EFFECT OF MACROMOLECULES ON ADP TRANSPORT INTO MITO-CHONDRIA

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INTRODUCTION

To obtain a better understanding of the bioenergetic basis of mitochondrial cytopathies [1] and pathologies as a result of ischemia and oxidative stress, improved knowledge of intracellular adenylate (AdN) transport is necessary. A working model regarding several intracellular ADP transport routes is shown in Fig. 1. We distinguish diffusion, channeling, and shuttling of AdN.

Energy transmitting kinases [2], such as mitochondrial creatine kinase (*2*, mi-CK; for a review see [3]) and mitochondrial adenylate kinase (*3*, mi-AK [4-7]) are localized within the mitochondrial intermembrane space. In conjunction with their extramitochondrial isoenzymes, mi-CK and mi-AK *shuttle* ADP from cytoplasmic ATP-splitting reactions into the intermembrane space at low cytoplasmic ADP concentrations. If ADP is formed via mi-CK and mi-AK, higher ADP concentrations are detected in the intermembrane space as compared to the bulk phase [4,7-9]. The mitochondrial outer membrane dynamically separates kinases of the intermembrane space from the extramitochondrial space by creating a diffusion resistance for adenine nucleotides and other metabolites crossing the porin pores ([10-14]; see also [15]).

Mitochondrial hexokinase (mi-HK) is an example for an energy consuming kinase [2]. These enzymes are not involved in ADP-*shuttles* but they *channel* ADP into the mitochondria (Fig. 1, *4*). Channeling counteracts a strong decrease of cytoplasmic phosphorylation potential at times of high energy flux. Although mi-HK is specifically bound to porin pores at the *outer* side of mitochondrial outer membrane [16], this enzyme is functionally linked to oxidative phosphorylation [17,18].

The contribution of these different ADP transport routes to total intracellular ADP transport depends on the specific enzyme pattern of the cell type and varies with metabolic state.

The intermembrane space of isolated mitochondria incubated in standard isotonic media is enlarged as compared to the *in vivo* situation due to the lack of oncotic pressure. To simulate the oncotic conditions of the intact cell, 10 to 15% macromolecules (dextrans, bovine serum albumin and others) may be added to incubation media [7,8,19-22]. Under these conditions the morphological properties of mitochondria appear similar to the *in vivo* situation [19]. It is now well established that these morphological effects of macromolecules are accompanied by changes of functional properties of mitochondria. These changes result from a number of factors including an increase of diffusion resistance of adenine nucleotides through the mitochondrial outer compartment
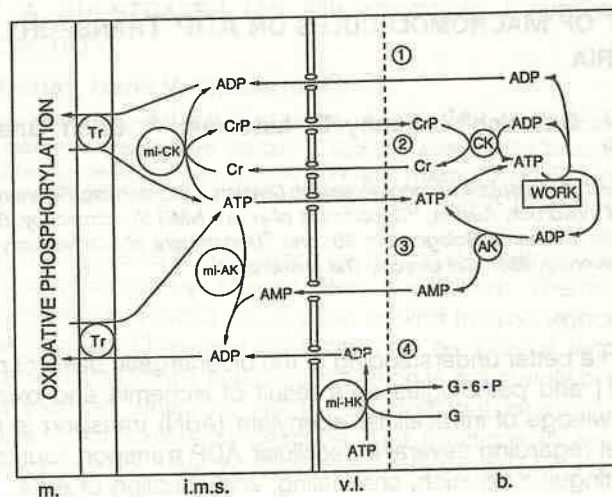
Fig. 1. Metabolic scheme of ADP transport routes into the mitochondria. (1) Direct ADP diffusion; (2) CrP-shuttle; (3) AK-shuttle; (4) mi-HK bound at the porin pores at the outside of mitochondrial membrane forms ADP which can be channelled to the AdN-translocator. b., bulk phase; u.l., unstirred layer; i.m.s., intermembrane space; m., matrix; Tr, AdN-translocator; G, glucose; G-6-P, glucose-6-phosphate.

and an increased number of contact sites [7,8,22]. Other properties of mitochondria, such as state 3 and 4 respiration, and kinetic constants of soluble enzymes are practically not affected under these conditions [7,8].

In the present study we investigated the effect of macromolecules on the main ADP-transport routes into isolated rat heart mitochondria (RHM) and rat liver mitochondria (RLM) by means of competition experiments between oxidative phosphorylation and pyruvate kinase.

METHODS

Isolation of mitochondria: RLM were prepared as described previously [23]. RHM were isolated in KCl-containing media. RLM were incubated in a medium containing 110 mM mannitol, 30 mM sucrose, 25 mM HEPES, 10 mM succinate, 5 mM KH_2PO_4, 10 mM $MgCl_2$, 20 mM glucose, 2.9 mM PEP, 4.7 mM ATP, 1 mM Na_2-EDTA, 2 μM rotenone, 10% dextran 20, with or without 1.37 mM AMP, pH 7.4. RHM were incubated in a modified physiological salt solution [24] consisting of 10 mM NaCl, 120 mM MES, 20 mM imidazole, 20 mM taurine, 10 mM glutamate and 2 mM malate, 8 mM $MgCl_2$, 5 mM KH_2PO_4, 0.5 mM dithiothreitol, 30 mM mannitol, 0.5 mM EDTA, 15% dextran, 4.7 mM ATP, 6.8 mM PEP, with or without 1.37 mM AMP, with or without 25 mM creatine, pH 7.4.

Respiration measurements: Oxygen flux was measured at 25 °C (RLM) and 30 °C (RHM) using an *Oroboros*® *Oxygraph* (Anton Paar, Graz, Austria). Oxygen concentration in the medium at air saturation and 101 kPa barometric pressure was considered to be 230 μM (25 °C) and 200 μM (30 °C).

RESULTS AND DISCUSSION

To compare the efficiency of the different ADP transport routes into mitochondria in the presence of macromolecules, competition experiments were performed with isolated mitochondria and pyruvate kinase, both competing for ADP formed in different microenvironments with respect to the mitochondrial outer membrane. Figure 2 shows the results of a representative experiment with RLM. In a first series of incubations, mitochondrial respiration was stimulated by mi-AK. About 2 min after starting the reactions by AMP addition, stationary rates of respiration were observed. Without pyruvate kinase, state 3 respiration was adjusted at 1.3 nmol $O_2 \cdot s^{-1} \cdot mg^{-1}$, indicating that the mi-AK activity was high enough to ensure the maximal ADP-phosphorylating activity of RLM (Table 1). The addition of pyruvate kinase reduced the rate of respiration to about 1 nmol $O_2 \cdot s^{-1} \cdot mg^{-1}$. In the second set of experiments, respiration was activated by addition of yeast HK instead of AMP. The activities of mi-AK and yeast HK were identical in both series of incubations. As expected respiration stimulated by hexokinase could be reduced to state 4 respiration by addition of excess PK activity. Extramitochondrial ADP concentrations are progressively reduced by increasing PK activity to critically low levels at which ADP cannot pass through the porin pores into the intermembrane space, irrespective of the high extramitochondrial ADP regeneration rate via added yeast HK. After 2 minutes of incubation samples were quenched and analyzed for the bulk phase ATP/ADP ratios (Fig. 2) which were different in both systems. At the same rate of respiration in both systems (ca. 1 nmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) the bulk phase ATP/ADP-ratio differs by a factor of 3. And at the same ATP/ADP ratio of about 530, the rate of
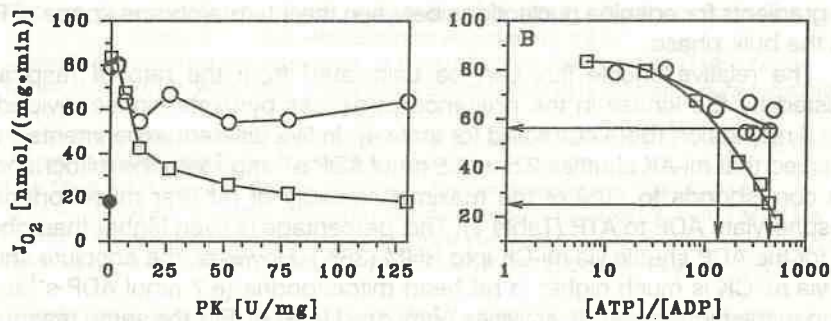

Fig. 2. Competition of oxidative phosphorylation of rat liver mitochondria and pyruvate kinase at ADP produced either by mi-AK (○) or soluble hexokinase (□) in the presence of 10 % dextran 20. Respiration was stimulated either by addition of 1.5 mM AMP (○) or of 795 mU yeast $HK \cdot mg^{-1}$ (□). Respiratory rates in the presence of 50 μM atractyloside (●). **A:** Rat liver mitochondria were incubated as described (Methods) with increasing activities of pyruvate kinase. **B:** 2 min after starting the reactions samples were quenched and analyzed fluorimetrically for the bulk phase ATP/ADP ratio. Each symbol represents one incubation.

TABLE 1
Maximal activities of kinases, ADP-transport routes through the kinases, and oxidative phosphorylation capacities in rat heart and liver mitochondria in the presence of dextran. Kinase(max): maximal ADP regenerating activities of kinases as determined spectrophotometrically under the same condition as respirometric experiments. Kinase+PK: rate of phosphorylation stimulated by the kinase in the presence of exceeding pyruvate kinase (minus state 4). State 3: maximal phosphorylation (minus state 4). [%]: relative shuttle or channelling flux expressed as percentage of the maximal phosphorylation (Kinase+PK divided by state 3). J_{ADP} was estimated from net oxygen fluxes (corrected for state 4) times the P:O stoichiometry (assuming 6 for heart and 4 for liver mitochondria). All data as means ± SD. The numbers in parentheses indicate the numbers of independent experiments.

Enzyme	J_{ADP} [nmol ADP·mg^{-1}·s^{-1}]			[%]
	Kinase(max)	Kinase+PK	State 3	
RHM/mi-CK	33.3 ± 3.3 (3)	6.7 ± 0.2 (5)	18.9 ± 4.0 (8)	35
RHM/mi-AK	4.0 ± 0.2 (3)	2.8 ± 1.0 (3)	- " -	15
RLM/mi-AK	13.3 ± 0.2 (4)	2.5 ± 0.5 (5)	4.2 ± 0.5 (5)	60
RLM/mi-HK	3.0 ± 0.3 (6)	1.1 ± 0.3 (6)	- " -	26

respiration in the AK system is about 3 times higher than in the HK system. Since the same rates of respiration in both systems require the same ATP/ADP ratios in the immediate vicinity of the AdN-translocator, these data point to concentration gradients for adenine nucleotides between the intermembrane space of RLM and the bulk phase.

The relative shuttle flux can be calculated from the rate of respiration adjusted by the kinase in the presence of excess pyruvate kinase divided by state 3 respiration (both corrected for state 4). In five different experiments it was observed that mi-AK shuttles 2.5 ± 0.5 nmol ADP·s^{-1}·mg^{-1} into the mitochondria. This corresponds to 60% of the maximal capacity of rat liver mitochondria to phosphorylate ADP to ATP (Table 1). This percentage is even higher than observed for the ADP shuttle via mi-CK into RHM (35%). However, the absolute shuttle flux via mi-CK is much higher in rat heart mitochondria (6.7 nmol ADP·s^{-1}·mg^{-1}) owing to the higher specific activities compared to RLM. For the same reason the absolute ADP shuttle flux via mi-AK in heart mitochondria was similar (2.8 nmol ADP·s^{-1}·mg^{-1}) to that in liver mitochondria. In contrast, the relative contribution of the ADP shuttle to maximal phosphorylating capacity was only 15 %.

The extent of relative shuttle fluxes via mi-AK and mi-CK increased with increasing dextran concentrations (not shown). In contrast to that, the direct ADP diffusion rate at non-saturating ADP concentrations decreased with increasing dextran concentrations. This can be deduced from the apparent Michaelis constants of oxidative phosphorylation of RHM (K_{ADP} = 20 and 120 μM ADP at 0% and 25% dextran, respectively).

As in the case for mi-CK and mi-AK, a remarkable ADP channeling activity was detected for mi-HK bound to rat liver mitochondria. 1.1 ± 0.3 nmol ADP·s^{-1}·mg^{-1} was fed into oxidative phosphorylation. This indicates that 30% of the ADP formed via mi-HK was not accessible for pyruvate kinase (see [25]).

Owing to the relatively low specific activity of bound hexokinase (200 mU/mg) this ADP flux was only 25% of the maximal phosphorylation capacity of liver mitochondria (Table 1).

In conclusion our results point towards remarkably effective ADP shuttles and channel fluxes via mi-CK, mi-AK and mi-HK into RHM and RLM in the presence of macromolecules. Macromolecules, however, diminish the direct ADP diffusion rates into the mitochondria. In tissues with sufficiently high activities of mitochondrial kinases ADP overcomes the diffusion barrier of the mitochondrial outer membrane via these enzymes.

ACKNOWLEDGEMENTS

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