Octamers of Mitochondrial Creatine Kinase Isoenzymes Differ in Stability and Membrane Binding*

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Octamer stability and membrane binding of mitochondrial creatine kinase (MtCK) are important for proper functioning of the enzyme and were suggested as targets for regulatory mechanisms. A quantitative analysis of these properties, using fluorescence spectroscopy, gel filtration, and surface plasmon resonance, revealed substantial differences between the two types of MtCK isoenzymes, sarcomeric (sMtCK) and ubiquitous (uMtCK). As compared with human and chicken sMtCK, human uMtCK showed a 23–34 times slower octamer dissociation rate, a reduced reoctamerization rate and a superior octamer stability as deduced from the octamer/dimer ratios at thermodynamic equilibrium. Octamer stability of sMtCK increased with temperature up to 30 °C, indicating a substantial contribution of hydrophobic interactions, while it decreased in the case of uMtCK, indicating the presence of additional polar dimer/dimer interactions. These conclusions are consistent with the recently solved x-ray structure of the human uMtCK (Eder, M., Fritz-Wolf, K., Kabsch, W., Wallimann, T., and Schlattner, U. (2000) Proteins 39, 216–225). When binding to 16% cardiolipin membranes, sMtCK showed slightly faster on-rates and higher affinities than uMtCK. However, human uMtCK was able to recruit the highest number of binding sites on the vesicle surface. The observed divergence of ubiquitous and sarcomeric MtCK is discussed with respect to their molecular structures and the possible physiological implications.

Mitochondrial creatine kinase (MtCK) belongs to the creatine kinase (CK) isoenzyme family occurring in vertebrates and some invertebrate species, especially in tissues with high or fluctuating energy requirements (reviewed in Ref. 1). Two tissue-specific isoenzymes are known in vertebrates, ubiquitous MtCK (uMtCK) and sarcomeric MtCK (sMtCK), but only the latter has been characterized in more detail (reviewed in Refs. 2 and 3). In contrast to the exclusively dimeric cytosolic CK isoenzymes, mitochondrial CK occurs in two different oligomeric forms: dimers and octamers. Although MtCK is mostly isolated in its octameric state, a dynamic octamer/dimer equilibrium has been found in vitro, depending on various parameters like MtCK concentration, pH, and temperature (3, 4). MtCK is localized in cristae and the intermembrane space of mitochondria (5), where the octamer binds to the membranes and interacts functionally and possibly also structurally with two transmembrane proteins, adenylate translocator (ANT) of the inner membrane and porin (VDAC) of the outer membrane (reviewed in Refs. 2 and 3). MtCK, ANT, and porin are enriched in the so-called contact sites between outer and inner mitochondrial membrane, which have been isolated and characterized (6, 7).

Two main functions of MtCK were described. First, MtCK is part of a unique temporal and spatial energy buffer system (reviewed in Refs. 9 and 10). ATP from oxidative phosphorylation, provided by ANT, is converted into phosphocreatine (PCr) by MtCK and channeled into the cytosol via porin (reviewed in Ref. 3). Cytosolic CK isoenzymes then use PCr in the reversible CK reaction to replenish ATP pools. Second, the presence of MtCK and creatine (Cr) can inhibit or at least delay the Ca2+-induced opening of the mitochondrial permeability transition pore (PTP) in isolated mitochondria (8). Although the appearance of a PTP in mitochondria has been known for a long time, its structural identity with ANT and possibly porin (VDAC) and its participation in the execution pathway of apoptosis have been postulated only recently (see Ref. 7; reviewed in Refs. 9 and 10). Both functions of MtCK are increasingly recognized to be important in human health and disease. Overexpression of uMtCK in many malignant cancers with especially poor prognosis (11, 12) may be related to high-energy turnover and failure to eliminate cancer cells via apoptosis. The protective effects of CK substrates like Cr and cyclocreatine in many muscular, neurodegenerative and age-related disorders (13–15) may also be linked to MtCK functions in energy buffering, transport, and PTP regulation.

The possible in vivo significance of the 10-nm large, cuboidal octameric structure of MtCK (4, 16) for the above mentioned physiological functions has been discussed extensively (see Refs. 4 and 17). Only recent studies have indicated that the membrane-bound, octameric state of MtCK is essential for the protein to function in the CK/PCr energy circuit and in regulating PTP. An exchange of charged residues at the N-terminal end of MtCK, leading to destabilized octamers and reduced membrane binding of the enzyme (18, 19), results in mutant proteins that are unable to sustain normal creatine-stimulated respiration when transfected into MtCK-free cells (19). Similarly, the protective function of MtCK and Cr in Ca2+-induced opening of mitochondrial PTP is abolished (8) if mitochondria are pretreated with the CK transition state analogue complex (TSAC, consisting of ADP, Mg2+, nitrate, and Cr) (20) known to dissociate MtCK octamers.
These findings, demonstrating distinct properties of MtCK octamers and dimers, suggest a role of octamer/dimer ratios in the regulation of MtCK in *vivo* (1, 2, 21–23). In contrast to *in vitro* experiments, which indicate a predominantly octameric state of MtCK, a decreased octamer/dimer ratio was observed in *vivo* under pathological conditions (24).

Despite the relevance of octameric state and membrane binding for correct functioning of MtCK, quantitative data are limited to chicken sarcomeric MtCK, restricted to striated muscle. Virtually no studies are available for ubiquitous MtCK, which is found in the brain, many other organs, and various malignant cancer tissues. Human MtCK isoenzymes have not been examined at all, despite their importance for health and disease. In this study, we analyze the transitions of octamERIC to dimeric MtCK and of free to membrane-bound MtCK in terms of kinetics and thermodynamic equilibrium. We compare both uMtCK and sMtCK from human origin, together with the already known chicken sMtCK, using fluorescence spectroscopy, gel filtration, and surface plasmon resonance (22, 25). It is shown for the first time that ubiquitous and sarcomeric MtCK octamers differ significantly in octamer stability and membrane binding properties.

**Experimental Procedures**

**Proteins**—Human uMtCK, human sMtCK, and chicken sMtCK were expressed in *Escherichia coli* and purified to homogeneity by column chromatography as described earlier (26, 27). CK activity was determined with a coupled enzymatic assay in a spectrophotometer (modified according to Refs. 28 and 29). Protein was quantified by determination of peptide bonds with the ESI protein assay (Roche Molecular Biochemicals) using bovine serum albumin as a standard (30).

**Fluorescence Spectroscopy and Gel Filtration Chromatography**—Fluorescence measurements were carried out with a SPEX Fluorolog-2 instrument, equipped with a 450-watt xenon arc lamp as excitation source and thermocooled, magnetically stirred quartz cells (22). Fluorescence emission at 340 nm was recorded in short intervals (10 s) with rectangular excitation at 295 nm, using slit widths of 1.7 nm (emission) and 0.7 nm (excitation) to avoid significant photobleaching during the measurements. The distribution of MtCK oligomeric species was determined by gel filtration chromatography of 10–50-μl samples with a Superose 12 column (Amersham Pharmacia Biotech) connected to a high pressure liquid chromatograph (BioCad, Perkin-Elmer). Separations were done in gel filtration buffer (50 mM sodium phosphate, 150 mM NaCl, 0.2 mM EDTA, 2 mM β-mercaptoethanol, pH 7.0) at a flow rate of 1 ml min⁻¹ and 22°C. Peak areas were calculated with the BioCad evaluation program (Perkin-Elmer).

**Octamer Dissociation and Reassociation Assay**—MtCK stock solutions in gel filtration buffer (6 mg/ml), containing >95% octamers, were diluted to various concentrations in gel filtration buffer and preincubated at the chosen temperature. Dissociation was initiated by the addition of TSAC substrates from a 4× concentrated, temperature-adjusted stock solution to a final concentration of 4 mM ADP, 5 mM MgCl₂, 20 mM Cr, and 50 mM KNO₃ (20, 22). The sample was stirred, and octamer dissociation kinetics were followed by fluorescence quenching or by gel filtration chromatography. Since dissociation of MtCK octamers leads to the solvent exposure of two tryptophans per dimer (Trp³⁴, sMtCK numbering), a distinct fluorescence quenching at 340 nm is observed (23). However, due to photobleaching, measurements are limited to about 30 min. For gel filtration, samples were taken at defined time points and immediately applied to a high pressure liquid chromatograph. The time elapsed between sampling and separation of TSAC components from MtCK on the column (which stops octamer dissociation) was determined to be 60 s. Dissociation of MtCK octamers follows an all-or-nothing rule, and the data could be fit with a single exponential rate law (22).

For octamer reassociation experiments, TSAC-dissociated MtCK dilutions were adjusted to the chosen temperature and incubated with excess EDTA (final concentration 25 mM), which complexes Mg²⁺ essential for constituting the TSAC (22). Kinetics of octamer reassociation were followed by gel filtration chromatography. The association of dimers follows a Bi Bi association mechanism of the type with dimers (D) forming highly unstable tetrameric intermediates (T) before associating into stable octamers (O). The corresponding set of differential rate equations was solved analytically (31) and can be simplified to describe just the disappearance of MtCK dimers (22).

**Results**

**Dissociation and Reassociation Kinetics of MtCK Octamers**—At low protein concentrations (e.g., 0.12 μM octameric MtCK, ~40 μg ml⁻¹ protein), octamers of all examined MtCK isoenzymes dissociated into dimers when the TSAC (20) was added (Fig. 1, A and B). Decay of human uMtCK octamers was, however, much slower than for human or chicken sMtCK (Fig. 1, A and B, Fig. 2). The kinetics of MtCK oligomeric transitions were determined by gel filtration chromatography and endogenous tryptophan fluorescence at 340 nm. Trp³⁴ is quenched upon octamer dissociation (22) and therefore very suitable for continuous on-line recording of octamer decay and further analysis of the dimer/dimer interface. The time course of TSAC-induced octamer decay in the concentration range of 0.06–0.6 μM octameric MtCK (~20–200 μg ml⁻¹ protein) was essentially identical with both methods (Fig. 2) and concentration-independent (not shown), indicating a first-order process. This allowed us to fit kinetics with a single exponential rate equation to obtain dissociation rates and half-life times (Table 1). Compared with chicken and human sMtCK, dissociation of human uMtCK was 23 and 34 times slower, respectively, and therefore not amenable to fluorescence spectroscopy due to photobleaching (Fig. 2, C and D). Fluorescence emission difference spectra of MtCK, recorded immediately after TSAC addition and 30 min thereafter, revealed maxima at 319–328 nm (not shown). The percentage of fluorescence quenching corresponding to complete octamer-dimer transition was isoenzyme-specific for the chicken sMtCK, 20%; human sMtCK, 8%; human MtCK, 5%.

Dimerized MtCK was treated with excess EDTA to complex Mg²⁺ and to disintegrate TSAC (Fig. 1, C and D). This led to rapid reoctamerization of dimers of both sMtCK isoenzymes. By contrast, human uMtCK reassociated only very weakly. This was probably due to the limited dimerization of uMtCK by TSAC, which did not allow studies of uMtCK with less than 40% octamers under the given conditions. With both human MtCK isoforms, dimerization was undetectable under the conditions used (Table 1). These findings, demonstrating distinct properties of MtCK octamers and dimers, suggest a role of octamer/dimer ratios in the regulation of MtCK in *vivo* (1, 2, 21–23). In contrast to *in vitro* experiments, which indicate a predominantly octameric state of MtCK, a decreased octamer/dimer ratio was observed in *vivo* under pathological conditions (24).
Mitochondrial Creatine Kinase isoenzymes, no tetrameric intermediates were detected during octamer association, as was reported for chicken sMtCK (22). Possibly, they escape detection by gel filtration chromatography because of a more rapid association to form octamers.

Kinetic analysis with gel filtration chromatography at protein concentrations over 1.2 mg/ml protein yielded concentration-dependent association kinetics. In Fig. 3, octamer reassociation is expressed as a decline in dimer concentration with time, depending only on dimer concentration (22). The calculated association rate constants of chicken MtCK isoenzymes, no tetrameric intermediates were detected during octamer association, as was reported for chicken sMtCK (22). Possibly, they escape detection by gel filtration chromatography because of a more rapid association to form octamers.

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Octamers of Mitochondrial Creatine Kinase

Fig. 1. Gel filtration chromatography of MtCK oligomers. Human uMtCK (A and C) and human sMtCK (B and D) were dissociated with TSAC substrates at 30 °C (A and B) and reassociated into octamers by the addition of EDTA (C and D), both at 30 °C. Protein concentrations were 0.12 mg/ml MtCK (~40 μg/ml) (A and B) and 1.9 mg/ml dimeric (dim) MtCK (~160 μg/ml) (C and D). Samples were removed at different time points and subjected to gel filtration chromatography as follows. A, 0 min (solid line), 2 h (thin dashed line), 4 h (dashed and dotted line), 8 h (dotted line). B, 0 min (solid line), 2 min (thick dashed line), 5 min (thin dashed line), 10 min (dashed and dotted line), 30 min (dotted line), C and D, 0 min (solid line), 30 min (dashed line), 150 min (dotted line). Note that peaks at 11.5 and 14 ml elution volume compare with octameric and dimeric MtCK, respectively.

Concentration and Temperature Dependence of the Octamer/Dimer Equilibrium—The octamer/dimer ratio at thermodynamic equilibrium is an important parameter for octamer stability and can yield valuable information on the strength of the dimer/dimer interface. After TSAC addition to octameric samples or EDTA addition to TSAC-dimerized samples, equilibrium was achieved by incubation at two different temperatures (4 and 30 °C; Fig. 4, A and B). The octamer/dimer equilibrium of all isoenzymes increased with protein concentration under all conditions examined, reaching >80% octamers in TSAC + EDTA-treated samples at >1 mg/ml protein and 30 °C. The main differences in octamer stability found between sarcomeric and ubiquitous MtCK were as follows: (i) uMtCK octamers were more stable than sMtCK octamers at 0.1–1 mg/ml protein; (ii) uMtCK octamers were more stable at 4 °C than at 30 °C, while the reverse was found for both sMtCK isoenzymes; and (iii) the addition of EDTA to TSAC-dimerized MtCK samples largely increased the octamer content of sMtCK but not so much for uMtCK.

The temperature dependence of octamer/dimer ratios at thermodynamic equilibrium was further examined between 4 and 30 °C (Fig. 4C). A rise in temperature led to a stabilization of sMtCK octamers, confirming earlier findings with chicken sMtCK (23). By contrast, human uMtCK showed a negative temperature dependence, with slightly less stable octamers at 30 than at 4 °C. The van’t Hoff plot of the octamer/dimer equilibrium constant K against 1/T revealed an inverse linear relationship of uMtCK and sMtCK in the temperature range of 4–22 °C (Fig. 4C, inset). In terms of thermodynamics, association enthalpy (ΔH°) as calculated from the van’t Hoff plot was constant and negative for uMtCK (~30.1 kJ mol⁻¹), favoring association, but strongly positive for human and chicken sMtCK (~72.9 and 81.7 kJ mol⁻¹, respectively), opposing association.
**TABLE I**

Rate constants for dissociation and reassociation of MtCK octamers

|        | $k_d$  | $t_{1/2}$ | $k_a$  | $t_{1/2}$a |
|--------|--------|-----------|--------|------------|
| Human uMtCK | 0.11 ± 0.04 | 105.0 | 158 ± 72 | -55 |
| Human sMtCK | 3.76 ± 1.05 | 3.1 | 823 ± 208 | -13 |
| Chicken sMtCK | 2.50 ± 0.75 | 4.6 | 501 ± 109 | -20 |

**Fig. 3. Kinetics of MtCK octamer reassociation.** 1.9 µM (−160 µg ml⁻¹) (A) or 3.8 µM (−320 µg ml⁻¹) (B) TSAC-dimerized human uMtCK (C), human sMtCK (D), and chicken sMtCK (E) were reassociated into octamers at 30 °C by the addition of EDTA to disintegrate the TSAC complex. Association was followed by gel filtration chromatography of samples taken at definite time points. The association rate equation was fit to the gel filtration data (line) (see Table I).

However, free energy $\Delta G^0$ of octamer formation at 22 °C, calculated from the equilibrium constant $K$, was favorable for octamerization of all three MtCK isoenzymes (human uMtCK, −96.9 kJ mol⁻¹; human sMtCK, −92.4 kJ mol⁻¹), and chicken sMtCK was reassociated into octamers at 30 °C by the addition of EDTA to disintegrate the TSAC complex. Association was followed by gel filtration chromatography of samples taken at definite time points. The association rate equation was fit to the gel filtration data (line) (see Table I).

**DISCUSSION**

Recent evidence strongly suggests that membrane binding and octameric structure of MtCK are essential for the specific functions of the enzyme in the CK/PCr circuit for energy homeostasis (19) and in regulation of the mitochondrial permeability transition pore (8). Kinetic and thermodynamic data on MtCK octamer formation and membrane binding are therefore highly relevant for the *in vivo* functions and regulatory targets. In the present study, we have quantitatively analyzed these properties in the two types of MtCK isoenzymes, ubiquitous and sarcomeric, and found significant kinetic and thermodynamic differences between them that relate to distinct structural features.

The kinetic stability of MtCK octamers is difficult to assess, since they are very stable at high protein concentrations. Changes in temperature, pH, protein, or substrate concentration can dissociate MtCK octamers into dimers, but with half-life times of days to weeks (2, 4). A more potent artificial dimerization trigger is the TSAC, which induces a fast dissociation of octamer within minutes (4, 20, 22, 33) by fixing the enzyme in a conformation that destabilizes the dimer/dimer interface. Using TSAC, we could show that rate and equilibrium constants of chicken and human sMtCK are very similar, while uMtCK has much slower rate constants (dissociation is 23–34 times slower) and an increased octamer/dimer ratio at equilibrium, especially at lower protein concentration. The latter was not only true for the equilibrium in the presence of TSAC (“destabilized” state, MtCK in a “closed” conformation) (34), but also for the equilibrium after disintegration of TSAC (“physiological” state, MtCK in an “open” conformation) (34). $\Delta G^0$ values calculated for octamer formation corroborate the higher stability of human uMtCK.

Two possible mechanisms may explain the clear cut divergence between sMtCK and uMtCK. Either TSAC interacts in a different way with the active site of uMtCK, inducing less pronounced conformational changes that cause the loosening of the dimer/dimer interface or, alternatively, uMtCK has a tighter dimer/dimer interface, which per se would be more resistant to perturbations. The first possibility is highly unlikely, since TSAC acts in a similar way as a competitive inhibitor for uMtCK and sMtCK, as well as for other CK isoenzymes. In addition, the $K_a$ of the enzymatic reaction for Cr and ADP is even lower for human uMtCK than for human sMtCK.² The concentrations of TSAC compounds used in our experiments were therefore not in a subsaturating range for MtCK dimerization, as they were in a previous study with chicken sMtCK (23). Thus, the divergent octamer stability of MtCK isoenzymes is more likely to reside at the dimer/dimer interaction site (35), which is, however, well conserved among MtCKs (36). The conserved Trp²⁶⁴, for example, was identified in chicken sMtCK as a key residue for octamer stability by pro-

² U. Schlattner and T. Wallimann, unpublished data.
Octamers of Mitochondrial Creatine Kinase

FIG. 4. Thermodynamics of MtCK octamer/dimer equilibrium. Concentration dependence (A and B) and temperature dependence (C) of octamer/dimer ratios at thermodynamic equilibrium with human uMtCK (C), human sMtCK (g), and chicken sMtCK (■) are shown. The relative octamer content is given for various protein concentrations at 4°C (A), 30°C (B), or for 800 μg ml⁻¹ MtCK at various temperatures (C). C, inset, van’t Hoff plot of the data in C with linear regressions in the range from 4 to 22°C. Samples were incubated for 1 day to reach equilibrium, either in the presence of complete TSAC (solid line) or after EDTA addition to TSAC-dimerized samples to complex Mg²⁺ and disintegrate TSAC (dashed line). MtCK oligomers were quantified by gel filtration. Note that reoctamerization of human uMtCK in the presence of EDTA is insignificant at 4°C and not included in Fig. 4A.

FIG. 5. Kinetics of MtCK membrane binding and dissociation. Representative SPR traces of contact phase (A) and dissociation phase (B) of 0.025 μm octameric human uMtCK (C), human sMtCK (g), and chicken sMtCK (■) are shown. Corresponding fits to the rate equations (see “Experimental Procedures”) are shown as thick lines, and residuals of every fit are shown in the panels below. SPR data were recorded at 25°C and a flow rate of 0.3 ml h⁻¹ with 10 mM TES and 50 mM NaCl as running buffer. Response units (RU) are proportional to the amount of MtCK bound at the vesicle surface.

Odd results with chicken sMtCK (23), while it slightly destabilized octameric uMtCK. Indeed, octamerization of sMtCK was exclusively driven by a very large negative entropic term −TΔS°, which overcame the positive enthalpy ΔF° that would oppose octamer formation. This can be explained by a large contribution of hydrophobic interactions, which are known to be predominantly entropy-driven. In the case of human uMtCK, the reverse temperature dependence is due to a negative enthalpy that adds to the favorable entropy for octamer formation. This is consistent with a stronger participation of polar interactions at the dimer/dimer interface of uMtCK.

A good candidate for such polar interactions is Arg151, which is conserved (or conservatively exchanged against lysine) in all known uMtCK proteins instead of a conserved asparagine residue in sMtCK (36). The recently solved x-ray structure of human uMtCK (27) confirms a number of additional ion pair bonds at the dimer/dimer interface that are not present in chicken sMtCK. Two of them, Arg151/Glu148 and Asp155/Arg151, would be buried inside the hydrophobic contact region and could contribute significantly to the stabilization of uMtCK octamers. Their presence could also explain the reduced susceptibility of the neighboring Trp²⁶⁴ for quenching upon solvent exposure. Additional polar interactions occur between the N termini of adjacent dimers, which assume an entirely different, clamplike conformation in case of uMtCK (27). Thus, our biochemical and physical data concerning differences in octamer stability between sMtCK and uMtCK are in line with x-ray structural data of the dimer/dimer interfaces of these MtCK isoenzymes (27).

A second property of MtCK octamers analyzed in this study is the ability to bind to membranes containing negatively charged phospholipids, especially cardiolipin. Membrane binding of MtCK is directly related to the octamer/dimer ratio, since dimers bind only very weakly (25, 33, 39). An in vitro assay, using SPR spectroscopy with avidin-biotin-immobilized vesicles (25, 32), allowed us to determine the rate and equilibrium constants for binding of octameric MtCK to 16% cardiolipin membranes. Fitting of the rate equations to the kinetic data suggested the presence of two independent binding sites. However, the data do not indicate whether the two binding sites are located at the surface of the liposomes or at the MtCK molecule itself. The two sites differ by their association and dissociation rates and were characterized as “fast” and “slow” binding sites (25). The quantitative data also revealed isoenzyme-specific differences. sMtCK did bind faster and with higher affinity than uMtCK, especially at the “slow” binding site, which may comprise partial penetration of MtCK into the membrane bilayer (25, 40). Such differences in affinity between ubiquitous...
Octamers of Mitochondrial Creatine Kinase

TABLE II

| MtCK isoenzyme     | $k_{a1}$ | $k_{a2}$ | $K_{d1}$ | $R_{eq}$  | $k_{d1}$ | $k_{d2}$ | $K_{d2}$ | $R_{eq}$ |
|--------------------|---------|---------|---------|-----------|---------|---------|---------|---------|
| Human uMtCK       | 6.9 ± 2.1 | 5.0 ± 0.1 | 86 ± 34 | 1020      | 4.5 ± 2.4 | 3.2 ± 0.9 | 72 ± 28 | 630      |
| Human sMtCK       | 13.2 ± 4.8 | 5.4 ± 0.3 | 44 ± 19 | 330       | 15.5 ± 8.3* | 3.1 ± 0.8 | 19 ± 6* | 150      |
| Chicken sMtCK     | 14.3 ± 4.0* | 7.0 ± 0.2 | 43 ± 15 | 800       | 20.8 ± 9.4* | 2.5 ± 0.6 | 16 ± 8* | 260      |

Association and dissociation of MtCK octamers were analyzed in a concentration range between 0.025 and 0.25 µM. Rate constants ($k_a$, $k_d$) were derived from a direct fit of dissociation and association rate equations to SPR data (25); values are averages ± S.D. of at least 10 measurements in the given protein concentration range. $K_d$ was calculated from Scatchard plots derived from the relationship between octamer concentration and the SPR signal at equilibrium (Fig. 6, C and D) (25); values are averages ± S.D. from the linear fit. The total number of binding sites at concentrations approaching $R_{eq}$ was also estimated from Scatchard plots. * values of $K_d$, $k_a$, and $k_d$ for sMtCK that are significantly different from those for uMtCK (Student’s test, $p < 0.01$): RU, response units.

and sarcomeric MtCK could be due to the distribution of positive charges at the main, C-terminal binding domain (3). The C terminus of sMtCK contains two terminal lysines (one lysine can be conservatively replaced by arginine). In uMtCK, just one lysine is conserved, while two additional histidines may only contribute to a minor degree to overall positive charge at pH 7. Finally, human uMtCK and also chicken sMtCK showed a higher maximal SPR response than human sMtCK, indicating a better recruitment of binding sites at the vesicle surface. This more complex behavior of MtCK isoenzymes has most probably multiple reasons. The difference between the two human MtCK isoenzymes may be determined by the flexibility of the C-terminal membrane binding domain, which is much higher in the case of human uMtCK as compared with human sMtCK (27). By contrast, the superior recruitment of binding sites by chicken sMtCK could relate to its more basic pH as compared with human MtCK, leading to a higher positive net charge at pH 7.5.

Binding of MtCK to cardiolipin-containing vesicles has no significant influence on the steady state kinetic properties of the enzymatic reaction (41). However, there is increasing evidence that octameric state and membrane binding are essential for MtCK to function in concert with the membrane proteins ANT and mitochondrial porin (VDAC) in the CK/PcR energy circuit (19) or as a regulator of the mitochondrial permeability transition (8). Our in vitro assays have clearly demonstrated that sMtCK and uMtCK octamers differ in octamer stability and membrane binding. In vivo, such kinetic and thermodynamic properties were suggested as regulatory parameters (reviewed in Refs. 1 and 2). Although octamer/dimer transitions seen with TSAC as an artificial dimerization trigger were too slow for fast metabolic adaptations, they could play a role in long term adaptations or modulation of energy metabolism. In addition, octamer/dimer transitions may be crucial in pathological situations, where potent dimerization triggers like radicals and peroxynitrite are involved (24). Both are known to modify CK isoenzymes in vitro (39, 42), and at least peroxynitrite is also able to induce octamer dissociation (24). A marked decrease of the sMtCK octamer/dimer ratio was recently observed in the infarcted heart of two different animal models for ischemia and reperfusion damage (24), and this could contribute to energy failure in this organ.

The increased octamer stability and the more efficient recruitment of membrane binding sites as found for uMtCK may confer an improved resistance against apoptosis or energy failure to cells and organs expressing the corresponding isoenzyme. Preliminary results indicate that human uMtCK is in fact more resistant to peroxynitrite-induced octamer dissociation than sMtCK. The specific properties of uMtCK may also explain, for instance, why tumors overexpressing this isoenzyme are renowned for their especially poor prognosis (11, 12). Further experiments will be necessary to confirm this hypothesis.

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Octamers of Mitochondrial Creatine Kinase

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