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Determination of mitochondrial creatine kinase fluxes in intact heart mitochondria using ^{31}P-saturation transfer nuclear magnetic resonance spectroscopy

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Abstract

Forward ($\rightarrow$ATP) and reverse ($\rightarrow$CrP) fluxes through the creatine kinase reaction were determined in isolated rat and bovine heart mitochondria and with soluble MM-CK from rabbit skeletal muscle, using ^{31}P-saturation transfer NMR. With *soluble MM-CK* forward and reverse fluxes were identical in the absence and presence of BSA or rat liver mitochondria. Addition of liver mitochondria decreased fluxes with increasing mitochondria concentration. The $flux_f/V_{max(f)}$ ratio was 0.006 with 10 mg BSA and 0.04 with 10 mg rat liver mitochondria, respectively. With heart mitochondria, $flux_r$ was considerably higher than $flux_f$ and the $flux_f/V_{max(f)}$ ratio was 1.7 for rat heart and 0.22 for bovine heart. It is concluded that in the presence of isolated mitochondria, the flux through the creatine kinase is driven by the mitochondrial ATP–ADP turnover. Therefore the $flux_f/V_{max(f)}$ ratio is highest for rat heart mitochondria with a high ATP–ADP turnover, intermediate for bovine heart mitochondria and low for MM-CK in the presence of liver mitochondria. It is lowest with MM-CK alone, where the creatine kinase reaction is at equilibrium and external ATP–ADP turnover is absent. The higher reverse than forward fluxes of mitochondrial creatine kinase determined at steady state by saturation transfer NMR, are caused mainly by a high ATP$\leftrightarrow$P$_i$ exchange in heart mitochondria preparations, having a high ATPase activity, compared to liver mitochondria.

Keywords: Saturation transfer NMR; Mitochondrial creatine kinase; Heart mitochondrion; Reaction rate

1. Introduction

The mitochondrial creatine kinase (Mi-CK) and the extramitochondrial creatine kinase (CK) systems play an important role in the energy transfer of cardiac and skeletal muscle [1]. Their concerted action

Abbreviations: AK, adenylate kinase; ANT, mitochondrial adenine nucleotide translocase; CrP, creatine phosphate; Cr, creatine; DTT, threo-1,4-dimercapto-2,3-butandiol; EDTA, ethylene dinitrilo tetraacetic acid; $flux_f$, forward flux of creatine kinase; $flux_r$, reverse flux of creatine kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid; k_f, pseudo first order forward reaction rate constant; k_r, pseudo first order reverse reaction rate constant; Mi-CK, mitochondrial creatine kinase; MM-CK, MM-creatine kinase; NMR, nuclear magnetic resonance spectroscopy; p_1p_5, p1,p5-di(adenosine-5′-)pentaphosphate; P_i, inorganic phosphate; RF, radiofrequency; Tris, tris-(hydroxymethyl)-aminoethane

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enables the effective channelling of mitochondrial ATP across the intermembrane space to the myofibrillae, where it is consumed in the contraction cycle. This shuttle mechanism rises the cytosolic phosphorylation potential above the level measured in non-excitable tissues, e.g. the liver [2]. Extramitochondrial CKs are associated with specific subcellular compartments or structures and use creatine phosphate (CrP) as an energy buffer to regenerate ATP at sites of high energy demand, e.g. actin/myosin ATPase, whereas Mi-CK, located at the outside of the inner mitochondrial membrane, uses mitochondrial ATP to replenish the cytosolic CrP pool. This is made possible by a functional coupling of the Mi-CK reaction to the oxidative phosphorylation at the inner mitochondrial membrane [3–5].

The pseudo first order reaction rate constants, k_f and k_r, for the phosphate exchange between ATP and CrP in the creatine kinase reaction, are defined as follows:

$$\mathrm{CrP} + \mathrm{ADP} \underset{k_r}{\overset{k_f}{\rightleftarrows}} \mathrm{ATP} + \mathrm{Cr}$$

They can be determined by application of saturation transfer NMR to the two-site exchange of phosphate between CrP and ATP; [^{31}P]CrP ↔ [γ-^{31}P]ATP.

It is not possible, while studying the creatine kinase reactions in heart tissue, to measure the Mi-CK activity separately from the total cytosolic CK activity, as the substrate phosphorus resonances are equivalent in both reactions. To avoid this problem, we measured the exchange rate constants and corresponding fluxes of Mi-CK separately in isolated mitochondria preparations from the highly active rat heart as well as from the less active bovine heart. In addition, phosphate exchange catalyzed by soluble CK was studied in the presence of liver mitochondria, where functional coupling of CK and oxidative phosphorylation within the intermembrane space is absent.

2. Theory

When studying the activity of creatine kinase using NMR, one selectively irradiates either [^{31}P]CrP or [γ-^{31}P]ATP while observing the effects of this saturation on the magnetisation of the other phosphate nucleus, i.e. γ-P-ATP or CrP, respectively. From the change in signal amplitude and the spin-lattice relaxation time (T_1) in the absence of chemical exchange, the pseudo first order reaction rate constants, k_f and k_r, can be calculated [6]:

$$k_f = \frac{1}{T_1^{CrP}} \times \left(\frac{M_0^{CrP}}{M_z^{CrP}(t)} - 1 \right) \quad (1)$$

$$k_r = \frac{1}{T_1^{ATP}} \times \left(\frac{M_0^{ATP}}{M_z^{ATP}(t)} - 1 \right) \quad (2)$$

where M_0 represents the equilibrium magnetisation and $M_z(t)$ the component of the magnetisation parallel to the static magnetic field after t seconds of saturation of the other resonance. The forward flux was calculated from the product $k_f \times$[CrP] and the reverse flux is the product $k_r \times$[ATP].

3. Materials and methods

Chemicals were purchased from Merck (Darmstadt, Germany) or from Sigma (Munich, Germany), biochemicals and enzymes were obtained from Sigma or from Boehringer (Ingelheim, Germany) and were of the highest purity available. MM-CK from rabbit skeletal muscle (300 U/mg) was from Boehringer (Ingelheim, Germany).

3.1. Isolation of mitochondria

Heart mitochondria from rats were isolated essentially according to Jacobus and Saks [7], using 1 mg trypsin for two hearts. Bovine heart mitochondria were prepared from fresh bovine hearts from the slaughterhouse according to the method of Smith [8]. Rat liver mitochondria were isolated according to the method of Klingenberg and Slenczka [9].

For isolation of the mitochondria, a medium containing 0.25 M sucrose, 10 mM Tris-HCl, 5 mM KH_2PO_4, 20 mM KCl and 0.2 mM EDTA, with pH adjusted to 7.2 with NaOH, was used. Respiratory control ratios were determined in an oxygraph chamber with a Clark-type oxygen electrode in the absence of Mg^{2+} ions and in the presence of 7.5 mM glutamate and 5.8 mM malate.

The specific activity of Mi-CK in mitochondrial

suspensions and of soluble MM-CK from rabbit skeletal muscle was measured as the rate of creatine production ($flux_f$) according to [10]. ATPase activity in isolated mitochondria was measured according to [11].

3.2. NMR experiments

All ^{31}P-NMR experiments were performed at 161.98 MHz in a 10 mm NMR tube (2.4 ml total volume) on a Bruker AMX 400 wide bore machine. A dedicated ^{31}P variable temperature probe with a 24 μs π/2 pulse length was used and the temperature within the probe could be regulated within ±0.3°C, using a Bruker Eurotherm K temperature controller. The temperature in the NMR tube was 295 K for all experiments. Magnetic field homogeneity was optimized on the $^{1}H_2O$ signal of each sample, resulting in a $^{1}H_2O$ line width of 15 Hz or smaller. Spin-lattice relaxation times were measured with an inversion recovery technique [12]. Typically, 10–12 time delays were measured, Fourier transformed and fitted to a single exponential, three parameter expression [13]. For these spin-lattice relaxation time experiments we used the same Mi-CK sample as for the saturation transfer techniques. However, prior to T_1 measurement CK was inactivated by a 15 min incubation at 60°C, then the sample was added to the incubation medium which contained CrP. Following this procedure, CrP breakdown was 5% within 3 h (not shown).

The creatine kinase fluxes were measured, using ^{31}P saturation transfer techniques. Selective 6 s saturation of the individual ^{31}P resonances was performed with low power continuous wave narrow band RF irradiation. The irradiated power was adjusted as to result in a saturation of a ^{31}P resonance in a narrow frequency range and it was assured that no spillover of saturation to neighbouring peaks was detectable. Details of these experiments have been published previously [6]. For each spectrum as many scans (8–24 π/2 pulses) as possible were collected with a repetition time of 35 s. Oxygenation of the mitochondrial suspensions was achieved with a hydrogen peroxide/catalase system (1–3 μM/500 U/ml). Catalase was added to the incubation medium and H_2O_2 was infused through a Teflon tube (Fig. 1). The H_2O_2 infusion rate was adjusted to the oxygen consumption rate of the samples (between 2 and 5 μl/min). This was tested in parallel incubations outside the NMR with an oxygen electrode, so that the oxygen concentration was held approximately constant. In phosphorus resonance spectra, insufficient oxygenation was detectable by an immediate decrease in the CrP peak.

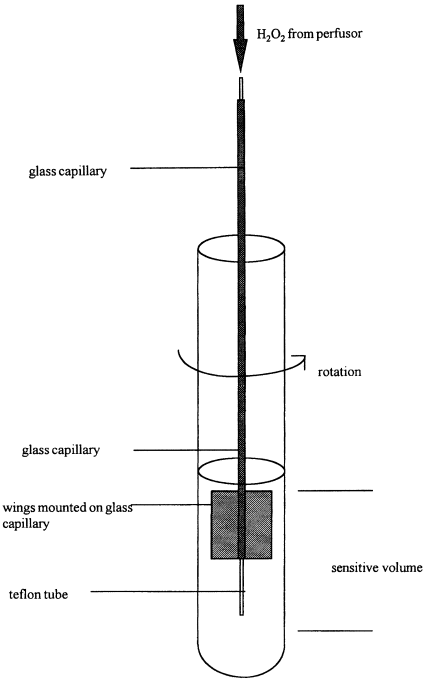

Fig. 1. Experimental set for the incubation of heart mitochondria in the NMR tube. Total volume, 2.4 ml; sensitive volume, ca. 2.0 ml; rotation velocity, 13 Hz; incubation conditions, see Section 3; catalase activity, 500 U/ml; H_2O_2 infusion rate, 2–5 μl/min.

Isolated mitochondria were incubated in a medium containing 120 mM sucrose, 70 mM HEPES, 5 mM KH_2PO_4, 20 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 5 mM glutamate, 5.8 mM malate, 1 mM DTT, 10 mM creatine and 3.5 mM ATP, which was adjusted to pH 7.2 with NaOH. Fluxes through soluble MM-CK (1–10 mg/ml corresponding to 100–1000 U/ml) were investigated using the same medium as for heart mitochondria. The time to reach steady state depended on the amount of mitochondria or soluble CK in the sample. During this time the mitochondria or the soluble CK produced CrP up to a steady state concentration. ATP and CrP concentrations were obtained from signal integrals which were calibrated using a standard capillary filled with a known amount of methylenediphosphonate.

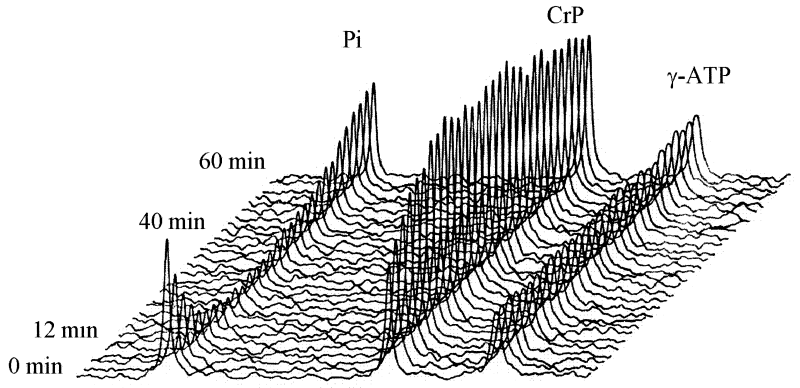

Fig. 2. Stacked plot of ^{31}P-NMR spectra of the development of CrP, ATP and P_i in rat heart mitochondria at a concentration of 3 mg/ml. Six scans/spectra. The time at which spectra were acquired is shown.

The mass action ratio of the creatine kinase reaction, [ATP]×[Cr]/[ADP]×[CrP] at pH 7.2, was determined from the concentrations of ATP, ADP, CrP and Cr using photometric analysis [11] in samples incubated under the same conditions as those used for NMR experiments. Protein concentrations were 1 mg of rat heart mitochondrial protein, 10 mg of rat liver mitochondrial protein together with 1000 U/ml MM-CK, 20 mg of bovine heart mitochondrial protein or 1000 U/ml MM-CK, respectively.

4. Results and discussion

4.1. Experimental design

Stable experimental conditions are essential for steady state measurements. Fig. 2 shows a typical stacked plot of a control experiment after addition of rat heart mitochondria (3 mg/ml) to the incubation medium. CrP is produced within 10–20 min, depending on the origin of the mitochondria and protein concentration, and steady state is maintained for at least 20 min. This time is sufficient for saturation transfer experiments, taking into account the signal to noise ratio. After this time, the CrP signal intensity decreased while P_i gained amplitude, indicating loss of mitochondrial integrity.

Hydrogen peroxide was infused at a rate to reach sufficient oxygenation of the samples, i.e. in a range between 1 and 3 μM, depending on type and concentration of mitochondria. Catalase was present in saturating amounts, so that complete conversion to H_2O was granted. Hydrogen peroxide in high con-

Table 1
Comparison of $V_{max(f)}$ and CK forward fluxes determined with soluble MM-CK and with Mi-CK in isolated heart mitochondria

	Respiratory control ratio	State 3 respiration (mU/mg)	ATPase V_{max} (U/mg)	CK $V_{max(f)}$ (U/mg)	$Flux_f$ (U/mg)	$Flux_f/V_{max(f)}$	CK mass action ratio
MM-CK	–	–	–	240.0 ± 10	14.8[b]	0.06	110[d]
Rat liver mitochondria (+MM-CK)	8.3 ± 0.6[a]	26.4 ± 1.0[a]	0.001[a]	240.0 ± 10	8.7[c]	0.04	47 ± 9
Rat heart mitochondria	6.3 ± 0.7	69.5 ± 3.5	0.40 ± 0.04	1.95 ± 0.07	3.4[b]	1.7	42 ± 2
Bovine heart mitochondria	3.5 ± 0.3	19.7 ± 1.0	0.38 ± 0.05	0.92 ± 0.04	0.20[b]	0.22	20 ± 2

V_{max} of ATPase and of creatine kinase was measured spectrophotometrically [10,11] under identical conditions.
[a]Measured in rat liver mitochondria in the absence of MM-CK. For $V_{max(f)}$ initial [CrP] was 30 mM, [Cr] zero; $V_{max(f)}$ of MM-CK was determined in the absence of BSA or liver mitochondria. $Flux_f$ was determined using saturation transfer ^{31}P-NMR (see Section 3).
[b]Evaluated from Fig. 4.
[c]From Fig. 3 at 10 mg/ml.
[d]From [6]; all measurements were performed at room temperature.

centration (1 mM) has been shown to cause inhibition of mitochondrial creatine kinase [14]. Therefore we preferred continuous infusion of limiting amounts of H_2O_2 yielding concentrations, which were three orders of magnitude below the level where it causes oxidative damage. Furthermore we added DTT for protection of reactive thiol groups of the enzyme. Consistently, Mi-CK activities measured with NMR were in the same range as the V_{max} obtained by photometric measurement (Table 1), and comparable to values found in the literature [15,16]. In previous experiments, where mitochondria were oxygenated by bubbling oxygen through the samples, the mitochondrial function declined much faster than with the hydrogen peroxide oxygenation (not shown).

The accurate determination of rate constants in the creatine kinase mediated phosphate transfer requires also the spin lattice relaxation time of the substrates. It is of great importance to determine T_1 in the absence of the relaxation effects due to the exchange process. For this purpose, we used samples where mitochondria or MM-CK were thermally inactivated at 60°C before performance of the T_1 measurements. Several experiments were performed with MM-CK in the presence of increasing amounts of bovine serum albumin. In general, one expects T_1 of metabolites to decrease with increasing protein concentration, as in concentrated protein solutions, the molecular reorientation slows down [17]. For this reason we measured T_1 for all protein concentrations used. We observed no change in the T_1 of γ-ATP (1.76 ± 0.016) and an overall small decrease in the T_1 of CrP (mean value 2.73 ± 0.045) upon increasing protein concentration (not shown), which did not affect the flux determinations (shown in Fig. 3A).

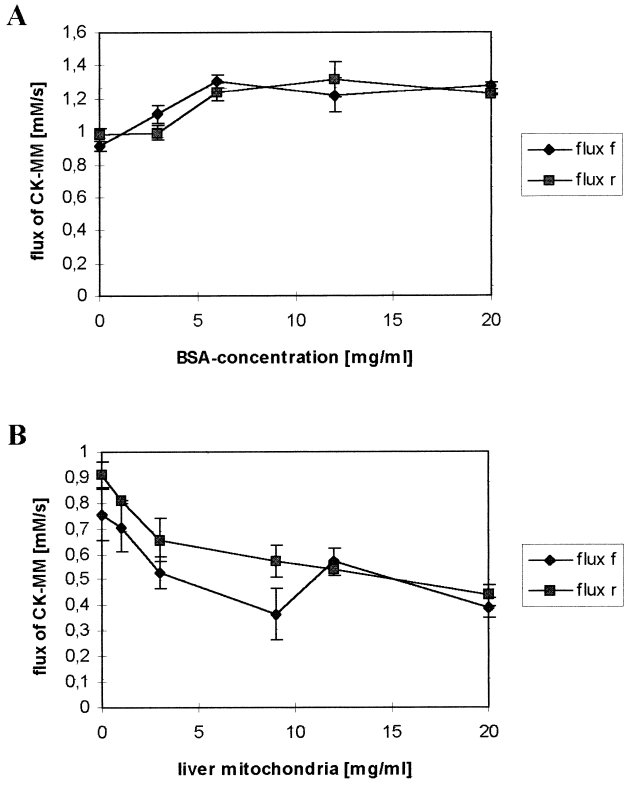

Fig. 3. Influence of BSA or rat liver mitochondria concentration on forward and reverse fluxes of soluble MM creatine kinase. (A) $Flux_f$ versus BSA concentration. (B) versus concentration of rat liver mitochondria. $n = 4$–8, mean ± S.E.

4.2. Determination of forward and reverse creatine kinase fluxes in intact heart mitochondria and in an in vitro system

Creatine kinase fluxes were determined in two different systems: (1) Mi-CK in intact rat and bovine heart mitochondria; and (2) soluble MM-CK from rabbit skeletal muscle in the presence of increasing amounts of rat liver mitochondria or bovine serum albumin.

A linear increase of forward flux of Mi-CK with increasing concentration of rat or bovine heart mitochondria, similar to the results with soluble CK, was obtained (Fig. 4A–C). From results obtained with the soluble enzyme [6] we expected non-measurable turnover rates for Mi-CK in intact heart mitochondria, as the mitochondrial preparations only contained about 1% of the creatine kinase activity used in the in vitro study. As the soluble enzyme in the closed incubation system reaches equilibrium very fast [6] the net flux through the reaction is zero, and the exchange rate of the enzyme should also be very low. Consistently, in our in vitro experiments, the forward flux was only 6% of $V_{max(f)}$ determined spectrophotometrically. On the other hand, in the heart mitochondria preparations, we observed the forward flux to be in the same order of magnitude as the $V_{max(f)}$ measured photometrically, being higher in rat heart mitochondria than in bovine heart mitochondria (Table 1). This is due to the presence of considerable ATPase activity in the heart mitochondria preparations driving the creatine kinase reaction to replenish ATP.

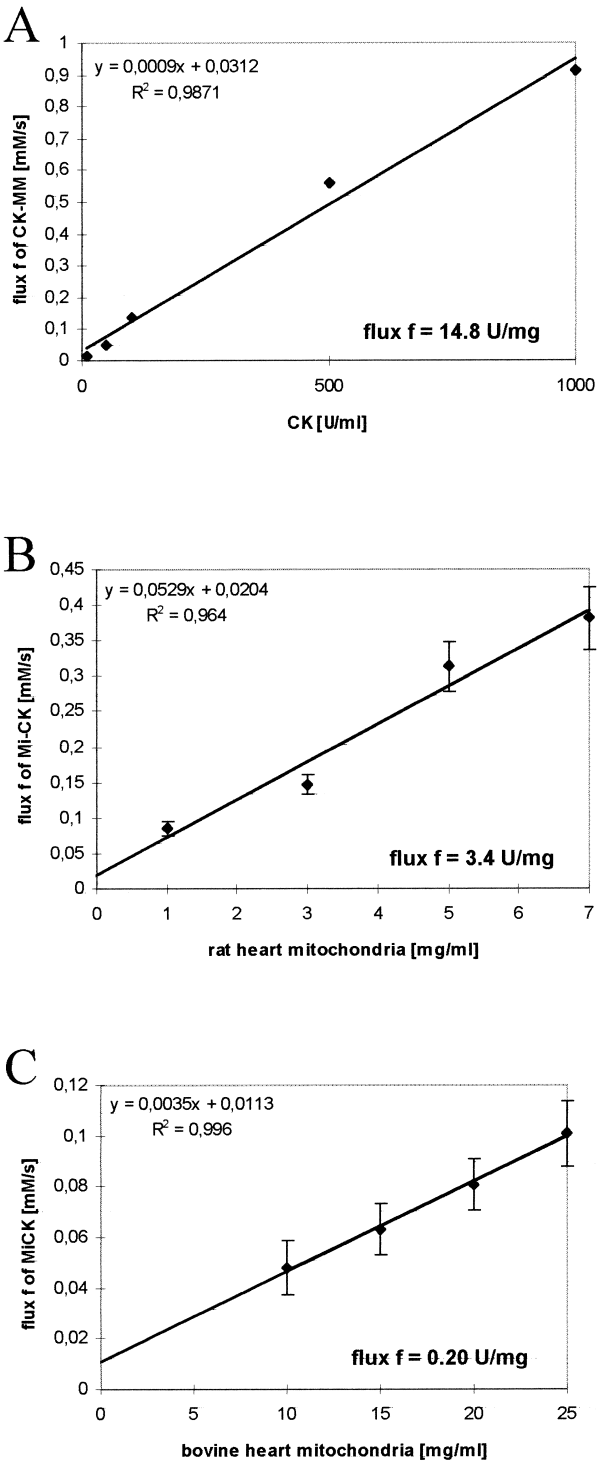

Fig. 4. Dependence of creatine kinase forward flux from $V_{max(forward)}$ with soluble MM-CK and of Mi-CK in isolated heart mitochondria. (A) Soluble MM-CK from rabbit skeletal muscle ($n=2$). (B) Rat heart mitochondria with a specific Mi-CK activity, $V_{max(f)}$ of 1.95 U/mg mitochondrial protein ($n=7$–13, mean ± S.E.). (C) bovine heart mitochondria with a specific Mi-CK activity, $V_{max(f)}$ of 0.92 U/mg mitochondrial protein ($n=10$–11, mean ± S.E.).
←

Although the forward reaction rates with bovine heart mitochondria are very low (Table 2), the 5-fold increase in protein concentration above the amount used for measurements in rat heart mitochondria, and the evaluation of $flux_f$ from the plot of $flux_f$ versus mitochondria concentration, allowed to get reasonably accurate values for $flux_f$ (Fig. 4C).

Since we are in a steady state where not only ATP is held constant, but also CrP (Fig. 2), CrP is resynthesized from ATP which is produced either by the reverse Mi-CK reaction or by oxidative phosphorylation (Fig. 5). ATP synthesis and breakdown are mediated by the Mi-CK and the ATP synthase/ATPase reactions of the mitochondria. These reactions are interconnected by the common ATP and ADP pools to yield a steady state where ATP and CrP are kept constant. The presence of considerable ATPase activity in heart mitochondria, together with oxidative phosphorylation establishes thus an internal creatine circuit where the turnover through the Mi-CK reaction is determined by the mitochondrial ATP turnover. Likewise ATP turnover is driven by the CK reaction. This was also observed in isolated rat skeletal muscle mitochondria using ^{31}P-NMR [18]. Consistently ATP hydrolysis estimated from state 4 respiration (low ATP turnover) is less than 10% of V_{max} of ATPase determined spectrophotmetrically in the isolated mitochondria (Table 1).

Mi-CK in this system operates in both directions playing the role of both, mitochondrial *and* extramitochondrial creatine kinases. This situation is similar to the situation in a recently developed transgenic mouse model where MM-CK had been knocked out [19]. The remaining very low ($<5\%$ of control mice) total CK activity measured with saturation transfer ^{31}P-NMR, accounted for the practically unchanged CrP, ATP and P_i in resting skeletal muscle, since the Mi-CK took over the function of mitochondrial and cytosolic creatine kinases. Their turnover rate was determined by the rate of total ATP consumption and by the oxidative phosphorylation rate.

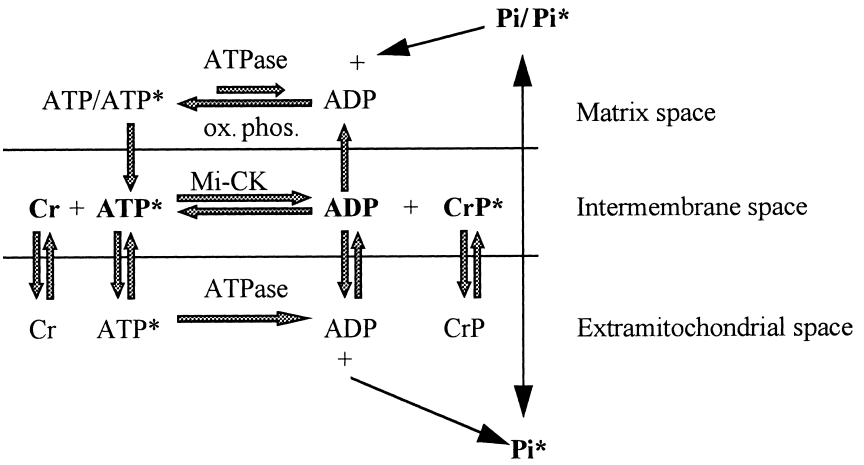

Fig. 5. Phosphate–ATP exchange in actively respiring heart mitochondria. *Presence of saturated phosphorus nuclei.

From Fig. 5, it is clear that in our system ATP exchanges γ-phosphate not only through the CK reaction but also through the ATPase/ATP synthase. When $flux_r$ of Mi-CK is determined by saturating the phosphate moiety of CrP and then calculating k_r from the effect on the γ-phosphate moiety of ATP, $flux_r$ = ($flux_f$+flux through oxidative phosphorylation flux through ATPase). Therefore when the reverse flux through the CK reaction in situ is measured using ^{31}P-saturation transfer NMR (i.e. steady state), reverse fluxes different from forward fluxes are observed [20–22]. In our experiments, the flux through the Mi-CK reaction is driven by the ATPase reactions in the forward direction and by oxidative phosphorylation in the reverse direction. As a steady state for the CrP and ATP pool has been established, the decrease in the γ-phosphate peak of ATP due to the action of the ATPases should be compensated by the increase due to oxidative phosphorylation. As a result, the observed decrease in magnetisation could then be accounted for sole by the saturation transfer from CrP to ATP via Mi-CK. But in our experiments $flux_f$ was found to be different from $flux_r$, the latter was much higher than the former (Table 2). The most reasonable explanation for this observation is a high $ATP \leftrightarrow P_i$ exchange in the isolated heart mitochondria preparation due to the presence of the high external ATPase activity (Table 1). If one assumes that phosphate transport across the mitochondrial membrane is not limiting in the overall reaction cycle depicted in Fig. 5, the magnetisation of the phosphate pool will become more and more saturated, depending on the $ATP \leftrightarrow P_i$ exchange rate and the saturation transfer rate of the Mi-CK reaction. Consequently the γ-phosphate moiety of ATP resynthesized by oxidative phosphorylation will not be fully detected in the phosphorus NMR spectrum and therefore the decrease in magnetisation of the γ-phosphate moiety of ATP is larger than would be expected from the saturation transfer due to the Mi-CK reaction alone. Likewise, an increase in $P_i \rightarrow ATP$ flux was observed in yeast with overexpressed adenine nucleotide translocase, using saturation transfer NMR, which was not reflected by an

Table 2
Forward and reverse fluxes of Mi-CK in isolated heart mitochondria

Protein (mg/ml)	Rat heart mitochondria		Protein (mg/ml)	Bovine heart mitochondria	
	$flux_f$ (mM/s)	$flux_r$ (mM/s)		$flux_f$ (mM/s)	$flux_r$ (mM/s)
1	0.085 ± 0.001	0.486 ± 0.101	10	0.048 ± 0.01	0.59 ± 0.16
3	0.147 ± 0.013	0.581 ± 0.065	15	0.063 ± 0.010	0.90 ± 0.18
5	0.314 ± 0.035	0.650 ± 0.135	20	0.081 ± 0.010	0.64 ± 0.16
7	0.382 ± 0.044	0.447 ± 0.025	25	0.100 ± 0.013	0.92 ± 0.34

Values are mean ± S.E., n = 7–13.

increase in respiration, but reflected an increase in $ATP \leftrightarrow P_i$ exchange. Although ATP transport is largely unidirectional from matrix to cytosol in energized mitochondria [23], at high external ATP the transport of ATP into the mitochondria is high in mutated yeast and therefore $ATP \leftrightarrow P_i$ exchange is stimulated. This enhancement induces the same effect on the magnetisation of P_i as in our experiments on the magnetisation of ATP [24].

However magnetisation of [γ-^{31}P]ATP could also be lost by phosphate transfer to AMP via AK. Even if ADP is then rephosphorylated by Mi-CK or oxidative phosphorylation, the label is on the β-site and not on the γ-site of ATP. In stimulated rat skeletal muscle it has been reported that total flux to ATP is due to CK by 70% and to AK by 30% using β-phosphorus magnetisation transfer NMR [25]. In our isolated rat heart mitochondria system, creatine stimulated respiration was only inhibited by about 6% by p_1p_5 (not shown). Therefore in isolated mitochondria AK appears to have only marginal influence on the overall ATP turnover. The overestimation of $flux_r$ of Mi-CK is, therefore, presumably mainly caused by the action of ATPases.

As mentioned in Section 1, efficient synthesis of CrP by Mi-CK is possible due to the coupling of Mi-CK and oxidative phosphorylation via their common substrates ATP and ADP. Mi-CK is located at the outer site of the inner mitochondrial membrane mainly within contact sites, where it is associated with the mitochondrial adenine nucleotide translocase (ANT) in the inner membrane and the porin channel in the outer membrane [26]. ATP from oxidative phosphorylation is channelled via ANT to the Mi-CK whereas CrP leaves the intermembrane space via porin. Mi-CK thus preferentially uses mitochondrial ATP for the synthesis of CrP, while ADP is shuttled back to mitochondrial ATP synthase. The close interaction between both processes becomes apparent in a shift of the mass action ratio of the Mi-CK reaction away from the equilibrium ratio determined for soluble CK ([27], Table 1). It has been frequently discussed whether channelling of substrates within contact sites is solely responsible for this shift [26,27], or whether it is, at least partially, dependent on the relative activities of all reactions involved in the phosphate–ATP turnover, i.e. a kinetic coupling [3].

To study this phenomenon in more detail, we also investigated soluble MM-CK in the presence of rat liver mitochondria which do not contain Mi-CK. In this system, all components discussed are present, ATPase, ATP synthase and CK; however, the functional compartmentation between CK and oxidative phosphorylation is absent. The results, shown in Table 1 and Fig. 3B were surprising:

(1) Despite the lack of functional coupling between oxidative phosphorylation of the rat liver mitochondria and the soluble CK, a shift in the mass action ratio of the CK reaction like in heart mitochondria was observed

(2) Forward and reverse fluxes were identical within experimental error.

(3) The forward fluxes measured with 1000 U/ml CK are in the same range as the Mi-CK fluxes in rat heart mitochondria, where only 25 U/ml total CK activity was present.

From (1) it is apparent that coupling of oxidative phosphorylation and CK reaction is not necessarily dependent on a close local interaction between CK and the proteins of oxidative phosphorylation, but is, under certain circumstances, effective solely via their common substrates ATP and ADP. The sharing of a common adenine nucleotide pool is supported by the observation that the fluxes measured in the presence of liver mitochondria are considerably lower than in the presence of equal amounts of bovine serum albumin (Fig. 3) and decreased when more liver mitochondria were added, indicating competition of CK and oxidative phosphorylation for ADP. It is noteworthy that oxidative phosphorylation has a K_m for ADP which is at least two orders of magnitude lower than that of CK [28,29] and therefore might compensate for the higher V_{max} of CK present in the in vitro, compared to the in situ experiments.

Despite the apparent kinetic interaction between the soluble CK and liver mitochondrial respiration, no difference between $flux_f$ and $flux_r$ could be observed, unlike the findings for Mi-CK in situ in heart mitochondria. Since there is only a very low ATPase activity detectable in isolated liver mitochondria preparations compared to heart mitochondria (about 1 mU/mg, Table 1), cycling of phosphate through ATP synthase/ATPase should be very low and does not affect the determination of $flux_r$ by phosphorus saturation transfer NMR (2). Furthermore, the low

activity of an ATP consuming pathway in the in vitro system does not allow the functioning of a creatine circuit and therefore the fluxes through the CK reaction are very low compared to the high V_{max} of soluble CK added (3).

It has been shown that the steady state creatine kinase forward flux is dependent also on the actual CrP/Cr ratio [30]. The ratio was 0.97 ± 0.14 in the rat liver mitochondria system and 0.51 ± 0.03 with rat heart mitochondria. In this range of ratios, the $flux_f/V_{max(f)}$ of the CK reaction differs only by 5–10% [30] and therefore the CrP/Cr cannot be responsible for the observed high $flux_f/V_{max(f)}$ ratio in heart mitochondria compared to liver mitochondria+MM-CK.

As mentioned above, the fluxes through the Mi-CK measured in rat heart and bovine heart mitochondria were of the same order of magnitude as their maximum specific activity. However, whereas $flux_f$ in bovine heart mitochondria is only 20% of $V_{max(f)}$, it is about twice $V_{max(f)}$ in rat heart mitochondria. This finding is of special interest in view of the proposed role of compartmentation of Mi-CK in the intermembrane space. Kinetic studies on creatine kinase reaction revealed phosphoryl group transfer, where both substrates (products) are bound to the enzyme, as the rate limiting step of the overall reaction [30]. Interaction of Mi-CK with the ANT in situ may optimise conformation of this complex to increase rate of phosphoryl group transfer, so that, due to compartmentation, V_{max} in situ is increased over V_{max} of Mi-CK in solution. In line with this proposal it was found in a recent in vitro study using isolated chicken sarcomeric Mi-CK, that the $flux_f$ was only 30% of $V_{max(f)}$ [29].

However, this advantage is only effective if the capacity of Mi-CK is not too high in relation to ATP–ADP turnover. With bovine heart mitochondria, $flux_f$ was measured to be smaller than the $V_{max(f)}$ of Mi-CK despite the advantage of a close local proximity of Mi-CK and the enzymes responsible for ATP turnover in the mitochondria. This can be explained by a comparably low adenine nucleotide turnover. Thus it is the fine tuning of all enzymes and pathways involved in ATP turnover within the heart cell which allows optimal rates of energy transfer. Compartmentation appears to be effective only when all reactions involved are very active.

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