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# Divergent Enzyme Kinetics and Structural Properties of the Two Human Mitochondrial Creatine Kinase Isoenzymes

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**The mitochondrial isoenzymes of creatine kinase (MtCK), ubiquitous uMtCK and sarcomeric sMtCK, are key enzymes of oxidative cellular energy metabolism and play an important role in human health and disease. Very little is known about uMtCK in general, or about sMtCK of human origin. Here we have heterologously expressed and purified both human MtCK isoenzymes to perform a biochemical, kinetic and structural characterization. Both isoenzymes occurred as octamers, which can dissociate into dimers. Distinct Stokes' radii of uMtCK and sMtCK in solution were indicative for conformational differences between these equally sized proteins. Both human MtCKs formed 2D-crystals on cardiolipin layers, which revealed further subtle differences in octamer structure and stability. Octameric human sMtCK displayed *p*4 symmetry with lattice parameters of 145 Å, indicating a 'flattening' of the octamer on the phospholipid layer. pH optima and enzyme kinetic constants of the two human isoenzymes were significantly different. A pronounced substrate binding synergism ($K_d > K_m$) was observed for all substrates, but was most pronounced in the forward reaction (PCr production) of uMtCK and led to a significantly lower $K_m$ for creatine (1.01 mM) and ATP (0.11 mM) as compared to sMtCK (creatine, 7.31 mM; ATP, 0.68 mM).**

*Key words:* 2D protein crystals / Energy metabolism / Enzyme kinetics / Guanidino kinase / Protein purification.

## Introduction

Mitochondrial creatine kinase (MtCK) is part of the creatine kinase (CK) isoenzyme family occurring in vertebrates and some invertebrate species, especially in tissues with high or fluctuating energy requirements (reviewed in Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Schlattner *et al.*, 1998). These isoenzymes catalyze the reversible phosphoryl transfer between ATP and creatine (Cr) to yield ADP and phosphocreatine (PCr). The CK family comprises exclusively dimeric cytosolic isoenzymes and dimeric, as well as octameric, mitochondrial isoenzymes. CK is often specifically localized at subcellular sites of ATP generation, where it builds up a PCr pool, or at sites of ATP consumption, where it uses PCr to restore proper local ATP/ADP. Thus, the CK/PCr system plays a central role in cellular energy buffering and energy transport.

The human genome, like other vertebrate genomes, encodes two tissue-specific MtCKs (Haas *et al.*, 1989; Haas and Strauss, 1990): ubiquitous MtCK (uMtCK, $M_r$ = 43 150), occurring in different organs like brain or kidney, and sarcomeric MtCK (sMtCK, $M_r$ = 43 360), which is restricted to striated muscle (Payne and Strauss, 1994). Both isoenzymes are synthesized in the cytosol as preproteins with a cleavable N-terminal targeting sequence for import into the mitochondrial intermembrane space. There, dimers and octamers are assembled, and the latter become the predominating oligomeric form *in vivo* (Wyss *et al.*, 1992). Both MtCKs are basic proteins, sharing 80% amino acid sequence identity.

Information on uMtCK is scarce, while sMtCK from several organisms has been characterized in some detail (reviewed in Schlattner *et al.*, 1998; Stachowiak *et al.*, 1998). sMtCK octamers show high-affinity binding to mitochondrial membranes (Rojo *et al.*, 1991) and are enriched in the so-called mitochondrial contact sites. There they functionally interact with two transmembrane proteins, adenylate translocator (ANT) in the inner, and porin in the outer, mitochondrial membrane (Beutner *et al.*, 1998). In addition, octameric MtCK, together with Cr, can delay the opening of the mitochondrial permeability transition pore (PTP, O'Gorman *et al.*, 1997a), which is considered to be a decisive step in the mitochondria-linked pathway to apoptosis (reviewed in Crompton, 1999). A destabilization of MtCK octamers leads to impairment of Cr-stimulated respiration in mitochondria (Khuchua *et al.*, 1998) and of the protective function for PTP opening (O'Gorman *et al.*, 1997a). Recently, the X-ray structures of chicken sMtCK (Fritz-Wolf *et al.*, 1996) and human uMtCK (Eder *et al.*, 2000) have been solved.

The functions of MtCK are also important for human health and disease. Overexpression of uMtCK is found in tumors with especially poor prognosis (*e.g.* Kanemitsu *et al.*, 1984; Pratt *et al.*, 1987; Kornacker *et al.*, 1997). The strong and specific growth inhibitory effect of CK analogs (Wyss and Kaddurah-Daouk, 2000) supports a role of uMtCK in cancer bioenergetics or the apoptotic elimination of cancer cells. The sMtCK isoenzyme is overexpressed in certain mitochondrial myopathies, possibly as

a compensatory mechanism for energy deficits, finally leading to the formation of crystalline inclusion bodies in the mitochondrial cristae (O'Gorman *et al.*, 1997b). On the other hand, radicals like NO or $O_2^-$, and their product peroxynitrite, inhibit CK activity and destabilize the MtCK octamer (Stachowiak *et al.*, 1997; Soboll *et al.*, 1999), which is important for MtCK functioning (Khuchua *et al.*, 1998; O'Gorman *et al.*, 1997a). This has been confirmed in animal models for ischemia and reperfusion damage *in vivo* (Soboll *et al.*, 1999). Disturbed mitochondrial energy metabolism, including free radical generation and mitochondrial permeability transition, also play an important role in many neuro-muscular, neuro-degenerative, and age-related disorders (Beal, 1998). This may explain the neuroprotective effect of Cr and certain Cr-analogs in animal models of Huntington or amyotrophic lateral sclerosis (ALS) (reviewed in Wyss and Kaddurah-Daouk, 2000).

In contrast to the known physiological and clinical relevance of MtCK, the ubiquitous isoenzyme, and also both MtCK isoenzymes of human origin, are very poorly characterized. Attempts to purify human sMtCK from tissue by using a multitude of purification steps gave very low yields or were prone to artifacts due to the long post-mortem time before extraction of the enzyme (Wevers *et al.*, 1982; Blum *et al.*, 1983; Grace *et al.*, 1983; Khuchua *et al.*, 1989; Walterscheid-Müller *et al.*, 1997). As a consequence, the reported properties of human MtCK show great discrepancies as far as specific activity (45 to 410 U/mg), isoelectric point (6.8 to 9.3) or oligomeric state (dimer or octamer) are concerned. The aim of our study was thus to obtain homogenous human MtCK by heterologous expression in *E. coli* and to characterize the two isoenzymes for further structural and functional studies. Both human MtCK isoenzymes have been cloned (Haas *et al.*, 1989; Haas and Strauss, 1990) and were already expressed in specific rat tissues (Miller *et al.*, 1997; Khuchua *et al.*, 1998). Here, we report the first heterologous expression of both human MtCK isoenzymes in *E. coli*, their purification to homogeneity, as well as their biochemical and kinetic characterization, including the generation of 2D crystals. This study, representing the first direct comparative study of the two isoenzymes of MtCK from the same species, revealed significant differences between ubiquitous and sarcomeric MtCK isoenzymes that are physiologically relevant.

## Results

### Expression and Purification of Human MtCK

The coding sequences of the mature human MtCK isoenzymes were separately transformed into *E. coli* cells under the control of the T7 promotor. Expression of both isoenzymes was induced with IPTG and maximal specific activity was obtained after about 4 hours of induction with a higher yield for human uMtCK as compared to sMtCK (data not shown). The enzymes were purified from the soluble fraction of bacterial lysates, where they already represented the predominate proteins (Figure 1, lanes a, e). A two-step column chromatography, including affinity chromatography with Blue Sepharose and cation exchange chromatography with HS resin, yielded MtCK preparations of high purity. uMtCK and sMtCK were both eluted at about 100 mM NaCl. Minor contaminants were eliminated by preparative gel filtration chromatography with Sephacryl S-200, resulting in homogenous MtCK with monomers of about 43 kDa (Figure 1, lane d, h; Figure 2a). Typical yield was 20–30 mg/l bacterial culture.

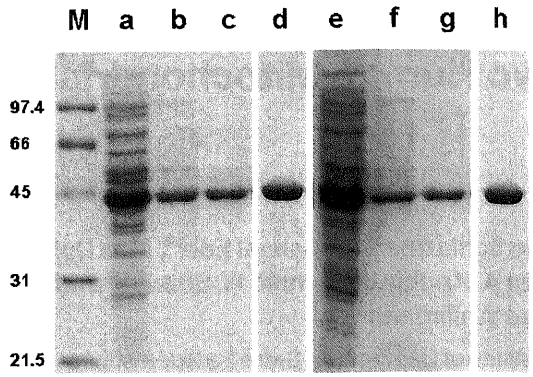


**Fig. 1** Monitoring of Purification Steps by SDS-PAGE. The progress of purification of (a–d) human sMtCK and (e–h) human uMtCK as analyzed by SDS-PAGE of the following fractions is shown: crude lysate, 12 μg protein (a, e), Blue Sepharose pool, 6 μg (b, f), HS ion exchanger pool, 3 μg (c, g), Superose 12 gel filtration pool, 10 μg (d, h). Positions of migration of molecular mass markers (M) are indicated at the left side of the Figure.

### Biochemical Characterization of Human MtCK

Freshly-purified human MtCK, as measured with calibrated analytical gel filtration chromatography, had Stokes' radii of 54.5 ± 0.2 Å (uMtCK) and 56.3 ± 0.2 Å (sMtCK) (Figure 2a), corresponding to the calculated $M_r$ of octamers, *i. e.* 345 200 (uMtCK) and 346 900 (sMtCK). Thus, similar to other known MtCKs, native human MtCK forms predominantly octamers. When octamers were diluted to a concentration of 1 mg/ml and incubated with transition state analog complex (TSAC), which causes octamer destabilization (Gross and Wallimann, 1993), a considerable part of the octamers dissociated into a lower $M_r$ form with a Stokes' radius of 39.2 ± 0.3 Å (uMtCK) and 41.2 ± 0.4 Å (sMtCK; Figure 2b). This corresponds to the calculated $M_r$ of dimers, *i. e.* 86 300 (uMtCK) and 86 700 (sMtCK). A difference in Stokes' radii of 1.8–2.0 Å was observed between uMtCK and sMtCK, both in octameric and dimeric molecules. This is much more than predicted by the small $M_r$ differences between the two isoenzymes. The TSAC experiments also showed that uMtCK octamers dissociate into dimers to a much smaller degree than sMtCK octamers (Figure 2b).

Isoelectric points (pI) of both oligomeric forms were determined with native agarose IEF, using TSAC to dissociate octamers into a mixture of octamers and dimers (Fig-

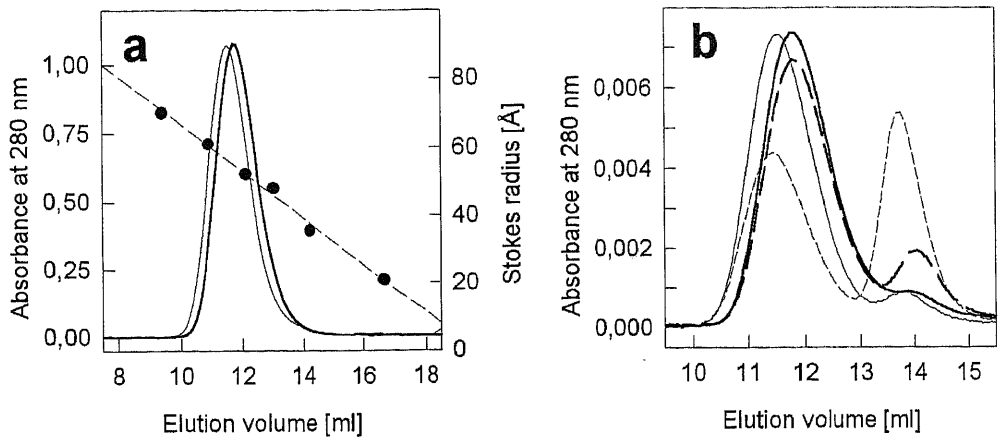


**Fig. 2** Oligomeric State of Purified MtCK.
Gel filtration chromatography with a Superose 12 column of (a) 250 µl aliquots of freshly-isolated MtCK (ion exchanger pool, 6 mg $ml^{-1}$ protein) and (b) 10 µl aliquots of diluted, purified MtCK (1 mg $ml^{-1}$ protein) with (dashed line) or without (solid line) pre-treatment with TSAC for 24 h at 4 °C. (—) sMtCK, (– – –) sMtCK + TSAC, (—) uMtCK, (— — —) uMtCK + TSAC. The column was calibrated for Stokes' radii by different marker proteins [see points and dashed line in (a); for details see Materials and Methods]. Note that in comparison to sMtCK, octamers and dimers of uMtCK have a somewhat smaller Stokes' radius and uMtCK octamers are more stable.

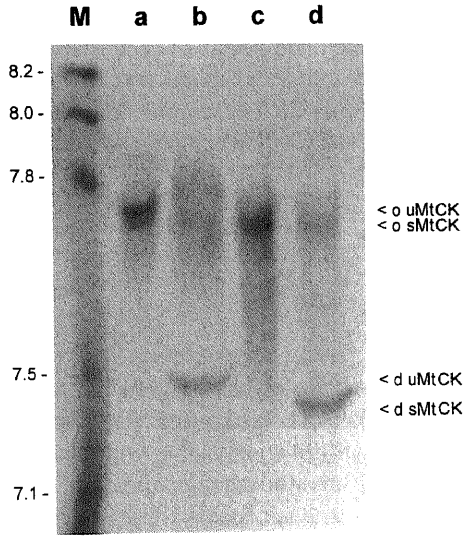


**Fig. 3** Isoelectric Point Determination of Human MtCK with Agarose-IEF.
Isoelectric focusing of 8 µg human uMtCK (a, b), and sMtCK (c, d) in the pH range 7 to 9. Purified, octameric MtCK (2 mg $ml^{-1}$) was treated overnight with (b, d), or without (a, c) TSAC at 4 °C. pI markers (M) are listed in Materials and Methods. o: octamers; d: dimers.

ure 3). The actual pH in the gel was determined with marker proteins and a pH electrode. Octamers of both human MtCK isoenzymes revealed a pI of about 7.7, while dimers showed a more acidic pI with 7.5 for uMtCK and 7.4 for sMtCK. The pI calculated from the cDNA sequence is 7.49 for uMtCK and 7.42 for sMtCK. Chicken sMtCK had a pI of 9.2 in the same IEF system.

### Electron Microscopy of Human MtCK

Single MtCK molecules, as well as two-dimensional (2D) crystals of both human MtCK isoenzymes, were visualized by transmission electron microscopy. This allowed comparing human MtCK to the well-characterized chicken sMtCK isoenzyme in several aspects, such as the general molecular shape and the capacity to form 2D crystals on a negatively-charged lipid surface. In a negative stain, single molecules appear as cube-shaped particles with a side-length of about 100 Å and a central cavity (inserts in Figure 4), corresponding to the cuboidal MtCK octamers known from the solved X-ray structures (Fritz-Wolf *et al.*, 1996; Eder *et al.*, 2000). 2D crystals of both human MtCK isoenzymes were prepared by the lipid-layer technique, using cardiolipin as an interface adhesion molecule (Schnyder *et al.*, 1994) (Figure 4). It is known from chicken sMtCK that crystallization of MtCK in a monolayer is favored by mainly electrostatic interactions between the negatively-charged phospholipid surface and positive charges located at the top and bottom faces of the MtCK octamers (Schnyder *et al.*, 1994; Schlattner *et al.*, 1998). Because correlation-averaged images from negatively-stained 2D crystals always showed identical top or bottom views of the MtCK octamers (Figure 4) with their characteristic four-fold symmetry (Fritz-Wolf *et al.*, 1996; Schnyder *et al.*, 1988), binding of human MtCK occurs in a similar way. However, the two human MtCK isoenzymes formed different 2D crystals under identical conditions. The parallelogram-shaped unit cell of uMtCK (Figure 4a) contained two octamers and exhibited *p*2 symmetry (lattice parameters a = 203 Å, b = 110 Å, $\gamma = 98°$), very similar to the packing in 3D crystals (Eder *et al.*, 2000). In the case of human sMtCK, 2D crystals displayed *p*4 symmetry with lattice parameters (a = b) of 145 Å and $\gamma = 90°$ (Figure 4b). The single octamer per unit cell clearly shows the central channel running along the four-fold symmetry axis and the four dimers arranged around this central axis. On top of each of these dimers, differences in the staining intensity indicate a cavity (darker) and an exposed stretch (brighter), which may correspond to the flexible C-terminal

stretch seen in the X-ray structures of MtCK (Fritz-Wolf *et al.*, 1996; Eder *et al.*, 2000).

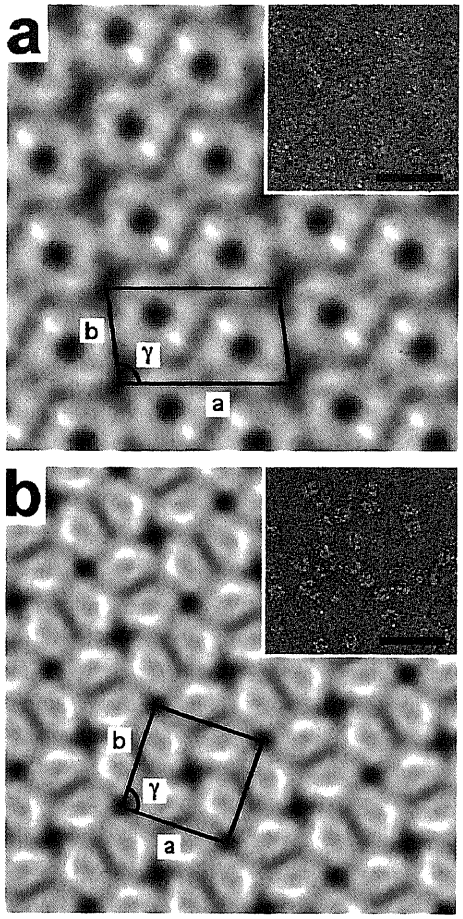


**Fig. 4** Single Molecules and Two-Dimensional Crystals of Human MtCK.
Electron micrographs of negatively-stained single molecules (insert images; the scale bar indicates 50 nm) and two-dimensional crystals (large images) of (a) octameric human uMtCK and (b) octameric human sMtCK. 2D crystals were formed by adsorption of single octamers on a cardiolipin layer and were negatively stained with uranylacetate. The signal-to-noise ratio was improved by correlation averaging. Unit cell parameters (outlined) are a = 203 Å, b = 110 Å and $\gamma = 98°$ ($p2$ symmetry) for human uMtCK and a = b = 145 ± 4 Å and $\gamma = 90°$ ($p4$ symmetry) for human sMtCK.

## Kinetic Characterization of Human MtCK

In the forward reaction (PCr synthesis, at pH 8), as well as in the reverse reaction (ATP synthesis, at pH 7), specific activities were higher for human sMtCK (105 U $mg^{-1}$ and 124 U $mg^{-1}$, respectively) than for human uMtCK (72 U $mg^{-1}$ and 108 U $mg^{-1}$, respectively) with a $v_{forward}/v_{reverse}$ ratio of 0.85 for sMtCK and of 0.65 for uMtCK. Both isoenzymes showed a very different pH dependency of enzymatic activity in a pH stat assay (Figure 5). Compared to sMtCK, pH maxima of uMtCK were shifted to more acidic (reverse reaction) or basic pH (forward reaction) and specific activites were lower, between pH 5.5 and 7.0, in the reverse reaction and between pH 6.5 and 9.0 in the forward reaction. However, in the range between pH 7 and 8, uMtCK showed a 5-fold activity increase in the physiologically relevant forward reaction (PCr synthesis), while sMtCK activity increased only 1.8-fold (Figure 5b).

Enzyme kinetic parameters were determined in detail with a photometric assay. Initial rate constants were measured by varying the concentrations of both CK substrates against a set of fixed concentrations of the second substrate for both directions of the CK reaction. Because there was no systematic deviation from linearity in Lineweaver-Burk plots (data not shown), we conclude that human MtCK, like other CK isoenzymes, follows Michaelis-Menten kinetics. Intersection of the regression lines iden-

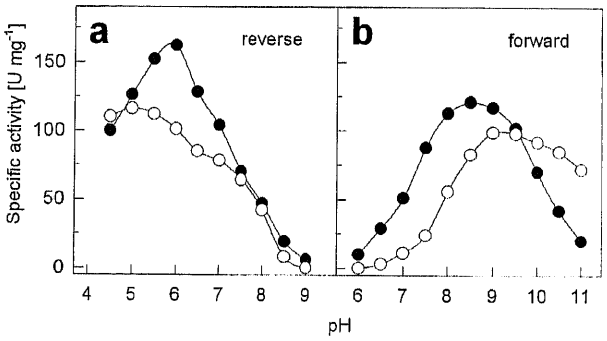


**Fig. 5** pH-Dependence of Enzymatic Activity of Human MtCK. Activity of human MtCK in (a) the reverse reaction (ATP production) and (b) the forward reaction (PCr production) was measured at different pH values with the pH-Stat method (see Materials and Methods). ○, human uMtCK; ●, human sMtCK.

**Table 1** Enzyme Kinetic Constants of Human MtCK.

| Substrate | Human uMtCK | | Human sMtCK | |
|---|---|---|---|---|
| | $K_d$ [mM] | $K_m$ [mM] | $K_d$ [mM] | $K_m$ [mM] |
| Forward reaction | | | | |
| MgATP | 4.04 ± 1.33 | 0.11 ± 0.02 | 4.06 ± 0.78 | 0.68 ± 0.21 |
| Cr | 45.8 ± 11.2 | 1.01 ± 0.13 | 43.9 ± 14.9 | 7.31 ± 1.27 |
| Reverse reaction | | | | |
| MgADP | 0.22 ± 0.03 | 0.13 ± 0.01 | 0.38 ± 0.09 | 0.15 ± 0.02 |
| PCr | 0.92 ± 0.13 | 0.55 ± 0.03 | 2.87 ± 0.68 | 1.16 ± 0.14 |

Constants for the CK forward and reverse reactions (at pH 8 and 7, respectively) were calculated from initial rate data by global fitting using computer software (see Materials and Methods).

tified a sequential reaction mechanism; the localization of the intersection points was consistent with a rapid equilibrium random mechanism at the pH values used (pH 8 in the forward and pH 7 in the reverse reaction; Rudolph and Fromm, 1979).

The values for $K_d$ and $K_m$ were calculated from a set of initial rate constants, using algorithms developed by Cleland (1979) (Table 1). $K_d$ and $K_m$ represent the dissociation constants of each substrate for the binary and ternary complexes, respectively. $K_m$ values were always significantly lower than $K_d$ values, indicating a substrate synergism that favors the binding of the second substrate (Price and Stevens, 1989). Substrate synergism was especially pronounced in the forward reaction. In the case of uMtCK, we found a 45-fold difference between $K_m$ and $K_d$ for ATP and Cr; in the case of sMtCK, the difference was only about 6-fold (Table 1). For the reverse reaction with ADP and PCr as substrates, this synergism was less pronounced for both human MtCK isoenzymes, with an only 2-fold difference between $K_m$ and $K_d$. When comparing human uMtCK with human sMtCK (Table 1) and sMtCK of other species (Wyss *et al.*, 1992), some marked differences in $K_m$ can be seen in the forward reaction (PCr production). The $K_m$ for Cr and MgATP is almost one order of magnitude lower for human uMtCK compared to human sMtCK. This is entirely due to the synergistic effects of the binary complex, since the $K_d$ values for Cr and MgATP do not differ between the isoenzymes.

## Discussion

Using heterologously expressed protein, we were able to study in detail the molecular properties of both human mitochondrial CK isoenzymes and to precisely characterize for the first time a ubiquitous MtCK. So far, preparations of MtCK from human tissue were always hampered by contamination with cytosolic isoenzymes, degradation or complex purification procedures (Wevers *et al.*, 1982; Blum *et al.*, 1983; Grace *et al.*, 1983; Khuchua *et al.*, 1989; Walterscheid-Müller *et al.*, 1997). By contrast, with our T7-based *E. coli* expression system and a simple 2-step purification procedure, we obtained up to 30 mg of pure MtCK per liter of culture. Recombinant human MtCK was identical to the native enzymes with respect to molecular mass and isoelectric point.

Freshly-purified human uMtCK was always octameric, but dilution and incubation in reagents inducing a transition state analog complex (TSAC) led to partial dissociation into dimers, similar to MtCK from other species (Wyss *et al.*, 1992). However, uMtCK was more resistant to TSAC-induced dissociation than sMtCK. This higher octamer stability of uMtCK octamers may be of physiological relevance, since only octamers show high affinity-binding to phospholipid membranes (Rojo *et al.*, 1991; Schlattner and Wallimann, 2000 a, b), delay opening of the PTP (O'Gorman *et al.*, 1997), or convey full Cr-stimulated respiration to isolated mitochondria (Khuchua *et al.*, 1998). In addition, Stokes' radii of uMtCK octamers and dimers of uMtCK were 1.8–2 Å smaller than those of sMtCK. This is more than predicted by the small difference in $M_r$ (*i. e.* 0.4 kDa for dimers) and indicative for conformational differences between uMtCK and sMtCK in solution.

For further details of the molecular structure of octameric MtCK, single particles and 2D crystals grown on negatively-charged cardiolipin layers were imaged by negative stain electron microscopy. Both human MtCK isoenzymes bound to the negatively-charged phospholipid surface by their identical top or bottom faces, showing the typical four-fold rotational symmetry of the cuboidal octamer, with four dimers, are arranged around a central channel (Fritz-Wolf *et al.*, 1996; Schnyder *et al.*, 1994). This is in support of a membrane-binding domain at these top and bottom faces, which expose several conserved, positively-charged residues, especially at the C-terminus (Schlattner *et al.*, 1998). These localized charges may, in contrast to overall pI, determine the binding to negatively-charged surfaces of biological membranes. In the crystal packing of 2D crystals, octameric uMtCK and sMtCK showed a remarkable difference. While the size of uMtCK octamers did not change significantly upon adsorption to the cardiolipin surface and the crystal packing was essentially the same as in 3D crystals (Eder *et al.*, 2000), the side length of human sMtCK octamers increased to 145 Å. This is much larger than the dimensions of the enzyme in solution (Stokes' radius of about 55 Å) or in 3D crystals of the equally-sized chicken sMtCK octamer (side length of 93 Å; Fritz-Wolf *et al.*, 1996). Obviously, upon membrane binding and subsequent air-drying, the sMtCK octamer is flattened to some degree, probably by a shearing movement between the dimers. This observation confirms that uMtCK has a more stable dimer/dimer interface as compared to sMtCK, in accordance with the higher octamer stability observed for uMtCK in solution. A flattening of MtCK octamers would explain how this bulky molecule, with a height of about 86 Å (Fritz-Wolf *et al.*, 1996), fits into the mitochondrial intermembrane space, which is only about 60–80 Å in width according to recent results with high-pressure-frozen samples (Frey and Mannella, 2000; Piendl and Wallimann, unpublished).

The determined pI of human MtCK isoenzymes agreed reasonably well with theoretically predicted values and most published data for MtCK isolated from human tissue (Wevers *et al.*, 1982; Walterscheid-Müller *et al.*, 1997; reviewed in Wyss *et al.*, 1992). Only two earlier studies reported a much higher pI for octameric human sMtCK than determined in our experiments (Khuchua *et al.*, 1989; Walterscheid-Müller *et al.*, 1997). This may be due to a cathodic drift in the pH gradient that may hamper exact pI determination at very basic pH. For this reason, we double-checked our pI values independently by IEF marker proteins as well as by a direct pH measurements of gel slices. The pI data finally revealed that human MtCK differs from MtCK of other species in several respects (see Wyss *et al.*, 1992): (i) human MtCK had a much lower pI (pH 7.4–7.7) than other MtCKs (pH 8.2–9.5); (ii) human sMtCK was

slightly more acidic than human uMtCK, while in other species sMtCK is the more basic isoenzyme; (iii) the octameric forms of human MtCK were more basic than the corresponding dimers, but this difference (0.2–0.3 pH units) was much less pronounced than with other MtCKs (0.5–1.0 pH units). The latter makes it rather unlikely that an overall surface charge difference between the two oligomeric forms is the only reason for the strong binding of octameric MtCK to cardiolipin membranes as compared to dimers (Rojo *et al.*, 1991; Schlattner and Wallimann, 2000 b). Our data rather support the existence of a localized, positively-charged cluster that is assembled upon the octamerization of the enzyme (Schlattner *et al.*, 1998).

Human MtCK isoenzymes differed significantly in various enzymatic properties, especially in the physiologically-relevant forward reaction (PCr synthesis): (i) pH-optima, (ii) substrate synergism and (iii) $K_m$ for Cr and ATP. With rising pH between 7 and 8, uMtCK activity showed a 5-fold increase from relatively low levels, while sMtCK activity increased only 2-fold. Thus, pH changes in this range would especially affect the capability of uMtCK to utilize mitochondrial ATP. Further, human uMtCK showed a more pronounced substrate synergism, that is, binding of the first substrate (binary complex) greatly facilitated binding of the second substrate (ternary complex). While dissociation constants for the binary enzyme-substrate complex ($K_d$) were similar for both isoenzymes, dissociation constants for the ternary binary enzyme-substrate complex ($K_m$) were up to 45-fold lower, especially with uMtCK in the forward reaction. Substrate synergism was also found for other CK isoenzymes, albeit to a much lower degree (Kaldis and Wallimann, 1995). In chicken sMtCK, substrate synergism can be suppressed by proteinase K digestion (Wyss *et al.*, 1993) or mutation of Cys 278 (Furter *et al.*, 1993). It is therefore most probably linked to hinge-bending domain movements during catalysis. Finally, due to the synergistic effect of substrate binding, uMtCK reached a 6–7 times lower $K_m$ for Cr and ATP than sMtCK. The especially low $K_m$ (Cr) of uMtCK is most probably an adaptation to the lower Cr and PCr levels in uMtCK-expressing tissue, *i.e.* brain, compared to sMtCK-expressing muscle. Total (Cr+PCr) concentrations are about 12 mM in brain (Tsuji *et al.*, 1996) and 25–50 mM in muscle, with a proportion of 20–30% of Cr (Wyss and Kaddurah-Daouk, 2000).

To summarize, we have identified numerous features that distinguish sarcomeric and ubiquitous human MtCK isoenzymes. As expected from the high sequence homology of vertebrate MtCK (Wyss *et al.*, 1992), some fundamental properties are similar and resemble MtCK from other species, *e.g.* the existence of active dimers and octamers and the affinity for acidic phospholipids. However, the numerous quantitative differences between human uMtCK and sMtCK comprise Stokes' radii, octamer stability, pH-dependence of activity, catalytic constants, substrate synergism in the forward reaction (PCr synthesis) and unit cell size in 2D crystals. The CK/PCr system has gained enormous clinical interest during recent years. Tumors with especially bad prognosis were often found to overexpress uMtCK (Kanemitsu *et al.*, 1984; Pratt *et al.*, 1987; Kornacker *et al.*, 1997) and the neuroprotective effect of Cr and its analogs (Wyss and Kaddurah-Daouk, 2000) may also be mediated by this enzyme. Some of the divergent properties described in this report, especially octamer stability and catalytic constants, may therefore be of (patho-) physiological significance *in vivo*.

## Materials and Methods

### Cloning of the Genes Encoding Mature MtCK

Cloned full-length cDNAs for human ubiquitous MtCK (uMtCK, GenBank J04469; Haas and Strauss, 1990) and human sarcomeric MtCK (sMtCK, GenBank J05401; Haas *et al.*, 1989) contain 5′-sequences coding for an N-terminal signal peptide. These sequences were removed by site-directed deletion *via* PCR. The PCR products were purified (Geneclean, Bio101, La Jolla, CA, USA), digested with flanking restriction enzymes and inserted into *Nde*I and *Bam*HI sites of a modified pET-3b vector (Studier *et al.*, 1990) under the control of a T7 promotor to yield expression vectors pUS01 (sMtCK) and pUS04 (uMtCK). Both strands of the MtCK coding region and the adjacent vector sequences were sequenced with the chain termination method (Sanger *et al.*, 1977) to check proper insertion into the vector and the absence of random mutations. Note that the N-terminus of mature human uMtCK is AASE, in contrast to SwissProt entry P12532.

### Expression and Purification of MtCK

Competent cells of *E. coli* strain BL21(DE3)pLysS were transformed with pUS01 or pUS04. Transformants were selected on $LB_{amp}$ plates and grown at 37 °C in 2 l bottles, each filled with 400 ml 2YT medium and placed on a rotary shaker at 250 rpm. Transcription of MtCK by T7 RNA polymerase was induced at a density of about 0.7 $OD_{600}$ by addition of 0.4 mM isopropyl β-D-thiogalactopyranoside. Maximal MtCK yield was achieved 4 h after induction according to pilot experiments. Cells were harvested and soluble protein was extracted by sonication according to Furter *et al.* (1992), the only exception being that an additional 2 μl Benzonase was added in the first lysis step to digest nucleic acids. The final extract was first purified by chromatography with Blue Sepharose (Pharmacia, Dübendorf, Switzerland). Active fractions were pooled and ultrafiltration (Amicon Diaflo PM30; Millipore, Volketswil, Switzerland) was used to concentrate MtCK and to exchange the buffer to cation exchange (CE) buffer (7 mM HEPES, 7 mM MES, 7 mM Na-acetate, 2 mM β-ME, 0.2 mM EDTA) at pH 6.5 (sMtCK) or pH 7.0 (uMtCK). The resulting concentrate was applied to a 4.6 × 100 mm column packed with the strong cation exchanger Poros HS (PerSeptive Biosystems, Perkin-Elmer, Rotkreuz, Switzerland), connected to a BioCAD Sprint HPLC (PerSeptive Biosystems) and equilibrated with the proper CE buffer. MtCK was eluted with a linear NaCl gradient at about 100 mM salt. Fractions with similar activities were pooled and concentrated if necessary with Centricon-30 (Millipore) to 6 mg/ml protein. Finally, 0.25 or 2.0 ml aliquots were applied to Superose 12 HR 10/30 (flow rate 1.0 ml/min) or HiPrep Sephacryl S-300 HR 16/60 (flow rate 0.4 ml/min), respectively, both equilibrated and run with GF buffer (50 mM Na-phosphate, 2 mM β-ME, 0.2 mM EDTA, and 150 mM NaCl, pH 7.0). Fractions were analyzed by standard 12% SDS-PAGE with a minigel apparatus and low-$M_r$ markers (Bio-Rad, Glattbrugg, Switzerland), stained with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany). Protein was routinely determined with the Bio-Rad reagent according to Bradford (1976) using BSA as a standard.

### Oligomeric State of MtCK

High-$M_r$ species of MtCK (octamers) were dissociated by dilution in gel filtration (GF) buffer pH 7 to 1 mg/ml and incubation for 1 d at 4 °C with the transition state analog complex (TSAC) substrates (4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM $KNO_3$; Milner-White and Watts, 1971; Gross and Wallimann, 1993). MtCK oligomers were separated and identified by HPLC, using 10 µl samples injected onto a Superose 12 gel filtration column (Pharmacia) in GF buffer at 1.0 ml/min and room temperature. The column was calibrated for Stokes' radii with the following marker proteins (Pharmacia): chymotrypsinogen A (20.9 Å, 25 kDa), albumin (35.5 Å, 67 kDa), aldolase (48.1 Å, 158 kDa), catalase (52.2 Å, 232 kDa), ferritin (61 Å, 440 kDa) and thyroglobulin (70.0 Å, 669 kDa).

### Agarose-IEF

Isoelectric focusing in horizontal agarose gels (Allen *et al.*, 1984) was optimized for the separation of MtCK oligomers. Gels consisting of 1.25% (w/v) agarose zero-$M_r$ (Bio-Rad), 9.6% (w/v) sorbitol, 3.6% (w/v) glycerol and 2% (v/v) ampholines pH 7–9 (Bio-Rad) were run on a Multiphor apparatus (Pharmacia) with filter paper electrodes soaked with 1 M NaOH (cathode) and 0.2 M histidine (anode). 15 µl samples (8 µg MtCK) and 2 µl colored IEF-marker (Bio-Rad), containing lentil lectin (pI 8.2, 8.0, 7.8), human hemoglobin C (pI 7.5), human hemoglobin A (pI 7.1) and equine myoglobin (pI 7.0), were applied onto 5 × 10 mm filter papers at 5 mm distance from the cathode. The gel was run at 10 °C and constant 6 W for 60 min at max. 500 V and, after removal of sample filter papers, for another 60 min at max. 800 V. The pH gradient was verified in an empty gel lane with a mini pH electrode (Metrohm, Herisau, Switzerland). The gel was washed for 10 min in $H_2O$ to remove ampholines and stained by a standard Coomassie procedure (Bio-Rad). Theoretical pI values of MtCKs were calculated from the sequences with PROTEAN software (DNAstar, Madison, WI, USA).

### Electron Microscopy and Image Processing

Imaging of single MtCK octamers by electron microscopy and negative staining with uranyl acetate has been described (Schnyder *et al.*, 1988). Crystallization of human MtCK on a single layer of negatively-charged cardiolipin, electron microscopy and correlation averaging of images was carried out according to Schnyder *et al.* (1994).

### Determination of Catalytic Properties of MtCK

CK activity was routinely determined with a photometer using a coupled enzyme assay modified after Wallimann *et al.* (1975). Briefly, PCr production (forward reaction) was coupled by pyruvate kinase (160 U/ml) and lactate dehydrogenase (800 U/ml) to NADH oxidation, using 4 mM ATP, 4.5 mM Mg-acetate, 20 mM Cr, 0.9 mM PEP and 0.45 mM NADH in 0.1 M triethanolamine buffer pH 8. The production of ATP (reverse reaction) was coupled by hexokinase (300 U/ml) and glucose-6-phosphate dehydrogenase (150 U/ml) to NADPH production, using 2 mM ADP, 5 mM $MgCl_2$, 20 mM PCr, 40 mM D-glucose and 1 mM NADP in 0.1 M triethanolamine buffer pH 7. The amount of enzyme activity required to reduce 1 µmol NADP per min, or to oxidize 1 µmol NADH per min, has been defined as 1 U. The pHs of the reactions were chosen in analogy to previous studies and according to established pH optima (Wyss *et al.*, 1992). The Mg salt concentrations selected assure that ADP and ATP are entirely in their $Mg^{2+}$-complexed form. Changes in the redox state of pyridine nucleotides were followed at 340 nm in a UV4 spectrophotometer (Unicam, Cambridge, UK) thermostated at 25 °C. For the determination of dissociation rate constants ($K_d$, $K_m$), a matrix of initial velocity data was recorded by varying 6–7 different concentrations of each substrate at 4 different, fixed concentrations of the second substrate. Fixed concentrations were 2, 5, 10 and 20 mM Cr; 0.2, 0.8, 2 and 4 mM MgATP; 0.5, 1, 4 and 20 mM PCr; and 0.02, 0.1, 0.4 and 2 mM MgADP. From the initial velocity data, rate constants were calculated according to Cleland (1979) with software written by R. Viola (Akron University, OH, USA). The pH-dependence of MtCK activity was determined with a pH stat (Radiometer Copenhagen, Villeurbane, France) at 25 °C, measuring proton consumption in the CK reverse reaction with 4 mM ADP and 10 mM PCr or proton release in the forward reaction with 4 mM ATP and 20 mM Cr (Wallimann *et al.*, 1984). CK activities at a pH lower than 6 were corrected for changes in stoichiometry between proton consumption and enzymatic activity.

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