Biochimica et Biophysica Acta, 593 (1980) 196—203

BBA 47964

INVESTIGATION OF THE DEPENDENCE OF THE INTRAMITOCHONDRIAL [ATP]/[ADP] RATIO ON THE RESPIRATION RATE

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(Received April 29th, 1980)

Key words: ATP/ADP ratio; Respiration rate; Adenine nucleotide pool; Oxidative phosphorylation; (Rat liver mitochondria)

Summary

The relationship between the respiration rate and the intra- and extramitochondrial adenine nucleotides was investigated in isolated rat liver mitochondria.

For the determination of adenine nucleotide patterns in both compartments a new procedure was developed, based on the evaluation of these metabolites from incubation of various amounts of mitochondria under identical stationary states of oxidative phosphorylation. These identical states were adjusted by addition of appropriate amounts of hexokinase to a glucose-containing incubation mixture.

Adenine nucleotides were measured in aliquots of the total extract of the incubation mixture without any separation. The concentrations of the adenine nucleotides in both compartments were obtained from a plot of the total concentration of these species versus mitochondrial protein. Disturbances of this method by unspecific efflux of adenine nucleotides could be excluded.

The results obtained for the total adenine nucleotide content (12 nmol · mg^{-1} protein) and the intramitochondrial [ATP]/[ADP] ratio (about 4 in the resting state) are in good agreement with data obtained by other methods.

Strong evidence is provided for a decrease of the intramitochondrial [ATP]/[ADP] ratio with increasing rate of oxygen consumption. Therefore it is not necessary to assume a microcompartmentation of the intramitochondrial

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adenine nucleotide pool in respect to the ATPase reaction and the adenine nucleotide translocation.

Introduction

The question of whether or not the intramitochondrial [ATP]/[ADP] ratio is constant or decreasing during transition of mitochondria from the resting to the active state is still under discussion [1—5]. It is possible that the results obtained after separation of intra- and extramitochondrial compartments are not relevant because of the time used for the separation process [5—8], and possible interconversion of adenine nucleotides even in the presence of inhibitors. Therefore, we developed a procedure for the determination of both intra- and extramitochondrial adenine nucleotides without the separation process. This method is based on an evaluation of adenine nucleotide patterns from incubations of various amounts of mitochondrial protein under identical metabolic conditions. By means of this new procedure it could be demonstrated that with an increasing rate of respiration the intramitochondrial [ATP]/[ADP] ratio decreases significantly.

Materials and Methods

Mitochondria were prepared from rat liver by a standard procedure [9]. The oxygraphic measurement of mitochondrial respiration was carried out with a Clark-type electrode at 25°C. The saline medium (50 mM KCl/20 mM $KHCO_3$/5 mM KH_2PO_4/10 mM glutamate/15 mM glucose/0.5 mM EDTA/2 mM $MgCl_2$/25 mM Tris-HCl, pH 7.2; in some experiments about 200 μM ATP was added) was gassed with 95% O_2 and 5% CO_2. The sucrose-containing medium (110 mM sucrose/60 mM KCl/60 mM Tris-HCl/10 mM potassium phosphate/5 mM $MgCl_2$/0.5 mM EDTA/15 mM glucose; about 150 μM ATP, pH 7.4) was gassed with pure oxygen. By means of a ratemeter the first derivative of the oxygen consumption was recorded. Stationary intermediate states of mitochondrial respiration between the resting state and the active state were adjusted by addition of suitable amounts of deionized hexokinase [10].

After 4 minutes of incubation, samples were taken from the oxygraphic cell, quenched with $HClO_4$ and neutralized with K_2CO_3 for adenine nucleotide determination in the whole extract. In some cases the mitochondria from parallel samples were sedimented (60 s, 10 000 × g) and the adenine nucleotides in the quenched supernatant were assayed. Determinations of protein and metabolites were carried out as reported elsewhere [10].

Biochemicals used were purchased from Boehringer GmbH, Mannheim. Other chemicals were of analytical grade.

Evaluation of adenine nucleotide patterns

Adenine nucleotides from the total extract consist of the sum of the extra- and intramitochondrial portions.

$$[AXP]_{total} = [AXP]_i + [AXP]_e; X = T,D,M \quad (1)$$

Since the pool of adenine nucleotides in mitochondria remains constant due to the 1 : 1 exchange character of the transport, the intramitochondrial fraction depends on the amount of incubated mitochondria (expressed in mg protein per ml).

$$[AXP]_i = \frac{\text{nmol } [AXP]_i}{1 \text{ mg mito.protein}} \cdot \frac{\text{mg mito.protein}}{\text{ml}} \quad (2)$$

It follows that the total content of adenine nucleotides of the sample is a linear function of the amount of mitochondrial protein

$$[AXP]_{total} = \frac{\text{nmol } [AXP]_i}{1 \text{ mg mito.protein}} \cdot \frac{\text{mg mito.protein}}{\text{ml}} + [AXP]_e \quad (3)$$

Eqn. 3, provides the basis for the estimation of intramitochondrial adenine nucleotides ($[AXP]_i$/mg protein $\triangleq$ slope) as well as the extramitochondrial ones ($[AXP]_e \triangleq$ ordinate intercept) from a plot of adenine nucleotide content vs. mg mitochondrial protein in the incubation medium.

Results and Discussion

Fig. 1 shows the adenine nucleotide content of total extracts from the incubations with increasing amounts of mitochondria respiring in the resting state. It is a prerequisite for the technique described above, that the [ATP]/[ADP] ratios in the medium as well as in the mitochondria, be identical for all incubations. This is achieved by ensuring an equal oxygen consumption rate/mg mitochondrial protein for all incubations (Fig. 1A). The linear dependence of total ATP, ADP and AMP (Fig. 1B) on the amount of mitochondrial protein in the sample indicates that the ratios are the same for all incubations. When the mitochondria were centrifuged after these incubations, the sum of adenine nucleotides in the supernatant was constant (Fig. 1A). This shows that unspecific efflux of adenine nucleotides from the mitochondria can be neglected. In fact, the content of adenine nucleotides in the supernatant (201 ± 3.6 μM) was identical with the amount of externally added ATP.

The maximum unspecific efflux of adenine nucleotides [11] was determined by incubation of mitochondria in absence of external ATP and subsequent separation of the mitochondria by centrifugation. Only about 10% of mitochondrial adenine nucleotides were found in the supernatant (Table I). From this kind of experiment we can conclude, that the [ATP]/[ADP] ratio, determined in the total extracts of incubations without externally added adenine nucleotides, yields an acceptable approximation to the real mitochondrial [ATP]/[ADP] ratio.

The low [ATP]/[ADP] ratio in the supernatant is not comparable with values observed earlier [10] under quite different conditions. This low ratio seems very likely to be a result of ATP splitting in the supernatant during centrifugation of mitochondria. The data taken from Fig. 1 and calculated according to Eqn. 3, of Materials and Methods are presented in Table II, part A. The values are in good agreement with the intramitochondrial [ATP]/[ADP]

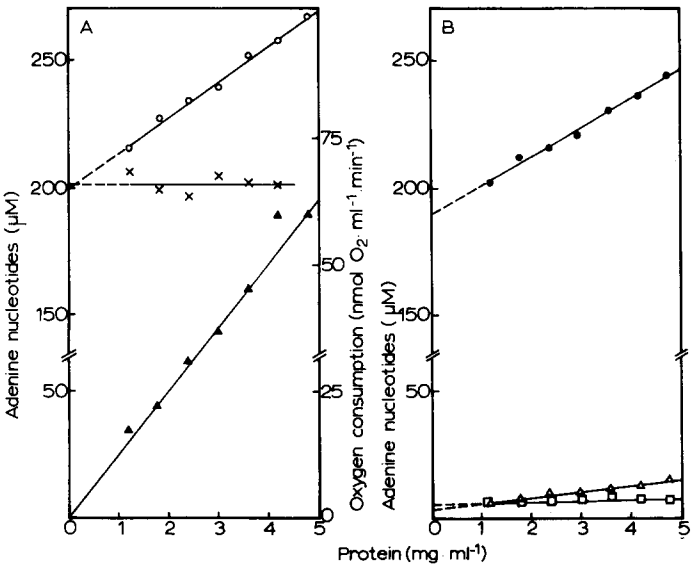

Fig. 1. Incubation of different amounts of mitochondria under resting state conditions. Increasing amounts of mitochondria were incubated in 6 ml of saline medium with the same concentration of approx. 0.2 mM ATP plus 10 mM glutamate. After 4 min two samples were withdrawn: (i) centrifuged for 60 s at 10 000 X *g* and the supernatant was quenched, and (ii) quenched immediately with $HClO_4$ (total extract). The results from one representative experiment out of five are given. (A) shows the rate of respiration (▲———▲), the sums of the adenine nucleotides in the total extract (○———○), and in the supernatant (X———X). (B) represents the concentrations of ATP (●———●), ADP (△———△) and AMP (□———□) in the total extract.

ratio of about 4 and the total content of about 12 nmol adenine nucleotides per mg mitochondrial protein as observed by Davis and Lumeng [1] and by Heldt et al. [12]. However, we reported significant changes in the intramitochondrial [ATP]/[ADP] ratio of rat liver mitochondria using citrulline synthesis [13]. Similar changes were observed by Heldt et al. [12] but were not found by Davis and Lumeng [1]. Part B of Table II shows the intra- and extra-

TABLE I

MITOCHONDRIAL ADENINE NUCLEOTIDE PATTERN AFTER INCUBATION UNDER RESTING STATE CONDITIONS

Mitochondria (29.8 mg protein) were incubated in 6 ml saline medium as described in the methods, without addition of adenine nucleotides. After 4 min samples were withdrawn for determination of adenine nucleotides in the whole $HClO_4$ extract ($[AXP]_{total}$) and for determination of adenine nucleotides in the quenched supernatant after centrifugation of the samples ($[AXP]_{supernatant}$). The adenine nucleotide contents are expressed in nmol · mg^{-1} protein.

Adenine nucleotides	$[AXP]_{total}$ mean of 3 samples	$[AXP]_{supernatant}$ mean of 2 samples
ATP	10.0	0.9
ADP	2.6	0.6
AMP	2.2	0.1
Σ AXP	14.8	1.6

TABLE II

INTRA- AND EXTRAMITOCHONDRIAL PATTERNS OF ADENINE NUCLEOTIDES AFTER INCUBATION OF MITOCHONDRIA IN THE RESTING STATE (A) AND AN INTERMEDIATE STATE (B) CORRESPONDING TO 72% OF MAXIMUM RATE OF RESPIRATION

Increasing amounts of mitochondria were incubated as in Fig. 1 in the presence of 10 mM glutamate and about 0.2 mM ATP. The intermediate state was adjusted by addition of limiting amounts of hexokinase. The data for the resting state were taken from Fig. 1. An adequate plot was received for the intermediate state, calculated according to Eqn. 3, and statistically checked [18]. In both states the intramitochondrial ATP and ADP are significantly different ($p = 0.01$).

Species	Correlation coefficient	Adenine nucleotides	
		intramitochondrial (nmol · mg^{-1} protein)	extramitochondrial (μM)
A ($n = 7$)			
ATP	0.995	11.2 ± 0.5	190.1 ± 1.7
ADP	0.985	2.3 ± 0.2	3.8 ± 0.6
AMP	0.661	0.4 ± 0.2	5.9 ± 0.7
Σ AXP	0.995	13.9 ± 0.6	199.7 ± 1.9
$\frac{ATP}{ADP}$	—	4.8 ± 0.6	50.0 ± 8.3
B ($n = 5$)			
ATP	0.982	5.6 ± 0.6	183.9 ± 1.7
ADP	0.933	5.5 ± 1.2	8.8 ± 1.2
AMP	0.588	0.11 ± 0.09	7.1 ± 0.2
Σ AXP	0.975	11.2 ± 1.5	199.8 ± 1.5
$\frac{ATP}{ADP}$	—	1.0 ± 0.3	21 ± 8.1

mitochondrial adenine nucleotide patterns for mitochondria respiring in a glucose-hexokinase system with 72% of maximum rate of respiration. It should be mentioned that the range between resting and fully active respiration is remarkably reduced in a bicarbonate-containing saline medium. However, this medium was used for comparison with experiments including citrulline synthesis. From the data presented it follows that the transition of the mitochondria from the resting state to this intermediate state of respiration produced a significant decrease in the mitochondrial [ATP]/[ADP] ratio from 4.8 to 1.0. Ratios of intramitochondrial [ATP]/[ADP] found in experiments with isolated hepatocytes [14] were within this range, too.

Results of experiments as described before provide strong evidence for an interrelationship of the rate of oxidative phosphorylation and the intramitochondrial [ATP]/[ADP] ratio. By this approach, however, no information can be obtained on the characteristics of this interrelationship in the range between the resting and the fully active state. For investigation of this dependence the same amount of mitochondria must be adjusted to different respiration rates, as shown in Fig. 2. Incremental addition of hexokinase allows a titration of the respiration rate up to the preset values. This must be immediately checked by the ratemeter trace. When the rate of oxygen consumption was stationary, a sample was withdrawn, quenched, neutralized and frozen in liquid nitrogen. After sampling, the next higher rate was adjusted. By repetition of this proce-

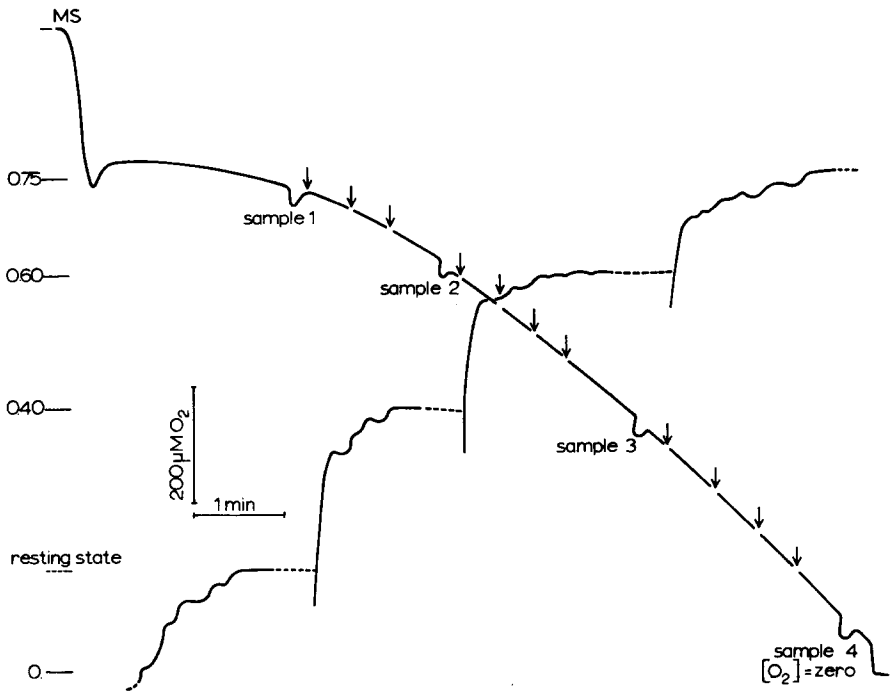

Fig. 2. Adjusting of various stationary states of mitochondrial respiration. Mitochondria (MS) (22 mg protein) were incubated in 6 ml sucrose-containing medium plus 10 mM glutamate. When the respiration rate was stationary, as checked by the ratemeter trace, the first sample was withdrawn and quenched. By addition of deionized hexokinase (↓) the next desired rate (mark: ——— portion of maximum respiration rate) is reached. In this way, at four different respiration rates samples can be taken before oxygen is exhausted. During sampling the ratemeter trace was interrupted.

dure for different protein equivalents of mitochondria, the internal and the external adenine nucleotide patterns of the corresponding respiration rates were obtained. The data summarized in Fig. 3 are the result of this procedure. This figure represents the respiration rate vs. the extra- and the intramitochondrial [ATP]/[ADP] ratios. The influence of the extramitochondrial ratio is in accordance with results published earlier [10]. The interrelationship of the intramitochondrial [ATP]/[ADP] ratio and the respiration rate shown in Fig. 3A are in contrast to the finding of Davis and Lumeng [1]. While the latter authors observed a constant intramitochondrial [ATP]/[ADP] ratio even in that range where the respiration is controlled by the extramitochondrial [ATP]/[ADP] ratio, the data in Fig. 3A demonstrate a reciprocal relationship between respiration rate and intramitochondrial [ATP]/[ADP] ratio. Therefore, it is not necessary to assume a microcompartmentation of the intramitochondrial adenine nucleotide pool in respect to the reaction of the ATPase or of the adenine nucleotide translocator [2,4]. The different findings of Davis and Lumeng [1], which would justify such a microcompartmentation, may be the result of internal turnover of adenine nucleotides during centrifugation through the silicone oil layer, as suggested by Brawand et al. [5]. Although the method presented is laborious in regard to the amount of mitochondria and the

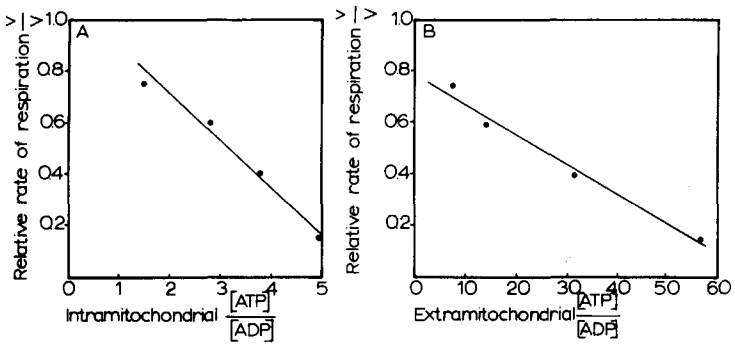

Fig. 3. Relative respiration rate vs. intramitochondrial (A) and extramitochondrial (B) [ATP]/[ADP] ratios. Increasing amounts of mitochondria (11.4—28.1 mg protein) were incubated in 6 ml sucrose-containing medium plus 10 mM glutamate and adjusted to four different respiration rates as shown in Fig. 2. After sampling the concentrations of adenine nucleotides were measured in the total extracts. These data were treated as shown in Table II and the resulting [ATP]/[ADP] ratios were plotted with the corresponding respiration rates.

number of metabolic determinations, it is a valuable tool for the steady state investigation of respiration and oxidative phosphorylation. It eliminates all disadvantages of the silicone oil layer method associated with the interconversion of adenine nucleotides during the centrifugation of mitochondria.

As recently reported [15], the intra- and the extramitochondrial [ATP]/[ADP] ratios can be used for a thermodynamic estimation of the free energy of the translocation of adenine nucleotides according to the following equation:

$$\Delta G = -F \cdot \Delta\Psi + 2.3RT \log \left(\frac{[ATP]_e}{[ADP]_e} \Big/ \frac{[ATP]_i}{[ADP]_i} \right)$$

For the calculation the following data taken from Fig. 3 were used: resting state: $[ATP]_e/[ADP]_e = 56$, $[ATP]_i/[ADP]_i = 5.0$; 75% of fully active state: $[ATP]_e/[ADP]_e = 7.6$, $[ATP]_i/[ADP]_i = 1.5$.

Assuming $\Delta\Psi$ as 150 mV and 135 mV for the resting state and the fully active state, respectively [16], the following ΔG for the translocation can be obtained: resting state: $\Delta G = -8.5$ kJ · mol^{-1}; 75% of the fully active state: $\Delta G = -8.9$ kJ · mol^{-1}. These values correspond to $|\Delta G/RT|$ values of 3.4 and 3.6, respectively, and do not fit to the relation $|\Delta G/RT| << 1$ which is required for close-equilibrium conditions [17]. It must be expected that the driving force is greater in the more active state than in the resting one due to the increased flux through the translocator. Our data point in this direction.

Acknowledgement

The excellent technical assistance of Mrs. U. Meyer, Mrs. G. Sackewitz and Miss I. Schmidl is gratefully acknowledged.

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