochondrial respiration is controlled by the extramitochondrial ATP/ADP ratio, the adenine nucleotide translocator being the rate-limiting step. Wilson and coworkers (review [10]) concluded that the first two sites of the respiratory chain are in near-equilibrium with the cytosolic phosphate potential and that regulation of respiration occurs at the cytochrome oxidase step. The adenine nucleotide translocator, as an integral part of the near-equilibrium system, would therefore also have to operate near to thermodynamic equilibrium.

It is clear that there is considerable difference of opinion on the question of whether the adenine nucleotide translocator is or is not in near-equilibrium [11,12]. In rat-liver mitochondria, ATP can be utilized not only extramitochondrially, but also in the intramitochondrial compartment [13]. We have therefore studied the control of respiration under conditions where the adenine nucleotide translocator is or is not operating. The results obtained have enabled us to measure the free energy difference of the adenine nucleotide translocator reaction under flux conditions.

2. Materials and methods

Rat-liver mitochondria were isolated from male Wistar rats (200–250 g), as in [14], using an isolation medium containing 250 mM mannitol, 5 mM Tris–HCl (pH 7.4) and 0.5 mM EGTA. The final pellet was taken up in 250 mM mannitol. Rats were fed a high-protein diet (80% casein) for 3 days prior to sacrifice. These mitochondria contain elevated levels of *N*-acetylglutamate [15].

Mitochondria (1–2 mg protein/ml) were incubated at 25°C in a thermostatically controlled oxygraph vessel (1.6 ml) equipped with a Clark electrode in a medium containing the following standard components: 100 mM KCl, 50 mM Tris–HCl, 1 mM EGTA, 10 mM potassium phosphate, 10 mM succinate, 1 mM malate, 20 mM glucose, 16.6 mM $KHCO_3$, 2.0 mM ATP, 10 mM $MgCl_2$ and 2 μg/ml rotenone. The final pH was 7.4. Further additions were made after a 30 s equilibration period. After a further 2.5 min incubation period reactions were terminated. Since ATP decays slowly to ADP under acidic conditions [7], which can lead to an underestimation of the ATP/ADP ratio when the latter is high, special care was taken in handling the samples. The procedure was as follows: a 1 ml sample was taken from the oxygraph vessel and added to 0.25 ml cold perchloric acid (final conc. 3.5%, w/v) in a small Eppendorf tube (1.6 ml). After thorough mixing for a few seconds, the denatured protein was removed by rapid centrifugation for 25 s

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in an Eppendorf microcentrifuge (model 3200). A 1 ml sample was taken from the supernatant and added with vigorous stirring to a predetermined volume of 2 M KOH–0.3 M morpholinopropane–sulphonic acid (Mops) so that the final pH was 6.8–7.2. Neutralization was completed within 1 min after adding acid. Samples were frozen in liquid nitrogen and stored at –80°C. ATP, ADP and glucose-6-phosphate were measured spectrophotometrically or fluorimetrically in the neutralized acid extracts according to standard procedures [16]. Citrulline was determined colorimetrically according to [17]. The rate of oxygen uptake was calculated using the O_2 solubility data in [18]. Mitochondrial protein was determined by a biuret method [19]. The enzymes and other biochemicals were obtained from Boehringer Mannheim.

3. Results and discussion

To examine the relationship between the rate of oxygen uptake and the intra- and extramitochondrial ATP/ADP ratios under conditions of both intra- and extramitochondrial ATP utilization, we have used citrulline synthesis and glucose-6-phosphate synthesis as intra- and extramitochondrial ATP-utilizing reactions, respectively. Fig.1 shows the results of an experiment in which mitochondria were incubated in the standard bicarbonate medium containing 10 mM succinate; 1 mM malate was also added to keep the redox pressure approximately constant. Different amounts of hexokinase were added in the absence or presence of ammonia or ammonia plus ornithine. The rate of glucose-6-phosphate production was a linear function of the hexokinase concentration up to 0.15 U/mg mitochondrial protein and was unaffected by the presence of ammonia or ammonia plus ornithine. The lack of effect of intramitochondrial ATP utilization on glucose-6-phosphate formation is probably due to the low sensitivity of the essentially irreversible hexokinase reaction to Mg^{2+}-ATP and Mg^{2+}-ADP under these conditions. The rate of citrulline synthesis progressively decreased as the amount of hexokinase was increased, due to the high sensitivity of carbamoylphosphate synthetase to the intramitochondrial ATP/ADP ratio [20]. Fig.1 also shows that ammonia on its own is able to stimulate respiration. This effect of ammonia is not a consequence of its potential uncoupling action as a protonophore (like in chloroplasts [21]) since it is dependent upon the presence of bicarbonate and since it is abolished by addition of oligomycin to block respiratory chain phosphorylation (fig.2). Moreover, the effect of ammonia cannot be duplicated by methylamine, a weak base with very similar characteristics to those of ammonia (not shown). A simple explanation compatible with all observations is that ammonia stimulates respiration due to the oligomycin-sensitive, bicarbonate-dependent synthesis of carbamoylphosphate. The data in [22] support this interpretation.

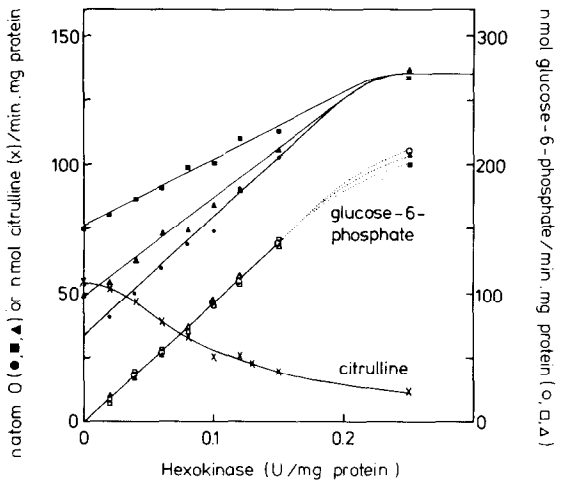

Fig.1. Effect of hexokinase concentration, ammonia and ornithine on oxygen uptake, glucose-6-phosphate formation and citrulline synthesis in rat-liver mitochondria. The mitochondria (1.53 mg protein/ml) were incubated in the standard reaction medium with different concentrations of hexokinase, without ammonia (○,●), with 5 mM ammonia (▲,△) or with 5 mM ammonia and 10 mM ornithine (□,■). O_2 uptake (closed symbols) was measured polarographically and glucose-6-phosphate (open symbols) and citrulline were measured in perchloric acid extracts. The results of a typical experiment are shown; similar results were obtained in 3 other experiments.

In fig.3A the rate of oxygen uptake (taken from fig.1) has been plotted as a function of the logarithm of the ATP/ADP ratio as calculated from the amounts of ATP and ADP measured in neutralized perchloric acid extracts of the incubation mixture (see section 2). A sigmoidal relationship is obtained. The observed sigmoidicity has been predicted both on kinetic [23] and on thermodynamic grounds [24,25]. It has been observed [26] in experiments with brown-adipose tissue mitochondria, but was not found [8,25] in rat-liver mitochondria. Part of the discrepancy may

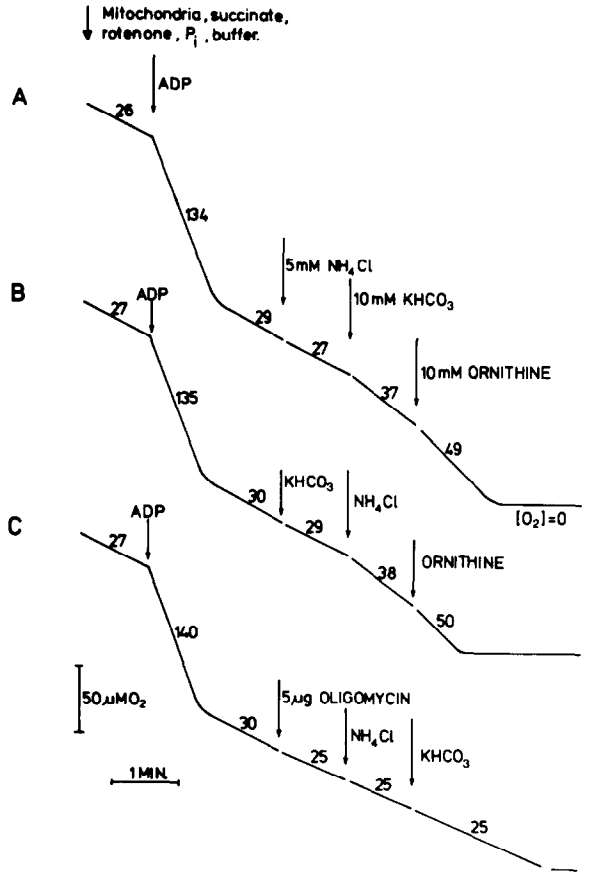

Fig.2.

Fig.2. Bicarbonate-dependence of stimulation of mitochondrial respiration by NH_4Cl and ornithine and sensitivity to oligomycin. Isolated rat-liver mitochondria (1.50 mg protein/ml) were incubated in the standard reaction mixture (see section 2) without bicarbonate. Aliquots of concentrated solutions of NH_4Cl, bicarbonate, ornithine and ADP were added as indicated to give the final concentrations shown in the figure. The numbers indicate rates of respiration in natom . min^{-1} . mg $protein^{-1}$.

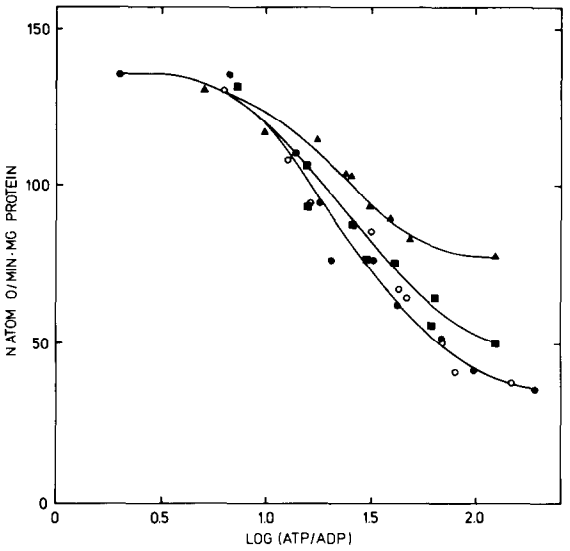

Fig.3A.

Fig.3. Relationship between rate of O_2 uptake and the intra- and extramitochondrial ATP/ADP ratio during intra- and extramitochondrial ATP utilization.

(A) Data from the experiment of fig.1. The extramitochondrial ATP/ADP ratio was calculated from the amounts of ATP and ADP found in the perchloric acid extracts of the reaction mixture (see text). The conditions were as follows: control (●); + ammonia (■); + ammonia + ornithine (▲); + methylamine + ornithine (○).

(B) Rat-liver mitochondria (1.93 mg protein/ml) were incubated in a reaction mixture containing the standard components, different concentrations of hexokinase either alone (○,●) or together with 5 mM NH_4Cl and 10 mM ornithine (△,▲). Intramitochondrial ATP and ADP were measured in neutralized perchloric acid extracts of the mitochondria after separation by centrifugation–filtration through silicone oil; the values were corrected for ATP and ADP present in adhering water. The left curve represents the intramitochondrial ATP/ADP ratio and the right curves the extramitochondrial ATP/ADP ratio, either uncorrected (closed symbols) or corrected for ATP and ADP in the mitochondria (open symbols).

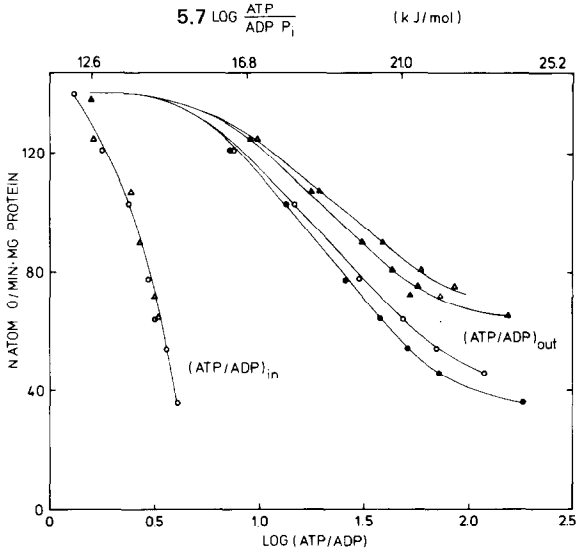

Fig.3B.

be due to underestimation of the ATP/ADP ratios (especially if the ratios are high) due to the slow hydrolysis of ATP to ADP that occurs if perchloric acid extracts are not rapidly neutralized (see section 2).

Fig.3A also shows that the curve relating oxygen uptake to the ATP/ADP ratio obtained in the presence of glucose plus hexokinase alone is shifted towards higher ATP/ADP ratios when, in addition, either ammonia or ammonia plus ornithine is present. In order to exclude the possibility that the shift observed was due to non-specific effects of ammonia and ornithine, control experiments were carried out with methylamine, a non-metabolizable analogue of ammonia. The curves obtained with ornithine plus methylamine coincided with the control curve (see fig.3A). Furthermore, in liver mitochondria from rats fed a standard low-protein diet the rate of citrulline synthesis is low (8–10 nmol . min^{-1} . mg $protein^{-1}$) and the shift is barely detectable (not shown).

An important distinction between the different sets of conditions in fig.3A is the extent to which the adenine nucleotide translocator is involved in the overall process of ATP utilization. Therefore, since the intramitochondrial adenine nucleotides behave as a common homogeneous pool [27] from which ATP is delivered for both adenine nucleotide translocation and carbamoylphosphate synthesis [20,28], it was of importance to determine the intramitochondrial ATP/ADP ratio under the different conditions. For this purpose the same experimental set-up was used as that in fig.1, except that at the end of the polarographic run an additional sample was taken and subjected to silicone oil centrifugation for the determination of the intramitochondrial adenine nucleotides. The measured intramitochondrial ATP/ADP ratios are slightly overestimated, particularly at low ATP/ADP ratios associated with high rates of oxidative phosphorylation, due to the fact that some phosphorylation occurs during centrifugation filtration through silicone oil [20]. The results of fig.3B show that the measured intramitochondrial ATP/ADP ratio is the same at one particular rate of respiration independent of the site(s) of ATP utilization (see also [29]).

In experiments such as that in fig.3A the concentration of mitochondrial protein was low and that of added ATP high. Thus the measured total amounts of ATP and ADP can be expected to give a good approximation of the extramitochondrial amounts of ATP and ADP (see also [8,25]. To determine the extramitochondrial ATP/ADP ratio more accurately the measured amounts of ATP and ADP in total extracts were corrected for the amounts found in the mitochondria after silicone oil centrifugation. This procedure could not be used to determine the correct extramitochondrial ATP/ADP ratio under state 4 conditions, since the mitochondrial contribution to ADP in the total extracts exceeded 95%. Fig.3B shows that the curve relating respiration to the extramitochondrial ATP/ADP ratio remains sigmoidal but is shifted slightly to higher ATP/ADP ratios compared to the curve obtained using uncorrected values.

In [30–34] it was shown that the translocation of adenine nucleotides across the mitochondrial membrane is dependent on the intra- and extramitochondrial ATP^{4-}/ADP^{3-} ratios and the membrane potential. Since the transport is fully electrogenic [34] the free-energy difference during adenine nucleotide translocation (ΔG_T) is given by:

$$\Delta G_T = F \,.\, \Delta\psi + 2.3\, RT \log \frac{[ATP^{4-}_{out}]/[ADP^{3-}_{out}]}{[ATP^{4-}_{in}]/[ADP^{3-}_{in}]} \quad (1)$$

To calculate ΔG_T directly, three parameters have to be determined: $\Delta\psi$; $(ATP^{4-}/ADP^{3-})_{out}$; and $(ATP^{4-}/ADP^{3-})_{in}$. However, the absolute magnitude of the membrane potential is a matter of controversy (discussed in [10]). Furthermore, the *free* intramitochondrial ATP^{4-} and ADP^{3-} concentrations have to be known. Uncertainties exist with regard to the free Mg^{2+} concentration in the matrix and the extent of binding of adenine nucleotides to mitochondrial proteins [35]. A method was therefore developed to circumvent these problems.

The principle of the method is the following. Two conditions were compared where the rate of O_2 uptake was equal:

(i) ATP was used extramitochondrially for glucose-6-phosphate formation, so that there was flux through the translocator.

(ii) ATP was used intramitochondrially for citrulline synthesis; furthermore, there was no Mg^{2+} present in the medium so that ATP hydrolysis by any extramitochondrial ATPases present in the preparation was not possible.

Thus in condition (ii) there was no flux through the translocator. Indeed, in condition (ii) carboxyatractyloside had no effect on the rate of respiration (not shown).

In condition (i) the equation for the translocator reaction is given by:

Table 1
Calculation of the free-energy difference of the adenine nucleotide translocator reaction

Parameter	Condition (i) (synthesis of glucose-6-phosphate)	Condition (ii) (synthesis of citrulline)
J_O (natom . min^{-1} . mg protein^{-1})	69.0	68.7
$([ATP_{out}]/[ADP_{out}])_{total}$	23.6	77.0
$([ATP^{4-}_{out}]/[ADP^{3-}_{out}])_{free}$	1.7	57.5

$\Delta G_T = 2.3\ RT \log (1.7/57.5) = -8.7$ kJ/mol

Mitochondria (1.53 mg protein/ml) were incubated in a reaction mixture containing the standard components (see section 2). In condition (ii), Mg^{2+} was omitted and 10 mM ornithine and 5 mM NH_4Cl were added. In condition (i), Mg^{2+} was present and sufficient hexokinase was added to give the same rate of respiration as in condition (ii)

$$(\Delta G_T)_1 = F\ .\ (\Delta\psi)_1 + 2.3\ RT\ \log \frac{([ATP^{4-}_{out}]/[ADP^{3-}_{out}])_1}{([ATP^{4-}_{in}]/[ADP^{3-}_{in}])_1} \quad (2)$$

In condition (ii) there is no flux through the translocator. Thus:

$$(\Delta G_T)_2 = 0 = F\ .\ (\Delta\psi)_2 + 2.3\ RT\ \log \frac{([ATP^{4-}_{out}]/[ADP^{3-}_{out}])_2}{([ATP^{4-}_{in}]/[ADP^{3-}_{in}])_2} \quad (3)$$

Since the rate of O_2 uptake under the two conditions is equal (see table 1), $\Delta\psi$ must be equal [29] and the intramitochondrial ATP/ADP must be the same (fig.3B and [29]) in both cases. Thus on subtracting eq. (3) from eq. (2) the following expression is obtained:

$$\Delta G_T = 2.3\ RT \log \frac{([ATP^{4-}_{out}]/[ADP^{3-}_{out}])_1}{([ATP^{4-}_{out}]/[ADP^{3-}_{out}])_2} \quad (4)$$

The actual calculation is shown in table 1. The free extramitochondrial ATP^{4-}/ADP^{3-} ratio in condition (i) was calculated using the ionization constants and the stability constants of the Mg^{2+} and K^+ complexes of ATP and ADP [36]. Thus when extramitochondrial ATP utilization leads to a flux through the translocator that is only ~30% of the maximal flux (measured in the presence of excess hexokinase), the adenine nucleotide translocator is already ~8.7 kJ/mol out of equilibrium.

4. Conclusions

The following conclusions can be drawn from these results:

(1) There is no direct, unequivocal control of respiration by the extramitochondrial ATP/ADP ratio.
(2) The rate of respiration is directly related to the intramitochondrial ATP/ADP ratio regardless of whether ATP is utilized in the mitochondrial matrix or the extramitochondrial compartment.
(3) The adenine nucleotide translocator is displaced from equilibrium during its operation in agreement with the conclusion in [37].

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