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The in vitro kinetics of mitochondrial and cytosolic creatine kinase determined by saturation transfer ^{31}P-NMR [1]

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Abstract

Michaelis- and dissociation constants of sarcomeric mitochondrial creatine kinase (Mi_b-CK) in solution were determined by enzyme assay and compared to those of cytosolic MM-CK under identical conditions at pH 7.4 and 25°C. Saturation transfer ^{31}P-NMR was used to determine the steady state fluxes mediated by Mi-CK and MM-CK in solution. The NMR detected fluxes of both Mi-CK and MM-CK exhibited, as expected, a linear dependence on V_{max} (V_{max} range 0–9 mM · s^{-1}). Interestingly, the oligomeric state of Mi-CK, with the Mi-CK octamer/dimer ratio ranging from 2 to 9, did not have a significant effect on the flux/V_{max} ratio. Furthermore, the flux/V_{max} ratio of Mi-CK was twice as high as that of MM-CK under similar conditions (flux/V_{max} for Mi-CK was 0.31 and for MM-CK was 0.15). This difference was primarily due to a 4-fold higher apparent affinity for MgADP of Mi-CK compared to MM-CK (K_m(MgADP) = 22 ± 9 μM and 80 ± 17 μM, resp.). The NMR observed fluxes were in agreement with the fluxes as calculated from the rate equation, using the appropriate metabolite concentrations and the kinetic constants from the spectrophotometric assays. Thus we conclude, that Mi-CK and MM-CK, when in solution, catalyse an exchange-reaction, the flux of which is fully observable by saturation transfer ^{31}P-NMR.

Keywords: Creatine kinase; P-31 NMR; Saturation transfer; Mitochondrion; Enzyme kinetics; Energy metabolism

1. Introduction

Creatine kinase (ATP: creatine phosphotransferase; EC 2.7.3.2) catalyses the reversible transfer of high-energy phosphate between ATP and phosphocreatine (PCr):

$$PCr^{2-} + MgADP^{-} + H^{+} \rightleftarrows Cr + MgATP^{2-}$$

The enzyme is present in tissues or cells with high and/or fluctuating energy requirements. Five different iso-enzyme species of creatine kinase (CK) are known and these are distributed in a tissue-specific fashion and are also subcellularly compartmentalised. The MM-CK isozyme is predominantly found in mature skeletal muscle, BB-CK in mammalian brain and smooth muscle, and MB-CK in adult mammalian heart and differentiating striated muscle. These three isozymes are dimeric molecules and are located mainly in the cytosol (for review see [1]). The remaining two are mitochondrial isoforms and, according to their tissue-specific expression, are named 'ubiquitous' and 'sarcomeric' mitochondrial creatine kinase (Mi-CK), respectively (for review see [2]).

Cytosolic MM-CK is not strictly a soluble enzyme, in that part of the total MM-CK pool is associated with different subcellular sites within a muscle fibre, e.g. with the sarcomeric M-line, with glycolytic enzymes at the I-band, with the sarcoplasmic reticulum and the sarcolemma [1]. In these cases CK seems to be functionally coupled to ATPases or to the glycolytic enzymes. The existence of different CK iso-enzymes and their subcellular distribution has led to the concept that CK and PCr and

Abbreviations: CK, creatine kinase; $MgAc_2$, magnesium acetate; MM-CK, cytosolic creatine kinase isoenzyme from rabbit skeletal muscle; Mi_b-CK, mitochondrial creatine kinase isoenzyme from chicken heart; PCr, phosphocreatine; Cr, creatine.

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[1] Note: Forward and reverse directions of the creatine kinase reaction follow the conventions common to the NMR literature, i.e. forward direction is PCr + MgADP → MgATP + Cr and reverse direction refers to PCr synthesis. This nomenclature is different from the conventions for CK.

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creatine are critically involved in energy transport and suggested an important role for CK isoenzymes in local energy buffering: the CK/PCr circuit model [1,3].

The mitochondrial isozymes [4,5] predominantly form octamers in vivo and are localised in the intermembrane space [2,6,7]. Mi-CK binds to the outer leaflet of the mitochondrial inner membrane [8] and has been suggested to be enriched in contact sites between inner and outer mitochondrial membrane [9–11]. Dissociation of Mi-CK octamers into dimers has been observed in solution, which may indicate a physiological role for the oligomeric state of Mi-CK in metabolic regulation [2,6,7,12]. A complete understanding of the CK isozyme system and the role of individual CK species requires detailed knowledge of the kinetic behaviour of the individual isozymes, preferably in an in vivo setting.

Magnetisation transfer ^{31}P-NMR techniques have proven to be valuable tools for assessing the kinetic properties, especially exchange fluxes, of specific enzymes, both under in vitro and in vivo conditions. The (MM-) CK catalysed reaction has been widely studied in vitro [13–20], in Langendorff perfused hearts [21–23] and for example in skeletal muscle in vivo [17,24]. An important aspect of in vivo magnetisation transfer studies on CK properties that should be recognised is that two or more isozymes may contribute to the observed 'total CK-flux', each to a different extent, depending on the relative amount of isoenzymes present, their kinetic properties and on the (local) substrate concentrations. The individual contributions of the different CK isozymes to the total CK-related flux and, in particular, that of the mitochondrial isoform, are still poorly understood.

Therefore, the objective of this study was to make a thorough comparison between the kinetic properties of isolated, purified Mi-CK in solution and those of MM-CK under similar conditions. Firstly, Michaelis–Menten and dissociation constants of both isozymes were determined by spectrophotometry. Secondly, saturation transfer ^{31}P-NMR was used to measure the steady state exchange fluxes of Mi-CK and MM-CK as a function of the respective maximal CK activities (V_{max}) and these fluxes were related to the theoretical values, as obtained from rate-equation calculations. Thirdly, the aim was to assess the effect of the oligomeric state of mitochondrial creatine kinase on the steady state Mi-CK flux, as measured by ^{31}P-NMR.

2. Materials and methods

2.1. Over-expression and purification of mitochondrial creatine kinase

Mature chicken sarcomeric mitochondrial creatine kinase (Mi_b-CK) was expressed in *Escherichia coli* and purified as described elsewhere [25]. Briefly, *E. coli* strain BL21(DE3)pLysS was transformed with plasmid pRF23 and grown to an A_{590} of 0.6 in 2 l of 2 × YT medium, supplemented with ampicillin (50 mg/l). Mi_b-CK expression was induced by adding 0.4 mM isopropylthiogalactoside. Thereafter, the cells were grown for another 4 h, harvested and washed. Next, the cells were lysed by sonication. The enzyme was subsequently purified in a two-step protocol involving Blue Sepharose CL6B affinity chromatography and cation-exchange chromatography on a Mono S HR 5/5 FPLC column (both from Pharmacia). Concentrated Mi_b-CK stocks were stored in the Mono S elution buffer (25 mM NaH_2PO_4, pH 7.0, 120 mM NaCl, 0.2 mM EDTA, 1 mM β-mercaptoethanol) at −20°C or in liquid nitrogen for long-term storage. The specific activity of preparations was between 50 and 90 U/mg protein. Purity of the enzyme preparations was routinely checked by polyacrylamide gel electrophoresis in the presence of SDS. Protein concentrations were determined by a dye-binding assay using BSA as standard [26].

2.2. Other reagents

Commercially available cytosolic MM-creatine kinase from rabbit muscle (Boehringer Mannheim, Germany) was used without further purification.

Glucose-6-phosphate dehydrogenase (NAD^+-specific type from *Leuconostoc mesenteroides*) was from Boehringer (Mannheim, Germany), while ATP, creatine and phosphocreatine were from Sigma Chemical Co. (St. Louis, USA). All other chemicals used were obtained from regular commercial sources and were of the highest purity available.

2.3. Determination of the Mi-CK octamer-to-dimer ratio

The octamer-to-dimer ratio of mitochondrial CK was measured at room temperature by gel-permeation chromatography on a Superose 12 HR10/30 FPLC column (Pharmacia) according to [7]. Typically, 30–50 μl aliquots of Mi-CK sample were loaded onto the column and eluted at 0.7 ml · min^{-1} in a buffer containing 50 mM NaH_2PO_4 (pH 7.2), 150 mM NaCl, 0.2 mM EDTA and 2 mM β-mercaptoethanol. Octamer-to-dimer ratios were calculated from the relative areas of the peaks detected at 280 nm, after cutting and weighing the peaks from the corresponding traces.

2.4. Determination of kinetic parameters by spectrophotometry

The CK activities were measured at 25°C on a Beckman DU65 spectrophotometer, both in the reverse direction (i.e. from ATP to PCr) and in the forward reaction (PCr to ATP) by a coupled enzyme assay, measuring the initial rate of formation or depletion of NADH from the absorbance change at 340 nm. One unit (U) of CK activity

corresponds to 1 μmol of phosphocreatine transphosphorylated/min at pH 7.4 and 25°C. Unless otherwise indicated, the assay medium A contained 30 mM Hepes-Na (pH 7.4), 200 mM mannitol, 25 mM sucrose, 1 mM EDTA and 0.5 mM β-mercaptoethanol. For determination of the forward reaction rate, 20 mM glucose and 10 mM $MgCl_2$ were added to this medium. The auxiliary enzymes hexokinase and glucose-6-phosphate dehydrogenase were both added at 5 U/ml, which is in large excess relative to the CK activity (~ 10 mU/ml). The reaction mixture (final volume of 1.0 ml) further contained 4 mM ADP, 20 mM PCr, 0.2 mM NAD^+, BSA (1 mg/ml) and, although not essential, 0.2 mM p^1,p^5-di(adenosine-5')pentaphosphate to inhibit possible contaminating adenylate kinase activity.

The reverse reaction rates (i.e. from ATP to PCr) were measured according to the same principle by monitoring the initial rate of NADH depletion at 340 nm. The incubation medium A at pH 7.4 was supplemented with 4 U/ml pyruvate kinase and 3 U/ml lactate dehydrogenase as auxiliary enzymes and with 0.2 mM NADH, 0.7 mM phosphoenolpyruvate and 13 mM $MgCl_2$. The apparent maximal reverse activity was determined with 90 mM creatine and 10 mM ATP as final concentrations. The $MgCl_2$ concentration always exceeded the ATP concentration by 3 mM.

Michaelis–Menten and binary complex dissociation constants were determined for each substrate by varying one substrate at four different concentrations of the second substrate.

2.5. ^{31}P-NMR measurements

^{31}P-NMR measurements were performed at 25°C on a Bruker MSL300 spectrometer with a 10 mm broad-band probe at a ^{31}P frequency of 121.5 MHz. Unless otherwise indicated, the incubation medium contained 100 mM Hepes-Na (pH 7.4), 0.5 mM EGTA and 1 mM β-mercaptoethanol and also 10 mM PCr, 10 mM Cr, 5 mM ATP, 5.7 mM $MgAc_2$ and 2 mM NaP_i. The $MgAc_2$ concentration was such that the free Mg^{2+} concentration was 1 mM as calculated from the dissociation constant for MgATP [27]. Typically 100 to 500 units CK per ml (2 ml total volume) were used for each measurement. Fully relaxed spectra were accumulations of 32 transients with 90° pulses (24 μs) and a 30 s repetition time. Unless otherwise indicated, a 10 Hz exponential line broadening was applied to the free induction decay prior to Fourier transformation.

2.6. Saturation transfer ^{31}P-NMR

Saturation transfer ^{31}P-NMR experiments were analysed assuming simple two-site exchange. When saturating either of the resonances of the exchanging spins, the fractional decrease in the resonance of the other spin is given by:

$$M_z/M_0 = (1 + k \cdot T_{1,\mathrm{intr}})^{-1} \quad (1)$$

where M_0 and M_z represent the equilibrium and steady state magnetizations, respectively, k is the pseudo first-order rate constant and $T_{1,\mathrm{intr}}$ is the intrinsic spin-lattice relaxation time.

The apparent spin-lattice relaxation time, $T_{1,\mathrm{app}}$, in the presence of the selective irradiation is determined separately:

$$T_{1,\mathrm{app}}^{-1} = T_{1,\mathrm{intr}}^{-1} + k \quad (2)$$

Combination of Eqs. 1 and 2 yields the apparent pseudo first-order rate constant k.

The method described here was applied to the CK reaction, selectively saturating either γ-ATP or PCr, the forward and reverse rate constants, k_{for} and k_{rev}, were determined, respectively. The steady state CK flux was obtained by multiplying the respective substrate concentrations with the rate constants:

$$\mathrm{Flux}_{\mathrm{for}} = k_{\mathrm{for}} \cdot [\mathrm{PCr}] \quad (3)$$

$$\mathrm{Flux}_{\mathrm{rev}} = k_{\mathrm{rev}} \cdot [\mathrm{MgADP}] \quad (4)$$

Selective saturation was achieved by directing the transmitter output to a fast high–low power switch unit (Bruker, Karlsruhe, Germany) resulting in a low power (150 μW) continuous wave radiofrequency pulse.

The steady-state saturation transfer spectra were collected in interleaved blocks of 8 scans (90° acquisition pulse; 20 s saturation time) with a total of 32 scans per spectrum. Direct spillover of saturation power to neighbouring resonances was generally negligable, as was tested routinely by performing the proper control experiments, i.e. by placing the saturation pulse on a mirror frequency relative to either γ-ATP or PCr. The apparent spin-lattice relaxation times, $T_{1,\mathrm{app}}$, were determined separately in the presence of the selective irradiation, using the saturation recovery method [28]. Each T_1 was determined from an exponential fit to the peak areas at 10 different delays (0.015–15 s).

2.7. Rate equation calculations

Michaelis–Menten constants, K_m, K_d and V_{max} were determined by analysing the initial velocity data using the software originally written in Fortran by W.W. Cleland [29] and rewritten in Basic by R. Viola (Akron University, Akron OH). All data are the means of at least two series of independent measurements and standard deviations are given.

The rate equation for creatine kinase that was used to relate the steady state flux to the maximal enzyme activity

V_{max} was derived from the original work by Morrison and Cleland [30] as amended by Kupriyanov et al. [13]:

$$\text{Flux}_{for} = \frac{V_{max}(for) \cdot [ADP] \cdot [PCr]}{D \cdot K_m(ADP) \cdot K_d(PCr)} \quad (5)$$

$$\text{Flux}_{rev} = \frac{V_{max}(rev) \cdot [ATP] \cdot [Cr]}{D \cdot K_m(ATP) \cdot K_d(Cr)} \quad (6)$$

where

$$D = 1 + \frac{[ADP]}{K_d(ADP)} + \frac{[PCr]}{K_d(PCr)} + \frac{[ATP]}{K_d(ATP)} + \frac{[Cr]}{K_d(Cr)} + \frac{[ADP] \cdot [PCr]}{K_m(ADP) \cdot K_d(PCr)} + \frac{[ATP] \cdot [Cr]}{K_m(ATP) \cdot K_d(Cr)} + \frac{[ADP] \cdot [Cr]}{K_m(ADP) \cdot K_d(Cr)}$$

V_{max} was determined by the spectrophotometric assay. Forward and reverse fluxes, Flux_{for} and Flux_{rev}, were determined from saturation transfer ^{31}P-NMR measurements. [PCr] and [ATP] concentrations were calculated from the relative areas of the peaks in fully relaxed ^{31}P-NMR spectra and related to total phosphorous determinations according to Fiske and Subbarow [31]. The total MgADP concentration was calculated by assuming an equilibrium constant for the CK reaction of 166 at pH 7.0 and 1 mM Mg^{2+} [27] and correcting for pH 7.4. Michaelis- and binary complex dissociation constants, K_m and K_d, were determined from the spectrophotometric assays as described above.

3. Results

3.1. Michaelis–Menten parameters for MM-CK and Mi-CK

The Michaelis–Menten parameters for MM-CK and Mi-CK were determined by coupled enzyme assays at pH 7.4 and 25°C. The ratio $V_{max,for}/V_{max,rev}$ was 1.4 for Mi-CK and 2.5 for MM-CK. By systematically varying the concentrations of all substrates and measuring the initial rates, the binary and ternary complex dissociation constants, K_d and K_m, were calculated using the method developed by Cleland [29]. The K_m and K_d values for PCr were obtained by varying [PCr] from 0.5–30 mM (ten different concentrations) at four different, fixed MgADP concentrations. These constants were not significantly different from those obtained by varying [MgADP] (0.01–4.0 mM) at different, fixed PCr concentrations. This was also true for the combination of creatine (range: 1–90 mM) and MgATP (range: 0.05–10 mM). The results of the kinetic analyses based on the spectrophotometric enzyme assays are shown in Table 1.

These data indicate that there were no major differences in the kinetic parameters of MM-CK and Mi-CK in solution, except for the dissimilar affinities for MgADP. The K_m(MgADP) of Mi-CK appeared to be almost 4-fold lower than that of MM-CK (22 and 80 μM, respectively).

3.2. Saturation transfer ^{31}P-NMR measurements of CK flux

Upon addition of either MM-CK or Mi-CK to the NMR medium, equilibrium was rapidly established. The relative areas of the resonances in fully relaxed ^{31}P-NMR spectra indicated that no major changes in the substrate concentrations took place. The areas of all peaks remained stable during the 2–2.5 h period required for the complete saturation transfer NMR experiment.

Fig. 1 shows a typical example of an in vitro steady state saturation transfer ^{31}P-NMR experiment with mitochondrial creatine kinase. Selective saturation of γ-ATP (Fig. 1D) results in a decrease in the PCr signal compared with the control spectrum 1C (notice the difference spectrum 1C–D), which is due to the PCr-to-ATP reaction. Likewise, saturation of PCr (Fig. 1A) leads to a decrease of the γ-ATP signal as a result of the reverse reaction (ATP-to-PCr).

Fig. 2 demonstrates that the forward and reverse fluxes, as measured by saturation transfer, were equal within error for the individual CK isoenzymes (Mi-CK and MM-CK) in the range of enzyme activities tested (0–500 IU/ml), but differed in absolute terms between the two (see below). The ratio of forward to reverse flux normally deviated no more than ~ 10%. These deviations seem to mainly reflect the accuracy with which the rate constants can be assessed from separate determinations of M_z, M_0 and $T_{1,app}$ and

Table 1
In vitro Michaelis–Menten parameters of soluble MM-CK and Mi-CK

CK substrate	MM-CK				Mi-CK			
	K_d (mM)		K_m (mM)		K_d (mM)		K_m (mM)	
PCr	4.2 ± 0.15	(5)	2.42 ± 0.22	(4)	7.8 ± 2.5	(5)	2.32 ± 0.82	(5)
MgADP	0.14 ± 0.03	(6)	0.080 ± 0.017	(6)	0.06 ± 0.02	(4)	0.022 ± 0.009	(5)
MgATP	2.35 ± 0.22	(3)	0.32 ± 0.08	(4)	1.70 ± 0.43	(4)	0.31 ± 0.12	(4)
Creatine	53 ± 4	(3)	9.7 ± 1.2	(4)	61 ± 21	(4)	13.0 ± 0.1	(3)

The kinetic parameters were determined by spectrophotometric coupled enzyme assays at pH 7.4 and 25°C. K_d and K_m are the dissociation constants for the binary and ternary complexes, respectively. Values are given as means ± standard deviation. The number of experiments n is given in parentheses.

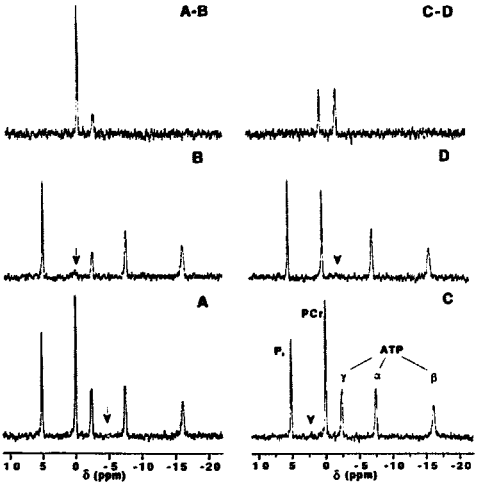

Fig. 1. ^{31}P-NMR saturation transfer of mitochondrial creatine kinase in solution. The NMR sample contained ~ 160 units Mi_b-CK/ml at pH 7.4 and 25°C. Irradiation frequencies for selective saturation are denoted by arrows. Panels A and C: control spectra; panels B and D: saturation of PCr (B) or γ-ATP (D); upper panels ((A–B) and (C–D)): difference spectra. The 121.5 MHz ^{31}P-NMR spectra are the sum of 32 transients, acquired with a 90° hard pulse (24 μs) and a 20 s low power saturation pulse (= repetition time). An exponential linebroadening of 4 Hz was used.

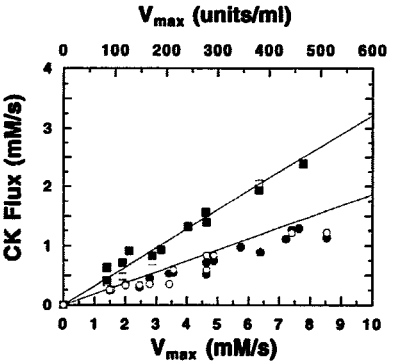

Fig. 2. In vitro Mi_b-CK flux and MM-CK flux measured by saturation transfer ^{31}P-NMR as a function of V_{max}. CK fluxes are shown as a function of the maximal unidirectional (forward) rate, V_{max}, at pH 7.4 and 25°C. Forward fluxes (–■– Mi-CK; –●– MM-CK) are equal to reverse fluxes (–□– Mi-CK; –○– MM-CK). Mi-CK fluxes (open and filled squares) are higher than MM-CK fluxes (open and filled circles) at the same V_{max}. Solid lines display the theoretical relationship between CK flux and V_{max} calculated from the rate equation (Eq. 5), using experimentally determined kinetic parameters from Table 1. The upper and lower solid line represent the theoretical flux/V_{max} relationships for Mi-CK and MM-CK, respectively.

corresponded roughly with the expected error (3–5%), calculated as described by Kuchel [32].

Fig. 2 demonstrates that there were absolute differences in the steady state fluxes of MM-CK and Mi_b-CK in solution, which was observed reproducibly by our saturation transfer measurements. The observed fluxes were related to the maximal forward CK activities ($V_{max,for}$), determined by spectrophotometry on 20 μl samples taken from the NMR tube. Under similar conditions, Mi-CK had a higher forward flux-to-V_{max} ratio than MM-CK. A linear regression fit to the experimental points (not shown) indicated, that the Mi-CK flux/V_{max} was 0.31 and the MM-CK flux/V_{max} was 0.15.

It should be noted, that the slightly different composition of the NMR medium and of the enzyme assay medium A did not in any way influence the above results. Assay medium A additionally contained mannitol and sucrose and was used in anticipation of future studies on isolated mitochondria. In 'NMR buffer', the K_m(MgADP)'s for both isoenzymes also differed and were identical to the values given in Table 1. Also the CK flux detected by saturation transfer remained unchanged when the enzymes were studied in the assay medium A.

The observation of linear flux-to-V_{max} relationships, with the flux/V_{max} ratio for Mi-CK being higher than that of MM-CK, was correlated to enzyme kinetic theory by using the Morrison–Cleland rate equation (Eq. 5) [13,30]. By using the dissociation constants and Michaelis constants, determined by enzyme assay at pH 7.4 and 25°C (Table 1), we calculated the expected forward flux for MM-CK and Mi-CK as a function of the $V_{max,for}$. The calculated fluxes are shown in Fig. 2 as solid lines. Under our experimental conditions, the calculated fluxes of both MM-CK and Mi_b-CK were in good agreement with the experimental data (Mi-CK flux/V_{max} was 0.32 and the MM-CK flux/V_{max} was 0.18, when calculated using Eq. 5). This demonstrates that, when the enzymes and their substrates are free in solution, CK catalysed fluxes are completely visible in saturation transfer ^{31}P-NMR experiments.

During prolonged experiments at 25°C, both MM-CK and Mi-CK samples showed a slow decrease in PCr signal and a concomitant increase in the inorganic phosphate (P_i). Aside from spontaneous, non-enzyme catalysed PCr hydrolysis, this may be ascribed to the presence of trace amounts of ATPase activity in the CK preparations ($(3–5)\cdot 10^{-3}$% of the CK activity in our Mi-CK preparations), since ATP levels are kept constant at the expense of PCr, due to CK activity. The CK flux determinations by saturation transfer were, however, not affected and thus gave no indication for significant ATPase activity in the sample.

3.3. Oligomeric state of Mi_b-CK

The dissociation of Mi-CK octamers into dimers is enhanced by the presence of equilibrium mixtures of sub-

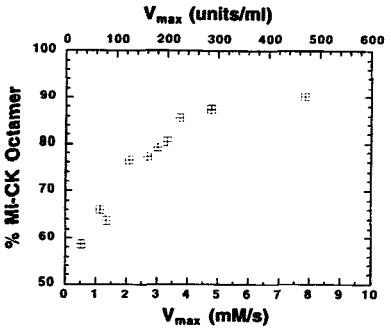

Fig. 3. The oligomeric state of mitochondrial creatine kinase as a function of Mi_b-CK activity (V_{max}). The Mi-CK octamer/dimer ratio was assessed from the relative areas of the peaks in the A_{280} pattern after gel-permeation chromatography on a Superose 12 FPLC column. FPLC patterns of aliquots from the NMR samples were recorded after 2–2.5 h of incubation of the enzyme in Hepes buffer (pH 7.4), at 25°C, in the presence of substrates. Data are plotted as the percentage of Mi-CK octamers in the same V_{max} range as the flux measurements of Fig. 2.

strates and by dilution of the enzyme to low protein concentrations [7,33,34]. We determined the oligomeric state of Mi-CK in each NMR sample, by means of gel-permeation chromatography, in order to establish whether the observed Mi-CK flux is influenced by the oligomeric state of the enzyme. During incubation of the enzyme at room temperature in the presence of substrates, the octamer/dimer equilibrium settled within 15–30 min (Van Dorsten, F.A., unpublished data; see also [2]). Small samples were removed from the NMR tube after each saturation transfer experiment, i.e. after approximately 2 h incubation of the enzyme in the presence of substrates, at 25°C and pH 7.4. Fig. 3 displays the relative percentage of Mi-CK octamers as a function of the Mi-CK activity, $V_{max,for}$. Over the entire range of enzyme activities that was studied, the relative percentage of Mi-CK octamers increased, according to mass action [7,33,34], from about 60% at 60 units/ml to more than 90% at 500 units/ml. This corresponded to a change in octamer/dimer ratio, proportional to the V_{max}, from about 2 to 9. In spite of the fact that at low Mi-CK activities a considerable proportion of Mi-CK was present as dimeric molecules in the NMR sample, this had no significant influence on the observed Mi-CK flux detected in the saturation transfer NMR experiment (Fig. 2).

4. Discussion

Magnetisation transfer ^{31}P-NMR spectroscopy has been used previously to determine the flux through the cytosolic MM-creatine kinase reaction in solution. The in vitro kinetic properties were subsequently used to explain the CK flux in isolated perfused hearts [21,23,41] or in vivo, e.g. in skeletal muscle [13,35]. The behaviour of the mitochondrial isoenzyme of CK in saturation transfer NMR experiments, which might be different from that of the cytosolic isoform, has received little attention. Notably, Perry et al. [23], studying neonatal rabbit hearts, made the interesting observation that the CK reaction velocity increased as Mi-CK content in the developing hearts increased, whereas the total CK activity in these hearts remained constant. Thus, particularly for studies of cardiac tissue, in which mitochondrial CK activity can amount to 30% of the total CK activity, a detailed knowledge of the 'NMR behaviour' of this isoenzyme is of critical importance for the interpretation of the in vivo data.

In this study we used saturation transfer ^{31}P-NMR to assess the Mi-CK exchange velocity in relation to the V_{max} and to the oligomeric state of the enzyme. Furthermore, we established whether the NMR detected Mi-CK flux in vitro can be completely explained by the kinetic parameters of the enzyme in solution. The more frequently studied cytosolic MM-CK isozyme was included in the kinetic analyses to allow for a direct comparison between the two CK species.

Table 1 shows that the major kinetic difference between cytosolic MM-CK and mitochondrial CK in solution was in the affinity for MgADP. K_m(MgADP) of Mi_b-CK was about four times lower (22 ± 9 μM) than that of MM-CK (80 ± 17 μM) at pH 7.4. The other kinetic parameters showed a great similarity. All parameters were similar to previously published data, as summarised in [21]. Our kinetic parameters for Mi-CK were generally comparable to the the data of Furter et al. [36] and Kaldis et al. [37], except that the K_d and K_m for creatine and the K_m(PCr) were higher in our case. These differences can probably be explained by differences in the pH of the assay media.

We assumed here that the Mi-CK catalysed reaction is a two-site exchange process [17] as has been shown to be valid for the MM-CK catalysed reaction in vitro [13,14,16]. The phosphoryl-exchange flux, measured by saturation transfer ^{31}P-NMR, represents the net flux through the reaction provided the interconversion of the ternary complexes is rate-limiting, i.e. the reaction mechanism is of the rapid-equilibrium type. The MM-CK reaction mechanism is of the rapid-equilibrium ordered type in both directions at pH 7 [39,40]. Analysis of the kinetic data (Table 1) suggested, that the Mi-CK reaction follows the rapid equilibrium random bi-bi mechanism at pH 7.4 and 25°C, since it was found that $K_d(A) \cdot K_m(B) = K_d(B) \cdot K_m(A)$ for both substrate pairs A and B and for both enzymes [29]. This was demonstrated earlier [38], and corroborated here by a graphical analysis of the initial rate data in primary and secondary Lineweaver–Burke plots (not shown). Thus, under our conditions, application of the saturation transfer method to establish the overall Mi-CK flux is valid. This represents an important reference point for the interpretation of in vivo measurements of CK flux by NMR.

Another prerequisite for obtaining reliable quantitative rate information from saturation transfer experiments is that the reaction is at steady state, implying that forward and reverse CK fluxes should be equal. Our saturation transfer data (Fig. 2) indeed indicate that, within error, the flux from PCr to ATP equaled the flux from ATP to PCr in both the MM-CK and the Mi-CK preparations. This suggests that contaminations of our CK preparations with e.g. ATPases did not significantly affect the NMR determined ratio of forward to reverse CK flux. Recently, Conrad et al. [20] suggested that ATPase activity in commercial MM-CK preparations could seriously affect the in vitro saturation transfer ^{31}P-NMR measurement of CK flux, but we found no evidence for this.

In Fig. 2 we further show that the forward CK flux was linearly related to the maximal activity added, as is expected for enzymes in solution. Interestingly, for Mi-CK the Flux/V_{max} appeared to be twice as high as for MM-CK (experimental Flux/V_{max} = 0.31 and 0.15, resp.). The explanation for this remarkable difference is presumably that K_m(MgADP) for Mi-CK is 4-fold lower than for MM-CK. [MgADP] in the MM-CK and Mi-CK samples was calculated from the equilibrium constant of 166 at pH 7.0 [27], to be 73 μM. For both Mi-CK and MM-CK this means that [MgADP] and also [creatine] are in the order of their respective K_m values (see Table 1) and thus will have a regulating effect on the reaction velocity. The kinetic constants of both isoenzymes for creatine are not significantly different. However, the distinctly higher affinity of Mi-CK for MgADP would indeed result in a higher Flux/V_{max} for Mi-CK, according to the rate equation (Eq. 5). The rate equation based on our experimental kinetic parameters also predicts that Mi-CK and MM-CK flux in vitro should vary with physiologically relevant MgADP concentrations of up to about 100–150 μM. This was corroborated by the finding that the in vitro MM-CK flux/V_{max}, detected by other investigators e.g. [20,35], who each used a variety of experimental conditions, i.e. different pH, PCr/Cr or PCr/ATP ratios and, consequently, variable MgADP levels, were in agreement with our calculations.

Using a [MgADP] of 73 μM and given the experimental conditions as described in the captions to Fig. 2 and the kinetic parameters that are outlined in Table 1, the relation between $Flux_{for}$ and $V_{max,for}$ was calculated using Eq. 5. A good agreement between the calculated and the experimental data was observed for both MM-CK and Mi-CK. These findings imply that the exchange activities of solubilised MM-CK and Mi-CK are both fully expressed in saturation transfer ^{31}P-NMR.

The mitochondrial CK isoform tends to dissociate from the octameric to the dimeric form upon dilution to low protein concentration. The rate of dissociation is also enhanced by the presence of all of the enzyme's substrates [12] or substrates and anions forming a so called transition-state-analogue-complex with the enzyme [7,33,41]. It has recently been shown [37,42] that both Mi-CK octamers and dimers are active, although the K_m and K_d for creatine of dimeric Mi-CK are lower than those of octameric Mi-CK by a factor of 2–3 [37].

In the course of the NMR experiment (Fig. 3), the octamer-dimer equilibrium shifted towards a predominantly octameric state when the Mi-CK concentration was increased. However, in our saturation transfer experiments (Fig. 2) at low Mi-CK concentrations, when 30–40% of the enzyme is dimeric, the flux/V_{max} ratio was indistinguishable from the overall linear flux/V_{max} dependency. Although the presence of significant quantities of dimeric Mi-CK during the spectrophotometric assay can not be ruled out completely, most likely these numbers are low since the enzyme was added directly from a concentrated Mi-CK stock solution to the assay-mix and octamer dissociation is generally slow compared to the time scale (4–5 min) of the assay [37]. The octamer/dimer ratio during our spectrophotometric assays was not established, due to the low protein concentration in the cuvette (approximately 0.1–0.2 μg/ml). With these points taken into consideration, we may conclude that alterations in the relative amounts of Mi-CK oligomers in solution, as shown in Fig. 3, do not produce significant variations in the overall flux through the enzyme, which could be of great regulatory importance. However, a regulatory function for Mi-CK octamer-dimer dissociation in vivo can of course not be excluded directly.

It has been suggested recently that saturation transfer ^{31}P-NMR experiments to determine CK flux in vivo might not reveal the total CK flux, but exclusively measure the flux that is mediated by free cytosolic CK, in free equilibration with its substrates. This was hinted at in particular by the experiments on the hind limb skeletal muscles of transgenic mice, with varying expression levels of MM-CK [43]. It appeared that CK flux in these muscles, as measured by in vivo inversion transfer NMR techniques, was negligible when up to 35% of wild-type MM-CK levels were expressed. Close to wild-type flux levels were reached when 50% of the MM-CK was expressed. This apparent non-linear correlation between NMR-detectable CK flux and total tissue enzyme activity led these authors to hypothesise that only the flux mediated by soluble MM-CK may be detected by magnetization transfer NMR, whereas the flux mediated by MM-CK which is bound to subcellular structures might not be detectable [43]. This seems at variance with recent conclusions of McFarland et al. [35], that the CK flux in the cat soleus muscle is adequately described by the kinetics of the enzyme in a homogeneous solution. Evidently, CK fluxes, as measured in vivo, must be interpreted with caution.

In conclusion, this paper presents the first in vitro saturation transfer ^{31}P-NMR analysis of the kinetic behaviour of mitochondrial creatine kinase in solution. Our present data clearly indicate that, when Mi-CK is free in solution, flux through the Mi-CK reaction is fully observ-

able by saturation transfer ^{31}P-NMR and in agreement with enzyme kinetic theory. These data therefore represent an important reference point for the interpretation of in vivo findings of the kind described above. The origin of the deviations from solution type kinetics suggested by in vivo magnetization transfer studies of skeletal muscle remains to be elucidated.

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