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Dynamic compartmentation of adenine nucleotides in the mitochondrial intermembrane space of rat-heart mitochondria

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To investigate whether or not the mitochondrial intermembrane space together with the extramitochondrial space form a homogeneous pool for adenine nucleotides, rat-heart mitochondria were studied in reconstituted systems with pyruvate kinase and ADP-producing enzymes with varied localization. In the hexokinase system, ADP is produced extramitochondrially by added yeast hexokinase, whereas in the creatine kinase system mitochondrial creatine kinase is responsible for ADP regeneration in the intermembrane space. The dependence of mitochondrial respiration on the extramitochondrial [ATP]/[ADP] ratio in both systems was investigated experimentally and by means of computer simulation. Near the resting state, higher [ATP]/[ADP] ratios were found in the creatine kinase system than in the hexokinase system at the same rate of respiration. This and the maintaining of a substantial creatine kinase-stimulated respiration in the presence of pyruvate kinase in excess is explained by a two-compartment model considering diffusion limitations of adenine nucleotides. A diffusion rate constant of $(8.7 \pm 4.7)\ 10^4\ \mu l \cdot mg^{-1} \cdot min^{-1}$ for ADP and ATP was estimated, resulting in rate-dependent concentration differences up to 13.7 μM AdN between the extramitochondrial space and the AdN-translocator at the maximum rate of oxidative phosphorylation of rat-heart mitochondria. The results support the assumption that ADP diffusion towards the AdN-translocator is limited if its extramitochondrial concentration is low, resulting in a dynamic compartmentation of adenine nucleotides in the mitochondrial intermembrane space.

Introduction

In the aerobic cell, most of the ADP formed by cytosolic ATP-utilizing reactions is rephosphorylated by mitochondrial oxidative phosphorylation. Mitochondrial and cytosolic pools of adenine nucleotides are interconnected by the AdN-translocator which operates far from the equilibrium [1–4] and contributes to the control of mitochondrial phosphorylation by the extramitochondrial [ATP]/[ADP] ratio [5] as a function of both system structure [6–8] and adenine nucleotide turnover [6–8]. Close functional relations between mitochondrial ADP phosphorylation and ADP regeneration by creatine kinase were first described by Bessman and Fonyo in

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Abbreviations: AdN-translocator, adenine nucleotide translocator; $[ADP]_i$ $[ATP]_i$, $[ADP]_e$ $[ATP]_e$, [ADP] [ATP], adenine nucleotide concentrations in the mitochondrial intermembrane compartment, in the extramitochondrial compartment, and total concentration, respectively.

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1966 [9] and thereafter confirmed by many investigators [10–13]. Whereas the AdN-translocator is an integral protein of the mitochondrial inner membrane [14,15], heart mitochondrial creatine kinase is reversibly bound at the outer surface of this membrane [16] probably at cardiolipin-containing domains [17,18]. It has been shown in kinetic studies [11,13] and by radioisotopic methods [19] that mitochondrial creatine kinase preferentially uses mitochondrial ATP [11,13,19]. It was also demonstrated that mitochondrial creatine kinase ensures a privileged ADP supply for oxidative phosphorylation [10], a direct coupling between AdN-translocator and creatine kinase being assumed as the mechanism [11,13]. Since an inhibition of the AdN-translocator has no influence on the kinetics of creatine kinase [20], a direct interaction between active centres of both enzymes should be ruled out. An alternative explanation of the functional relations between creatine kinase and AdN-translocator is the assumption that both are localized in the same microcompartment of mitochondrial intermembrane space which is dynamically separated from the extramitochondrial space. This should be possible if the adenine nucleotides do not completely equilibrate between intermembrane space and the extramitochondrial compartment, resulting in different AdN-patterns in both pools.

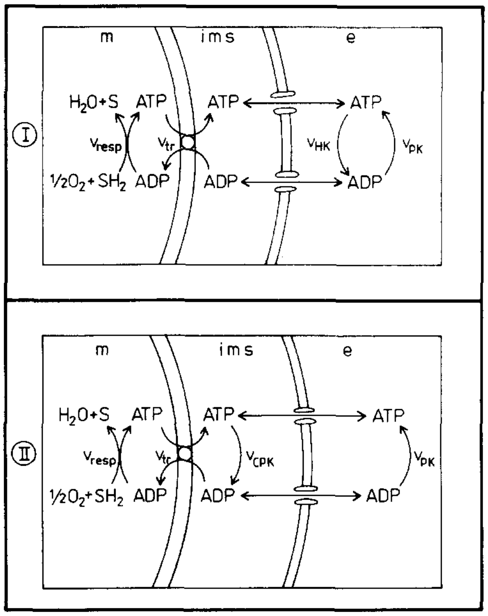

Fig. 1. Scheme of reconstituted systems consisting of rat-heart mitochondria plus pyruvate kinase complemented with hexokinase and glucose, or with creatine. I, Hexokinase system: extramitochondrial ADP regeneration by added yeast hexokinase; II, creatine kinase system: ADP regeneration in the intermembrane compartment by creatine kinase. m, mitochondrial matrix space; ims, intermembrane space; e, extramitochondrial compartment; v_p, v_{CPK}, v_{PK}, v_{HK}: Reaction rates of oxidative phosphorylation, creatine kinase, pyruvate kinase, and hexokinase, respectively.

Two reconstituted systems as shown in Fig. 1 were used for experimental investigation into this problem. In the first system (I), ADP was formed extramitochondrially by added yeast hexokinase, whereas in the second system (II) creatine kinase was responsible for ADP regeneration in the microcompartment. Both systems were further extended to include a competing ADP-trapping system: pyruvate kinase plus phospho*enol*pyruvate, which allows to diminish mitochondrial respiration at a constant high activity of creatine kinase or hexokinase by increasing the pyruvate kinase activity [6,10,21]. Mitochondrial respiration vs. extramitochondrial [ATP]/[ADP] ratio was experimentally investigated in both systems. Furthermore, the differences observed in the relations between mitochondrial respiration and extramitochondrial ADP concentrations were analyzed by means of computer simulation taking into account diffusion limitations of adenine nucleotides between cytosol and AdN-translocator. It was thus possible to estimate a diffusion rate constant of ADP and ATP for their exchange between the intermembrane compartment and the extramitochondrial bulk phase.

Materials and Methods

Chemicals and enzymes. Yeast hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), lactate dehydrogenase (EC 1.1.1.27), creatine kinase (EC 2.7.3.2), pyruvate kinase (EC 2.7.1.40), phospho*enol*pyruvate, carboxyatractyloside, ATP, ADP, glutamate, malate, TRIS, NADP$^+$ and NADH were purchased from Boehringer, Mannheim (F.R.G.), creatine from Merck, Darmstadt (F.R.G.), trypsin from Fritz Leidholdt Biochemie, Kleinmachnow (G.D.R.), dithiothreitol from Calbiochem, Lucerne (Switzerland), and oligomycin as well as trypsin-inhibitor from Serva, Heidelberg (F.R.G.). Other chemicals were analytical grades produced in the G.D.R.

Isolation of mitochondria. Rat-heart mitochondria were isolated from eight rats. After decapitation, hearts were removed and immediately cooled in 0.3 M sucrose. The tissue was minced with scissors and pressed through a syringe [22] into 20 ml of isolation medium containing: 300 mM sucrose/10 mM Tris-HCl/0.2 mM EDTA (pH 7.2)/3.0 mg trypsin and incubated for 15 min. After 7 min it was slightly homogenized with a Potter-Elvehjem homogenizer. The tryptic incubation was finished by addition of 1.3 mg trypsin-inhibitor dissolved in 20 ml isolation medium and a profound homogenization with a Potter-Elvehjem homogenizer. Then, mitochondria were isolated by differential centrifugation as described earlier [10,13]. The following activities of ATP-splitting enzymes were measured in the present mitochondrial preparations: 0.54 ± 0.19 μmol · mg^{-1} · min^{-1} ($n = 5$) extramitochondrial ATPase as contamination including: (i) 0.42 ± 0.17 μmol · mg^{-1} · min^{-1} ($n = 5$) oligomycin-sensitive ATPase; (ii) 0.069 ± 0.015 μmol · mg^{-1} · min^{-1} ($n = 5$) oligomycin-insensitive ATPase; furthermore, 0.014 ± 0.006 μmol · mg^{-1} · min^{-1} ($n = 6$) mitochondrial hexokinase, and 1.2 ± 0.27 μmol · mg^{-1} · min^{-1} ($n = 6$) mitochondrial creatine kinase.

Incubation conditions. The standard medium for incubation contained: 250 mM sucrose, 10 mM Hepes, 4 mM glutamate, 2 mM malate, 8 mM acetate, 0.37 mM dithiothreitol, 4 mM potassium phosphate, 0.3 mM EDTA, 5 mM phospho*enol*-pyruvate, 0.8–4 mM adenine nucleotides (pH 7.4). Further additions were as indicated in the legends. The respiratory rates and their first derivatives were measured in a thermostatically controlled (25°C) closed vessel by a rate meter equipped with a Clark-type oxygen electrode.

Assays. Mitochondrial protein was determined by a biuret procedure [23]. ATP [24], ADP [25], creatine phosphate [24], hexokinase [26], creatine kinase [11], and ATPase [27] were measured by standard enzymatic methods.

Denaturation procedure. A modified phenol-chloroform-isoamylalcohol method according to Slater et al. [28] and Wanders et al. [29] was used: a 1 ml sample was withdrawn from the oxygraphic cell and immediately added to 4 ml phenol/chloroform/isoamylalcohol mixture (38 : 24 : 1) (v/v/v), saturated before with water, and 0.6 ml 66 mM EDTA in a glass tube and intensively shaken by means of a lab shaker for 90 s. The tubes were then put on ice until the end of phase separation. About 1.3 ml from the upper phase were centrifuged ($12\,000 \times g$ for 60 s) and 1.1 ml supernatant were added to 1 ml water-saturated diethylether in a glass tube and mixed for 60 s. This step was necessary to remove the organic compounds of the stop mixture dissolved in the water phase in order to avoid disturbances in the enzymatic assays. 1 ml of the aqueous phase was frozen in liquid nitrogen. Before measurement, the diethylether still present in the aqueous phase was removed by air bubbling through the solution. Unless water-saturated organic phases were used, the adenine nucleotide concentration increased during denaturation due to water uptake into the organic phases.

Model computations. The computer model was developed for simulation of experimental data and for calculation of diffusion rate constants of adenine nucleotides. The structure of the computer model is shown in Fig. 2. The ADP concentration in the intermembrane compartment increased by the action of creatine kinase and decreased by both oxidative phosphorylation and diffusion into the extramitochondrial compartment. The diffusion rate (v_d) must be equal to the difference between ADP production in the intermembrane space by creatine kinase (v_{CPK}) and the rate of mitochondrial ADP phosphorylation (v_P).

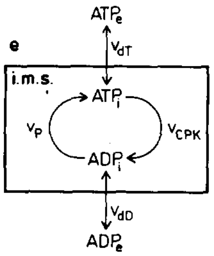

Fig. 2. Scheme of the computer model. ATP$_i$ and ADP$_i$, ATP and ADP in the intermembrane space (ims); ATP$_e$ and ADP$_e$, ATP and ADP in the extramitochondrial bulk phase (e); v_{dT} and v_{dD}, diffusion rate of ATP and ADP from one compartment to the other; v_P and v_{CPK}, reaction rates of oxidative phosphorylation and creatine kinase.

$$v_d = v_{CPK} - v_p \tag{1}$$

The ADP diffusing into the extramitochondrial compartment together with the ADP generated by extramitochondrial ATPase is phosphorylated by pyruvate kinase which, for simplicity, was not included in the computer model. In the hexokinase system, ADP diffuse in the opposite direction, and v_d is equal to the rate of mitochondrial ADP phosphorylation ($v_{CPK} = 0$ in Eqn. 1). The diffusion-linked exchange of ATP and ADP between the intermembrane space and the extramitochondrial compartment was described in terms of equations analogous to Fick's first law of diffusion assuming equal diffusion coefficients for ADP and ATP:

$$v_d = k_d([ADP]_i - [ADP]_e) = k_d([ATP]_e - [ATP]_i) \tag{2}$$

where k_d is the rate constant of diffusion for ADP and ATP. k_d includes the diffusion coefficient D, the area A through which diffusion takes place, and the diffusion distance Δl

$$k_d = \frac{AD}{\Delta l} \tag{3}$$

It follows from Eqn. 2 that the sum of concentrations [ADP] + [ATP] in both compartments must be equal

$$[ADP]_i + [ATP]_i = [ADP]_e + [ATP]_e = A_o \tag{4}$$

where A_o is the sum of AdN concentrations in one compartment, the contribution of AMP being neglected. The relation observed between the activity of creatine kinase (v_{CPK}) and the extramitochondrial adenine nucleotide pattern can be described by an empiric rate equation

$$v_{CPK} = \frac{V_{CPK}[ATP]_e}{[ATP]_e + K_M(ATP)\left(\frac{1+[ADP]_e}{K_I(ADP)}\right)} \tag{5}$$

where V_{CPK} is the maximum rate, K_M(ATP) the Michaelis constant for ATP, and K_I(ADP) the inhibition constant for ADP. The competitive inhibition by ADP expressed in Eqn. 5 is in agreement with the complete rate equation developed by Saks et al. [11].

The dependence of mitochondrial respiration and ADP phosphorylation on the $[ATP]_i/[ADP]_i$ ratio was described according to the rate equations 6 and 7 [30]

$$v_r = V_r \frac{1 + \frac{V_l}{V_p}\left(1 + \frac{\frac{[ATP]_i^n}{[ADP]_i^n}}{K^n_{[ATP]/[ADP]}}\right)}{(\text{den})} \tag{6}$$

$$v_p = mV_r \frac{1 - \frac{[ATP]_i^n/[ADP]_i^n}{K^n_{eq}}}{(\text{den})} \tag{7}$$

with

$$(\text{den}) = 1 + \frac{mV_r + V_l}{V_p}\left(1 + \frac{\frac{[ATP]_i^n}{[ADP]_i^n}}{K^n_{[ATP]/[ADP]}}\right) + \frac{mV_r\frac{[ATP]_i^n}{[ADP]_i^n}}{V_l K^n_{eq}}$$

where V_r, V_p and V_l are the maximum velocities of respiration, phosphorylation and proton leak, respectively; $K_{[ATP]/[ADP]}$ the ratio of apparent half saturation constants with respect to ATP and ADP; K_{eq} is the [ATP]/[ADP] ratio when the net phosphorylation stops; n is an apparent cooperativity coefficient with respect to the [ATP]/[ADP] ratio; m is the stoichiometric coefficient of mol ATP formed per mol O_2 consumed.

Using Eqns. 1, 2 and 4–7 it was possible to describe the relations between mitochondrial respiration and the extramitochondrial $[ATP]_e/[ADP]_e$ ratio. For this purpose, Eqn. 6 was solved for $[ATP]_i/[ADP]_i$ as function φ_1 of v_r:

$$\frac{[ATP]_i}{[ADP]_i} = \varphi_1(v_r) \tag{8}$$

Then, v_p and $[ADP]_i$ are obtained from Eqns. 7 and 4, respectively. Substituting Eqns. 2, 4 and 5 in Eqn. 1 yields an equation permitting to compute $[ADP]_e$ as a function φ_2 of $[ADP]_i$ and the rate v_p:

$$[ADP]_e = \varphi_2([ADP]_i, v_p) \tag{9}$$

The computations were performed on a desk computer (8 Kbyte; EMG 666). The model parameters were estimated from the measured experimental data by nonlinear regression [31].

Results

To investigate whether or not there is a dynamic separation of adenine nucleotides between the intermembrane compartment and the extramitochondrial compartment, rat heart mitochondria were studied in parallel experiments in both the hexokinase system (I) and the creatine kinase system (II) (Fig. 1).

The general approach of these experiments is illustrated in Fig. 3. The most interesting results obtained with these two systems are compared here. Mitochondrial respiration was stimulated by either mitochondrial creatine kinase in the presence of creatine, or added yeast hexokinase in the presence of glucose. The mitochondrial creatine kinase activity was not sufficient to stimulate the fully active respiration; therefore, the sample with yeast hexokinase plus glucose allowed to obtain higher rates of respiration. In both systems, the addition of increasing activities of pyruvate kinase in the presence of phospho*enol*pyruvate caused decreased stationary rates of mitochondrial respiration. In the hexokinase system, the respiratory rate was decreased by pyruvate kinase to 29.7 nmol O_2 per mg per min. In the creatine kinase system, however, higher rates of respiration remained at similar activities of pyruvate kinase, indicating an increased supply of ADP to oxidative phosphorylation via mitochondrial creatine kinase, which could obviously not be suppressed even by an excess of pyruvate kinase. As already demonstrated [10,32], the addition of carboxyatractyloside inhibits the respiration in both systems to the same resting state of respiration, indicating that the differences without carboxyatractyloside are not due to any partial uncoupling in the creatine kinase system.

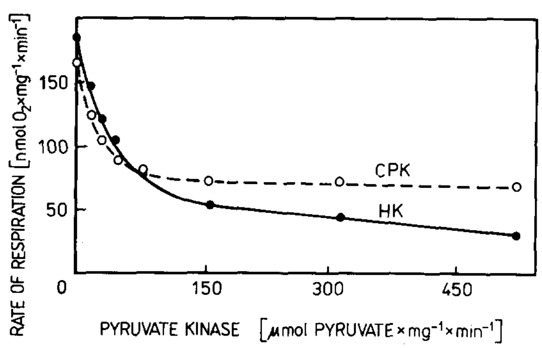

Fig. 3. Respiration rate of heart mitochondria stimulated by added yeast hexokinase or by mitochondrial creatine kinase, vs. pyruvate kinase activity. Rat-heart mitochondria (0.18 mg protein/ml) were incubated in a medium as described in Materials and Methods, containing additionally 1 mM adenine nucleotides and either 25 mM creatine (O------O) or 10 mM glucose and yeast hexokinase (1.86 μmol · mg^{-1} · min^{-1}) (●———●) as well as increasing activities of pyruvate kinase as indicated. Each data point is from a separate incubation.

As mentioned above, no direct interaction was found to exist between creatine kinase and the AdN-translocator [20]. Therefore, a uniform dependence of mitochondrial respiration on the $[ATP]_i/[ADP]_i$ ratio in the immediate vicinity of the AdN-translocator must be assumed for both

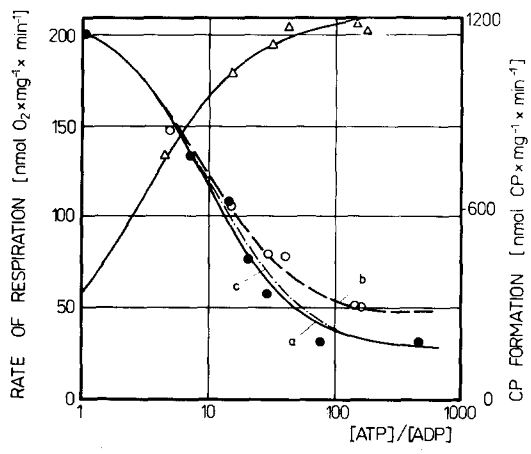

Fig. 4. Control of mitochondrial respiration by the extramitochondrial [ATP]/[ADP] ratio for different localization of the ADP-regenerating enzyme. Mitochondria (0.14 mg protein per ml) were incubated as described in Fig. 3. Curve (a): hexokinase system (2.16 μmol·min^{-1}·mg^{-1} yeast hexokinase); curve (b): creatine kinase system. 90 s after starting the reactions by addition of 0.8 mM adenine nucleotides, samples were withdrawn and analyzed as described in Materials and Methods. The formation of creatine phosphate was calculated while assuming stationary reaction velocity. Curve fitting of data points was accomplished by nonlinear regression. V_{CPK} = (1.485 ± 0.022) μmol·mg^{-1}·min^{-1} and K_I(ADP) = (66 ± 8) μM were estimated from the measured formation of creatine phosphate using Eqn. 5 with K_M(ATP) = 0.20 mM [13]. These values were used for curve fitting of data points of respiration in the presence and absence of creatine with $m = 6$ mol ATP/mol O_2; $K_{eq} = 10^3$; $V_p = 10^4$ nmol·min^{-1}·mg^{-1}, and A_0 = 0.8 mM. The estimated parameter values were: V_r = (240 ± 23) nmol·mg^{-1}·min^{-1}; V_1 = (202 ± 50) nmol·mg^{-1}·min^{-1}; $K_{[ATP]/[ADP]}$ = 2.1 ± 1.8; n = 1.2 ± 0.5; k_d = (8.7 ± 4.7) 10^4 μl·mg^{-1}·min^{-1}. Line (c) represents the computed dependence of respiration on the [ATP]/[ADP] ratio in the intermembrane compartment.

experimental systems. The different respiration rates at higher pyruvate kinase activities suggest differences between the $[ATP]_i/[ADP]_i$ ratios in the intermembrane compartment of both systems, resulting from gradients produced by the varied localization of the ATP-utilizing enzymes.

To study this problem experimentally, samples were withdrawn at each respiratory rate and quenched immediately to determine phosphorylated compounds. The results of one such experiment are shown in Fig. 4. Assuming a mitochondrial adenine nucleotide content of 16 nmol per mg mitochondrial protein [33], the respective contributions of the amounts of mitochondrial and extramitochondrial adenine nucleotides to the total concentration in the incubation mixture were calculated to be 2.2 and 800 μM AdN for this experiment, i.e., the total [ATP]/[ADP] ratio represents mainly the extramitochondrial adenine nucleotides. Additionally, the formation of creatine phosphate was determined in each sample as the marker of the actual creatine kinase activity. The results showed creatine phosphate formation to be highly dependent on the [ATP]/[ADP] ratio, probably due to ADP inhibition of creatine kinase [11]. For an [ADP]/[ADP] ratio of about 6, the activity was only 60% (0.775 μmol CP/mg per min) of that at excess pyruvate kinase activity, corresponding to a respiration rate of about 160 nmol O_2/mg per min. No further stimulation of respiration by creatine kinase was possible under these conditions. It was, however, possible by addition of an excess hexokinase activity (2.160 μmol/mg per min). As demonstrated, both control characteristics are comparable in the range of high respiratory rates. However, at [ATP]/[ADP] ratios higher than 20, a curve splitting was observed. At high [ATP]/[ADP] ratios suppressing the phosphorylating mitochondrial respiration in the hexokinase system, a remarkably elevated rate of respiration remained in the creatine kinase system, which is suggestive of differences between the compositions of the adenine nucleotide pools in the microcompartment and in the extramitochondrial bulk phase.

Assuming a diffusion-limited equilibration of adenine nucleotides between these two pools, the experimental results were analyzed by means of a computer simulation as described in Materials and Methods. Since the rate of ATP utilization due to the creatine kinase in the intermembrane compartment was known from the measured rate of creatine phosphate formation, the exchange rate of ADP and ATP between the extramitochondrial and the intermembrane compartments could be estimated while assuming complete suppression of mitochondrial net phosphorylation of ADP at an $[ATP]_i/[ADP]_i$ ratio of 1000 in the microcompartment. A diffusion rate constant of $(8.7 \pm 4.7) \cdot 10^4$ μl/mg per min (mean ± S.D.) was estimated from the data in Fig. 3 by nonlinear regression.

Furthermore, the model allowed the corresponding $[ATP]_i/[ADP]_i$ ratios in the intermembrane compartment to be calculated (curve c). These ratios must be identical for both systems at the same respiratory rate, but higher than in the extramitochondrial compartment of the hexokinase system and lower than that of the creatine kinase system.

Fig. 5 demonstrates the simulated effects of changed diffusion-rate constants in the range from infinity to $1 \cdot 10^4$ μl/min per mg on computed control characteristics in the creatine kinase system. Lowered diffusion-rate constants produce increased rates of respiration at an equally high [ATP]/[ADP] ratio due to an elevated independence of the adenine nucleotides in the microcompartment.

In further experiments, it was attempted to

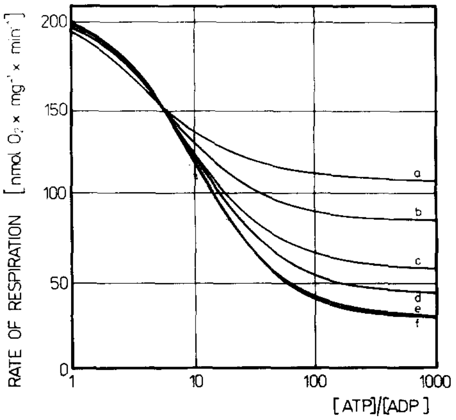

Fig. 5. Simulated effect of diffusion limitation on the mitochondrial respiration vs. extramitochondrial $[ATP]_e/[ADP]_e$ ratio. Curves (a)–(f) were computed with $k_d = 1 \cdot 10^4, 2 \cdot 10^4, 5 \cdot 10^4, 10 \cdot 10^4, 100 \cdot 10^4$ and ∞ $\mu l \cdot min^{-1} \cdot mg^{-1}$. The other parameters were as in Fig. 4.

TABLE I

INFLUENCE OF LOCALIZATION OF ADP-REGENERATING ENZYMES ON STATIONARY [ATP]/[ADP] RATIOS AT DIFFERENT TOTAL AdN-CONCENTRATIONS

Rat-heart mitochondria (0.17–0.32 mg protein per ml) were incubated in a medium as described in Materials and Methods containing additionally 25 mM creatine, pyruvate kinase (600 μmol·mg^{-1}·min^{-1}) and adenine nucleotides as indicated. Once stationary respiratory rates were obtained, two or three samples were quenched from one incubation. Then, in parallel experiments, mitochondria were incubated without creatine, but in the presence of 10 mM glucose and yeast hexokinase (0.81 μmol·mg^{-1}·min^{-1}). Almost the same rates of respiration as in the corresponding creatine kinase experiment were adjusted by addition of pyruvate kinase. Data are as mean ± S.D. for AdN, ADP and ATP (n = 4–6) as well as for the average ratios ϕ (n = 4).

System	[AdN] (μM)	v_{resp} nmol O_2 per mg per min	[ADP] (μM)	[ATP] (μM)	$\frac{[ATP]}{[ADP]}$	$\frac{[ADP]_{CPK}}{[ADP]_{HK}}$	$\frac{[ATP]_{CPK}}{[ATP]_{HK}}$	$\frac{([ATP]/[ADP])_{HK}}{([ATP]/[ADP])_{CPK}}$
CPK	1016.7 ± 40.1	24.3	7.4 ± 3.0	991.2 ± 33.7	134	0.35	0.98	0.36
HK	1054.8 ± 55.1	24.3	20.9 ± 1.6	1012.3 ± 50.3	48			
CPK	1453.2 ± 60.2	27.2	4.5 ± 0.6	1444.1 ± 58.1	321	0.57	1.01	0.56
HK	1457.0 ± 90.4	25.7	7.9 ± 0.9	1429.1 ± 89.1	181			
CPK	2874.0 ± 63.4	30.4	6.7 ± 2.3	2858.0 ± 60.4	427	0.53	0.99	0.53
HK	2898.0 ± 180.7	28.8	12.7 ± 2.9	2882.1 ± 17.7	227			
CPK	4182.8 ± 59.5	34.5	8.2 ± 1.5	4164.0 ± 56.6	508	0.63	1.03	0.61
HK	4079.5 ± 138.2	34.5	13.0 ± 0.9	4062.0 ± 13.7	312			
					ϕ	0.52 ± 0.12	1.00 ± 0.002	0.52 ± 0.11

analyze whether or not the diffusion limitation of ADP or ATP is responsible for the effects shown in Figs. 3 and 4. At an identical rate of respiration, the limiting nucleotide should reveal different extramitochondrial concentrations in both systems. The results are presented in Table I. Mitochondria were incubated first in the creatine kinase system at high pyruvate kinase activity. Stationary rates of respiration obtained, samples were withdrawn and quenched for determination of adenine nucleotides. Then, in parallel experiments, virtually the same rates of respiration were adjusted by addition of appropriate activities of pyruvate kinase in the presence of hexokinase. In all experiments, the total stationary ADP concentration was higher in the hexokinase system than in the creatine kinase system. The $[ADP]_{CPK}/[ADP]_{HK}$ ratios averaged 0.52 which was similar to the $([ATP]/[ADP])_{HK}/([ATP]/[ADP])_{CPK}$ ratio of 0.52, but substantially different from the $[ATP]_{CPK}/[ATP]_{HK}$ ratio of 1.00. This indicates that the different [ATP]/[ADP] ratios in both systems are caused by different stationary ADP concentrations and not by differences in ATP concentrations.

Discussion

Due to the barrier function of the mitochondrial outer membrane for enzymes such as hexokinase, creatine kinase or pyruvate kinase, the enzyme pattern of the mitochondrial intermembrane space differs essentially from that in the extramitochondrial space. To investigate whether or not both compartments are also different with respect to the adenine nucleotide, information is required about their concentrations in these compartments.

To date, however, no method exists for direct determination of adenine nucleotide concentrations in the mitochondrial intermembrane space. Therefore, the mitochondrial respiratory rate was used as an indirect marker for the $[ATP]_i/[ADP]_i$ ratio in the immediate vicinity of the AdN-translocator. We assume that, at the same respiratory rate, identical $[ATP]_i/[ADP]_i$ ratios exist in the neighbourhood of the AdN-translocator in both systems, since the total adenine nucleotide concentrations were exactly the same. The only difference under both incubation conditions was the use of 10 mM glucose in the hexokinase system instead of 25 mM creatine in the creatine kinase system. These additions are not known to have any influence on the functional properties of isolated mitochondria.

Using the first derivative of the oxygen-time curve and the phenol-chloroform-isoamylalcohol stop procedure [28,29], it was possible to ensure higher [ATP]/[ADP] ratios in the extramitochondrial compartment of the creatine kinase system than in the hexokinase system at identical rates of mitochondrial respiration near the resting state. Table I and Fig. 4 clearly demonstrate that this can be attributed to higher extramitochondrial ADP concentrations in the hexokinase system, whereas the extramitochondrial ATP concentrations in both systems do not differ from each other.

Different $[ADP]_e$ concentrations in both systems at the same respiratory rate are indicative of different [ATP]/[ADP] ratios in the intermembrane space and the bulk phase. Obviously, the $[ATP]_e/[ADP]_e$ ratio is higher in the creatine kinase system and lower in the hexokinase system than in the intermembrane spaces due to the varied localization of ADP-regenerating enzymes and the resulting transport fluxes for ADP.

The main feature of the computer model used for this work is its ability to simulate the shift of the dependence on the extramitochondrial [ATP]/[ADP] ratio of respiration after the diffusion of adenine nucleotides is included in the model.

The model opens up the opportunity to estimate a diffusion rate constant for adenine nucleotides of $k_d = (8.7 \pm 4.7) \cdot 10^4\ \mu l \cdot min^{-1} \cdot mg^{-1}$ for diffusion from one compartment to the other. The calculation of this constant did not require any assumptions on the nature and geometry of the compartment boundary. Using Eqn. 2, this diffusion rate constant allows a concentration gradient to be calculated which should exist between the microcompartment and the extramitochondrial bulk phase. In the hexokinase system, the mitochondrial phosphorylation rate is equal to the diffusion rate of ADP to the AdN-translocator (Eqn. 1; $v_{CPK} = 0$). For half-maximal and maximal phosphorylation rates of 600 and 1 200 nmol ADP/mg per min, the concentration gradients calculated were 6.8 and 13.7 μM ADP, respectively. Since the concentration gradient is a function of the rate of oxidative phosphorylation and disappears in the absence of net transport through the outer membrane, this kind of compartmentation is referred to as dynamic compartmentation. In the stationary state, the diffusion rate for ATP must be the same as for ADP, but in the opposite direction. This requires an ATP gradient of the same value and with a sign opposite to that for ADP. A diffusion limitation occurs if the ATP or ADP concentration has a similar value as the concentration gradient required for a given diffusion velocity. This was the case under the present conditions for ADP in the near-resting state. Diffusion limitation diminishes with increasing ADP concentrations and does not occur for ATP because of its high absolute concentration in comparison with the concentration gradients.

The biological importance of mitochondrial localization of ATP-utilizing enzymes such as hexokinase or creatine kinase was discussed as a possibility of a privileged supply of the bound enzyme with mitochondrial ATP [34]. Though hexokinase is bound at the outer side of the mitochondrial outer membrane [34,35] it does not ensure an increased supply of ADP for oxidative phosphorylation. This was shown for rabbit reticulocyte mitochondria (Kunz, W., Gellerich, F.N. and Bohnensack, R., unpublished results). An increased ADP supply to the AdN-translocator in the presence of excess pyruvate kinase activity occurs only if ADP is generated in the intermembrane space, e.g., by creatine kinase or adenylate kinase (Ref. 42; and Kunz, W., Gellerich, F.N. and Bohnensack, R., unpublished results). The generally accepted view that adenylate kinase is a soluble enzyme of the mitochondrial intermembrane space [41] supports the interpretation of the present results which does not require the assumption of a direct interaction between AdN-translocator and creatine kinase. Dynamic compartmentation of adenine nucleotides seems to be a general phenomenon attributed to limitation of diffusion of adenine nucleotides at low concentrations and a high turnover.

It is interesting to note that in the creatine kinase system at minimum respiration, a much higher ADP flux occurs towards the bulk phase than under comparable conditions in the hexokinase system towards the AdN-translocator. This high ADP outflow is further accompanied by a high creatine phosphate flux in the same direction. As the high creatine phosphate flux might compete to some extent with the ADP outflow, similar experiments were performed with an adenylate kinase system (Ref. 42; and Kunz, W., Gellerich, F.N. and Bohnensack, R., unpublished results) in which only adenine nucleotides were transported through the outer membrane. Since a similar compartmentation of ADP in the mitochondrial intermembrane space was observed in the adenylate kinase system, too, we believe that the diffusion limitation is not restricted to our creatine kinase system.

Results of the present work did not provide information about the dimensions of the microcompartment; however, it can be speculated that it is identical with the intermembrane space, as other workers concluded from their experimental findings [19]. Since porine was found in the mitochondrial membrane, forming water-filled pores of 2 nm diameter [37] suitable for molecules up to a molecular weight of 8000 [38] to pass, it is reasonable to assume that the limited number of porine pores [39] is the reason for the diffusion limitation. However, additional unstirred layer effects in the cristae are not excluded, since compartmentation of adenine nucleotides was also demonstrated with mitoplasts [40].

Our results are in line with the explanation of the creatine phosphate shuttle [11,13] by facilitated diffusion [36], since the creatine concentration is high enough for a high-speed diffusion to mitochondrial creatine kinase and a high rate of ADP regeneration in the microcompartment.

The diffusion coefficient for adenine nucleotides in intact cells was estimated to be about 0.1 times than in aqueous solutions (for a review, see Ref. 43). Therefore, we should expect that concentration gradients and dynamic compartmentations of adenine nucleotides may remarkably contribute to the control of energy metabolism in the intact cell.

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