ADP delivery from adenylate kinase in the mitochondrial intermembrane space to oxidative phosphorylation increases in the presence of macromolecules

Fanny D. Laterveer[a,b,*], Klaas Nicolay[a], Frank N. Gellerich[a,**]

[a] *Department of in vivo NMR Spectroscopy, Bijvoet Center for Biomolecular Research, Utrecht University, Bolognalaan 50, 3584 CJ Utrecht, The Netherlands*

[b] *Center for Biomembranes and Lipid Enzymology, Department of Biochemistry of Membranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*

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Abstract Macromolecules were added to isolated rat liver mitochondria to mimic cytosolic macromolecules and tested for their effects on the ADP delivery from adenylate kinase in the intermembrane space to oxidative phosphorylation. In the presence of 10% (w/v) dextran M20 or bovine serum albumin, approximately 60% of the maximal ADP flux from adenylate kinase to oxidative phosphorylation was not accessible to an extramitochondrial ADP scavenger. In the absence of macromolecules this was 34%. ADP determinations from incubations with macromolecules demonstrated the existence of flux-dependent ADP concentration gradients across the outer membrane which can be as high as 12 μM.

Key words: Adenylate kinase; Macromolecule; Mitochondria, rat liver; Intermembrane space; Oxidative phosphorylation; Compartmentation

1. Introduction

In the cell, mitochondria are embedded in a concentrated protein solution, i.e. the cytosolic protein content may be as high as 20–30% (w/v) [1]. In most studies on isolated mitochondria, isotonic media without macromolecules have been used. Under these conditions, the intermembrane space is much larger than in the in vivo situation. The structural changes in the mitochondrial periphery which occur upon isolation can partly be explained by missing colloid osmotic pressure and can be counteracted by the addition of proteins (like albumin) but also by the addition of other macromolecules like dextrans and polyvinyl pyrrolidone [2-5]. Furthermore, macromolecules increase the number of contact sites between outer and inner mitochondrial membranes [4].

In the mitochondrial periphery, several kinases are present [6,7]. These include adenylate kinase and creatine kinase which are localized between the two envelope membranes. Others, like certain mammalian hexokinase isozymes and glycerol kinase, are bound to the outer surface of the mitochondrion by interaction with porin [7]. The biological relevance of the mitochondrial localization of the kinases has been proposed as an advantage in supplying these enzymes with mitochondrially formed ATP and by analogy supplying oxidative phosphorylation with ADP generated by kinase activity [8,9].

This study focuses on mitochondrial adenylate kinase (Mi-AK) (ATP:AMP phosphotransferase, EC 2.7.4.3), which is highly abundant in liver and certain muscles [10]. There are also indications that large amounts of Mi-AK are present in bovine spermatozoa [11]. The aim of this study, which used rat liver mitochondria, was twofold. First we determined the effects of macromolecules on the ADP delivery from Mi-AK to oxidative phosphorylation under in vitro conditions. This was measured in a reconstituted system in which mitochondrial oxidative phosphorylation, in the absence and the presence of BSA or dextran M20, competed with extramitochondrial pyruvate kinase (PK) for ADP generated through Mi-AK activity. With this approach it was demonstrated that macromolecules increase the fraction of ADP that is delivered from Mi-AK into the mitochondrial matrix. Secondly, we investigated the consequences of the coupling between Mi-AK activity and mitochondrial activity for the extramitochondrial ADP and ATP levels. This was done by ADP and ATP determinations in incubation mixtures containing mitochondria in which ADP was generated either by Mi-AK or by an extramitochondrial kinase, i.e. yeast hexokinase (HK). The latter studies were performed in the presence of macromolecules and showed that at relatively low bulk ADP levels higher respiratory rates were maintained when ADP was generated by Mi-AK compared to yeast HK.

2. Materials and methods

2.1. Materials

Rat liver mitochondria were prepared as described previously [12] and resuspended in 0.25 M sucrose to give a protein concentration of 55–65 mg·ml^{-1}.

Lyophilized PK which was essentially salt free, lyophilized yeast HK and essentially fatty acid free BSA were obtained from Sigma. Dextran M20 (15–20 kDa) was from Serva. All other chemicals used were of the highest grade available and were obtained from regular commercial sources.

2.2. Protein determination

The protein concentration of the mitochondria preparation was measured by the BCA assay (Pierce). The BCA reagent was supplemented with 0.1% (w/v) sodium dodecylsulfate. BSA was used as a standard.

*Corresponding author. Fax: (31) (30) 2535561.

***Present address:* Neurologische Klinik und Poliklinik, Muskellabor, Martin-Luther-Universität Halle-Wittenberg, Julius-Kühn Str. 7, D-06097 Halle/Wittenberg, Germany.

Abbreviations: AK, adenylate kinase; BSA, bovine serum albumin; HK, hexokinase; Mi-AK, mitochondrial adenylate kinase; PK, pyruvate kinase

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2.3. Respiration measurements

Respiratory rates were measured at 25°C in an Oroboros oxygraph in a medium containing 110 mM mannitol, 30 mM sucrose, 25 mM HEPES, 10 mM succinic acid, 10 mM $MgCl_2$, 20 mM glucose, 1 mM disodium EDTA, 5 mM KH_2PO_4, 2 μM rotenone and, where indicated, dextran M20 or BSA (pH 7.4 with KOH). The oxygen content of the air-saturated oxygraph medium was taken from [13]. The specific oxygen consumption rates (nmol O_2·mg mitochondrial protein^{-1}·min^{-1}) were calculated from the first derivative of the oxygraph trace.

Mitochondrial respiration was measured in the presence of varying amounts of PK. Single incubations were performed for each different PK addition. The following components were added together: (a) 1520 μl oxygraph medium; (b) 15 μl rat liver mitochondria; (c) 32 μl ATP (200 mM pH 7.0; final concentration 3.8 mM); (d) 32 μl phospho*enol*pyruvate (100 mM, tricyclohexylammonium salt; final concentration 1.9 mM) and (e) 0–100 μl PK (1200 U·ml^{-1} in oxygraph medium) and 100–0 μl oxygraph medium. After closing the oxygraph chamber to a final volume of 1.5 ml, the Mi-AK-induced respiration was initiated by the addition of 50 μl 50 mM AMP (final concentration 1.7 mM). Stimulation of the respiration by extramitochondrial HK was achieved by addition of yeast HK (0.8 U/mg mitochondrial protein) instead of AMP.

The different states of respiration indicated in the text (states 3 and 4) are as defined by Chance and Williams [14]. The respiratory control index of the mitochondria was higher than 5.

2.4. Spectrophotometric assay of Mi-AK and HK activity

The activities of Mi-AK and yeast HK were determined in the direction of ADP formation with a coupled enzyme assay at 25°C under conditions comparable to the oxygraph experiments. In these measurements, the oxygraph medium was supplemented with NADH (0.26 mM), phospho*enol*pyruvate (1.9 mM), PK (2.3 U·ml^{-1}) and lactate dehydrogenase (2.6 U·ml^{-1}). For determination of the yeast HK activity, the reaction was started by addition of ATP (3.8 mM). For determination of Mi-AK activity, first ATP (3.8 mM) was added and then the reaction was started by addition of AMP (1.7 mM). The ADP production rates in these assays are linearly related to the NADH utilization rate which was measured at a wavelength of 340 nm. The kinase activities are expressed as nmol ADP·mg mitochondrial protein^{-1}·min^{-1}.

2.5. Metabolite determinations

For determination of metabolite levels, samples were taken 2.5 min after addition of AMP or yeast HK when the respiratory rate was stationary. All reactions were terminated by immediate dilution of the samples in an organic solvent mixture as described [13,15].

ATP, ADP, AMP and glucose-6-phosphate levels were measured spectrophotometrically or fluorimetrically using standard enzymatic assays [16]. In some samples, the ADP determinations were hampered by the presence of large amounts of pyruvate, because pyruvate is an intermediate in the ADP assay. In those samples, pyruvate was first converted to lactate by addition of an equimolar amount of NADH, and by addition of lactate dehydrogenase.

2.6. Calculation of the ADP fluxes and ADP concentration gradients

The ADP concentration gradient across the outer mitochondrial membrane was calculated basically as described in [10,17]. The ADP production flux by Mi-AK (v_{AK}) was calculated from the decrease in AMP concentration, assuming that the rate was stationary throughout the 2.5 min period. The ADP production flux by yeast HK (v_{HK}) was likewise calculated from the increase in glucose-6-phosphate. The ADP flux to oxidative phosphorylation (v_p) was calculated from the difference between the Mi-AK- or HK-induced respiration and state 4 respiration (i.e. rate of respiration prior to addition of AMP or HK), and the P/O ratio of 2 (Gellerich, F.N., unpublished results) for respiration on succinate. All fluxes are expressed as nmol ADP·mg mitochondrial protein^{-1}·min^{-1}. The extramitochondrial ADP concentration $[ADP]_e$ was obtained by correcting the bulk ADP concentration for the contribution of intramitochondrial ADP. The latter was estimated from the total amount of 12–14 nmol ATP+ADP/mg mitochondrial protein in rat liver mitochondria [18] and the reciprocal relationship between respiratory rate and intramitochondrial ATP/ADP ratio [19].

3. Results

3.1. Effects of macromolecules on ADP delivery from Mi-AK to oxidative phosphorylation

The ADP delivery from Mi-AK to oxidative phosphorylation was studied in oxygraph experiments. The stimulation of respiration by ADP formed by Mi-AK was measured after addition of a saturating amount of AMP in the presence of increasing amounts of extramitochondrial PK. The ADP formed by Mi-AK in the intermembrane space can either (a) be utilized by oxidative phosphorylation or (b) be consumed by external PK.

As shown in Table 1, the maximal ADP generating activity of Mi-AK in rat liver mitochondria was 795 ± 13 nmol ADP ·mg^{-1}·min^{-1} (n=3) in the absence and 800 ± 12 nmol ADP ·mg^{-1}·min^{-1} (n=6) in the presence of 10% (w/v) dextran. This Mi-AK activity exceeds about two times the maximal ADP phosphorylation activity of rat liver mitochondria and therefore was able to stimulate the mitochondria to state 3 respiration. In the absence and in the presence of 10% (w/v) dextran, Mi-AK stimulated the respiratory rate to the same extent: from 16 ± 1 to 95 ± 7 nmol O_2·mg^{-1}·min^{-1} (n=3) in the absence and from 20 ± 4 to 89 ± 7 nmol O_2· mg^{-1}·min^{-1} (n=6) in the presence of 10% (w/v) dextran M20. State 4 respiration was not affected by the presence of dextran. Fig. 1 shows that increasing activities of PK decreased the Mi-AK-induced respiratory rate. Although PK was added externally up to 100 U/mg mitochondrial protein, i.e. an approximately 200-fold higher ADP consuming activity than the maximal ADP consumption rate of oxidative phosphorylation, PK did not completely reduce the respiration to resting state levels. Importantly, the effect of PK was considerably less in the presence of 10% (w/v) dextran. PK reduced the oxygen consumption to

Table 1
ADP delivery from Mi-AK or extramitochondrial yeast HK to oxidative phosphorylation

Kinase	Dextran M20 (%, w/v)	Kinase V_{max}	ADP flux to oxidative phosphorylation		PK inaccessible proportion (%)
			no PK	excess PK	
		(nmol ADP·mg mitochondrial protein^{-1}·min^{-1})			
Mi-AK (n=3)	0	795 ± 13	315 ± 31	107 ± 28	34
Mi-AK (n=6)	10	800 ± 12	261 ± 39	167 ± 35	64
yeast HK (n=3)	10	795 ± 7	248 ± 35	9 ± 10	4

Mitochondrial respiration as stimulated by either Mi-AK or yeast HK was measured with or without an excess of PK (100–130 U·mg mitochondrial protein^{-1}). The maximal Mi-AK and HK activities (kinase V_{max}) were measured spectrophotometrically in separate incubations. The flux of ADP from kinase to oxidative phosphorylation was caluclated as described in section 2. The PK inaccessible proportion was calculated from the ratio of ADP flux from kinase to oxidative phosphorylation in the presence of an excess of PK compared to that in the absence of PK. The values represent mean ± standard deviation of 3–6 independent experiments.

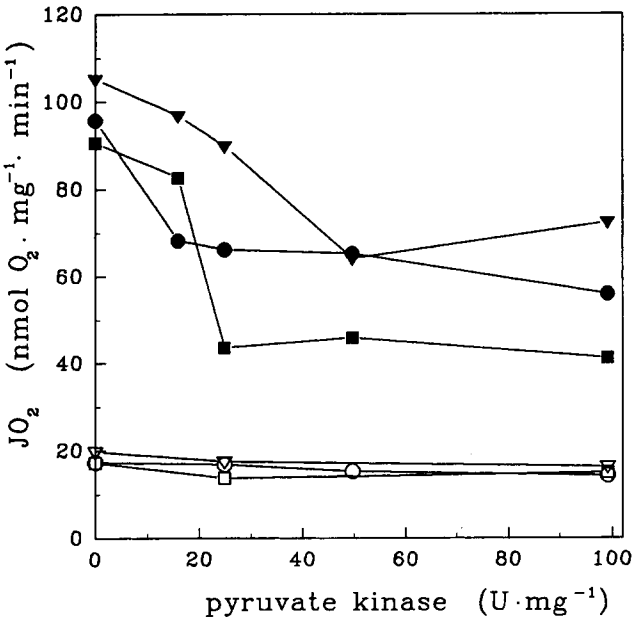

Fig. 1. Effects of macromolecules on the competition between oxidative phosphorylation and PK for ADP produced by Mi-AK in rat liver mitochondria. Mitochondrial respiration (JO_2) was measured in the presence of phospho*enol*pyruvate and increasing amounts of externally added PK. The open symbols represent resting rates of respiration prior to addition of AMP and the filled symbols represent Mi-AK-induced stationary rates of respiration, for measurements in the absence of macromolecules (□,■), in the presence of 10% (w/v) dextran M20 (○,●) or in the presence of 10% (w/v) BSA (▽,▼). Data from a single typical experiment are shown.

41 ± 7 (n=3) nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ in the absence of macromolecules and to 60 ± 7 (n=6) nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ in the presence of 10% (w/v) dextran M20. These results imply that part of the ADP generated by Mi-AK in the intermembrane space was not accessible to extramitochondrial PK and that the PK inaccessible fraction increased in the presence of 10% (w/v) dextran M20. Addition of atractyloside, an inhibitor of the nucleotide translocator, suppressed the rate of respiration to 12 ± 2 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ (n=2) in the absence of macromolecules and to 14 ± 4 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ (n=2) in the presence of 10% (w/v) dextran M20. Earlier studies [4,13] had already shown that macromolecules including dextran M20 and BSA had only minor effects on the basic respiratory properties of rat liver mitochondria like state 3 and 4 respiration. The kinetic properties of several kinases were also not affected by macromolecules [13,20].

The ADP fluxes from Mi-AK to oxidative phosphorylation (v_p) are shown in Table 1. In the absence of macromolecules, the PK inaccessible flux of ADP from Mi-AK to the mitochondrial matrix was 107 ± 28 nmol ADP·$mg^{-1} \cdot min^{-1}$. This corresponds to 34% of the maximal ADP flux from Mi-AK to oxidative phosphorylation. In the presence of 10% (w/v) dextran M20, the PK inaccessible flux of ADP from the intermembrane space into the matrix increased to 167 ± 35 nmol ADP·$mg^{-1} \cdot min^{-1}$, which was 64% of the maximal ADP flux from Mi-AK to oxidative phosphorylation.

BSA was also tested for its effects on the ADP delivery from Mi-AK to oxidative phosphorylation. Fig. 1 shows that in the presence of 10% (w/v) BSA, Mi-AK stimulated the respiration to a similar extent as in the absence or the presence of 10% (w/v) dextran, i.e. from 21 to 105 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$. PK had considerably less effect on the Mi-AK-induced respiration in the presence of 10% (w/v) BSA compared to in the absence of macromolecules. The PK inaccessible flux of ADP from the intermembrane space into the matrix corresponded to 191 nmol ADP·$mg^{-1} \cdot min^{-1}$, which is 57% of the maximal flux of ADP from Mi-AK into the mitochondrial matrix.

3.2. *Mi-AK versus extramitochondrial yeast HK in the presence of 10% (w/v) dextran M20*

In the following, we compared the ADP delivery from Mi-AK to oxidative phosphorylation with that from an extramitochondrially located kinase, i.e. yeast HK. Yeast HK is not able to bind to mitochondria. In the latter studies, yeast HK was added in an equal ADP generating activity as Mi-AK (see Table 1). The experiments were performed in the presence of 10% (w/v) dextran M20 because the more native structure of the mitochondria under those conditions is probably a better representation of the in vivo situation.

In Fig. 2, the difference between the kinase-induced stationary rate of respiration and state 4 respiration is plotted as a function of the amount of PK which was present. This figure demonstrates that the decrease of the kinase-induced respiration by PK was considerably stronger when the ADP was formed by yeast HK (see also Table 1). At sufficiently high PK activities, respiration was suppressed to the resting state indicating that all ADP formed by yeast HK was completely phosphorylated extramitochondrially.

To obtain quantitative insight into the compartmentation

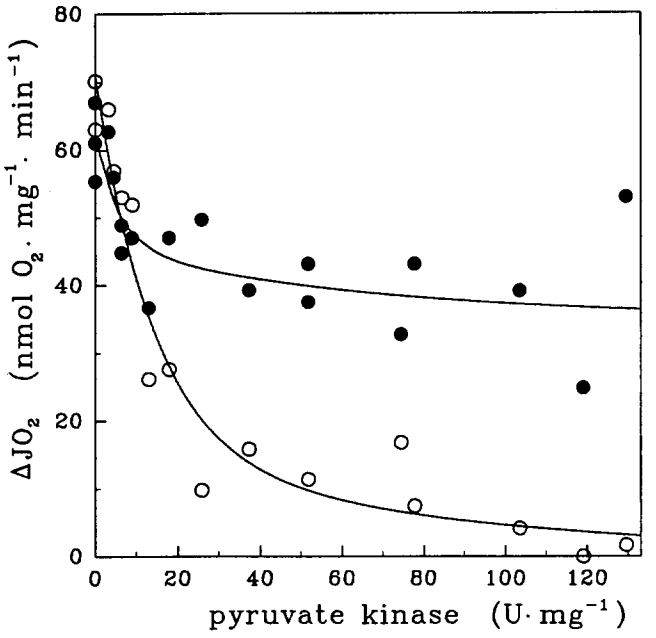

Fig. 2. Competition between oxidative phosphorylation and PK for ADP generated by Mi-AK or by yeast HK in the presence of 10% (w/v) dextran M20. The respiratory rates of rat liver mitochondria induced by Mi-AK or yeast HK were measured in the presence of 10% (w/v) dextran M20, phospho*enol*pyruvate and increasing amounts of PK. The difference between kinase-induced stationary rate of respiration and resting rate of respiration (i.e. prior to addition of AMP or yeast HK) (ΔJO_2) is depicted for Mi-AK (●) and yeast HK (○). Both plots contain data points of two independent experiments. Lines were drawn to guide the eye.

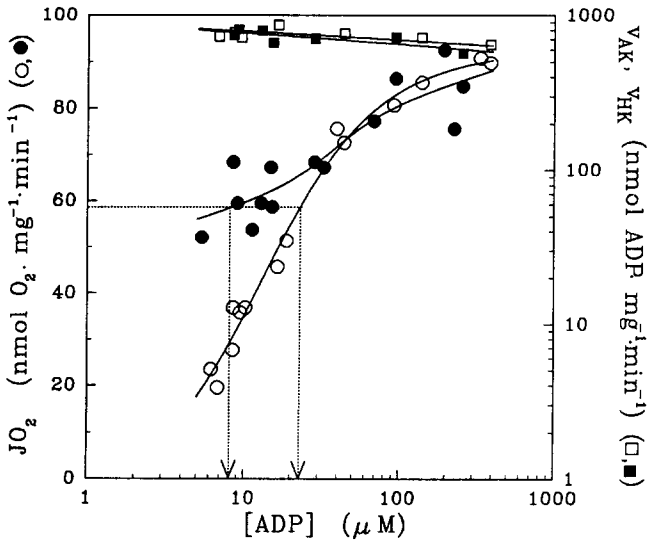

Fig. 3. Effect of the localization of ADP generating enzymes on the dependence of respiration on bulk ADP concentrations. ATP, ADP, AMP and glucose-6-phosphate levels during stationary respiratory rates of the experiment, depicted in Fig. 2, were determined as described in section 2. The respiratory rates (JO_2) induced by Mi-AK (●) or yeast HK (○) are plotted against the bulk ADP levels. The squares represent the rates of ADP formation by Mi-AK (v_{AK}) (■) or yeast HK (v_{HK}) (□) which were calculated from the decrease in AMP levels and the increase in glucose-6-phosphate levels, respectively. All plots depicted in this graph contain data points of two independent representative experiments. The lines were drawn to guide the eye only. The vertical arrows mark the bulk ADP concentrations at a respiratory rate of 58 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$.

of adenine nucleotides, ATP and ADP levels during stationary rates of respiration were determined in total incubation mixtures. Such ATP and ADP determinations include contributions from both the intramitochondrial and the extramitochondrial compartments. Taking into account a mitochondrial adenine nucleotide content of 12–14 nmol ATP+ADP·mg mitochondrial $protein^{-1}$ [18] and the reciprocal relationship between respiratory rate and intramitochondrial ATP/ADP ratio [19], the contribution of intramitochondrial ATP to total ATP levels was negligible. The intramitochondrial contribution to bulk ADP increased from about 1 μM in the resting state to 5 μM during state 3 respiration. In Fig. 3, the Mi-AK-induced and yeast HK-induced respiratory rates at the various PK additions are plotted against the bulk ADP levels. Interestingly, at low ADP levels the rate of respiration appeared considerably higher when the ADP was generated in the intermembrane space by Mi-AK compared to extramitochondrial ADP regeneration by yeast HK. The ATP levels in the total incubation mixtures were not different when comparing Mi-AK with yeast HK (not shown).

Fig. 3 also shows that the actual rates of ADP formation by Mi-AK or yeast HK were not significantly different. This implies that the observed differences in bulk ADP levels in the Mi-AK and HK system were not caused by differences in ADP production rates.

4. Discussion

In this study, we investigated the effects of macromolecules on the ADP delivery from Mi-AK in the intermembrane space to oxidative phosphorylation. The most important finding was that macromolecules increased the ADP supply from Mi-AK into the mitochondrial matrix (Table 1), i.e. the coupling between Mi-AK activity and mitochondrial activity had increased. Because dextran and BSA had similar effects, the macromolecule effect presumably is a general phenomenon exerted by the colloid osmotic pressure.

Earlier studies [10,17,21,22] in the absence of macromolecules had shown that a significant part of ADP generated by intermembrane space kinases was not accessible to extramitochondrial ADP consumers. This phenomenon can partially be explained by action of the outer membrane as a diffusion barrier for nucleotides [10,22]. The latter was convincingly demonstrated in studies with rat heart mitochondria from which the outer membrane had been removed by digitonin treatment and which still contained the majority of mitochondrial creatine kinase bound to the inner membrane [10,22]. PK was considerably more effective in suppression of the creatine kinase-induced respiration in these mitoplasts compared to that in intact mitochondria. The outer membrane of mitochondria is permeable to nucleotides because of the presence of porin pores [23]. Nevertheless, the limited number of pores may pose a diffusion limitation, in particular to ADP [10].

The increased ADP supply from Mi-AK to oxidative phosphorylation in the presence of macromolecules can be explained neither by direct effects of macromolecules on the respiratory properties of rat liver mitochondria nor by direct effects of macromolecules on the kinetic properties of Mi-AK and PK because these effects are only marginal [4,13,20]. Two aspects must be considered to explain the increased ADP delivery from Mi-AK to oxidative phosphorylation. First, several studies have shown that a small distance between enzymes is kinetically advantageous [24,25]. Mi-AK is a soluble enzyme in the intermembrane space [6]. The reduction of volume of the intermembrane space as brought about by macromolecules might bring the ADP consuming component (oxidative phosphorylation) and the ADP producing component (Mi-AK) closer together, and the effective ADP concentration in the intermembrane space might be higher. Secondly, a number of studies have demonstrated that macromolecules not only decrease the efflux of ADP from the intermembrane space to the extramitochondrial compartment, but reduce the nucleotide exchange between the two compartments in general [10,20,26]. The possibility that macromolecules may alter the outer membrane permeability by directly influencing the pore permeability seems less likely since dextrans had no effect on the apparent affinity for ADP of Mi-AK enclosed in outer membrane vesicles (Bijvoet, A. and Laterveer, F.D., unpublished results). Brdiczka et al. [27] have postulated that in contact sites, the voltage-dependent porin molecules may sense the membrane potential across the inner mitochondrial membrane due to capacitive coupling. According to this concept, a macromolecule-induced change in intermembrane contact site frequency may alter the membrane permeability. Another possibility is that the change in structure of the mitochondrial periphery as brought about by addition of macromolecules may affect the resistance for diffusion within the intermembrane space, thereby also influencing the exchange of intramitochondrial nucleotides with their extramitochondrial pools. To obtain insight into the physiological relevance of the localization of Mi-AK in the intermembrane space, the functioning of the mitochondrial enzyme was compared to that of extramitochondrial (non-bindable) yeast HK.

Those studies were performed in the presence of macromolecules because under those conditions the mitochondria are in a more native configuration. The competition between intramitochondrial and extramitochondrial ADP phosphorylation was strongly influenced by the localization of the ADP generating enzymes. PK completely suppressed the kinase-induced respiration when the ADP was formed by external yeast HK both in the absence [10,17] and in the presence (this study) of macromolecules. ADP measurements showed that the dependence of oxidative phosphorylation on bulk ADP levels was clearly different in the Mi-AK system compared to the HK system. At relatively low ADP levels the respiratory rates were higher in the Mi-AK system. As an example, at a respiratory rate of 58 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ (Fig. 3) the bulk ADP levels were 8 μM and 23 μM for Mi-AK and yeast HK, respectively. Since intramitochondrial (matrix and intermembrane space) ADP levels can be assumed to be equal at equal rates of respiration [10], this observation shows that the extramitochondrial ADP level is lower when ADP is formed in the intermembrane space. Because ATP levels were not different between the Mi-AK and HK systems, this also means that the extramitochondrial ATP/ADP ratios were higher in the Mi-AK system. Furthermore, these data demonstrate the existence of concentration gradients of ADP across the outer mitochondrial membrane. At a respiratory rate of 58 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ the ADP concentration gradient across the outer membrane in the Mi-AK system was 12 μM. Fig. 3 shows that the concentration gradients are dynamic, i.e. they depend on the fluxes of ADP consuming and ADP producing enzymes on both sides of the membrane.

Data presented in this paper lend support to the hypothesis of an AK shuttle [10,21,28]. In tissues with high activities of mitochondrial and cytoplasmic AK, like liver, these enzymes can contribute to the ADP transport into the mitochondria at least under conditions of elevated cytoplasmic AMP concentrations. AMP formed by cytoplasmic AK diffuses into the intermembrane space and is converted to ADP via Mi-AK, after which the ADP is used for oxidative phosphorylation. Since the cytoplasmic ADP concentrations are very low (in the μM range) the AK shuttle might be needed to overcome the diffusion resistance of the outer membrane for ADP [10]. This study demonstrates that the ADP flux from Mi-AK to oxidative phosphorylation increases in the presence of macromolecules when isolated mitochondria retain their native conformation. In vivo, the AK shuttle might be important for maintaining low extramitochondrial ADP levels and high extramitochondrial ATP/ADP ratios which is favorable for the action of several cellular ATPases [10].

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