Functional differences between dimeric and octameric mitochondrial creatine kinase

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Mitochondrial creatine kinase (Mi-CK) consists of octameric and dimeric molecules that are interconvertible. In the present study, the kinetic properties of purified chicken heart Mi-CK (Mi_b-CK) dimers and octamers were investigated separately under highly controlled conditions. Gel-permeation chromatography was performed before and after kinetic measurements in order to clearly define the proportions of octamers and dimers. 'Dimeric' Mi-CK solutions consisted of $\geqslant 90\%$ dimers throughout the experiment whereas 'octameric' Mi-CK solutions consisted in the beginning of 90% octamers, but upon measuring with the highest concentrations of creatine (Cr) and ATP approximately one-third of the octamers dissociated into dimers. These proper controls enabled us to pinpoint the observed kinetic differences between dimers and octamers solely to the oligomeric state of Mi_b-CK. Both dimeric and octameric Mi-CK displayed synergism in substrate binding (K_d values are higher than K_m values), meaning that binding of the first substrate facilitates subsequent binding of the second substrate. Most interestingly, K_m(Cr) and K_d(Cr) values are both 2–3 times higher for octameric than for dimeric Mi-CK. Thus, at low Cr concentrations, the dimer is kinetically favoured for the forward direction of the reaction (phosphorylcreatine synthesis) compared with the octamer. The possible physiological significance of the lower K_d(Cr) value of dimeric versus octameric Mi_b-CK, as well as the apparent negative cooperativity of ATP binding at higher [Cr], are discussed within the context of a possible functional role for dimeric Mi_b-CK *in vivo*.

INTRODUCTION

Creatine kinase (CK, EC 2.7.3.2) isoenzymes catalyse the reversible transfer of the N-phosphoryl group of phosphorylcreatine (PCr) to ADP in order to regenerate ATP (for a review see [1]). CK isoenzymes are expressed in tissues with high and fluctuating energy demands, e.g. in skeletal and cardiac muscle, brain, retina and spermatozoa. In most tissues, cytosolic and mitochondrial CK isoenzymes are co-expressed. The three cytosolic CK isoenzymes always exist as dimeric molecules (MM-CK, MB-CK, and BB-CK) composed of two types of subunits (M standing for the 'muscle' isoform; B standing for the 'brain' isoform). They are found as soluble enzymes in the cytosol, as well as in association with subcellular structures, e.g. the myofibrillar M-line or the sarcoplasmic reticulum in muscle, where they are functionally coupled to the acto-myosin ATPase [2,3] and the Ca^{2+}-ATPase [4,5] respectively.

In contrast, the mitochondrial CK (Mi-CK) isoenzymes form either octameric or dimeric molecules [6,7], with the dimer as the basic stable building block for the octamer [8]. Mi-CK is bound to the outer side of the inner mitochondrial membrane [9] and has been localized both along the cristae membrane and at peripheral sites where inner and outer mitochondrial membranes are in close proximity [10,11]. Mi-CK dimers and octamers are readily interconvertible, with several factors influencing the dimer/octamer ratio *in vitro* [12]. The octameric form is favoured at high Mi-CK concentrations and at low pH [6,8,12,13]. On the other hand, equilibrium substrate combinations (MgADP + MgATP + Cr + PCr) or formation of a transition-state-analogue complex (TSAC, composed of Mi-CK, MgADP, Cr and nitrate) result in the complete relatively fast dissociation of octamers into dimers within 20–30 min [12,14–16]. Reassociation of dimers into octamers is only observed at protein concentrations higher than 0.1 mg/ml and is rather slow (taking many hours to complete) compared with the dissociation in the presence of the TSAC substrates [15].

Imaging of Mi-CK by electron microscopy revealed a banana-shaped structure for the dimer, while the octamer displays a cube-like shape with a P422 symmetry, a side width of 10 nm, and a central cavity or channel running through the molecule along the fourfold symmetry axis [17]. Based on the membrane interaction properties [12,18] and the molecule's shape, the octameric molecule would seem to be well suited to supporting metabolite channelling [19] at the mitochondrial contact sites. Studies on isolated mitochondria have shown that mitochondrial oxidative phosphorylation and the Mi-CK reaction are functionally coupled, that is Mi-CK preferentially utilizes ATP synthesized through oxidative phosphorylation, for PCr synthesis (for reviews see [20,21]).

The kinetic properties of the cytosolic CK isoenzymes have been characterized in detail [1,22–24]. In contrast, for the Mi-CK isoenzymes, only a few studies are available [13,25,26], and in only one of these was the oligomeric state of Mi-CK taken into consideration [13].

In order to define more clearly the functional properties of dimeric and octameric Mi-CK, we carried out a series of experiments for the determination of the kinetic constants of dimeric and octameric chicken heart Mi-CK (Mi_b-CK) under identical, properly controlled conditions.

Abbreviations used: CK, creatine kinase; B-CK, cytosolic brain-type CK isoenzyme; M-CK, cytosolic muscle-type CK; Mi-CK, mitochondrial CK isoenzymes; Mi_b-CK, Mi-CK isoenzyme from chicken heart; Cr, creatine; PCr, phosphorylcreatine; 2-ME, 2-mercaptoethanol; TSAC, transition-state-analogue complex; CK forward reaction, $MgATP + Cr \rightarrow MgADP + PCr + H^+$; CK reverse reaction, $MgADP + PCr + H^+ \rightarrow MgATP + Cr$.

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MATERIALS AND METHODS

Protein preparation

Escherichia coli strain BL21(DE3)pLysS and expression vector pET-3b [27] were used for the expression of Mi_b-CK as has been described in detail previously [28]. Protein purification was performed according to Furter et al. [26].

Enzyme assays

CK activity was determined using the pH-stat method by measuring H^+ release in the forward direction of the reaction, $Cr + MgATP \rightarrow PCr + MgADP + H^+$, and H^+ consumption in the reverse direction of the reaction, $PCr + MgADP + H^+ \rightarrow Cr + MgATP$ [2,29]. One enzyme unit corresponds to 1 μmol of ATP or PCr transphosphorylated per min at 25 °C. For the reverse direction of the reaction (ATP synthesis), Mi_b-CK activity was determined in an assay mixture containing 65 mM KCl, 8.5 mM $MgCl_2$, 85 μM EGTA, 1 mM 2-mercaptoethanol (2-ME) and 4 mM ADP at pH 7.0. [PCr] was varied from 0.5 mM to 20 mM to determine K_m(PCr) and $V_{max.,rev}$. For the forward direction of the reaction (PCr synthesis), the assay mixture contained 75 mM KCl and 1 mM 2-ME at pH 8.0. In order to determine K_m(MgATP), K_d(MgATP), K_m(Cr), K_d(Cr) and $V_{max.,for}$, [ATP] was varied from 0.1 mM to 8 mM and [Cr] from 6 mM to 45 mM. The concentration of Mg^{2+} (added as magnesium acetate) always exceeded that of ATP by 1 mM. For the determination of all kinetic parameters, initial velocity data were analysed using the program package written by W. W. Cleland [30] as adapted for personal computers by R. Viola (the program was obtained through R. Viola, Akron University, OH, U.S.A.). For technical reasons, the pH-stat method is not suited for an accurate determination of the K_m(MgADP) of CK, since this K_m value is far below 100 μM (for a review, see [21]). Therefore, the K_m values for PCr were determined at a constant MgADP concentration of 4 mM. Unless otherwise stated, the values are given as mean ± S.E.M.

The pH-stat method produced most reliable results because it measures directly the proton consumption (reverse CK reaction) or production (forward CK reaction) of the CK reaction and in contrast to enzyme-coupled colorimetric CK assays does not involve auxiliary enzymes which are also sensitive to the conditions used. In addition, for the analysis of the kinetic constants we only used initial velocity data (30–60 s) in order to ensure that changing concentrations of buffering substrates, which may act as long-term buffers, had no influence on the measurements. The measurements were done at pH 7.0 in the reverse direction of the reaction, whereas in the forward direction of the reaction, they were done at pH 8.0. There were two reasons for this, first the pH optima in the two directions of the reaction are different [26] and secondly in the forward reaction the reaction mechanism is changing from pH 7.0 (rapid equilibrium ordered, [31,32]) to pH 8.0 (rapid equilibrium random, [22,25]) which is the mechanism proposed for Mi-CK [13,25]. A temperature of 25 °C for *in vitro* measurements was chosen as the actual body temperature of chickens (42 °C) led to partial denaturation of Mi_b-CK *in vitro*.

Generation of Mi_b-CK solutions with defined proportions of dimers and octamers

For dimeric Mi_b-CK solutions (90–100 % dimers), concentrated Mi_b-CK (> 4.5 mg/ml) was diluted with buffer A (50 mM NaH_2PO_4, 0.5 mM EDTA, pH 7.2, 150 mM NaCl, 1 mM 2-ME, at 4 °C) additionally containing the TSAC substrates (4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, 50 mM nitrate) to give a final protein concentration of 50–60 μg/ml. After incubation for at least 2 h at 4 °C to dissociate all octamers into dimers, the solution was dialysed for 2 days against four changes of buffer A at 4 °C in order to remove the TSAC substrates.

For octameric Mi_b-CK solutions (90–100 % octamers), concentrated Mi_b-CK was diluted to 50–60 μg/ml with buffer A and used within a few hours.

Gel-permeation chromatography

Mi_b-CK solutions were analysed for dimer and octamer contents by gel-permeation chromatography on an FPLC Superose 12 HR 10/30 column (Pharmacia) at 4 °C, using buffer A as eluent. The proportion of octamers and dimers was estimated by measuring the area of the absorption peaks (280 nm) with a digital planimeter (Placom KP-90, Japan).

Protein determination

Protein concentrations were determined by the method of Bradford [33] using the Bio-Rad assay and BSA as standard. At least three measurements were averaged to get a mean ± S.E.M.

RESULTS

Gel-permeation chromatography of Mi_b-CK

Mi_b-CK consists of readily interconvertible octameric and dimeric molecules that can be separated easily by gel-permeation chromatography [6,12,16]. Figure 1 shows a typical gel-filtration run where elution of Mi_b-CK octamers and dimers was monitored by measurements of CK activity, of the absorbance at 280 nm (A_{280}), and of protein content by the method of Bradford [33]. The CK activity of all dimer fractions was measured by the pH-stat method within 3 min of elution from the gel-permeation column. This was crucial for the avoidance of any re-octamerization of Mi_b-CK in the dimer fractions. The time point for the activity measurements of the octamer fractions, instead, were not so critical since the dissociation of octamers into dimers upon dilution in the absence of the TSAC substrates takes place only after prolonged incubation times [16]. As can be seen in Figure 1,

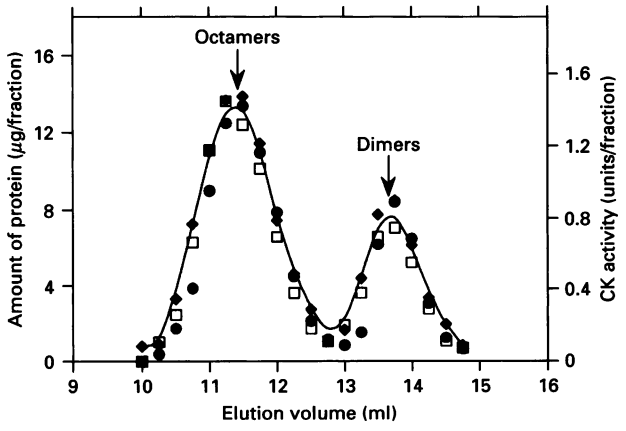

Figure 1 Separation of Mi_b-CK octamers and dimers by gel-permeation chromatography

Mi_b-CK was chromatographed on a Superose 12 FPLC column (Pharmacia), and 250 μl fractions were collected. The CK activity (□) was determined in the reverse direction of the reaction (see the Materials and methods section), and the amount of protein (◆) was measured by the method of Bradford [33]. The absorbance (●) was monitored at 280 nm, and the octamer and dimer peak areas were estimated by planimetry.

the curves for the amount of protein, for CK activity and for the absorbance (A) at 280 nm were almost perfectly superimposable. In the illustrated case (Figure 1), the octamer peak included 70.9% of the A area, 69.5% of the total amount of protein measured, and 70.9% of the CK activity, while the remainder of approximately 30% was found in the dimer peak. These results indicate first, that octamers and dimers might have the same specific activity and secondly, that the absorption coefficients at 280 nm of both CK oligomers are in the same range. Therefore, for purified Mi_b-CK, the A_{280} is a good measure for the amount of Mi_b-CK protein (absorption coefficient, $\epsilon^{1\%}_{280\,nm} = 8.8$, [8]) as well as for the CK activity, provided that the enzyme is fully active. This method for analysis allowed us to determine the exact proportion of octamers and dimers present in each sample before and after kinetic measurements.

Generation of defined dimeric or octameric Mi_b-CK preparations

For measurements of the kinetic parameters of the CK oligomers, well-defined dimeric or octameric Mi_b-CK solutions had to be generated. Conditions were needed where at the same Mi_b-CK protein concentration and in the absence of CK substrates, the protein solutions mainly consisted either of dimers or of octamers. Octameric Mi_b-CK is rather stable upon dilution, even at concentrations of less than 50 μg/ml, and it dissociates into dimers only after prolonged incubation times [16], if no substrates are present. In the case of dimeric Mi_b-CK, it was somewhat more difficult to obtain homogenous dimer preparations because Mi_b-CK dimers tend to re-associate quickly into octamers in the absence of the TSAC substrates, unless the protein concentration is less than 0.1 mg/ml [15]. Therefore, dimeric Mi_b-CK was prepared by diluting concentrated Mi_b-CK stock solutions to 50–60 μg/ml with buffer A containing additionally the TSAC substrates. After incubation for at least 2 h at 4 °C, the TSAC substrates were removed by dialysing extensively against buffer A (see the Materials and methods section). Analytical gel-permeation chromatography of this 'dimeric' Mi_b-CK solution immediately before and after the kinetic measurements revealed negligible amounts of TSAC substrates left and an Mi_b-CK octamer content in such pure preparations of only 10.1 ± 1.0%. To obtain octameric Mi_b-CK at the same protein concentration as dimeric Mi_b-CK (see above), concentrated Mi_b-CK stock solutions were simply diluted to 50–60 μg/ml just before use, resulting in a solution consisting of 89.6 ± 1.2% octamers. For pH-stat measurements, 6.8 ± 0.2 μg ($n = 18$) and 3.0 ± 0.3 μg ($n = 6$) of 'octameric' Mi_b-CK, and 5.5 ± 0.1 μg ($n = 18$) and 2.5 ± 0.1 μg ($n = 6$) of 'dimeric' Mi_b-CK were used in the forward and reverse directions of the reaction respectively (in a total volume of 3 ml). Following these procedures, we were able to generate highly enriched octameric and dimeric solutions at very similar final protein concentrations and under the very same buffer conditions. This was a prerequisite for a direct kinetic comparison of octamers versus dimers.

Changes in the proportions of Mi_b-CK octamers and dimers during the pH-stat measurements

In the pH-stat assay, Mi_b-CK had to be used highly diluted (0.8–2.3 μg of protein/ml) and the enzyme was additionally exposed to CK substrates, conditions which are known to significantly shift the dimer/octamer equilibrium towards the dimer. Therefore, it had to be considered that part of the octameric Mi_b-CK dissociates into dimeric Mi_b-CK during the kinetic measurements in the pH-stat, although only initial velocities were taken into account (first 30 s). As a control, after the pH-stat assay, aliquots of the assay mixture were directly

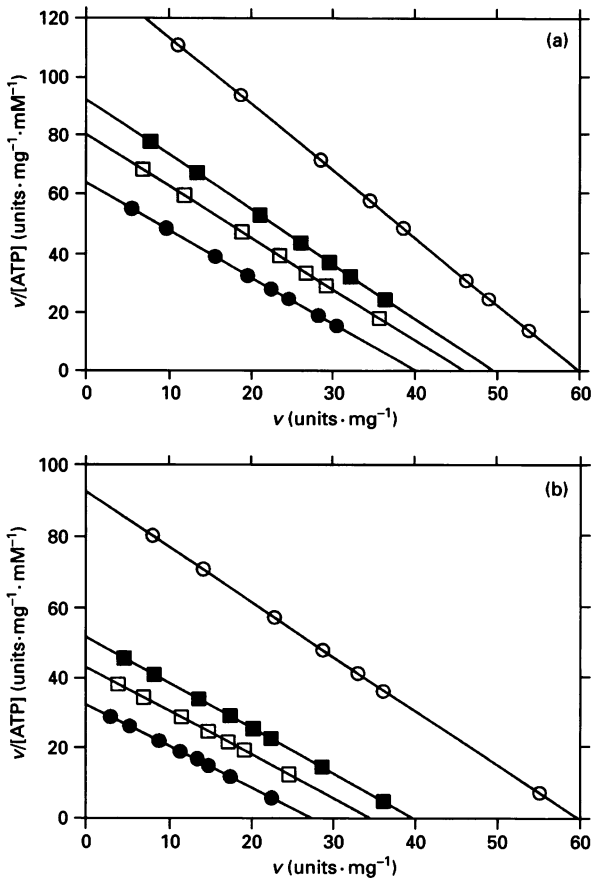

Figure 2 Eadie–Hofstee plots for the forward CK reaction (PCr synthesis) catalysed by dimeric (a) and octameric Mi_b-CK (b)

CK activity measurements were performed at pH 8.0 and 25 °C as described in the Materials and methods section. [ATP] was varied from 0.1 to 8 mM at [Cr] of 6 mM (●), 9 mM (□), 12 mM (■), and 45 mM (○). The concentration of Mg^{2+} (added as magnesium acetate) always exceeded that of ATP by 1 mM. For each experimental value, six independent measurements were averaged. From such complete sets of data the K_m as well as the K_d values both for ATP and Cr could be calculated (see Table 1, and the Materials and methods section).

analysed by gel-permeation chromatography. In assay mixtures containing 6 mM Cr and 0.1 mM ATP, 'octameric' Mi_b-CK still consisted of 89.6 ± 1.2% octamers ($n = 4$), indicating that under these conditions, and within this short period of time, dissociation was negligible. If, however, 45 mM Cr and 8 mM ATP were used, the proportion of octamers dropped to 62.0 ± 2.9% octamers ($n = 4$). It is important to point out that the conditions used for gel-permeation chromatography [i.e. highly diluted protein, time scale (elution of Mi_b-CK after 20–25 min), and, in the beginning, the presence of substrates] were generally rather favouring dissociation of octamers into dimers during the gel-filtration run. Therefore, the octamer percentage values indicated represent the absolute lowest limit levels for the proportion of octamers present at the end of the pH-stat measurements. Thus, during the first 30 s considered for initial CK velocity determination the octamer content must have been significantly higher than 62.0%.

In the case of dimeric Mi_b-CK, a possible re-octamerization during the pH-stat assay, normally taking place in a significant way on this time-scale only at protein concentrations above 0.1 mg/ml, might have influenced the kinetic measurements, although this is very unlikely at the low Mi_b-CK protein

Table 1 Kinetic constants of dimeric and octameric Mi_b-CK in the direction of PCr synthesis (A, forward CK reaction) and in the direction of ATP synthesis (B, reverse CK reaction)

CK activity measurements in the forward direction were performed at pH 8.0 and 25 °C (see the Materials and methods section). [ATP] was varied from 0.1 to 8 mM, and [Cr] from 6 to 45 mM. The concentration of Mg^{2+} (as magnesium acetate) always exceeded that of ATP by 1 mM. All values represent the mean ± S.E.M. of six independent sets of measurements. CK activity measurements in the reverse direction were performed at pH 7.0 and 25 °C. [PCr] was varied from 0.5 to 20 mM at constant [ADP] of 4 mM. The values given represent the mean ± S.E.M. of 15 and five independent measurements for dimeric and octameric Mi_b-CK respectively.

A	$MgATP + Cr \rightarrow MgADP + PCr + H^+$	
	Dimeric Mi_b-CK	Octameric Mi_b-CK
K_m(Cr)	3.69 ± 0.40 mM	10.1 ± 0.8 mM
K_d(Cr)	9.56 ± 1.28 mM	18.3 ± 2.1 mM
K_m(MgATP)	0.39 ± 0.03 mM	0.56 ± 0.04 mM
K_d(MgATP)	1.01 ± 0.18 mM	1.01 ± 0.11 mM
$V_{max,for}$	64.7 ± 1.3 units/mg	72.9 ± 1.9 units/mg
B	$MgADP + PCr + H^+ \rightarrow MgATP + Cr$	
	Dimeric Mi_b-CK	Octameric Mi_b-CK
K_m(PCr)	1.12 ± 0.03 mM	1.69 ± 0.14 mM
$V_{max,rev}$	111.0 ± 0.9 units/mg	121.0 ± 3.3 units/mg

concentrations (0.8–2.3 μg/ml) used [15]. Nonetheless, aliquots of 'dimeric' Mi_b-CK were also analysed by gel-permeation chromatography directly after the pH-stat measurements. In an assay mixture containing 45 mM Cr and 8 mM ATP, no octamers could be detected at all (0%; $n = 5$), indicating that at these substrate concentrations the small percentage of octamers (≈ 10%) present before dilution had dissociated completely into dimers during the assay.

Similar findings were made in the reverse direction of the reaction. In assay mixtures containing 0.5 mM PCr and 4 mM ADP, dissociation of octameric Mi_b-CK was negligible, i.e. the proportion of octamers was still 89.3 ± 0.3% ($n = 3$). In assay mixtures containing 20 mM PCr and 4 mM ADP, 78.6 ± 0.7% octamers were measured, demonstrating that under the most extreme conditions only a small proportion of octameric Mi_b-CK dissociated into dimers during the time of the pH-stat measurements in the presence of high concentrations of PCr and ADP.

Determination of kinetic constants of dimeric and octameric Mi_b-CK

In a rapid-equilibrium random mechanism of substrate binding, as proposed for cytosolic CK [22,24,31] and Mi-CK [13,25] at pH 8.0, the CK substrates have equal access to the respective binding sites within the enzyme. If the K_d values are higher than the K_m values [$K_d > K_m$] for any of the substrates, this indicates 'synergism' in substrate binding. In such a case, as shown to be true for MM-CK [23,24] and Mi_b-CK [26], binding of the first substrate facilitates binding of the second one.

In Figure 2 the results of the kinetic measurements in the forward direction of dimeric and octameric Mi_b-CK are shown as Eadie–Hofstee plots and by using the kinetic analysis program of Cleland ([30], see also the Materials and methods section) $V_{max.}$, K_m and K_d values could be calculated (Table 1A). Dimeric as well as octameric Mi_b-CK displayed synergism in substrate binding, that is, in both cases, the K_d values for MgATP and Cr were higher than the corresponding K_m values. Interestingly, both the K_d and K_m values for Cr (2–3 times) and the K_m value for ATP (1.5 times) were higher for octameric compared with dimeric Mi_b-CK, pointing to a significant kinetic difference of octameric versus dimeric Mi_b-CK in the binding of Cr as a substrate. As judged from the slopes of the Eadie–Hofstee plots (with the slope representing $-1/K_m$; Figures 2a and 2b) it can be seen that, since the data are very accurate, the Mi-CK enzyme kinetics is not as simple as suggested because the K_m(MgATP) at 45 mM Cr is obviously lower than that at 6 mM Cr. This fact, indicating an apparent negative cooperativity, is true for dimeric as well as octameric Mi_b-CK and therefore seems to be characteristic for Mi-CK activity.

In the reverse direction of the reaction, K_m for PCr and $V_{max.}$ for dimeric and octameric Mi_b-CK were determined at pH 7.0, since, at pH 8.0, the activity in the reverse direction of the reaction is approximately three times lower than at pH 7.0 ([26], see also the Materials and methods section). K_m(PCr) and $V_{max.}$ values were determined by averaging several sets of independent measurements (see the Materials and methods section) and were lower for dimeric compared with octameric Mi_b-CK (Table 1B).

DISCUSSION

The kinetic constants measured in this study under defined conditions concerning the octamer/dimer ratio were lower for dimeric compared with octameric Mi_b-CK, with the binding constants for creatine [K_m(Cr) and K_d(Cr)] differing by a factor of 2–3. Since the kinetic measurements in the pH-stat were done under absolutely identical conditions for dimers and octamers, i.e. identical protein concentrations, pH, temperature and substrate concentrations, except for the oligomeric state of Mi_b-CK, the observed kinetic differences most likely originated from the different oligomeric state of Mi_b-CK.

Synergism in substrate binding was found for both oligomers, and $V_{max.}$ of dimeric and octameric Mi_b-CK were very similar, being in agreement with earlier findings [12]. The kinetic constants K_m(PCr), K_m(MgATP), K_d(Cr), and K_m(Cr) derived from sets of experiments shown in Figure 2 are 1.5–3 times lower for dimeric than for the octameric Mi_b-CK (Table 1), meaning that dimers have higher affinities for the substrates than octamers. This difference, albeit rather small, may become physiologically relevant especially at low, local substrate concentrations in the cell. The difference of the kinetic constants relating to the substrate Cr in the forward CK reaction is especially interesting, since Mi-CK was shown to be coupled to oxidative phosphorylation via ATP/ADP translocase, a process enhancing the forward CK reaction [34,35]. In addition, from the Eadie–Hofstee plots (Figure 2) it can be seen that both for dimers and octamers, the K_m(MgATP) is lower at high [Cr]. This fact would indicate that in a low-energy state (low, that is at a high Cr/PCr ratio) Mi-CK has a higher affinity for ATP transported through the inner mitochondrial membrane by the ATP/ADP translocase. Such behaviour of Mi-CK would favour the production of 'high-energy' phosphate, e.g. PCr by mitochondria in cells which are in a state of low phosphorylation potential. This apparent negative cooperativity of CK could also be a hint that the ATP- and the Cr-binding sites are somehow structurally connected, which allows communication with each other.

Lipskaya et al. [13] found similar K_m values compared with our results, but did not determine the K_d values for the substrates and, in addition, these authors determined the kinetic constants of octameric and dimeric Mi-CK each under very different experimental conditions. In theory, the enzyme concentration

used in the pH-stat assay should have no influence on the kinetic values, but in practice we noted that it was necessary to use very similar protein concentrations of dimeric and octameric Mi_b-CK to be able to directly compare their K_m values (P. Kaldis and T. Wallimann, unpublished work). Based on our experience, differences in kinetic constants obtained by using protein concentrations which were 25 times higher for the octamer compared with those taken for the dimer [13] should therefore be interpreted with caution.

The proportion of dimeric Mi-CK present *in vivo* in the mitochondrial intermembrane space was estimated, by extraction experiments, to be at least 10 % of the total Mi-CK for chicken, depending on the species and the tissue [12,21]. Considering the fact that in resting muscle, the concentration of Cr is between 5 and maximally 10 mM (see [20]), the observed differences in K_d(Cr) and K_m(Cr) for Mi_b-CK dimers and octamers (9.56 versus 18.3 and 3.69 versus 10.1 mM, respectively) seem to be physiologically relevant. That is, at resting levels of [Cr] during early phases of muscle activity, only Mi_b-CK dimers, but not octamers, might be saturated by Cr. Thus, owing to their higher affinity for Cr, the low proportion of Mi_b-CK dimers (10 %) may be responsible for approximately 20 % of the reaction flux from Cr to PCr at 1 mM Cr, but only for 10 % of the reaction flux at 20 mM Cr. This estimation indicates that besides Mi_b-CK octamers, the dimers may also play a role in PCr synthesis and in energy metabolism in general, as already postulated [16,20], especially at early phases of activation or at low workloads when [Cr] still is or remains low, respectively. However, at high cellular workload, when [Cr] may rise to 20–25 mM, Mi_b-CK octamers will also be mostly saturated by Cr. As octamers are able to induce contacts between mitochondrial membranes [18] and are postulated to form 'functionally coupled channels' with porin of the outer mitochondrial membrane [36] and ATP/ADP-translocator of the inner mitochondrial membrane, they will probably enhance the efficiency of substrate channelling and vectorial transport of PCr more than dimeric Mi_b-CK [19]. Since, *in vitro*, (i) the dimer/octamer equilibrium is governed in part by pH, by nucleotides, and by Cr and/or PCr levels; (ii) a pH-dependent differential interaction of octamers and dimers with the inner mitochondrial membrane has been demonstrated [12,37]; and (iii) a clear difference in important kinetic parameters between octamers and dimers has been shown here, it is reasonable to speculate that such a dynamic equilibrium between dimers and octamers, as well as between free and membrane-bound Mi_b-CK observed *in vitro*, may also be of some physiological importance in modulating cellular energetics *in vivo* (see [20,21]). However, the *in vivo* conditions, e.g. the high Mi-CK concentrations and the temperature in the intermembrane space seem rather to favour a strong prevalence of Mi-CK octamers.

In the present study, we show for the first time that under properly controlled conditions, Mi_b-CK dimers differ significantly in the K_d(Cr) and K_m(Cr) values from Mi_b-CK octamers. Based on the facts presented here, we postulate that dimeric Mi_b-CK may, nevertheless, also play an important physiological role in contributing to the generation of PCr by the mitochondria, especially at rest or during early phases of cellular activity. Secondly, the apparent negative cooperativity of Mi-CK, showing a lower K_m(MgATP) at higher [Cr], would favour PCr production by mitochondria in cells which are in a low-energy (high-Cr/PCr) state.

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