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The quantitation of ADP diffusion gradients across the outer membrane of heart mitochondria in the presence of macromolecules

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Abstract

We have previously provided evidence that diffusion of metabolites across the porin pores of mitochondrial outer membrane is hindered. A functional consequence of this diffusion limitation is the dynamic compartmentation of ADP in the intermembrane space. These earlier studies were done on isolated mitochondria suspended in isotonic media without macromolecules, in which intermembrane space of mitochondria is enlarged. The present study was undertaken to assess the diffusion limitation of outer membrane in the presence of 10% (w/v) dextran M20, in order to mimick the action of cytosolic macromolecules on mitochondria. Under these conditions, mitochondria have a more native, condensed configuration. Flux-dependent concentration gradients of ADP were estimated by measuring the ADP diffusion fluxes across the porin pores of isolated rat heart mitochondria incubated together with pyruvate kinase (PK), both of which compete for ADP regenerated by mitochondrial creatine kinase (mtCK) within the intermembrane space or by yeast hexokinase (HK) extramitochondrially. From diffusion fluxes and bulk phase concentrations of ADP, its concentrations in the intermembrane space were calculated using Fick's law of diffusion. Flux-dependent gradients up to 23 μM ADP (for a diffusion rate of J_{Dif} = 1.9 μmol ADP/min/mg mitochondrial protein) were observed. These gradients are about twice those estimated in the absence of dextran and in the same order of magnitude as the cytosolic ADP concentration (30 μM), but they are negligibly low for cytosolic ATP (5 mM). Therefore, it is concluded that the dynamic ADP compartmentation is of biological importance for intact heart cells. If mtCK generates ADP within the intermembrane space, the local ADP concentration can be clearly higher than in the cytosol resulting in higher extramitochondrial phosphorylation potentials. In this way, mtCK contributes to ensure optimal kinetic conditions for ATP-splitting reactions in the extramitochondrial compartment.

Keywords: Macromolecule; Oxidative phosphorylation; Creatine kinase; Hexokinase; Compartmentation; Concentration gradient; Rat

1. Introduction

There is increasing evidence that ADP transport from the cytosol into the intermembrane space of the mitochondrion occurs both indirectly via ADP shuttle systems consisting of creatine kinase (CK) [1–3] or adenylate kinase [4–6] isoenzymes, and directly by diffusion of ADP itself. In these ADP shuttles, different members of isoenzyme families are located on both sides of the mitochondrial outer membrane, where they presumably work in opposite ways: in the direction of ATP formation in the extramitochondrial space and in the direction of ADP formation in the intermembrane space. This can only be achieved if the concentrations of the relevant substrates are sufficiently different in the two compartments.

We have shown that metabolite diffusion through the porin pores is limited, resulting in the formation of diffusion gradients in the micromolar range [7]. These studies have been performed with isolated rat heart mitochondria in classical isotonic media, in which no significant oncotic pressure is imposed on the mitochondria [7]. It is known that under those conditions, mitochondria are in an artificially swollen state [8,9], while having a condensed configuration in situ [10]. By using measurements of the kinetic constants of the mitochondrial isoenzyme of CK [11] and of oxidative phosphorylation [12] as a sensor, the permeability of outer membrane of heart mitochondria has

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been investigated. The apparent Michaelis constant of mtCK in intact mitochondria was increased compared to the solubilized enzyme [11] and the addition of macromolecules, including dextrans and serum albumin, to the incubation medium led to a further significant increase, indicating a further decrease of permeability [11,13–15]. These studies provided *qualitative* evidence for diffusion hindrance imposed by the outer membrane and how this is modulated by the composition of the incubation medium. However, a detailed understanding of the regulation of oxidative phosphorylation within intact tissues requires *quantitative* information on the extent of dynamic compartmentation. Presently, however, techniques are lacking to obtain these data for intact tissues or whole cells. Therefore, we have introduced reconstituted systems, which consist of isolated mitochondria, pyruvate kinase (PK) (as an extramitochondrial ADP scavenging device, which represents a substitute for glycolysis) and an ADP-generating kinase [2,7,11]. We have studied several kinases, which were positioned at different locations with respect to the outer membrane [11]: (a) mitochondrial creatine kinase (mtCK) [7] and mitochondrial adenylate kinase (mtAK) [5], which are both localized in the intermembrane space; (b) mitochondrial hexokinase (mtHK) [13], which is bound at the external surface of the outer membrane; and (c) yeast hexokinase (HK), which lacks the ability to bind to the mitochondrion and thus serves as a nonbound, extramitochondrial control.

Fig. 1 represents a schematic view of the system used in this work. The rationale of the reconstituted system is that the mitochondria and PK compete for kinase-generated ADP and that the balance between ADP utilization by oxidative phosphorylation or PK activity depends on the location of the ADP-generating kinase with respect to outer membrane. The intracellular oncotic pressure was simulated by addition of 10% dextran to the incubation media. These model systems were used to quantitatively measure the flux-dependent diffusion gradients across the porin pores under conditions of simulated cytosolic oncotic pressure on rat heart mitochondria.

2. Materials and methods

2.1. Materials

Rat heart mitochondria were isolated as described previously [12]. The final mitochondrial pellet was resuspended in 300 mM sucrose, 10 mM Tris, 0.2 mM disodium EDTA, 2 mM MgO (pH 7.4) to give a protein concentration of approximately 30 mg/ml. Lyophilized PK, which was essentially salt-free and lyophilized yeast HK were obtained from Sigma. Dextran M20 (15–20 kDa) was from Serva. All other chemicals used were of the highest grade available and obtained from regular commercial sources.

2.2. Protein determination

The protein concentration of the mitochondrial preparation was measured by the BCA assay [16]. The BCA reagent was supplemented with 0.1% (w/v) sodium dodecylsulfate. Bovine serum albumin was used as a standard.

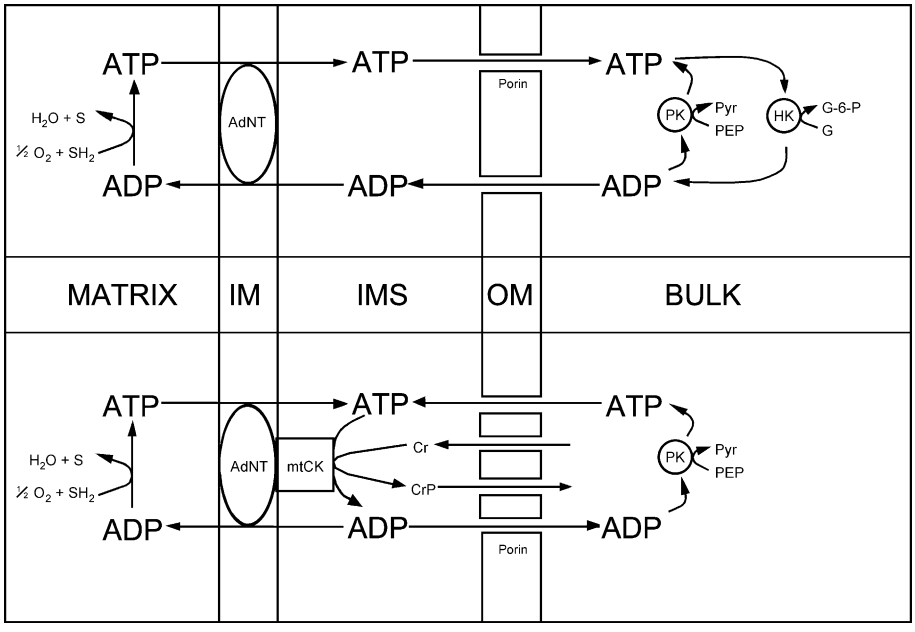

Fig. 1. Metabolic scheme of the reconstituted systems used for experimental determination of diffusion gradients across the outer membrane of isolated rat heart mitochondria. HK system (top), CK system (down). G, glucose; G-6-P, glucose-6-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Cr, creatine; CrP, creatine phosphate; SH_2, substrate; S, reduced substrate; AdNT, adenine nucleotide translocator; IM, inner membrane; IMS, inner membrane space; OM, outer membrane. For explanations, see text.

2.3. Respiration measurements

Respiratory rates of rat heart mitochondria were measured at 30 °C in an OROBOROS oxygraph [17] in a medium containing 120 mM MES, 5 mM KH_2PO_4, 0.5 mM mannitol, 10 mM NaCl, 20 mM imidazole, 20 mM taurine, 8 mM $MgCl_2$, 5 mM KH_2PO_4, 0.5 mM disodium EDTA, 0.5 mM dithiothreitol, 8.8 mM glutamate, 1.8 mM malate, 25 mM creatine and, where indicated, dextran (pH 7.4 with KOH). The oxygen content of the air-saturated oxygraph medium was measured as described previously [12]. Oxygraph medium without, with 10% (w/v) dextran M20 and with 15% (w/v) dextran M20 appeared to contain 248 ± 5 μM ($n=7$), 219 ± 8 μM ($n=6$) and 199 ± 8 μM ($n=4$) O_2, respectively. The specific oxygen consumption rates (nmol O_2/mg mitochondrial protein/min) were calculated from the first derivative of the oxygraph trace.

The mitochondrial respiration as induced by mtCK was measured in the presence of varying amounts of PK. Single incubations were performed for each PK addition. The following components were added together: (a) 1520 μl oxygraph medium; (b) 10 μl rat heart mitochondria; (c) 75 μl phosphoenolpyruvate (100 mM tricyclohexylammonium salt; final concentration 4.2 mM); (d) 0–200 μl PK (1200 U/ml in oxygraph medium) and 200–0 μl oxygraph medium. After closing the oxygraph chamber to a total volume of 1.5 ml, the mtCK-induced respiration was initiated by the addition of 32 μl of 250 mM ATP (pH 7.0; final concentration 5.3 mM). For stimulation of the respiration by yeast HK, creatine was omitted from the oxygraph medium and 22 mM glucose was added instead. Yeast HK was added to a final concentration of 2 U/mg mitochondrial protein, and the HK-induced respiration was started by addition of ATP. The respiratory control index of the rat heart mitochondria was always higher than 5.

2.4. Spectrophotometric assay of CK and HK activity

The activities of mtCK and yeast HK were measured in the direction of ADP formation with a coupled enzyme assay at 30 °C under conditions comparable to the oxygraph experiments. The oxygraph medium was supplemented with NADH (0.25 mM) and oligomycin (17 μM). For the determination of the activity of mtCK in rat heart mitochondria, the reaction was started by addition of ATP (5.3 mM). The measurements of mtCK activity were corrected for the contribution of mitochondrial ATPase activity that was not completely blocked by oligomycin. For determination of HK activity, the creatine in the oxygraph medium was replaced by 22 mM glucose and the reaction was initiated by addition of ATP. The ADP production rate in these assays is linearly related to the NADH utilization rate, which was measured spectrophotometrically at a wavelength of 340 nm. The kinase activities are expressed in nmol ADP/mg mitochondrial protein/min.

2.5. Metabolite determinations

To determine metabolite concentrations, samples were taken 2.5 min after addition of ATP when the mtCK-induced or HK-induced respiratory rates were stationary. All reactions were terminated by immediate dilution of the samples in organic solvent mixtures as described earlier [13,19]. ATP, ADP, glucose 6-phosphate, phosphocreatine and pyruvate concentrations were measured spectrophotometrically or fluorimetrically using standard enzymatic assays [20]. In a number of 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, the pyruvate was first converted to lactate by addition of an equimolar amount of NADH and by addition of lactate dehydrogenase.

2.6. Calculation of ADP fluxes

The ADP production fluxes by mtCK (J_{CK}) and HK (J_{HK}), as well as the ADP flux to PK (J_{PK}), were calculated from the increase in the concentrations of phosphocreatine, glucose 6-phosphate and pyruvate, respectively, assuming that the activities were stationary throughout the 2.5-min incubation period. The ADP fluxes from mtCK or yeast HK to oxidative phosphorylation (J_P) were calculated from the differences between the kinase-induced respiration and state 4 respiration, taking into account the theoretical P/O ratio of 2.5 for respiration on glutamate and malate. All fluxes are expressed as nmol ADP/mg mitochondrial protein/min.

3. Results

3.1. Dynamic ADP compartmentation in intermembrane space of heart mitochondria in dextran-containing media

At first, the effects of dextran addition on the basic properties of isolated rat heart mitochondria were studied. The J_{max} of mtCK was 1953 ± 199 nmol ADP/mg mitochondrial protein/min ($n=3$) in the absence of dextran. In agreement with earlier studies [11,14], this activity was not changed in the presence of dextran (1903 ± 224 nmol ADP/mg mitochondrial protein/min; $n=4$). The mtCK activity is about 1.5 times the maximal ADP phosphorylating activity of rat heart mitochondria, and therefore mtCK was able to stimulate mitochondrial respiration to state 3 levels. Table 1 shows that dextran M20 at a concentration of 10% (w/v) had no effect on state 3 respiration or the respiratory rate in the presence of atractylate, which inhibits the adenine nucleotide translocator. At 15% (w/v) dextran, state 3 respiration tended to decrease, in agreement with earlier studies on rat liver mitochondria [12,14]. State 4 respiration was not affected by dextran (data not shown; see also Refs. [12,14]).

Next, the effects of addition of PK on the rate of respiration were measured for the case of ADP generation

Table 1
Effects of dextran M20 on the respiratory properties of rat heart mitochondria

Dextran (%, w/v)	Respiratory rate (nmol O_2/mg/min)	
	State 3	AT-state
0	226 ± 3	27 ± 4
10	234 ± 44	31 ± 9
15	190 ± 5	18 ± 1

State 3 respiration was induced by endogenous mtCK activity. The atractylate (AT) state represents the rate of respiration after addition of 50 μM AT. The values represent mean ± S.D. of at least three different experiments.

by either mtCK in the intermembrane space or yeast HK in the extramitochondrial compartment. Typical results are compared in Fig. 2 for an experiment in dextran-containing medium. Respiration was first activated via mtCK (Fig. 2, top) in the absence of PK (left). Addition of PK (50 U/mg mitochondrial protein) reduced respiration only to ca. 60% of the state 3 level, indicating that part of ADP generated by mtCK is preferentially delivered to oxidative phosphorylation. The bottom part of Fig. 2 depicts the corresponding experiments for the case of ADP generation by extramitochondrial yeast HK, which also stimulated respiration to state 3 levels in the absence of PK (left). Importantly, however, in this case, addition of PK reduced the respiratory rate nearly to state 4 levels, which was deduced from the fact that subsequent inhibition of the adenine nucleotide translocator only led to a minor further decrease in oxygen uptake. These results indicate that most of the ADP produced by HK in the extramitochondrial compartment is phosphorylated by PK (see also below), as expected.

3.2. Measurement of ADP concentration gradients across the outer membrane

In the second set of experiments the PK activity was gradually titrated and the rate of respiration was measured for each PK addition (Fig. 3). In these experiments we also assessed the effect of dextran on the dynamic compartmentation of ADP. For the HK system, PK activity diminished the rate of respiration down to resting state levels both in the absence (data not shown) and in the presence of 10% (w/v) dextran M20. For the mtCK system, however, respiratory activity remained well above state 4 values at saturating PK activity. In the presence of dextran the phosphorylating rate of PK-insensitive respiration was significantly increased

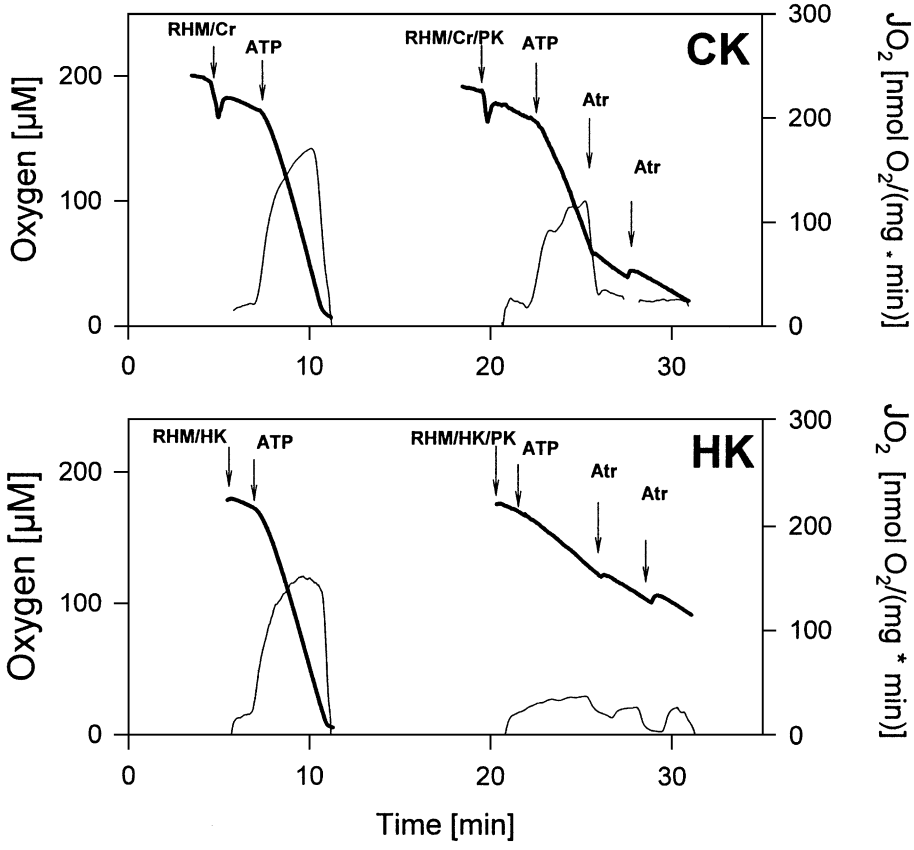

Fig. 2. Effect of localization of ADP regenerating enzyme on competition between rat heart mitochondria and pyruvate kinase for ADP. Recordings of oxygen uptake by rat heart mitochondria stimulated by mtCK (CK) or by extramitochondrial HK, without (left) or with added PK in a 10% (w/v) dextran-containing medium as described in Materials and methods. Oxygen concentration (thick lines) and the rate of respiration (thin lines) as a function of time. Additions: RHM, 0.3 mg mitochondrial protein; Cr, 25 mM creatine; PK, 50 U PK/ml; HK, 2.2 U yeast hexokinase/mg mitochondrial protein; ATP, 4.7 mM ATP; Atr, 50 μM atractylate.

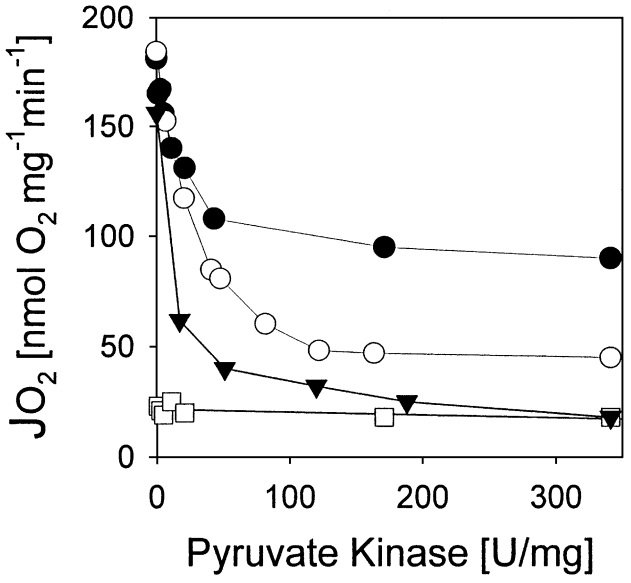

Fig. 3. Effect of dextran M20 (10% w/v) on the competition between oxidative phosphorylation and PK for ADP generated by mtCK or yeast HK in suspensions of rat heart mitochondria. Respiration, as stimulated by endogenous mtCK (●, ○) or by extramitochondrial yeast HK (▼), was measured in the presence of phosphoenolpyruvate and increasing PK activities as indicated. Experiments were carried out as described in the legend to Fig. 2. Stationary rates of respiration were observed 2.5 min after starting the reactions by addition of 4.7 mM ATP. In some incubations with creatine and PK, respiration was measured after inhibition of AdN-translocator with atractylate (□). All incubations were done in the presence of 10% dextran except (○). Each symbol represents one separate incubation.

(+160%). These data clearly show that macromolecules increase the compartmentation of adenine nucleotides in the intermembrane space.

In order to quantify the flux-dependent concentration gradients across the outer mitochondrial membrane in the presence of dextran, single incubations were performed as shown in Fig. 2 and samples were quenched for determinations of ATP, ADP, pyruvate, glucose 6-phosphate and creatine phosphate. From these concentrations and the measured rates of respiration, the fluxes of ADP through the pertinent processes in the reconstituted systems (i.e., the mtCK, yeast HK, and PK reactions as well as oxidative phosphorylation) were calculated. In Fig. 4, these ADP fluxes are plotted as a function of bulk phase ADP concentration. The data points at the highest ADP concentration were obtained from incubations without PK. Under these conditions, J_{CK} (the ADP production flux through mtCK) was submaximal due to pronounced ADP inhibition of mtCK but the ADP phosphorylation equals ADP formation. On the contrary, product inhibition of yeast HK by ADP is much less pronounced, as expected on the basis of its known kinetic properties [7]; therefore, ADP generation by HK exceeded the ADP utilization by oxidative phosphorylation, indicating no stationary conditions in this single incubation without PK. In all other incubations, however, the ADP generation by either mtCK or HK was balanced by ADP utilization via oxidative phosphorylation *and* PK activity. The metabolic differences between both systems are visible at bulk phase concentrations lower than 10 μM ADP. In this concentration range J_{O_2} tends to zero in the HK system but remains remarkable in the CK system. Fig. 5 demonstrates this difference more clearly. In the HK system the bulk phase ADP concentration was 212 μM under state 3 conditions, and half maximal respiration occurred at 60 μM ADP. The state 3 concentration of ADP in the CK system was 161 μM and also the half maximal rate of respiration occurred at a significantly lower bulk phase ADP concentration (23 μM ADP) as compared to the HK system. Fig. 5 also shows that the differences in the steady-state ADP concentrations between the two sys-

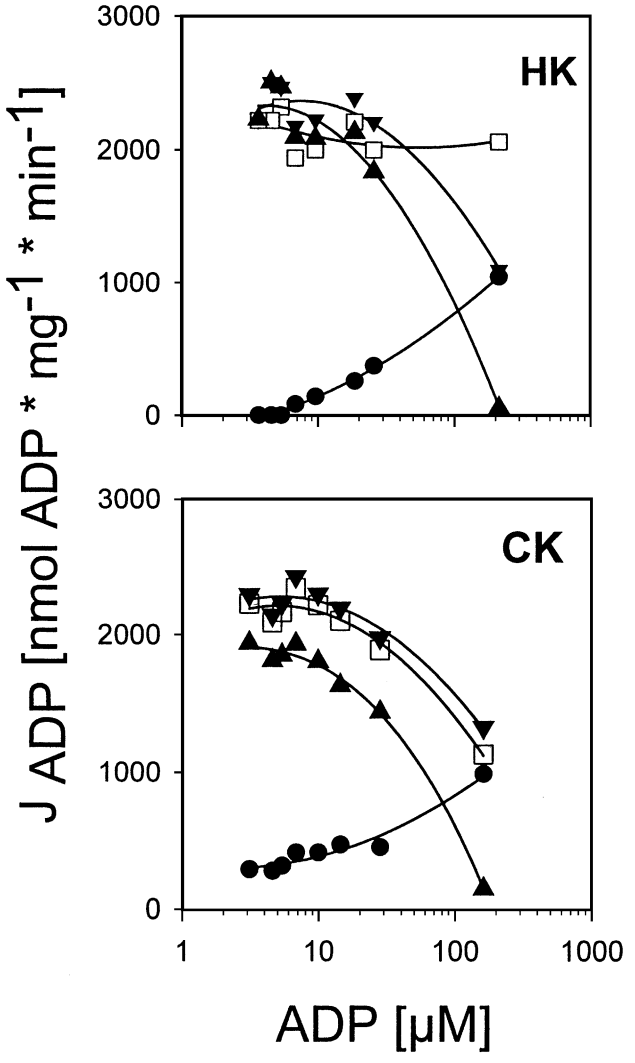

Fig. 4. Effect of localization of ADP regenerating enzyme on ADP fluxes in heart mitochondria. Experiments were performed as described in Figs. 2 and 3 and in Materials and methods. ADP regenerating fluxes J_{CK} (bottom, □) and J_{HK} (top, □), and ADP phosphorylating fluxes J_{OP} (●), J_{PK} (▲), $J_{PK}+J_{OP}$ (▼) were plotted versus the bulk phase ADP concentration. The data are from one (out of three) typical experiment.

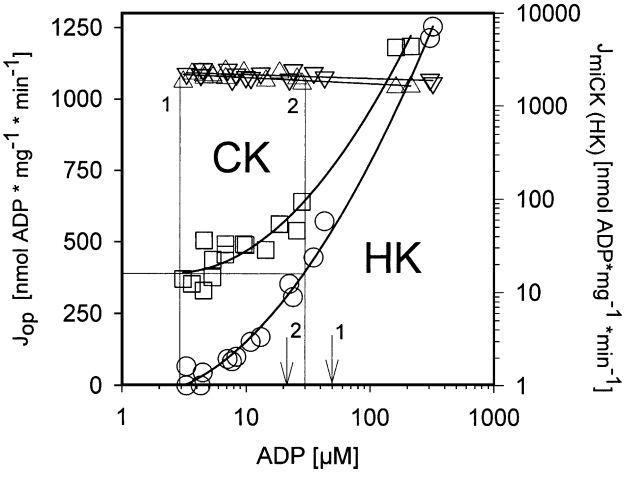

Fig. 5. Direct demonstration of ADP gradients across the outer membrane of heart mitochondria. ADP fluxes to oxidative phosphorylation, J_{OP}, in the CK system (□) and in the HK system (○) as well as ADP regenerating fluxes by HK, J_{HK} (▽), and mtCK, J_{CK} (△), were plotted versus the bulk phase ADP concentration. The data points with the highest bulk phase ADP were obtained in incubations without PK. Thin horizontal lines (1,2) mark the bulk phase concentrations of ADP in both systems at the minimum rate of respiration measured in CK system in the presence of excess PK activity (vertical line). The small arrows mark K_{ADP} values for HK (1) and CK system (2).

tems were not caused by differences in the rates of ADP formation by mtCK and HK, since these were essentially identical.

The key point of the data presented in Fig. 5 is that the relationship between the rate of oxidative phosphorylation and the extramitochondrial ADP concentration is shifted to substantially lower ADP concentrations in the mtCK system as compared to the HK system. Moreover, it was not possible to decrease J_{OP} in the CK system lower than 388 nmol ADP/mg/min as marked by the vertical line in Fig. 5. This minimal respiration occurred at 3 μM ADP bulk phase concentration (horizontal line 1). If in the HK system mitochondria have the same activity, then the bulk phase ADP concentration is 30 μM (horizontal line 2). The ADP formation fluxes by HK and mtCK under these conditions can be seen from the cross points of the vertical lines with regression lines ($J_{CK}=2255$ ADP/mg/min; $J_{HK}=2348$ ADP/mg/min). Since the mitochondrial ADP concentrations (i.e., in the matrix and intermembrane space compartments) must be considered equal at equal rates of respiration, these data clearly imply that ADP gradients exist across the outer membrane. ATP concentrations were identical in the two systems (data not shown), as expected.

3.3. Calculation of ADP concentrations in intermembrane space

The experimentally determined ADP fluxes and bulk phase ADP concentrations can be used to estimate the magnitude of the ADP concentration gradients across the outer membrane. According to a model described previously [7,11] and the metabolic scheme in Fig. 1, the ADP diffusion flux (J_{Dif}) through the porin pores in the mtCK system must be equal to the difference between the mtCK-based ADP production flux in the intermembrane space (J_{CK}) and the ADP flux from mtCK to oxidative phosphorylation (J_{OP}). Furthermore, in the CK system J_{Dif} is also equal to the phosphorylation flux by PK (J_{PK}). This results in the following relation:

$$J_{Dif} = J_{CK} - J_{OP} = J_{PK} \quad (1)$$

The ADP diffusion flux is linearly related to its concentration gradient across the outer membrane, which can be described by an equation analogous to Fick's first law of diffusion:

$$J_{Dif} = k_{Dif}([ADP]_{IMS} - [ADP]_{BP}) \quad (2)$$

in which $[ADP]_{IMS}$ and $[ADP]_{BP}$ are the ADP concentrations in the intermembrane space and bulk phase, respectively; k_{Dif} is the rate of diffusion for ADP.

In the HK system, the net ADP diffusion flux occurs in the opposite direction, and J'_{Dif} is equal to the ADP flux to oxidative phosphorylation (J'_{OP}). For distinguishing the fluxes in the different systems, we mark the fluxes through the HK system by a prime symbol. Both fluxes are equal to the difference between J'_{HK} and J'_{PK}:

$$J'_{Dif} = J'_{OP} = J'_{HK} - J'_{PK} \quad (3)$$

J'_{Dif} is linearly related to $[ADP]'_{BP} - [ADP]'_{IMS}$, leading to:

$$J'_{Dif} = k_{Dif}([ADP]'_{BP} - [ADP]'_{IMS}) \quad (4)$$

In these equations, k_{Dif}, $[ADP]_{IMS}$ and $[ADP]'_{IMS}$ are the only unknowns. As mentioned above, the $[ADP]_{IMS}$ can be considered equal in the mtCK and HK systems at equal rates of respiration (i.e., $[ADP]_{IMS}=[ADP]'_{IMS}$). When assuming that the rate constants for ADP diffusion through the outer membrane pores are equal in the outward and inward directions, the $[ADP]_{IMS}$ concentrations and consequently the ADP concentration gradients across the outer membrane can be calculated by combining Eqs. (2) and (4).

As an example, the measured ADP fluxes and ADP concentrations at the minimal rate of respiration in the CK system at maximal PK activity (see lines in Figs. 4 and 5) are presented in Fig. 6. From these data collected in the CK and the yeast HK system, it was calculated that $[ADP]_{IMS}$ was 26 μM under these conditions. This implies that the maximal ADP concentration gradient across the outer membrane was 23 μM in the mtCK system for a J_{Dif} of 1925 nmol ADP/mg/min. At the same rate of respiration in the HK system, $[ADP]_{BP}$ amounted to 30 μM. The gradient across the porin pores is only 4 μM under these conditions,

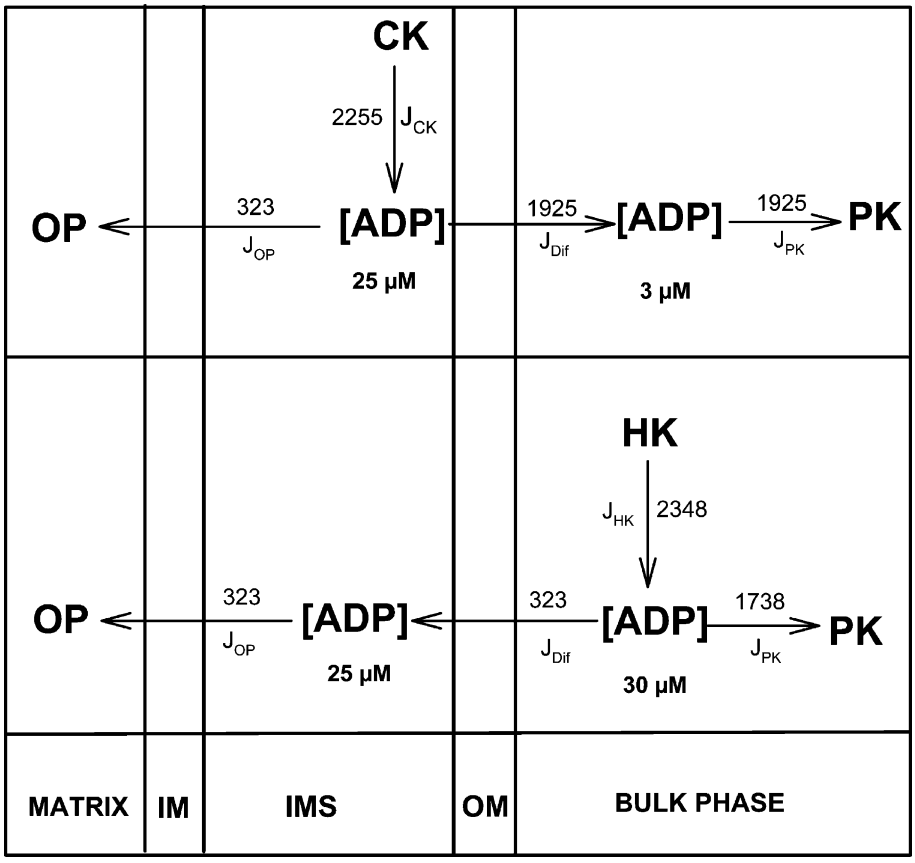

Fig. 6. Calculation of ADP concentrations in the intermembrane space from experimentally determined ADP diffusion fluxes across the OM of heart mitochondria. Fluxes and bulk phase concentrations were taken from Figs. 4 and 5. The intermembrane space (IMS) concentrations of ADP were estimated as detailed in the text. Fluxes are denoted in units of nmol ADP/mg/min. IM, inner membrane; OM, outer membrane.

which is due to the relatively low J_{Dif} of 323 nmol ADP mg/min.

4. Discussion

4.1. The use of dextran for mimicking the intracellular oncotic conditions

In several studies, we presented evidence that ADP is dynamically compartmented in the intermembrane space of isolated heart and liver mitochondria [2,4–7,11–15]. It is, however, important to know to what extent ADP compartmentation occurs within intact cells. Therefore, we tried to simulate the oncotic conditions of intact cells in reconstituted systems consisting of isolated mitochondria and kinases especially designed for investigation of fluxes across the porin pores [2,14]. In vivo, mitochondria are embedded in cytoplasm, in which the protein content may be as high as 20–30% (mass/vol.) [21]. After isolation in commonly employed isotonic media devoid of proteins, mitochondria exhibit a strongly enlarged intermembrane space [8–10] and a decreased number of contact sites [15]. In the macromolecule-free media, the colloid-osmotic balance across the outer membrane is disturbed, resulting in water uptake into the intermembrane space [8]. Addition of macromolecules like albumin, dextrans, ficoll and polyvinylpyrrolidone to isolated mitochondria results in reversal of the morphological changes [8,9,15]. The macromolecule-induced restoration of the in vivo conformation of mitochondria is accompanied by functional changes [5,11–15]. It has been shown that macromolecules have in general two effects on the functioning of isolated mitochondria. (a) Macromolecules increase the coupling between several mitochondrial kinases and oxidative phosphorylation. Thus, it was demonstrated that the ADP supply from mtAK [5,6], mtCK (this study) and externally associated HK isoenzyme I [6,13] to oxidative phosphorylation is increased in the presence of macromolecules like dextran and albumin. (b) Macromolecules increase the diffusion resistance for nucleotide transport between the extramitochondrial compartment and the intermembrane space [5,11,12,14]. The latter was demonstrated in studies showing that macromolecules decrease the apparent affinity of mtAK [14] for externally added ADP and/or ATP. Since the kinetic properties of kinases under solubilized and homogeneous conditions (including mtAK and mtCK but also PK and HK) are not affected by the presence of macromolecules [14], the

effects on the apparent affinities are most likely explained by a reduced accessibility of the substrates to intramitochondrial processes. Also, the decreased apparent affinity of oxidative phosphorylation for externally added ADP in the presence of macromolecules ([12] and this study) might be explained by an increased diffusion resistance for nucleotide transport between the extramitochondrial compartment and the intermembrane space. The K_{ADP} of oxidative phosphorylation increased from 20 μM ADP over 50 μM ADP at 10–15% (w/v) dextran up to 120 μM ADP, if the dextran content of the incubation medium was further increased to 25% (w/v). This indicates that it is possible to further increase the dynamic compartmentation. For the present experiments, however, we used the dextran concentration of 10% (w/v), which is similar to the conditions (12% ficoll and 0.5% F-actin) as used by Hou et al. [22] to establish in vitro the same diffusion rates for fluorescent dyes as measured within intact cells. It may be therefore concluded that within intact cells, the gradients across the porin pores are at least in the same extent of those detected in our model systems. However, due to the complex cellular structure, probably further gradients exist as indicated by the detection of functional units in heart [23] and skeletal muscle [23,24]. These additional gradients may further increase the dynamic ADP compartmentation within intact cells.

4.2. Mechanism of dextran effects on dynamic ADP compartmentation

Several lines of evidence show that the outer membrane is the main diffusion barrier for ADP on its way to the adenine nucleotide translocator [11,14,25,26]. Due to this barrier function, the main part of ADP generated by mtAK or mtCK in the intermembrane space is not accessible to extramitochondrial processes [4,7,25].

The effect of dextran on the permeability of the outer membrane was demonstrated at the level of isolated porin molecules in reconstituted black lipid membranes [14,27]. Apart from a decreased conductivity, dextran [14] or other macromolecules [27] induced a change in the voltage sensitivity of pores. The mechanism of the dextran effect on the porin permeability on molecular level was explained by a decreased pores diameter due to the oncotic stress [27]. This effect on the molecular level could explain also the increased dynamic compartmentation in the CK/HK model system.

However, dextran also increases the compartmentation in mtHK/HK model systems, where mtHK is localized at the cytosolic side of the porin pores [13]. Here a reduction of the pores permeability should reduce the diffusion of ADP into the mitochondria too. Therefore, the reinforcing effect of dextran on channelling of mtHK formed ADP to the AdN-translocator point at the formation of multienzyme complexes consisting of AdN-translocator, porin pores and kinases as mitochondrial HK or CK. The tendency of those proteins to form multienzyme complexes is long known [28]. Evidences are accumulated showing that these enzyme complexes are components of the permeability transition pore [29,30] and that they are involved in mechanisms to release the apoptotic signal cytochrome *c* [31,32]. One advantage of such multienzyme complexes is a rigorous reduction of diffusion distances for metabolites as ADP. Several studies have shown that a short distance between enzymes of one metabolic sequence is kinetically advantageous [33,34].

In conclusion, it seems that dextran acts via compressing the intermembrane space and increasing the frequency of contact sites, which favours the formation of multi enzyme complexes. ADP formed within these complexes has easy access to the AdN-translocator but ADP formed outside of these complexes is separated by a diffusion barrier.

4.3. Gradients and compartmentation

In the intact heart, ATP concentration (5 mM [36]) is much higher than the concentration gradients across the porin pores. Therefore ATP as well as other metabolites of CK as creatine (15 mM [36]) and creatine phosphate (15 mM [36]) can not be dynamically compartmentalized. Cytosolic ADP concentration (30–50 μM [36]), however, is in the same order of magnitude than the gradient. To ensure half maximal respiration (J_{OP} = 0.57 μmol ADP/min/mg protein) in the HK system, extramitochondrial ADP concentration (60 μM) must be 6 μM *higher* than in the intermembrane space (54 μM). When ADP regeneration occurs within the intermembrane space by mtCK, than extramitochondrial ADP concentration is 22 μM (K_{ADP}), 19 μM *lower* than in the intermembrane space (41 μM). These different ADP concentrations are optimal for both, the stimulation of oxidative phosphorylation and for ensuring high extramitochondrial phosphorylation potentials [35]. This advantage disappears, however, if the outer membrane is partially or completely disrupted [25] as it can occur after ischemia [18] or due to other cytotoxic events.

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References

[1] T. Wallimann, M. Wyss, D. Brdiczka, K. Nicolay, H.M. Eppenberger, Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands:

the 'phosphocreatine circuit' for cellular energy homeostasis, Biochem. J. 281 (1992) 21–40.
[2] F.N. Gellerich, V.A. Saks, Control of heart mitochondrial oxygen consumption by creatine kinase: the importance of enzyme localization, Biochem. Biophys. Res. Commun. 105 (1982) 1473–1481.
[3] S.P. Bessman, A. Fonyo, The possible role of the mitochondrial bound creatine kinase in regulation of mitochondrial respiration, Biochem. Biophys. Res. Commun. 22 (1966) 597–602.
[4] F.N. Gellerich, The role of adenylate kinase in dynamic compartmentation of adenine nucleotides in the mitochondrial intermembrane space, FEBS Lett. 297 (1992) 55–58.
[5] F.D. Laterveer, K. Nicolay, F.N. Gellerich, ADP delivery from adenylate kinase in the mitochondrial intermembrane space to oxidative phosphorylation increases in the presence of macromolecules, FEBS Lett. 386 (1996) 255–259.
[6] F.D. Laterveer, K. Nicolay, F.N. Gellerich, Experimental evidence for dynamic compartmentation of ADP at the mitochondrial periphery: coupling of mitochondrial adenylate kinase and mitochondrial hexokinase with oxidative phosphorylation under conditions mimicking the intracellular colloid osmotic pressure, Mol. Cell. Biochem. 174 (1997) 43–51.
[7] F.N. Gellerich, M. Schlame, R. Bohnensack, W. Kunz, Dynamic compartmentation of adenine nucleotides in the mitochondrial intermembrane space of rat-heart mitochondria, Biochim. Biophys. Acta 890 (1987) 117–126.
[8] L.E. Bakeeva, Y.S. Chentsov, A.A. Jasaitis, V.P. Skulachev, The effect of oncotic pressure on heart muscle mitochondria, Biochim. Biophys. Acta 275 (1972) 319–332.
[9] K. Wrogemann, E.G. Nylen, I. Adamson, S.V. Pande, Functional studies on in situ-like mitochondria isolated in the presence of polyvinyl pyrrolidone, Biochim. Biophys. Acta 806 (1985) 1–8.
[10] K. Schwerzmann, H. Hoppeler, S.R. Kayar, E.R. Weibel, Oxidative capacity of muscle and mitochondria: correlation of physiological, biochemical, and morphometric characteristics, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 1583–1587.
[11] F.N. Gellerich, M. Kapischke, W. Kunz, W. Neumann, A. Kuznetsov, D. Brdiczka, K. Nicolay, The influence of the cytosolic oncotic pressure on the permeability of the mitochondrial outer membrane for ADP: implications for the kinetic properties of mitochondrial creatine kinase and for ADP channelling into the intermembrane space, Mol. Cell. Biochem. 133–134 (1994) 85–104.
[12] F.N. Gellerich, F.D. Laterveer, B. Korzeniewski, S. Zierz, K. Nicolay, Dextran strongly increases the Michaelis constants of oxidative phosphorylation and of mitochondrial creatine kinase in heart mitochondria, Eur. J. Biochem. 254 (1998) 172–180.
[13] F.D. Laterveer, F.N. Gellerich, K. Nicolay, Macromolecules increase the channeling of ADP from externally associated hexokinase to the matrix of mitochondria, Eur. J. Biochem. 232 (1995) 569–577.
[14] F.N. Gellerich, M. Wagner, M. Kapischke, U. Wicker, D. Brdiczka, Effect of macromolecules on the regulation of the mitochondrial outer membrane pore and the activity of adenylate kinase in the inter-membrane space, Biochim. Biophys. Acta 1142 (1993) 217–227.
[15] U. Wicker, K. Bucheler, F.N. Gellerich, M. Wagner, M. Kapischke, D. Brdiczka, Effect of macromolecules on the structure of the mitochondrial inter-membrane space and the regulation of hexokinase, Biochim. Biophys. Acta 1142 (1993) 228–239.
[16] K.J. Wiechelman, R.D. Braun, J.D. Fitzpatrick, Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation, Anal. Biochem. 175 (1988) 231–237.
[17] T. Haller, M. Ortner, E. Gnaiger, A respirometer for investigating oxidative cell metabolism: toward optimization of respiratory studies, Anal. Biochem. 218 (1994) 338–342.
[18] F.N. Gellerich, S. Trumbeckaite, J.P. Opalka, E. Seppet, H.N. Rasmussen, C. Neuhoff, S. Zierz, Function of the mitochondrial outer membrane as a diffusion barrier in health and diseases, Biochem. Soc. Trans. 28 (2000) 64–69.
[19] L. Schild, F.N. Gellerich, Effect of the extramitochondrial adenine nucleotide pool size on oxidative phosphorylation in isolated rat liver mitochondria, Eur. J. Biochem. 252 (1998) 508–512.
[20] J.R. Williamson, B.E. Corkey, Assay of citric acid cycle intermediates and related compounds—update with tissue metabolite levels and intracellular distribution, Methods Enzymol. 55 (1979) 200–222.
[21] A.B. Fulton, How crowded is the cytoplasm? Cell 30 (1982) 345–347.
[22] L. Hou, F. Lanni, K. Luby-Phelps, Tracer diffusion in F-actin and Ficoll mixtures: toward a model for cytoplasm, Biophys. J. 58 (1990) 31–43.
[23] V.A. Saks, T. Kaambre, P. Sikk, M. Eimre, E. Orlova, K. Paju, A. Piirsoo, F. Appaix, L. Kay, V. Regitz-Zagrosek, E. Fleck, E. Seppet, Intracellular energetic units in red muscle cells, Biochem. J. 356 (2001) 643–657.
[24] U. Braun, K. Paju, M. Eimre, E. Seppet, E. Orlova, L. Kadaja, S. Trumbeckaite, F.N. Gellerich, S. Zierz, H. Jockusch, E.K. Seppet, Lack of dystrophin is associated with altered integration of the mitochondria and ATPases in slow-twich muscle cells of MDX mice, Biochim. Biophys. Acta 1505 (2001) 258–270.
[25] F.N. Gellerich, Z.A. Khuchua, A.V. Kuznetsov, Influence of the mitochondrial outer membrane and the binding of creatine kinase to the mitochondrial inner membrane on the compartmentation of adenine nucleotides in the intermembrane space of rat heart mitochondria, Biochim. Biophys. Acta 1140 (1993) 327–334.
[26] L. Kay, Z. Daneshrad, V.A. Saks, A. Rossi, Alteration in the control of mitochondrial respiration by outer mitochondrial membrane and creatine during heart preservation, Cardiovasc. Res. 34 (1997) 547–556.
[27] J. Zimmerberg, V.A. Parsegian, Polymer inaccessible volume changes during opening and closing of a voltage-dependent ionic channel, Nature 323 (1986) 36–39.
[28] D. Brdiczka, Contact sites between mitochondrial envelope membranes: structure and function in energy- and protein-transfer, Biochim. Biophys. Acta 1071 (1991) 291–312.
[29] D. Brdiczka, G. Beutner, A. Ruck, M. Dolder, T. Wallimann, The molecular structure of mitochondrial contact sites: their role in regulation of energy metabolism and permeability transition, Biofactors 8 (1998) 235–242.
[30] P. Bernardi, Mitochondrial transport of cations: channels, exchangers, and permeability transition, Physiol. Rev. 79 (1999) 1127–1155.
[31] Y. Tsujimoto, S. Shimizu, VDAC regulation by the Bcl-2 family of proteins, Cell Death Differ. 7 (2000) 1174–1181.
[32] P.M. Kerr, M.S. Suleiman, A.P. Halestrap, Reversal of permeability transition during recovery of hearts and its enhancement by pyruvate, Am. J. Physiol. 276 (1999) H496–H502.
[33] D. Burstein, E.T. Fossel, Nuclear magnetic resonance studies of intracellular ions in perfused frog heart, Am. J. Physiol. 252 (1987) H1138–H1146.
[34] K. Mosbach, B. Mattiasson, Multistep enzyme systems, Methods Enzymol. 44 (1976) 453–478.
[35] H. Kammermeier, Why do cells need phosphocreatine and a phosphocreatine shuttle, J. Mol. Cell. Cardiol. 19 (1987) 115–118.
[36] J.A. Bittl, J.S. Ingwall, Reaction rates of creatine kinase and ATP synthesis in the isolated rat heart. A 31P NMR magnetization transfer study, J. Biol. Chem. 260 (1985) 3512–3517.