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The role of adenylate kinase in dynamic compartmentation of adenine nucleotides in the mitochondrial intermembrane space

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To investigate the existence of a dynamic adenine nucleotide (AdN) compartment in the mitochondrial intermembrane space, we used reconstituted systems consisting of (i) functional intact liver and heart mitochondria and (ii) pyruvate kinase plus phosphoenolpyruvate, both competing for ADP either formed in the intermembrane space by adenylate kinase or added directly into, or regenerated by ATPase within, the extramitochondrial space. It is shown that ADP formation in the mitochondrial intermembrane space is a prerequisite for a dominating oxidative phosphorylation in reconstituted systems, suggesting dynamic ADP compartmentation in that space.

Adenylate kinase; Rat heart mitochondrion; Rat liver mitochondrion; Compartmentation; Adenine nucleotide; Intermembrane space

1. INTRODUCTION

In the past, the biological relevance of mitochondrially localized ATP-splitting enzymes such as creatine kinase or hexokinase has been discussed as an advantage in supplying these enzymes with mitochondrially formed ATP [1–4]. A new approach to this problem area became possible through experiments with reconstituted systems [5–8] in which mitochondria and muscle pyruvate kinase compete for ADP produced by ATP-splitting enzymes in varied localization. For rat heart mitochondria it was shown that the ADP supply to oxidative phosphorylation via mitochondrial creatine kinase is priviliged in comparison to the ADP supply with hexokinase [7,8]. Therefore, channelling of the extramitochondrially formed ADP into the mitochondria seems to be the crucial problem [7,8]; this is accomplished in creatine kinase-containing tissues by means of the creatine phosphate shuttle (for a recent review, see [9]). Functional [10] or dynamic reasons [8] have been assumed to account for the obvious compartmentation of adenine nucleotides in the mitochondrial intermembrane space [7,8,10,11]. The dynamic compartmentation is probably caused by a limitation of diffusion for adenine nucleotides through the restricted number of porine pores in the mitochondrial outer membrane [8]. If so, the adenine nucleotide (AdN) compartmentation should be also demonstrable with the help of other ATP-splitting enzymes localized in the intermembrane space in a soluble form.

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Therefore, it was the purpose of this study to investigate AdN compartmentation in the intermembrane space of rat heart and rat liver mitochondria using adenylate kinase, the soluble marker enzyme of this space [12].

It is shown that ADP generated from AMP and ATP within the mitochondrial outer membrane is important for a dominating oxidative phosphorylation in reconstituted systems.

2. MATERIALS AND METHODS

Rat liver [13] and rat heart [7,8] mitochondria were isolated as described previously. Liver mitochondria were incubated in medium I (110 mM sucrose, 60 mM KCl, 15 mM glucose, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM phosphoenolpyruvate (PEP), 10 mM succinate, and 1 μM rotenone at pH 7.4). Rat heart mitochondria were incubated in medium II (250 mM sucrose, 10 mM HEPES, 4 mM glutamate, 2 mM malate, 5 mM MgO, 0.37 mM dithiothreitol, 5 mM PEP, and 0.3 mM EDTA at pH 7.4). Using a Clark-type electrode and a custom-built rate meter, the oxygen uptake was measured and calculated assuming an initial oxygen concentration of 230 μM [14]. The specific oxygen consumption following ADP and AMP additions was calculated from the area under the peaks of the first derivative of the oxygen–time curve. Mitochondrial protein was determined by a biuret procedure [15]. Samples were quenched by a modified phenol-stop as described in [8]. Adenine nucleotides were measured spectrophotometrically as described previously [8,16].

3. RESULTS

Fig. 1 demonstrates the metabolic system used to investigate the role of adenylate kinase in dynamic ADP compartmentation. Pyruvate kinase and mitochondria compete for the phosphorylation of ADP formed in, or added to, the different compartments. The general experimental approach is shown for rat liver mi-

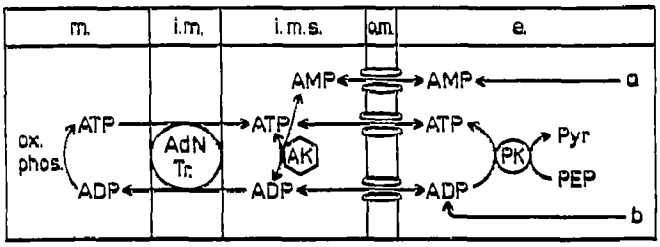

Fig. 1. Scheme of the reconstituted systems used, consisting of isolated heart or liver mitochondria and pyruvate kinase: (i) ADP formation by adenylate kinase in the mitochondrial intermembrane space from ATP and added AMP; (ii) direct ADP addition into the extramitochondrial compartment or its formation via extramitochondrial ATPase.

tochondria in Fig. 2. We measured the oxygen consumption and the first derivative of this signal. The experiments were started by the addition of 5 mM ATP. The marked stimulation of mitochondrial respiration was caused by an obligatory contamination of ATP with ADP and AMP. The following ADP and AMP additions induced the alternating transitions between states 3 and 4. Since adenylate kinase produces, from one AMP molecule, two ADP molecules, the effective amounts of ADP were 404 and 420 nmol ADP after the ADP and AMP additions, respectively. Then, in the presence of pyruvate kinase (319 U/mg), the same amounts of ADP and AMP were added as before. The oxygen consumption after ADP addition was now negligible, indicating that virtually all of the ADP added was phosphorylated by the pyruvate kinase. If, however, the ADP is formed in the intermembrane space, substantially more ADP can be used for oxidative phosphorylation despite the presence of high pyruvate kinase activity. Under these conditions, 83% of the ADP formed in the intermembrane space diffused through the porin pores to the pyruvate kinase, but 17% remained for oxidative phosphorylation.

The experiment shown in Table I demonstrates that different amounts of oxygen consumption after equivalent ADP and AMP additions to the completely reconstituted system also existed for heart mitochondria. If, furthermore, soluble de-ionized adenylate kinase was added to this system, then this extramitochondrial enzyme should convert AMP into ADP, and this ADP will be phosphorylated by pyruvate kinase without any participation of the mitochondria. Indeed, the AMP-induced oxygen consumption in the presence of adenylate kinase diminished from 117 to 2 nmol O_2. These findings provide evidence of the dynamic inhomogeneity of the intermembrane and the extramitochondrial space.

To demonstrate that pyruvate kinase phosphorylates the ADP formed by mitochondrial adenylate kinase rather than being used for oxidative phosphorylation, we performed experiments as shown in Table II. A total of 327 nmol AMP were added to rat heart mitochondria in the presence of exceeding pyruvate kinase activity. The increasing pyruvate concentration clearly indicates the activity of pyruvate kinase. Since that enzyme also phosphorylated the ADP formed by extramitochondrial ATPases the total increase in pyruvate was somewhat higher than expected. The time course of respiratory rates did not markedly correlate with the expected changes in the total ATP/ADP ratios.

To detect adenylate kinase-induced concentration gradients between the intermembrane and the extramitochondrial space the respiration of rat heart mitochondria was activated by either mitochondrial adenylate kinase or extramitochondrial ATPase under stationary conditions (Table III). To this end, identical rates of respiration were adjusted by varying the activity of soluble pyruvate kinase. Furthermore, in additional incubations, the same rate of respiration was adjusted via mitochondrial creatine kinase. For extramitochondrial ADP regeneration, a total ADP concentration of 30 μM was found, whereas this value decreased to 15 μM for ADP regeneration in the intermembrane space.

In the adenylate kinase system, mitochondrial adenylate kinase works in the direction of ADP formation, but in the other two systems this enzyme equilibrates the adenine nucleotides of the intermembrane space (Fig. 1). Due to the absence of an extramitochondrial AMP-converting enzyme, the AMP concentrations (30 μM) in both the creatine kinase and the ATPase system indicated the same ATP and ADP concentrations in the intermembrane space despite different extramitochondrial ADP concentrations. These results clearly point to concentration gradients for adenine nucleotides between the intermembrane and the extramitochondrial space at sufficiently high fluxes.

Table I

Different amounts of oxygen consumption after equivalent AMP and ADP additions to rat heart mitochondria and the prevention of AMP-induced oxygen consumption by addition of soluble adenylate kinase

Additions	Mitochondrial oxygen consumption (nmol O_2/incubation)
AMP (– PK)	350.4 ± 12.8
ADP (– PK)	332.3 ± 10.8
AMP (+ PK)	116.8 ± 16.3
ADP (+ PK)	8.4 ± 2.3
AMP (+ PK + AK I)	12.9 ± 1.8
AMP (+ PK + AK II)	5.3 ± 0.9
AMP (+ PK + AK III)	2.3 ± 0.6

Incubation of rat heart mitochondria (0.64 mg/ml) in medium II containing an additional 4 mM ATP. The mitochondrial oxygen consumption after addition of 595 nmol AMP and 810 nmol ADP (containing 188 nmol AMP) were determined before and after addition of 121 U pyruvate kinase (PK)/mg. Then, the AMP-induced oxygen consumption was determined in the additional presence of increasing activities of adenylate kinase (I, II, III = 22, 55 and 88 U/incubation, respectively). Data are means ± S.D. of 4 incubations.

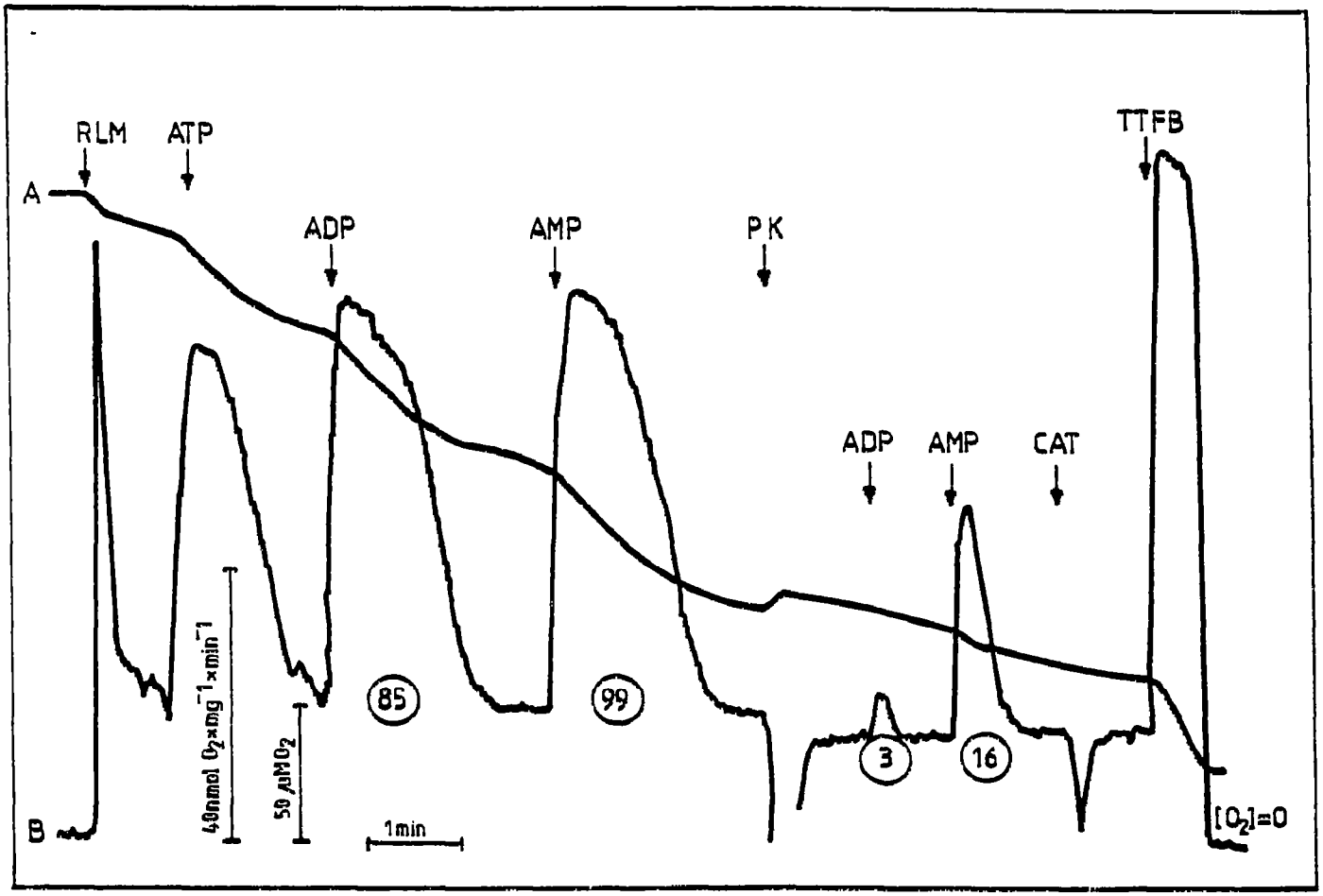

Fig. 2. Different amounts of oxygen consumption after equivalent AMP and ADP additions to rat liver mitochondria in the presence of exceeding pyruvate kinase activity. Incubation of 1.4 mg rat liver mitochondria to 3 ml medium I. Additions: (ATP) 5.5 mM ATP; (ADP) 263 μM ADP (containing 71 μM AMP); (AMP) 210 μM AMP; (PK) 319 U pyruvate kinase/mg; (CAT) 1 μM carboxyatractyloside; (TTFB) 10 μM 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole. The numbers in the circles represent the oxygen consumption in nmol O_2/mg mitochondrial protein.

4. DISCUSSION

In the experiments presented in this paper mitochondrial adenylate kinase rather than mitochondrial creatine kinase was used for ADP regeneration in the intermembrane space, and compared with extramitochondrial ADP regeneration by ATPase or with direct ADP addition to the extramitochondrial compartment. Similar to the creatine kinase system, the ADP produced by adenylate kinase was preferentially used

Table II

Time course of total adenine nucleotide and pyruvate concentrations following AMP addition to heart mitochondria plus pyruvate kinase

Time after AMP addition (min)	V_{resp} (nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$)	AMP (μM)	ADP (μM)	ATP (μM)	Pyruvate (μM)	ATP/ADP
before	16.0	47 ± 7	23 ± 9	2136 ± 18	647 ± 52	92
0.5	24	243 ± 30	87 ± 4	2222 ± 97	1018 ± 23	25
1.0	28	227 ± 14	32 ± 9	2215 ± 15	1138 ± 9	68
1.5	31	146 ± 8	36 ± 1	2226 ± 35	1251 ± 9	61
2.2	28	65 ± 1	23 ± 1	2251 ± 58	1310 ± 32	79
4.0	16	55 ± 3	21 ± 7	2323 ± 73	1629 ± 58	111

Incubation of 4.4 mg rat heart mitochondria in 5 ml medium II containing 2.2 mM adenine nucleotides and 106 U pyruvate kinase/mg, similar to the experiment shown in Fig. 3. Before and after addition of 327 nmol AMP samples were quenched to determine metabolites. Concentrations are given as means ± S.D. (n = 4–6 determinations from 2 samples).

Table III

Effect of a different localization of ADP-regenerating enzymes on the total ADP concentration

System	V_{resp} (nmol O_2/mg)	PK (U/mg)	AMP (μM)	ADP (μM)
AK[a]	34.2	133	72.5 ± 9.0	14.8 ± 2.8
CPK[b]	33.8	133	30.2 ± 1.1	16.3 ± 2.4
ATPase[c]	33.8	9.7	29.4 ± 1.1	30.9 ± 3.9

Rat heart mitochondria (1.71 mg/ml) were incubated in medium II which additionally contained 3.3 ± 0.2 mM ATP and soluble kinase (PK). State 3 and state 4 respiration rates were 157.1 and 15.8 nmol O_2/mg, respectively. Data are means ± S.D. of 4 determinations in 2 different incubations.

[a] Respiration was activated by addition of 330 μM AMP. 2 min after AMP addition samples were quenched to determine adenine nucleotides.

[b] Respiration was activated via mitochondrial creatine kinase and adjusted by variation of creatine concentration (33 mM).

[c] Respiration was activated by extramitochondrial ATPase present in normal heart mitochondrial preparations (0.54 U/mg) and adjusted by variation of pyruvate kinase activity.

for oxidative phosphorylation. The concept of dynamic compartmentation assumes a rate-dependent diffusion limitation of adenine nucleotides between adenine nucleotide translocase and the extramitochondrial space, causing remarkable diffusion gradients of up to 13 μM between both pools [8]. These might be attributable to the limited number of porin pores in the outer membrane [8], unstirred layer effects in the intermembrane space [17], and the decreased diffusion coefficient of molecules in protein-containing solutions [18]. Whereas the creatine kinase is reversibly bound to the mitochondrial inner membrane [19,20], it is generally accepted that adenylate kinase is a soluble enzyme of the intermembrane space [12]. Therefore, the finding of an adenylate kinase-induced ADP compartmentation supports the importance of the outer mitochondrial membrane for the dynamic AdN compartmentation in the intermembrane space. Furthermore, the results show that the adenylate kinases might contribute to the channelling of ADP equivalents into the mitochondria through facilitated diffusion as proposed in [21]. This might be true of tissues in which creatine kinase is not present. In the normal muscle, however, the AMP concentration is probably too low (20 μM) [22] for an effective ADP channelling to be mediated by adenylate kinase, except possibly in ischaemic states [23]. Therefore, in tissues containing creatine kinase, the creatine phosphate shuttle is believed to greatly account for transport of cytosolic ADP to the mitochondrial adenine nucleotide translocator.

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