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Why is creatine kinase a dimer? Evidence for cooperativity between the two subunits

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Abstract

The dimeric chicken brain type isoenzyme of creatine kinase (BB-CK) was mutated by a C283S amino acid exchange in the catalytic site to produce a basically inactive dimer (B*B*-CK). The mutated enzyme showed a residual activity of about 4% compared to the wild-type, whereas substrate binding parameters were not altered. The inactivated dimer was hybridized with native dimeric muscle enzyme (MM-CK) to produce a partially inactivated MB*-CK heterodimeric hybrid and also to a his-tagged BB-CK (hBhB-CK) resulting in a partially inactive hBB*-CK homodimer. The generated hybrids were purified by chromatography. The V_{max} and substrate binding parameters K_m and K_d were determined for both directions of the CK reaction and compared to the parameters of the wild-type enzymes (MM-, BB-, hBhB-, MB-CK). In the direction of ATP synthesis (reverse reaction), the MB*- and hBB*-CK hybrids showed a decrease of V_{max} to 34% and 32%, respectively, compared to the unmodified wild-type isoform. The inactivation of a single subunit in MB*-CK led to an increase in the K_d value resulting in an significant substrate synergism, not seen with the MB-CK wild-type enzyme. In the direction of phosphocreatine synthesis (forward reaction), the modified hybrids showed a decrease of V_{max} to 50% of the wild-type enzymes and no significant alterations of the K_m and K_d parameters. These results strongly suggest an enzymatic cooperativity of the two subunits in the reverse reaction but independent catalytic function in the forward reaction.

Keywords: Creatine kinase; Heterodimer; Subunit cooperativity; K_m; Isoenzyme; Site-directed mutagenesis

1. Introduction

Creatine kinase (CK, EC 2.7.2.3) is a key enzyme of the cellular energy metabolism, catalyzing the reversible phosphoryl transfer from phosphocreatine (PCr) to ADP (for a review, see [1]). CKs constitute a group of different oligomeric isoforms with tissue-specific expression and isoenzyme-specific subcellular localization. Three dimeric isoforms, ubiquitous brain type BB-CK, sarcomeric muscle type MM-CK and the MB-CK heterodimer [2], as well as two octameric mitochondrial isoforms, ubiquitous Mi_a-CK and sarcomeric Mi_b-CK, are synthesized in a tissue-specific manner [3,4]. CK is a member of the family of guanidino kinases (ATP:guanidino-phosphotransferases), enzymes with closely related primary amino acid sequences and structural similarities [5]. PCr is the only phosphagen compound found in vertebrates [6], whereas in invertebrates, at least six

Abbreviations: CK, creatine kinase (EC 2.7.3.2); AK, arginine kinase (EC 2.7.3.3); PCr, phosphocreatine; MM-CK, muscle type isozyme of creatine kinase; BB-CK, brain type isozyme of creatine kinase; B*B*-CK, inactivated brain type isoenzyme; hBhB-CK, brain type isoenzyme fused to a penta his-tag

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other unique phosphagens appeared as energy carriers during evolution.

Arginine kinase (AK, EC 2.7.3.3), another member of this family, utilizes phospho-arginine as high energy compound and is found exclusively in invertebrates. Although CK and AK show high sequence and structural similarities, AK is mainly found as a monomer with a single 40 kDa subunit, occasionally forming dimers in some species. CK appears always as a dimer, composed of two 40 kDa subunits. Interestingly, monomeric AKs are found in arthropods and mollusks while higher molecular weight forms of AK occur in echinoderms and annelids [7]. The homologous amino acid sequences of CKs and AKs suggest that they have evolved from a common ancestor whereas AK is thought to be more closely related to the ancestral phosphagen kinase than CK [5,8]. The change from the monomeric into the dimeric state was suspected to have evolutionary and physiological advantages [9]. Earlier investigations on a cooperativity between the two CK subunits, as a possible evolutionary advantage of the dimeric over the monomeric state, led to controversial results. Wang and co-workers suggested that the two subunits of a MB-CK hybrid dimer work fully independently [10], whereas others showed some evidence for a cooperativity between the two subunits [11,12]. However, no detailed investigation of the kinetic parameters has been performed yet. In this work, we present evidence suggesting that the two enzymatically active subunits of the CK dimer show indeed cooperativity, influencing the maximal initial velocity and to a certain extent also the substrate binding parameters of the subunits.

2. Materials and methods

2.1. Escherichia coli strains, plasmids and DNA manipulation

E. coli strain BL21 (DE3) pLysS and expression vector pET3b [13] were used as described earlier for mitochondrial CK [14] or M- and B-CK [15]. pRF5 is identical to pET3b except for a deleted *Eco*RV/*Eco*RI fragment. *E. coli* XL1 blue [16], media and standard DNA manipulations were used as already described [17]. The construction of plasmid pT17, containing the chicken M-CK cDNA [18,19], and pT23, containing the chicken B_b-CK cDNA [20], has also been described earlier [21]. For the generation of the B-CK isoform, containing an N-terminal penta his-tag, the coding region of pT23 was subcloned into a pET14b expression vector (Novagen).

2.2. Site-directed mutagenesis and construction of the CK point mutation

Polymerase chain reaction was used for site-directed mutagenesis [22] of chicken B-CK. The site-specific mutations were introduced by using the inverse PCR method [23] and appropriate oligonucleotides that were 5′-phosphorylated. As template for the B-CK mutant, plasmid pT23 was used [15]. Site-directed mutagenesis was performed using Pfu DNA polymerase (Stratagene). The DMSO concentration was varied between 0 and 10% to increase yield and specificity of the PCR reaction. The resulting PCR products were extracted by phenol/chloroform [17] and subsequently purified by agarose gels using the Geneclean kit (Bio101 Inc., La Jolla, CA, USA). The purified PCR products where self-ligated using T4 DNA ligase (FPLCpure, Pharmacia Biotech) and transformed into *E. coli* XL1 blue. The mutants were finally checked by DNA sequencing.

2.3. Protein sources

Chicken MM-CK, chicken BB-CK and the mutant derivatives thereof were expressed in *E. coli* as described elsewhere [14]. Active BB-CK and the B-CK-derived inactive B*B*-CK mutant could be purified out of the soluble fraction of the cell lysates in a two-step procedure similar to an earlier published protocol [24].

MM-CK was purified by solubilization of the insoluble cell fraction in urea, subsequent refolding and anion exchange chromatography as already described [25]. Hybridization of the subunits was done by mixing stoichiometric amounts of the purified CK isoforms and mutants thereof, e.g. inactive B*B*-CK and his-tag BB-CK (hBhB-CK) at a final concentration of 3–5 mg/ml. The proteins were denatured by adding solid urea to a final concentration of 6 M. After incubation (1 h at room temperature), the protein was dialyzed against renaturation buffer (50 mM

Tris–HCl pH 9.5, 150 mM NaCl, 10 mM EDTA, 2.5 mM mercaptoethanol, 1 mM PMSF) for 12 h (4°C). To separate the resulting dimers of MM-, MB- and BB-CK, the protein was dialyzed against anion exchange buffer (10 mM Tris–HCl, 10 mM bis-Tris–propane, 0.5 mM β-mercaptoethanol, pH 8.0) and applied on a Poros HQ column (PerSeptive Biosystems Inc., Framingham, MA, USA) equilibrated with anion exchange buffer pH 8.0. Proteins were eluted with a linear NaCl gradient from 0 mM to 250 mM. The MB-CK heterodimer eluted as a first sharp peak at about 50 mM NaCl and BB-CK as a second peak at 150 mM. MM-CK did not bind to the column under the chosen conditions and therefore was found in the flow trough.

To separate the mixture of his-tagged and non-his-tagged BB-CK isoforms, the refolded proteins were dialyzed against buffer H (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5 mM β-mercaptoethanol) and applied on a metal chelate Ni^{2+}-NTA agarose column (Qiagen, 4.6 mm × 100 mm) previously charged with 100 mM $NiSO_4$ and equilibrated in buffer H. The proteins were eluted with a linear imidazole gradient from 0 to 250 mM. The hBB* heterodimer containing a single his-tag eluted between 20 and 50 mM imidazole, the hBhB homodimer eluted at 100–150 mM imidazole. The untagged B*B*-CK did not bind to the column.

2.4. Enzyme activity assays and K_m/K_d determination

CK activity was determined with a photometer (UNICAM UV4, thermostated to 25°C) using a coupled enzyme assay modified after [26] in the forward reaction (pH 8.0) and reverse reaction (pH 7.0) varying both substrate concentrations in a 5 × 5 matrix.

For the determination of the constants K_m, K_d and V_{max}, initial velocity data were analyzed using the program package written by W.W. Cleland [27] and adapted for personal computer by R. Viola (the program was obtained through R. Viola, Akron University, Akron, OH, USA). Mean values and standard errors are given for the calculated values. Each set of constants represents the mean of three independent measurements.

2.5. Other methods

Protein concentrations were determined by the method of Bradford [28] using the Bio-Rad reagent and bovine serum albumin as a standard. Cellulose polyacetate (CPA) electrophoresis was performed as described earlier [29].

3. Results

3.1. C283S BB-CK point mutation

The highly conserved cysteine residue C283 is located near the catalytic site of CK and is important for full enzymatic activity [30]. It probably interacts with the guanidino group of the creatine substrate and is most likely responsible for its correct orientation within the active site [31]. C283 was identified to be a main target for chemical inactivation of CK by a large number of reagents [32–34]. This inactivation is partly reversible due to reactivation of the thiol groups by reducing agents. The exchange of the cysteine 283 in BB-CK to a structurally homologous serine reduced the specific activity of the mutant dramatically to about 4% in the reverse reaction (pH 7.0) and 2.5% in the forward reaction (pH 8.0) compared to the BB-CK wild-type (Tables 1 and 2). The same mutation in the mitochondrial isoform showed

Table 1
Enzyme activities and kinetic parameters of CK wild-type and mutant isoforms, determined for the forward reaction (mean ± S.D.)

Enzyme	Specific activity (μmol/min/mg)	K_m (ATP) (mM)	K_d (ATP) (mM)	K_m (Cr) (mM)	K_d (Cr) (mM)
MM	50 ± 1.6	0.33 ± 0.075	3.26 ± 0.44	4.63 ± 0.49	45.6 ± 11.4
BB	60 ± 1.4	0.27 ± 0.034	1.99 ± 0.30	1.55 ± 0.15	11.4 ± 1.9
MB	**43 ± 2.1**	**0.25 ± 0.074**	**3.29 ± 1.38**	**1.00 ± 0.31**	**13.0 ± 5.0**
MB*	**21 ± 0.7**	**0.14 ± 0.054**	**8.30 ± 3.92**	**0.73 ± 0.30**	**45.4 ± 20.9**
B*B*	1.6 ± 0.3	0.25 ± 0.033	1.68 ± 0.23	1.95 ± 0.16	12.8 ± 2.2

Table 2
Enzyme activities and kinetic parameters of CK wild-type and mutant isoforms, determined for the reverse reaction (mean ± S.D.)

Enzyme	Specific activity (μmol/min/mg)	K_m (ADP) (mM)	K_d (ADP) (mM)	K_m (PCr) (mM)	K_d (PCr) (mM)
MM	304 ± 7	0.44 ± 0.042	0.50 ± 0.10	3.14 ± 0.33	3.59 ± 0.62
BB	375 ± 15	0.19 ± 0.015	0.19 ± 0.035	2.17 ± 0.16	2.20 ± 0.40
MB	**378 ± 8**	**0.25 ± 0.016**	**0.34 ± 0.046**	**2.17 ± 0.15**	**2.84 ± 0.37**
MB*	**130 ± 4**	**0.28 ± 0.028**	**0.68 ± 0.14**	**1.65 ± 0.19**	**3.93 ± 0.71**
B*B*	16 ± 0.4	0.26 ± 0.020	0.22 ± 0.04	1.89 ± 0.16	1.61 ± 0.33
hBhB	**238 ± 8**	**0.18 ± 0.019**	**0.14 ± 0.031**	**1.74 ± 0.18**	**1.36 ± 0.29**
hBB*	**78 ± 3**	**0.16 ± 0.013**	**0.17 ± 0.22**	**1.50 ± 0.11**	**0.16 ± 0.21**

a similar degree of inactivation [30]. During purification and on native CPA electrophoresis, the mutant behaved identically to the wild-type proteins and was equally stable during extended storage at 4°C. The structural integrity of the mutant after substitution of the cysteine residue by serine was also confirmed earlier by circular dichroism (CD) spectra and X-ray [30].

3.2. Formation and separation of the hybrids

The denaturing of CK in 6 M urea leads to an unfolding of the protein and a dissociation into monomers. After the removal of the denaturant by dialysis, the monomers refold and reassociate with quantitative recovery of the enzyme activity [35]. The reassociation of a mixture of M- and B-CK monomers resulted not only in the reformation of the MM- and BB-CK homodimers but also in the formation of MB-CK heterodimers (Fig. 1a). The three isoenzymes MM-, MB- and BB-CK could be separated by anion exchange chromatography to purified homo- and heterodimers and analyzed by CPA gel electrophoresis (Fig. 1b). No differences in the purification and electrophoretic behavior were seen between BB- and the mutant B*B*- or between MB- and MB*-CK. The purified heterodimers were stable and no rearrangement of the subunits to the homodimers was observed (data not shown).

Since the BB-CK wild-type and the B*B*-CK mutant behaved identically during purification, a separation of the BB*-CK heterodimer from the BB and B*B* homodimers was not possible with this protocol. To overcome this problem, we expressed the wild-type B-CK isoform with an additional N-terminal penta his-tag (hB-CK) and used metal chelate chromatography to separate the heterodimeric hBB*, expressing one his-tag, from the homodimeric forms containing either two (hBhB-CK) or no tag (B*B*-CK). The additional tag extension led to a slightly altered electrophoretic mobility on the CPA gel and enabled us to monitor the separation of hBB* from the homodimeric forms (Fig. 1c).

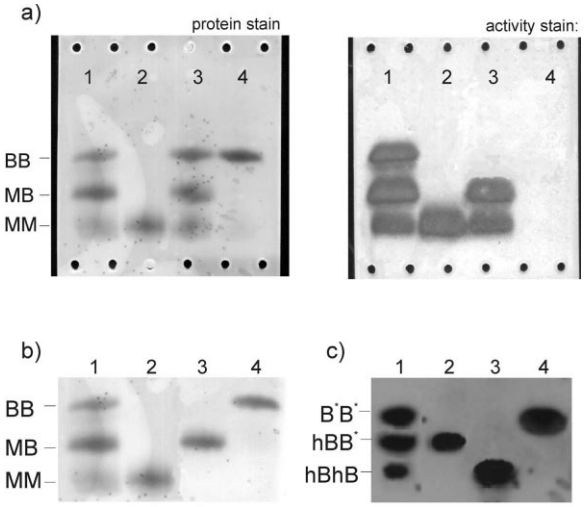

Fig. 1. Separation of CK isoforms by CPA gel electrophoresis. (a) Isoenzymes separated by CPA gel electrophoresis and stained with Coomassie blue for protein (left) or with an enzyme-coupled color reaction for CK activity (right). Lanes 1, mixture of MM, MB and BB after renaturation; 2, MM; 3, mixture of MM, MB* and B*B* after renaturation; 4, B*B*. (b) Purified CK isoenzymes stained for protein with Coomassie blue. Lane 1, MM, MB, BB; 2, MM; 3, MB; 4, BB. Note that the heterodimer consisting of an active M- and an inactive B* monomer shows enzymatic activity, whereas the B*B* homodimer is basically inactive. (c) Purified his-tagged B-CK isoforms, stained for protein with Coomassie blue. Lane 1, mixed hBhB, hBB*, B*B*; lane 2, hBB*; lane 3, hBhB; lane 4, B*B*.

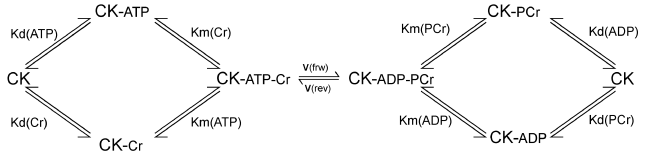

Fig. 2. Scheme of the random BiBi type substrate binding mechanism for CK, according to Cleland [46].

3.3. Enzymatic and kinetic properties of the wild-type and mutant CKs

Substrates of CK bind specifically in a rapid equilibrium random BiBi mechanism (Fig. 2) for both directions [36,37]. For a complete analysis of the kinetic parameters, it was necessary to determine the initial velocity (v_0), varying one substrate concentration [A] at a constant concentration of the second substrate [B] and vice versa. The values determined can be fitted according to Cleland [27]:

$$v_0 = (V_{max} \times [A] \times [B])/(K_{dA} \times K_{mB} + K_{mA} \times [B] + K_{mB} \times [A] + [A] \times [B])$$

resulting in values for V_{max}, K_{dA}, K_{dB}, K_{mA} and K_{mB}. Since the determined initial velocity (v_0) approaches V_{max} asymptotically with increasing substrate concentrations and for example reaches only about 90% of V_{max} even at a substrate concentration of 10-fold K_m, the mathematical determination of V_{max} is the more precise method.

The initial velocity values (v_0) for the native and mutant homodimers (MM, BB, B*B*, hBhB) and heterodimers (MB, MB*, hBB*) were determined by varying the substrate concentrations in a 5×5 matrix ($n = 3$) and for both reactions (forward and reverse) except for hBhB and hBB* which was only measured in the reverse reaction. The results are summarized in Tables 1 and 2.

As could be seen, the mutation C283S in B*B*-CK reduces the V_{max} value dramatically. However, a clear residual activity of 2.5% in the forward and 5% in the reverse direction could still be detected, which enabled us to determine the K_m and K_d constants for this mutant. This was in contrast to results of Lin et al. [11] who did not see any activity after mutating B-CK at the very same residues. However, it was in line with earlier results showing a similar basal activity after the mutation of this residue in the mitochondrial isoform [14], indicating that cysteine 283 of BB-CK is very important for its activity but not absolutely required for catalysis per se. In discrepancy to these earlier investigations in which the mutated Mi-CK showed a significantly increased K_m for creatine, no influence of the mutation on the substrate binding parameters K_m and K_d was observed with the BB-CK mutant. The wild-type BB- and the mutant B*B*-CK showed similar values for all four substrates. This observed discrepancy might be caused by isoform-specific differences between the octameric Mi-CK and the dimeric BB-CK isoform or different activity assay conditions, used in this study.

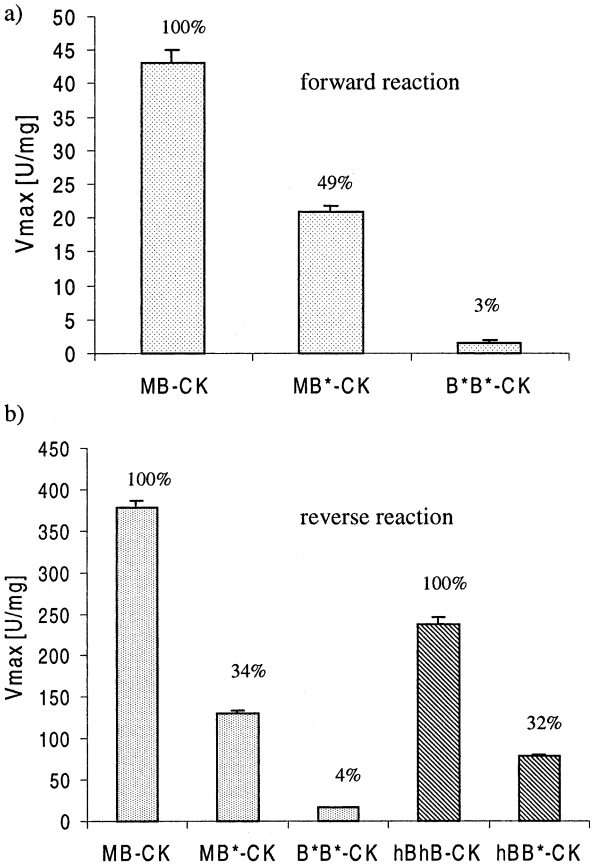

Fig. 3. V_{max} of CK isoforms with none, one or both enzyme subunits inactivated. The wild-type activity was set to 100%. The relative activities are indicated. (a) V_{max} determined for the forward reaction. (b) V_{max} determined for the reverse reaction. Note the general reduction of V_{max} to about one third (34%) in the reverse reaction after inactivation of one subunit only. A similar behavior is seen also with his-tagged hBB*-CK heterodimers (32%).

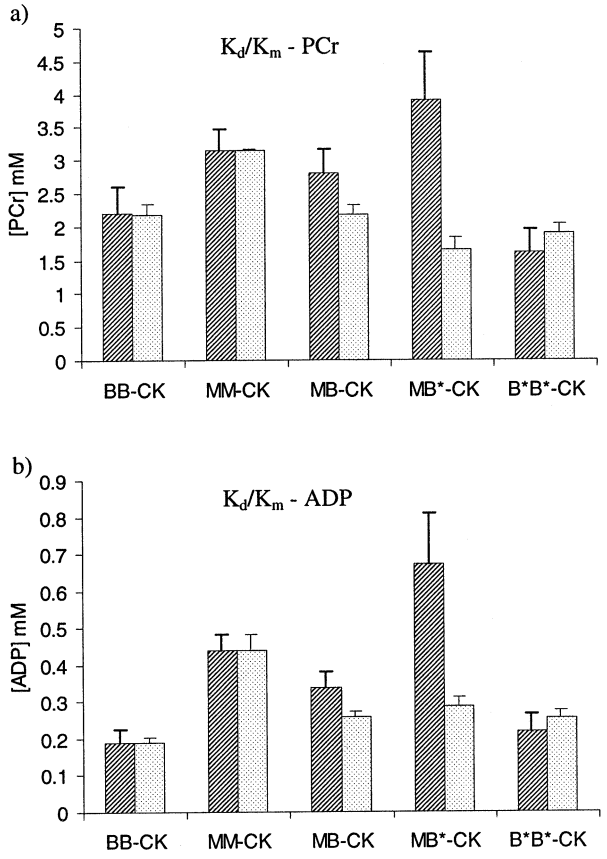

Fig. 4. K_d (hatched bars) and K_m (gray bars) values determined for PCr (a) and ADP (b). Please note the significantly increased K_d values, for both substrates after inactivation of one subunit in the heterodimer (MB*-CK) compared to the wild-type isoform (MB-CK), whereas the K_m was not affected within the experimental errors.

Comparing the kinetic parameters of the three cytosolic CKs (MM-, MB-, BB-), a higher substrate affinity of the brain type isoform is evident for all four substrates, compared to the muscle isoform. Interestingly, the values for the heterodimeric MB-CK isoform were not intermediate between the two homodimeric MM- and BB-CK isoforms but resembled more the higher affinity brain type isoform. Concerning the V_{max} values, all cytosolic isoforms showed comparable values, whereas the brain type tended to have the highest specific activity followed by MM- and MB-CK. In the forward reaction, a striking substrate synergism, indicated by much higher K_d than K_m values (Table 1), was obvious and was also reported earlier [36,38]. This behavior was not seen in the reverse reaction with the wild-type enzymes (Table 2).

3.4. Cooperativity between the CK subunits

The maximal initial velocity of the wild-type MB-CK and mutant MB*-CK hybrid was determined for both reactions. Concerning the forward reaction (Fig. 3a), the mutation of one subunit led to a decrease in activity to about 50% of the wild-type as it would be expected if there were no cooperativity between the subunits. This was different, however, in the reverse direction (Fig. 3b). The inactivation of one subunit in the dimer consistently reduced the maximal initial velocity (V_{max}) of the mutant hybrid to 34% compared to the fully active MB-CK wild-type enzyme. The kinetic parameters showed similar K_m values between MB- and MB*-CK, but a significantly increased K_d for PCr and ADP with the hybrid containing an inactive subunit (Fig. 4a,b, see MB*-CK). Whereas the active heterodimer did not show a significant substrate synergism, the inactivation of one subunit led to a clear synergism.

Regarding the situation with the his-tagged hBhB homodimer, we saw a general decrease in the enzyme activity after adding the tag extension. However, the substrate binding parameters remained the same. Comparing the situation with the hBB* hybrid which contained an inactivated subunit, a reduction of the V_{max} to about one third of the unmodified dimer occurred. In contrast to the MB*-CK heterodimer, we saw no increase of the K_d for PCr and ADP.

4. Discussion

In this study, we determined and compared the kinetic properties of all cytosolic CK isoforms MM-, MB- and BB-CK from chicken. Furthermore, the influence of inactivation of a single subunit on the catalytic properties of homo- and heterodimers consisting of an active and inactive subunit was studied as well.

The evolutionary advantage of developing three tissue-specific dimeric cytosolic CK isoenzymes is not obvious at first glance. An analysis of the kinetic parameters, described in the present work, points out various differences between the CK isoforms. In general, the brain type isoenzyme, the most ubiquitous cytosolic CK isoform expressed in a variety of tissues [39], showed the highest V_{max} and lowest K_m values

for all substrates. This potentially enables the enzyme to work more efficiently even at low substrate concentrations. Compared to BB-CK, the muscle type of CK generally showed a decrease in substrate affinity and also lower V_{max} values. This might be an adaptation to the relatively high concentrations of PCr and Cr (up to 25 and 15 mM, respectively) in skeletal muscle. It is important to mention in this context that MM-CK has the capacity to interact specifically with intracellular structures like the sarcomeric M-line, and the sarcoplasmic reticulum [1,40]. The enzyme is functionally coupled to various energy consuming processes like the myosin ATPases and ion pumps [41,42]. In fully differentiated tissues, the heterodimer MB-CK is found only in cardiac muscle. Interestingly, the kinetic parameters of this hybrid isoform are not an intermediate between the muscle and the brain type CK isoform, but resemble more the BB-CK isoform. CD spectra indicate that the MB-CK hybrid has an overall secondary structure which is not an intermediate between the MM- and BB-CK isoform as well [12]. Since MB-CK is specifically found in adult cardiac muscle, the higher substrate affinities might help to guarantee the appropriate function of this important organ. Together with the higher substrate affinities, the ability to interact with intracellular structures, mediated by the M-type subunit, could be a further advantage for expressing this heterodimer in the heart muscle.

In the forward reaction, a generally much higher K_d than K_m value was seen for all substrates. For a random BiBi substrate binding mechanism, the K_d value can be interpreted as the affinity of the enzyme to the first substrate whereas the constant K_m describes the affinity to the second substrate. A lower K_m compared to K_d value means that the binding of the second substrate is facilitated by the prior binding of the first one to the enzyme. This substrate synergism is known for MM-CK in the forward reaction [36] but also true for the other cytosolic isoforms (Table 1) as well as for Mi-CK. No substrate synergism is seen in the reverse reaction with the wild-type enzymes.

There is considerable controversy as to whether cooperativity exists between the two subunits of the CK dimer. Wang et al. [10] could not detect a kinetic influence between subunits when comparing the activity of an active CK dimer with a dimer containing a chemically inactivated subunit. These authors reported a reduced activity of the hybrid dimer to exactly 50% in the direction of PCr synthesis which would be expected if the subunits act fully independently. However, others reported an activity reduction of greater than 50% after hybridization of an active monomer with an either chemically [12] or genetically inactivated subunit [11]. To clarify this issue, we compared the kinetic parameters of a CK homo- and heterodimer containing an inactivated B-subunit with the wild-type enzymes. It was shown that the introduced exchange of cysteine 283 to serine did not affect the substrate binding of the inactivated enzyme (Tables 1 and 2). Concerning the forward reaction, the activity reduction of the MB* hybrid was almost exactly half (49%) compared to the MB-CK wild-type, as reported earlier. No changes in the substrate affinity parameters were seen between MB* and MB-CK, suggesting that the subunits act fully independently in the forward direction. However, the situation changes if one analyzes the enzyme kinetics in the reverse reaction. We found a significantly higher reduction of V_{max} with the MB*-CK and hBB*-CK hybrid to 34% and 32%, respectively, after inactivation of one subunit. These data strongly suggest that a subunit interaction between the CK monomers must exist when kinetic parameters are analyzed in the direction of ATP synthesis.

Interestingly, the substrate binding parameters of MB-CK and MB*-CK showed a significant increase in the K_d value for PCr and ADP, whereas the corresponding K_m remained more or less unchanged. This would mean that the inactivation of one subunit results in an elevated substrate synergism. In other words, the presence of an enzymatically active subunit improves the binding of the first substrate to the MB-CK dimer. This seems to be a feature of the MB-CK heterodimer since the same could not be seen with the hBB*-CK homodimer. Although the fusion of B-CK with a his-tag resulted in a highly active enzyme, the tag clearly influences the maximal activity of the enzyme. Nevertheless, we observed a subunit cooperativity on the activity level but not on the substrate binding. However, it cannot be excluded that the difference between hBB*- and MB*-CK is due to an effect of the his-tag extension.

The three dimensional X-ray structure of the AK

transition state implies a rather large domain movement of the monomer upon catalysis [31]. The high structural homology between AK and CK permits us to suggest that this is also true for CK [43]. The binding of the Mg-nucleotide substrate to CK results in a change of the enzyme diameter as seen by small angle X-ray scattering [44]. The tight monomer–monomer interface between the two CK subunits might be involved in transforming these structural changes to the opposite monomer, resulting in an improvement of the catalytic properties. This flip-flop cooperativity between the monomers within the stable dimer could be a major advantage of the dimeric state of CK over the monomeric AK and thus might provide a selective rationale for why this group of enzymes can be found as a dimer or an assembly of four dimers as in Mi-CK [45] in more advanced organisms.

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