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Chapter 5

MOLECULAR STRUCTURE AND FUNCTION OF MITOCHONDRIAL CREATINE KINASE

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ABSTRACT

Mitochondrial creatine kinases (MtCK) are members of the creatine kinase (CK) isoenzyme family that are localized in the peripheral intermembrane space and the cristae of mitochondria. Besides the conversion of mitochondrially produced ATP into phosphocreatine and its export as "high energy currency" into the cytosol, additional functions have been proposed for these kinases. More recently, the solved X-ray structures of all four vertebrate CK isoenzymes, as well as mutagenesis, biochemical and biophysical *in vitro* studies have significantly advanced our understanding of this class of enzymes. This review summarizes in a first part the molecular structure, substrate binding and catalysis of MtCK, as well as genuine MtCK properties like octamer formation or membrane binding, with an emphasis on differences between the two MtCK isoenzymes, sarcomeric and ubiquitous MtCK. A second part describes location and function of MtCKs in their organellar environment, in particular the putative topology of MtCK in proteolipid complexes. Finally, the review discusses the implications of MtCK in human health and disease.

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MtCK - Mitochondrial Creatine Kinase in the "CK/phosphocreatine Shuttle"

The existence of tissue and compartment-specific isoenzymes of creatine kinase (CK) is an important property of this long-known enzyme [1] and key to its functions in cellular energy metabolism. Most vertebrate tissues express two CK isoenzymes, a dimeric cytosolic and a mostly octameric, mitochondrial CK (MtCK) that is localized in cristae and intermembrane space [2-5] (Figure 1). Mitochondrial and cytosolic CK have diverged very early during evolution, at least 670 million years ago [6], suggesting that compartmentalized CK isoenzymes have evolved to fulfill specific functions (see also Ellington and Suzuki, this book). In vertebrates, liver seems to be the only organ that is devoid of both, cytosolic and mitochondrial CK. By contrast, the occurrence of two tissue-specific MtCK isoenzymes [7] is a rather late phylogenetic event. In vertebrates, sarcomeric MtCK (sMtCK) is found in striated muscle, while ubiquitous MtCK (uMtCK) has been detected in most other tissues like brain, kidney, reproductive organs or skin [8-12].

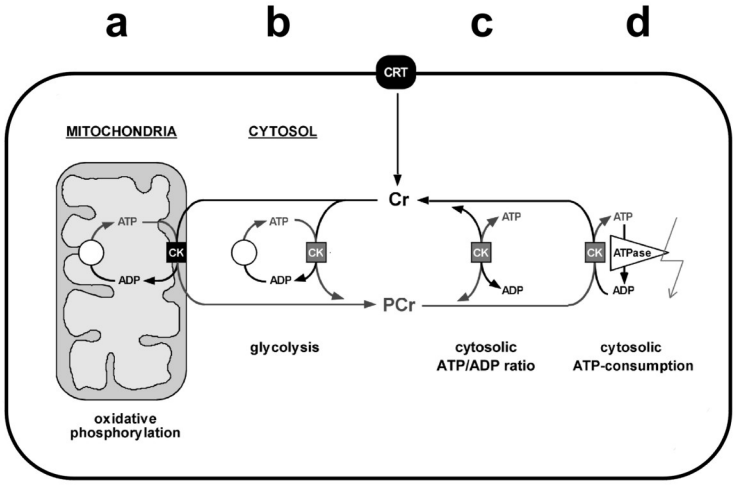

Figure 1: The CK/phosphocreatine system and the concepts of temporal and spatial energy buffering. (a) Mitochondrial CK (CK, black box) in the peripheral intermembrane space and the cristae of mitochondria uses ATP, generated by oxidative phosphorylation and exported via ANT, to phosphorylate creatine (Cr) to phosphocreatine (PCr). In turn, phosphocreatine leaves the mitochondria via VDAC into the cytosol. **(b)** In less oxidative tissues, e.g. in fast-twitch glycolytic muscle, phosphocreatine is mainly synthesized by cytosolic CK (CK, gray box), which is in part associated with glycolytic enzymes. **(c)** A highly concentrated cytosolic phosphocreatine pool (up to 30 mM) is built up. This pool constitutes an efficient temporal energy buffer, which assures a constant cytosolic ATP/ADP ratio over a wide range of workloads. **(d)** Cytosolic CK is often found associated and functionally coupled with ATP-consuming and ATP-regulated processes. There, CK uses phosphocreatine to maintain locally high ATP concentrations and ATP/ADP ratios. Note: Creatine is synthesized in only a few types of cells (mainly in liver and kidney); other cells rely on creatine uptake from the blood stream by a specific creatine transporter (CRT). In cells that are polarized and/or have very high and localized ATP consumption, CK associated with ATP-providing and -consuming processes may facilitate spatial energy buffering or energy transport working as a CK/phosphocreatine-shuttle or -circuit, thus connecting sites of energy consumption with those of energy production **(a-d)**..

The interplay between cytosolic and mitochondrial CK isoenzymes fulfills multiple roles in cellular energy homeostasis [13-20] (Figure 1). First, both isoenzymes contribute to the built-up of a large intracellular pool of phosphocreatine that represents an efficient temporal energy buffer and prevents a rapid fall in global [ATP]. Further functions of CK are based on the concepts of subcellular compartmentation of CK isoenzymes and limitations of free diffusion even of smaller molecules within the cell, as e.g. adenine nucleotides. CK isoenzymes are not strictly soluble, but they are associated to a variable degree with subcellular structures. This involves direct or indirect association of CK with ATP- providing or -consuming processes. Such associations represent microcompartments which often facilitate a direct exchange of ADP and ATP between the association partners without mixing with bulk solution, the so-called metabolite channeling or functional coupling [21, 22]. This microcompartmentation allows to maintain high local [ATP]/[ADP] ratios in the vicinity of cellular ATPases for a maximal ΔG of ATP, and on the other hand a relatively low [ATP]/[ADP] ratio in the mitochondrial matrix to stimulate oxidative phosphorylation. Due to the specific localization of mitochondrial and cytosolic isoenzymes and the slightly faster diffusion rate of phosphocreatine as compared to ATP, the CK/phosphocreatine-system can in principle provide a spatial "energy shuttle" [3, 23, 24] or "energy circuit" [13], bridging sites of energy generation with sites of energy consumption (Figure 1). Although some of these functions have been a matter of debate [25-28], there is growing evidence for CK and creatine compartments in the cell, as well as for the presence of a CK/phosphocreatine shuttle at least in large and polar cells. Convincing evidence comes from Ca^{2+}-handling deficiencies in CK knockout mice [29, 30], *in vivo* analysis of polar cells like spermatozoa [31, 32], direct *in vivo* $^{18}0$ labeling of phosphoryl moieties in intact muscle [33, 34], ^{31}P- and ^{1}H-NMR in perfused heart [35-37], experiments with chemically skinned muscle fibers [38, 39], as well as *in silico* modeling approaches [40-42]. Moreover, MtCK and the CK/phosphocreatine system seem to contribute to an intricate, metabolic energy transfer network in the cell, connecting mitochondria with myofibrils, sarcoplasmic reticulum, and even nuclei [17, 36, 38, 43-46].

Although MtCK is much less studied as compared to cytosolic CK, several aspects have been subject of topical reviews during recent years and will not be discussed in detail here. These include MtCK evolution in the guanidino kinase family [6], MtCK genes [47], sequence relationships [48], the large body of earlier biochemical and histochemical work [4, 13], classical concepts of CK functioning [14, 15, 17], and MtCK transgenic mice [49]. This review will concentrate on the progress in essentially three fields, (i) deciphering molecular structure and structure/function relationships of MtCK [5, 50], (ii) their implications for the molecular physiology of MtCK in mitochondria [13, 23, 51-53], as well as (iii) the role of MtCK in human health and disease, including molecular damage of the protein [54, 55].

MtCK - From Molecular Structure to Molecular Function

Creatine kinase is part of a larger enzyme family, the guanidino (phosphagen) kinases [6]. They all reversibly phosphorylate different guanidino substrates to produce a large pool of ‘high-energy phosphate’, like e.g. phosphoarginine with arginine kinase or phosphocreatine with CK. Their similar function is reflected by a high degree of sequence

homology. Thus, after the molecular structure of octameric mitochondrial CK from chicken was solved as the first guanidino kinase structure [56], this new knowledge allowed conclusions on all CK isoenzymes and also other guanidino kinases. Considered as a milestone, the sMtCK structure let not only to a deeper understanding of the many important residues identified in earlier studies by chemical modification, site-directed mutagenesis or spectroscopic analysis, but also to the knowledge-based design of new studies aimed at specific molecular properties and functions of guanidino kinases. Similarly, the solution of the first transition state structure with monomeric arginine kinase allowed more insight into the catalytic mechanism of CK [57]. Since then, a number of other X-ray structures have followed, allowing some detailed structure-function analysis. Here, we mainly concentrate on MtCK-specific properties. For clarity, residues are consistently numbered according to human uMtCK throughout the text.

From 2D-crystals to 3D Crystals

Single molecule imaging [58, 59] and 2D-protein crystals [60] have given early insights into the overall structural symmetry and functional properties of octameric MtCK. For a long time, the protein resisted to form 3D-crystals suitable for X-ray crystallography, and it was only in 1996 when octameric chicken sMtCK complexed with ATP was solved at 3Å resolution [50, 56] (Figure 2a). Keys for the successful crystallization and structure determination were probably the choice of chicken enzyme, the use of highly pure recombinant protein, as well as a complex theoretical approach involving phase extension based on the fourfold redundancy of electron density, which improved the original resolution obtained with isomorphous replacement [50, 56]. The coordinates of chicken sMtCK have subsequently been used for the molecular replacement solution of further guanidino kinase structures, including dimeric cytosolic CK (rabbit and human MMCK [61, 62], human and bovine BBCK [63, 64]), as well as the transition state structures of monomeric arginine kinase from horseshoe crab [57, 65] and dimeric CK from *Torpedo californica* [66]**.** Human uMtCK, the second MtCK isoenzyme, was solved by molecular replacement to 2.7 Å [67] (Figure 2b).

Figure 2 – See end of the section for colour presentation.

Overall Structure and Chain Fold

In contrast to mostly monomeric arginine kinase and exclusively dimeric cytosolic CK, MtCK not only forms dimers, but also associates into octamers. The octameric species is the predominant oligomeric form *in vitro* as well as *in vivo*. It is favored by high enzyme concentrations and thus the only oligomeric form found in MtCK protein crystals. The highly ordered, large cuboidal octamer has overall dimensions of about 105 × 105 × 86 Å and displays 422 point group symmetry (Figure 2). It assembles from four elongated "banana-shaped" dimers that are arranged around its fourfold axis. Along this axis, a central channel of about 20 Å diameter extends through the entire octamer. The N-termini of all monomers protrude into this channel. The top and bottom faces that are perpendicular to the fourfold

octamer axis each expose the C-termini of four monomers. The general fold of MtCK, which was found to be highly conserved among the guanidino kinase family, is unique [67] (Figure 3a). The monomer consists of a small N-terminal domain (residues 1-100) and a larger C-terminal domain (residues 113-end) connected by a long linker region without secondary structure (residues 101-112). The small N-terminal domain is exclusively α-helical. It contains 5 conserved α-helices and one 3_{10}-helix (chicken sMtCK) or an additional α-helix (human uMtCK). The core of the C-terminal large domain is formed by an eight-stranded antiparallel β-sheet, which is flanked by seven α-helices. The β-sheet has a saddle-like shape and is bended to about 120°. All α-helices except α8 are on the convex side of the β-sheet, while the enzyme's active center is on the concave side. The β-sheet and the α8-helix form the common core of CK isoenzymes that is characterized by a conserved amino acid sequence and contains the enzyme's active site. All these structural elements are conserved throughout the guanidino kinase family and only small differences were found between the crystallographically independent monomers in the MtCK octamer, mainly restricted to flexible, solvent accessible regions. Despite the absence of any significant sequence similarity, the large domain may be evolutionary related to the C-terminal domain of glutamine synthetase by its duplicated βαββα folding element and the similar position of the ATP binding site [50, 68].

Figure 3 – See end of the section for colour presentation.

Some aspects of the MtCK structures indicate a high degree of flexibility, which has been confirmed by the transition state structures of CK and arginine kinase [57, 66]. Two loops in the region of residues 61-65 and 316-326 are disordered in both known MtCK structures, but close down on the active site during catalysis (Figure 3a). Consistent with the capability of CK to undergo flexible hinge-bending between large and small domain during catalysis, the "banana-shaped" dimers of MtCK are less curved than in BBCK, possibly because of stronger packing interactions in the MtCK crystals [63].

Since CK isoenzymes have an almost identical, conserved structural fold (Figure 3b), they differ mainly in their primary structure, i.e. differences in electrostatic potential and hydrophobicity. On the first sight, CK and other guanidino kinases also show a high degree of sequence homology, with MtCK isoenzymes having a particularly high sequence identity of 82-85%. However, most homologies are concentrated in 6 highly conserved regions [48] that form a compact cluster in the common core of the enzyme and are involved in substrate binding and catalysis. Some other regions differ in their sequence between cytosolic and mitochondrial CK isoenzymes, in particular at the N-terminus, the linker region between the two domains, parts of α-helices 11, 12 and 13, as well at as the C-terminus. These isoenzyme-specific sequences [48] are located in solvent accessible regions at the surface of the enzyme and therefore potential candidates for interaction domains. In MtCK isoenzymes, the divergence of the amino acid sequence is particularly pronounced in the N- and C-terminal stretches. Indeed, these sequences fulfil specific isoenzyme-specific functions in stabilization of the octamer and membrane binding (see below).

Subunit Interactions and Octamer/dimer Transitions

Dimers are the basic building block of all creatine kinases and appeared already very early during metazoan evolution [6]. These dimers are very stable and disintegrate into monomers only under chaotropic conditions along with partial unfolding of the monomer [4, 69]. The monomer/monomer interface of MtCK comprises three contact regions at the N-terminus (residues 5-20 and 31), the small domain (residues 34 , 44-45, and 47-58), and the large domain (residues 142-147, 172, 191, 204-206), including the strictly conserved Trp206 that was identified to be important for monomer/monomer interaction by site-directed mutagenesis, fluorescence spectroscopy [70] and unfolding experiments [71]. Mutations of four conserved residues in the interface of MMCK dimers led to a partially active MMCK monomers, showing that dimers are not essential for activity, but may rather stabilize the CK structure [72]. As compared to cytosolic CK, the monomer/monomer interface of MtCK is smaller, comprises less polar interactions and is less dependent on the N-terminal region. The solvent inaccessible monomer/monomer interface of MtCK is only ~2200-2690 $Å^2$, in contrast to ~3700 $Å^2$ with cytosolic chicken BBCK [63]. These data seem to indicate that MtCK dimers are less stable, but direct experimental evidence for this hypothesis in form of unfolding or stability studies is lacking. The numerous interactions that stabilize the MtCK dimer include mainly hydrophobic interactions and a smaller number of polar interactions. The polar interactions involve hydrogen bonds, but also some salt bridges depending on the isoenzyme. Although dimers are a common feature of CK, the hydrogen-bond forming residues at the monomer/monomer interface differ among isoenzymes. Only Asp57, Arg143, Arg148, and Asp205 are conserved throughout the entire CK family. Additional hydrogen bond interactions mediated by a water molecules at the monomer/ monomer interface may explain the remarkable stability of CK dimers [73].

The ability to form highly symmetrical cuboidal octamers is a unique feature of all MtCKs [74-76]. The octamer is held together by several interactions of two rather small regions, comprising some residues in the small domain (N-terminal contact region, in addition residues 44-45 in uMtCK) and a predominantly hydrophobic patch in the large domain around Trp264 (residues 134-136 and 261-264 in sMtCK, in addition residues 140-155 in uMtCK; Figure 4) [56]. Earlier studies had already shown a key role of N-terminal amino acids and Trp264 for octamer stability, using site directed mutagenesis [70, 77, 78] and thermodynamic analysis of octamer stability [69]. The basis of the dimer/dimer interface is a hydrophobic patch, which is strengthened by a number of polar interactions. The latter vary between MtCK isoenzymes due to a divergent N-terminal fold and sequence differences in the contact regions. Because contacts between MtCK dimers are limited, a dynamic conversion between the octameric and dimeric state of the enzyme can be observed *in vitro* and *in vivo*. Biophysical analysis revealed that octamer dissociation can be triggered by changes in temperature, ionic strength, pH, protein or substrate concentration, but shows half-life times of days to weeks [69, 74]. Disassembly of the octamer happens in an all-or-nothing fashion, while the reformation of the octamer takes place via a short-lifetime tetramer intermediate [79]. A more potent artificial dimerization trigger is the dead end substrate mixture (transition state analogue complex, TSAC; creatine, Mg^{2+}, ADP, NO_3^-; [80]). It leads to octamer dissociation within minutes by fixing the enzyme in a conformation that destabilizes the dimer/dimer interface [74, 79, 81]. With TSAC, rate constants are in the range of minutes to hours and equilibrium constants show the presence of a considerable amount of

dimeric MtCK [69]. Interestingly, kinetic and equilibrium constants of octamer/dimer transitions differ between MtCK isoenzymes [76]. The relevance of such octamer/dimer transitions for the enzyme *in vivo* remains controversial. They could play a regulatory role, since only octameric MtCK localizes to the so-called mitochondrial contact-sites that are involved in energy transduction from mitochondria to the cytosol, while dimeric MtCK does not [4] (see also below). However, the MtCK octamer is stabilized by a high MtCK concentrations in the mitochondrial intermembrane space and its binding to mitochondrial membranes [82], as shown by the extraction of mostly octameric MtCK from tissue or heterologous expression in *E. coli* cultures. Octamer/dimer transitions are also too slow to play a regulatory role during fast metabolic changes, although a contribution to long term adaptation or modulation of energy metabolism cannot be ruled out. However, a crucial role of octamer/dimer transitions has been observed in pathological situations that are characterized by oxidative stress (see below).

Figure 4 – See end of the section for colour presentation.

Catalytic Mechanism, Conformational Changes and Active Site Topology

Enzymatic mechanism and active site of CK and other guanidino kinases have been among the most thoroughly studied by classical methods, even before a molecular structure had been solved. The availability of X-ray CK structures with and without bound substrates has increased our understanding of substrate binding and catalysis on a molecular level [57, 66]. In fact, CK has acquired a specific fold to achieve phosphoryl transfer and seems to be unique in missing essential catalytic residues. Enzymatic catalysis of CK is discussed in detail in earlier reviews [4, 83] and in other contributions to this book (Kenyon and McLeish, Chapman). Here, we focus on properties that were first discovered with MtCK or are specific to this class of isoenzymes.

In comparison to cytosolic CK, much less is known about enzymatic catalysis of MtCK. In addition, most existing knowledge has been obtained with MtCK in solution, often ignoring the oligomeric state and in particular the enzyme's native environment, i.e. its association into proteolipid complexes in mitochondrial intermembrane space or cristae. The general catalytic mechanism is identical to all guanidino kinases and does neither diverge between octameric and dimeric MtCK [84, 85]. However, the two oligomeric states differ in K_m and K_d for creatine, which is 2-3 times higher for the octamer as compared to the dimer [84]. Further significant differences in K_m and V_{max} were observed between sMtCK and uMtCK isoenzymes and will be discussed later [85].

Much more than cytosolic CK, both MtCK isoenzymes are characterized by a pronounced substrate binding synergism for all substrates, but most pronounced in the forward reaction (phosphocreatine production) that is relevant in the native environment. This indicates a cross-talk between the two substrate binding sites in the active center of a CK monomer [85]. A further cooperativity between the different active sites in a dimer or octamer could partially explain why assembly of CK into such oligomeric forms is advantageous. However, there is contradictory experimental evidence and the issue remains controversial [86-89]. Finally, *in vivo* analysis with isolated heart mitochondria and skinned muscle fibers suggested differences in substrate specificity between MtCK and cytosolic CK

[90]. The latter was able to phosphorylate and to slowly dephosphorylate β-guanidinopropionic acid, methyl-guanidinopropionic acid, guanidinoacetic acid, and cyclocreatine *in vivo*, while dephosphorylation of these analogues *in vitro* is much slower. In contrast, out of these four analogs, MtCK could only use cyclocreatine as a substrate [90].

Each monomer in the octameric structure has its own catalytic center, buried in the cleft between the N-and C-terminal domains of CK [56], as already suggested earlier by mapping with monoclonal antibodies [91] (Figure 3a). The molecular structures of arginine kinase and of a cytosolic CK, both obtained in presence of substrates that form the dead-end transition state analogue complex (TSAC), now precisely located the active site in the highly conserved region of all guanidino kinases [57, 66]. Except for the binding site of the guanidino substrate, both structures are almost identical, such that a CK transition state could already be modeled earlier on the basis of the arginine kinase structure [63]. These "closed" conformations, as opposed to the "open" conformations without substrates or with only the Mg-free nucleotides bound, are more rigid and compact. They show the two flexible loops (residues 61-65 and 316-326) closed down on the active site, and indicate a hinge-bending movement between the two domains during catalysis [57, 66]. Hinge-bending of domains often occurs with enzymes that catalyze two-substrate reactions, especially kinases [92]. Here, domain-cleft closure and flexible loops may act as a lid to exclude water during phosphoryl group transfer, thus avoiding hydrolysis of the phosphoryl groups. Already earlier studies indicated conformational changes during substrate binding [93] and catalysis [94]. A reduced radius of gyration upon substrate binding was consistently observed with small angle X-ray scattering for monomeric arginine kinase, dimeric cytosolic and octameric mitochondrial CK in solution [95-97]. These conformational changes seem to depend on binding of Mg-nucleotide or complete TSAC substrate mixture, with no effects observed with nucleotide or creatine alone. However, in the transition state structure of dimeric *Torpedo* CK which contains one monomer bound to TSAC and one bound to sole MgADP, the Mg-nucleotide does not induce a closed conformation, although it is bound at about the same position in both monomers [66]. Protein secondary structure remains basically preserved during these conformational changes, e.g. during Mg-nucleotide binding [98, 99]. The flexible elements of the CK structure are probably also involved in the observed substrate binding synergism. Nicking of MtCK at Asp330 near the highly flexible loop 316-326 did not affect substrate binding *per se*, but abolished substrate synergism [100]. In another study, two sMtCK protein fragments corresponding approximately to N- and C-terminal domains and yielding insoluble protein when expressed separately in *E. coli* gave fully functional protein when coexpressed [101]. However, the positive cooperativity of substrate binding was abolished, suggesting that indeed the covalent domain linker plays a role in the cross-talk between the two substrate binding sites.

The ATP binding site of CK is located in a cleft of the large subunit, while the creatine binding site is rather situated between the two CK domains. A large number of residues are involved in binding and correct positioning of the substrates. In the past, several residues have been proposed as to act as acid-base catalyst, including a histidine [102, 103] or even two conserved acidic residues (Glu227 and Asp321) [104, 105]. However, site-directed mutagenesis experiments [106-108] and the transition state structures [57, 66] have clearly demonstrated that none of these conserved residues is really essential. Other residues that were earlier claimed to be essential, including Cys278 [109-111] and Trp223 [112] in the active site, were later also shown by site-directed mutagenesis and the transition state

structures to play a rather accessory role in binding synergism, correct positioning of the substrates, or stabilization of the transition state [70, 113-115]. It seems as if phosphoryl transfer by CK is not accomplished by essential catalytic residues, but rather facilitated by a large number of enzyme-substrate interactions that involve multiple mechanisms, including not only acid-base catalysis, but also correct positioning and alignment of both substrates. Stroud has coined the term "conzyme" for such an enzyme [116]. However, even after knowing two transition state structures of guanidino kinases, many details of their bisubstrate reaction are still far from being fully understood.

Interaction of MtCK with Mitochondrial Membranes

MtCK differs from cytosolic CK not only by its organellar localization and octameric structure, but also by its membrane-binding properties. Cytosolic CK isoenzymes are mainly soluble and only a small fraction is bound to particular subcellular structures like myofibrils, sarcoplasmic reticulum or plasma membrane [13]. By contrast, octameric MtCK behaves as a typical peripheral membrane protein. It occurs in cristae and peripheral intermembrane space of mitochondria and shows a strong affinity to the outer leaflet of the inner mitochondrial membrane [3, 76, 117-119]. In addition, octameric MtCK bridges the intermembrane space by its molecular dimensions and its capability to simultaneously interact with inner and outer mitochondrial membrane, thus virtually crosslinking these membranes [120]. The binding of MtCK to mitochondrial membranes occurs mainly through electrostatic interactions [121-123]. The principle binding partners are negatively charged phospholipids [118, 119], in particular cardiolipin [124, 125]. Cardiolipin (diphosphatidyl glycerol) is unique in being mainly restricted to the inner mitochondrial membrane [126] and having a large dianionic headgroup. These properties may explain the high affinity and the preferential binding of MtCK to this phospholipid.

In contrast to octamers, dimeric MtCK interacts only weakly with anionic phospholipids or crosslinks membranes [82, 118, 120, 123]. The slow binding kinetics of dimeric MtCK could not be fitted by common rate equations, indicating a complex binding mode where the rate limiting step may be octamerization of dimers while attached to the membrane [82, 123]. Indeed, when N-terminal charges of sMtCK were replaced by uncharged amino acids, the mutant protein was predominantly dimeric and showed reduced membrane binding [77, 78]. Interestingly, when expressed in almost MtCK-deficient neonatal cardiomyocytes, this otherwise fully enzymatically active mutant sMtCK displayed a strong functional impairment as evidenced by much lower rates of creatine-stimulated oxidative phosphorylation compared to wild-type enzyme [78]. Thus, the octamer/dimer ratio of MtCK has a direct influence on the proportion of membrane-bound, functional MtCK. A more detailed description of earlier work on MtCK/membrane interaction may be found in previous reviews [5, 127].

The availability of X-ray structures for MtCK and the application of different spectrometric methods during recent years has shed some new light on membrane interaction of this isoenzyme [76, 98, 119, 123, 125, 128, 129]. Fluorescence spectroscopy was applied to determine structural changes of MtCK or the lipid membrane upon interaction, using either endogenous fluorescence of MtCK tryptophan residues or the fluorescent membrane probe Laurdan that is sensitive to the polarity of the environment [98]. These experiments indicated structural changes in both binding partners, with the lipid membrane becoming less fluid and

MtCK undergoing subtle conformational changes. In addition, MgADP, MgATP and even phosphocreatine may be able to detach MtCK from phospholipid membranes *in vitro* [129]. The introduction of surface plasmon resonance (SPR) spectroscopy for the analysis of MtCK-membrane interaction finally allowed a very detailed and quantitative characterization ([76, 123, 125, 128], see below). In this biophysical assay, biotinylated liposomes are immobilized on an avidin-coated sensor chip and binding and dissociation kinetics of the MtCK are followed on-line. The setup allows a free choice of lipid composition or the reconstitution of membrane proteins and can be calibrated with positive and negative controls (e.g. cytochrome c and serum albumin in case of cardiolipin).

There is good evidence for a functional interaction of MtCK with the transmembrane proteins adenine nucleotide translocator (ANT) in the inner membrane and mitochondrial porin (VDAC, voltage dependent anion channel) in the outer membrane [4]. ANT is an obligatory antiporter for ATP/ADP exchange across the inner mitochondrial membrane, while VDAC is a non-specific, potential-dependent pore in the outer membrane. Functional proteolipid complexes containing these proteins could be isolated [130-132]. In case of VDAC, its addition *in vitro* to a partially octamerization-incompetent MtCK mutant triggered the octamer formation, suggesting that both proteins interact at some stage of octamerization [77, 133]. However, no evidence for a persistent, physical interaction between these proteins could be obtained, e.g. by chemical crosslinking experiments [134, 135]. Recently, SPR experiments with reconstituted VDAC finally provided definitive proof for a direct binding to MtCK [136]. The interaction is of very high affinity (K_D < 10 nM), mostly of electrostatic nature, and further increased by micromolar concentrations of calcium [136]. Thus, MtCK interacts with the outer membrane not only via different anionic phospholipids, but also via the high affinity receptor VDAC. No such direct interaction was detected by SPR between MtCK and reconstituted ANT, confirming earlier studies [124]. The association between these two proteins may therefore be indirect and mediated by cardiolipin [4, 5, 76, 119, 137]. ANT and MtCK both show a high affinity for cardiolipin (K_D<100 nM) [74, 76, 138, 139], which would lead to a close co-localization in cardiolipin membrane patches. In fact, it is well known that cardiolipin-protein interactions are important not only for activity and structural integrity of mitochondrial inner membrane proteins, but also for subunit assembly and complex formation [140-142]. Here, cardiolipin can act as a "glue" for supercomplex formation [141, 142].

Structural information was essential to identify the phospholipid/cardiolipin interaction domain of MtCK. A first indication came from single molecule imaging and 2D-protein crystals grown on cardiolipin membranes. These electron micrographs revealed that the cuboidal MtCK octamer binds to membranes via their identical "top" or "bottom" faces [58-60] (Figure 2). The existence of two membrane binding faces per octamer also explained how MtCK can simultaneously bind to two different membranes [120, 125]. The X-ray structures of MtCK finally revealed two putative membrane binding motifs per monomer, which are MtCK-specific, properly exposed at the membrane binding faces, and carry a positive net charge to interact with acidic cardiolipin: a C-terminal stretch (Asp357 - Lys380) and a short internal stretch (Ala107 - Gln115). The C-terminal motif consists of two basic residues in tandem at the very C-terminus and another one 10 amino acids further upstream (Figure 4). This motif lacks any secondary structure and is less ordered in the MtCK crystals; it may therefore be well suited as a flexible docking domain. Indeed, a recent site-directed mutagenesis study, using SPR, light scattering and fluorescence methods, revealed that the

three lysines in the C-terminal motif of human sMtCK largely determine MtCK/cardiolipin interaction [143], leaving only a minor contribution to other binding motifs. However, the lysines were only partially responsible for the decreased membrane fluidity that occurs upon MtCK/cardiolipin binding. Here, a moderately hydrophobic, proline-rich stretch of 6-7 amino acids may be involved that separates the basic charges in the C-terminal motif. Alternatively, this hydrophobic motif may participate in protein/protein interactions at mitochondrial contact sites (see below). Finally, SH-group reagents [119, 144] and oxidation of the enzyme (Schlattner *et al.*, unpublished data) were found to inhibit MtCK membrane binding, indicating that the conserved Cys258 near the C-terminal binding motif affects MtCK/cardiolipin interaction. From a phylogenetic perspective, ancient MtCK like in the marine chordate *Ciona intestinalis* or the invertebrate annelid *Chaetopterus variopedatus*, differ from vertebrate MtCK discussed here. Although they still present a high sequence homology of at least 71%, their C-termini miss the cardiolipin binding domain and show homology to cytosolic CK isoenzymes [143], which are known to bind only weakly to cardiolipin-containing membranes [127]. Very likely, such octamers would not interact with cardiolipin in a way vertebrate MtCK is doing.

SPR spectroscopy allowed for the first time a detailed quantitative analysis of binding parameters of both octameric human MtCK isoenzymes. Association and dissociation kinetics fit well to a model for heterogeneous interaction, suggesting two independent binding sites [76, 123]. The two sites differ in their rate constants by one order of magnitude, and we may therefore distinguish "fast" (uMtCK: $k_{on} = 5.4 \pm 1.1\ 10^5\ M^{-1}\ s^{-1}$) and "slow" binding sites (uMtCK: $k_{on} = 4.1 \pm 0.1\ 10^4\ M^{-1}\ s^{-1}$). The affinity of both sites, however, is very similar and in the range of 80-100 nM, although some subtle differences were observed between the two MtCK isoenzymes. The presence of two binding sites for MtCK was already described earlier [122], although these sites differed in affinity. One may speculate that they represent two different binding sites at the MtCK surface or, *vice-versa*, two different kinds of cardiolipin-patches on the liposomes. However, their phenomenological occurrence may also reflect a two-step binding mode of MtCK involving different binding mechanisms (Figure 5). While fast binding could occur via the basic residues at the MtCK C-terminus and would be purely electrostatic, slow binding could involve the formation of specific cardiolipin-patches, possibly into non-bilayer structures [145] and MtCK undergoing structural changes to further expose the hydrophobic stretch at the C-terminus [5]. This process could finally lead to a partial penetration of the hydrophobic C-terminal moiety of MtCK into the lipid bilayer. Although earlier studies favored an entirely electrostatic binding process at the lipid surface [146], a number of data are in support of the latter hypothesis. Besides the structural features of MtCK and its known hydrophobic character [4], different methodological approaches have shown a disturbance of the lipid bilayer upon MtCK binding [98, 118, 119, 143] and *vice versa* some subtle structural changes in the β-sheet structures of MtCK upon lipid binding [98]. A partial integration of MtCK into the membrane would contribute to the reinforcement of the MtCK-membrane interaction. It would also explain the resistance of MtCK against full detachment by high ionic strength buffers [147] or the presence of such a bulky enzyme in the narrow mitochondrial intermembrane space [5] (Figure 6). A model for such a binding mechanism may be found with cytochrome c, which also shows high affinity for cardiolipin [148] and binds to mitochondrial membranes via electrostatic and hydrophobic interactions [149].

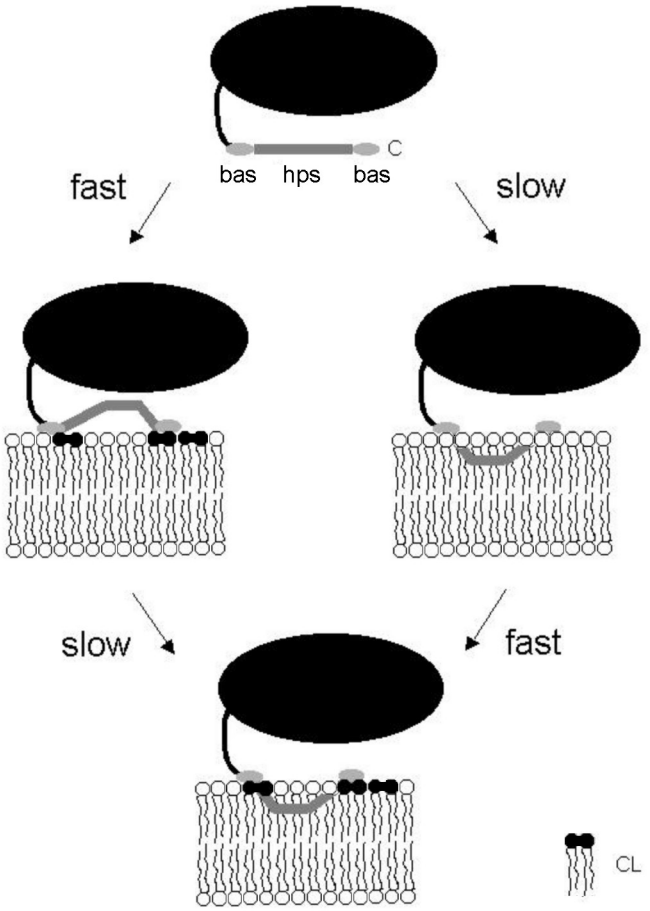

Figure 5: Putative model for membrane binding of MtCK. The model is based on (i) a biphasic binding process of MtCK to phospholipid vesicles, involving two interaction sites [122, 125] with fast (depending on polar interactions) and slow (probably depending on hydrophobic interactions) association as seen with surface plasmon resonance spectroscopy [76, 123], (ii) effects on the lipid bilayer as observed by independent methods [98, 118, 119, 143], and (iii) the temperature-dependence of binding, indicating two different association mechanisms with a participation of electrostatic and hydrophobic interactions (Schlattner and Wallimann, unpublished data). The model shows membrane binding of one out of the four monomers at each MtCK binding face. Electrostatic attraction between C-terminal basic residues (bas) and acidic cardiolipin (CL) would lead to fast binding at the membrane surface. A parallel, slower association of MtCK may involve insertion of the C-terminal hydrophobic stretch (hps) into the lipid bilayer. This could anchor MtCK more firmly in the membrane, making it also more difficult to detach from membranes and thus explaining the slow dissociation rate observed for this binding site.

ISOENZYMES OF MTCK - MOLECULAR DIVERGENCE FOR DIFFERENT FUNCTIONS?

Compartmentalized CK isoenzymes have evolved to fulfill specialized functions [6]. In fact, MtCKs exhibit several specific properties, which include octamer formation, phospholipid binding and functional or structural interaction with ANT and VDAC, respectively. In addition, there are some differences in enzyme catalysis. As compared to cytosolic isoenzymes, MtCKs have lower specific activities, different pH-profiles of enzymatic activity, as well as a pronounced substrate binding synergism [84]. The occurrence of two different tissue-specific mitochondrial CK isoenzymes, sarcomeric sMtCK and ubiquitous uMtCK, however, is a rather recent event of vertebrate evolution [6]. Accordingly, sMtCK and uMtCK show the highest amino acid sequence identity (82-85% [48]) and structural homology among all CK isoenzymes. Despite this similarity, a detailed quantitative comparison of both human MtCK isoenzymes revealed some substantial and instructive structural and functional differences [76, 85, 123]. They concern flexibility and stability of the octamer, membrane binding, enzyme catalysis, and susceptibility to molecular damage.

Overall Molecular Structure

Despite an almost identical X-ray structure of both MtCK isoenzymes (Figure 3b), some conformational differences were observed in solution as well as in 2D protein crystals. Both isoenzymes have about the same molecular mass, but differ in their Stokes radii, with sMtCK being slightly larger than uMtCK [85]. 2D-crystals of sMtCK and uMtCK obtained under identical crystallization conditions show a remarkably different packing. The parallelogram-shaped unit cell of human uMtCK contains two octamers in *p*2 symmetry with lattice parameters of a=203 Å, b=110 Å, γ=98°. This is very similar to the dimensions in solution or in 3D-crystals [67], showing that uMtCK octamers remain unchanged upon adsorption to the surface and subsequent air-drying. By contrast, the unit cell of human sMtCK comprises one octamer in *p*4 symmetry with lattice parameters of a=b=145 Å, γ=90° [85], corresponding to a flattening as compared to 3D-crystals. These differences in octamer dimension are probably due to shearing movements between the dimers, which would indicate a weaker dimer/dimer interface of human sMtCK as compared to uMtCK.

Octamer Stability

Direct observations of octamer-dimer transitions by gel filtration chromatography and tryptophan fluorescence confirmed differences between u- and sMtCK [76, 79]. The sMtCK isoenzymes showed an about 30-fold faster octamer dissociation rate and a considerably lower octamer stability [76]. Furthermore, octamer stability of sMtCK increased with temperature up to 30°C, indicating a predominant contribution of hydrophobic interactions, while it decreased with uMtCK, characteristic for the presence of additional polar dimer-dimer contacts. A molecular explanation for this functional divergence was finally found in the corresponding X-ray structures. In sMtCK, the dimer/dimer interface is almost entirely

hydrophobic, while in uMtCK it is efficiently stabilized by 12 additional hydrogen-bonded or charged interactions [67]. This is mainly due to a completely different conformation of the first 9 N-terminal residues (Figures 2 and 3b) and distinct sequence differences. The two N-termini of an uMtCK dimer extend from the monomer core, intertwine with the N-termini of the two neighboring dimers, and form extensive clamp-like contacts including Arg6, Arg7 and Tyr9. In chicken sMtCK, the N-termini fold back to their own monomer surface and the arginine residues do not form any contacts with neighboring dimers. The second uMtCK-specific region involves Arg151, which participates in two salt bridges with Glu148 and Asp155 located in the center of the hydrophobic dimer/dimer interface. In addition, the dimer/dimer interface of uMtCK is considerably larger (~1900 Å) as compared to chicken sMtCK (~900 Å) and contains additional hydrophobic interactions. These include Tyr9, Pro10, Pro11, Tyr15, Pro31, and Trp264, which are all located at the edge of the contact area, thus shielding the central electrostatic interactions of Arg151 from solvent. Interestingly, the octameric MtCK of the polychaete *Chaetopterus variopedatus* contains homologues of Arg151, Glu148 and Asp155 and thus most probably forms similar salt bridge pairs likely to stabilize the dimer/dimer interface of *Chaetopterus* MtCK [75].

Interaction with Anionic Phospholipids

An SPR analysis of MtCK/cardiolipin interactions suggested two independent binding sites in case of both MtCK isoenzymes, which differ in their association and dissociation rates and were termed “fast” and “slow” sites [76, 123, 128]. However, these sites showed slightly faster on-rates and higher affinities in case of sMtCK. This may be due to a different pattern of positive charges in the C-terminal membrane binding motif, where the C-terminal histidine of uMtCK is replaced by a lysine in sMtCK (Figure 4). By contrast, human uMtCK was able to recruit a higher number of binding sites at the vesicle surface. This may be related to the "floppy end" of uMtCK, a stretch of five C-terminal residues that is completely disordered in the crystals of human uMtCK, but not in those of chicken sMtCK [67]. Since this stretch carries the two basic residues of the C-terminal binding motif, it may be used as a highly adaptive membrane anchor for the interaction with acidic phospholipids [67].

Enzyme Catalysis

MtCK isoenzymes differ in various enzymatic properties, in particular in the physiologically relevant forward reaction (phosphocreatine synthesis), comprising pH-optima, substrate synergism, and K_m for creatine and ATP [85]. Enzymatic activity of uMtCK shows a 5-fold increase in the pH-range of 7 to 8, while sMtCK activity increased only 2-fold. Thus, pH-changes in this range would in particular affect the utilization of mitochondrial ATP by uMtCK. A pronounced substrate binding synergism was observed for all substrates, but was most pronounced for uMtCK. Here, dissociation constants for the ternary enzyme-substrate (ATP and creatine) complex were up to 45-fold lower than for the binary enzyme-substrate (ATP or creatine) complex. Substrate binding synergism is probably linked to a cross-talk between the two CK domains and the hinge-bending domain movement during catalysis (see above). The highly synergistic effect of substrate binding in uMtCK led to

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). The especially low K_m of uMtCK for creatine is most probably an adaptation to the lower creatine and phosphocreatine levels in uMtCK-expressing tissue like brain, kidney or skin, as compared to sMtCK-expressing muscle. Total (creatine + phosphocreatine) concentrations are about 12 mM in brain [150] and 25-50 mM in skeletal muscle with a proportion of 20-30% of creatine [16].

Susceptibility to Molecular Damage

Reactive oxygen species lead to various modifications of MtCK residues that affect the key properties of the kinase, such as enzymatic activity, octameric state and membrane interaction (see also below). As could be demonstrated *in vitro* with peroxynitrite and anthracycline anti-cancer drugs, sMtCK and uMtCK differ substantially in their susceptibility to these oxidative agents [151, 152]. The sMtCK isoenzyme is affected at much lower peroxynitrite and anthracycline concentrations than uMtCK. Possibly, uMtCK is protected by its superior octamer stability and recruitment of membrane binding sites.

Since the enzymatically active, membrane-bound MtCK octamer is required for full functionality of the kinase *in vivo* [78, 153], the differential susceptibility of MtCK isoenzymes would have direct functional consequences for the corresponding tissue. A more resistant uMtCK could have cell protective effects by maintaining its physiological functions even in pathological situations that generate oxidative stress. On the other hand, in tumors over-expressing uMtCK, the higher stability of this isoenzyme may contribute to the very poor prognosis of this type of cancer. Finally, molecular damage of the more susceptible sMtCK isoenzyme in heart would contribute to the pronounced tissue-specific toxicity of anthracycline drugs, which is characterized by impaired “high energy phosphate“ metabolism (see also below, “MtCK in Human Health and Disease”).

MtCK-containing Mitochondrial Microcompartments – A Dual Role in Energy Transfer and Permeability Transition?

Astonishingly enough for an organelle studied for decades, some paradigms about mitochondria have changed during recent years. Mitochondria are no longer only the cellular "powerhouse", but they are increasingly recognized as key-players in cell regulatory systems like Ca^{2+}-handling and apoptosis [154, 155]. Basic to these functions is the organization of mitochondrial membranes and sub-compartments, the distribution of proteins therein, as well as transport and diffusion pathways across the mitochondrial membranes and compartments. Many specific functions rely on large proteolipid complexes, and MtCK seems to participate in a particular type of complex with multiple functions. Here, we mainly discuss the involved structure-function relationships of MtCK. A more physiological look at these aspects is given in another contribution to this book by Brdiczka et al., while the importance of MtCK for the entire CK/phosphocreatine system and cellular metabolic networks in general is discussed in the contributions of Dzeja et al. and Saks et al.

Dual Localization of MtCK

MtCK is localized in both, the peripheral intermembrane space (IMS) and the cristae space, as observed with immunogold electron microscopy [156, 157]. Relevant to this dual localization is recent progress in fast or high pressure freezing techniques and electron microscopic tomography, which have given a new three-dimensional view of the mitochondrial organelle [158-160]. This morphology is probably much closer to the *in vivo* situation than the classical picture still found in most textbooks [161]. The IMS seems to be much narrower than generally assumed, usually smaller than 10 nm. With a height of about 9 nm, the MtCK octamer would just fit in-between the two mitochondrial membranes [158, 162-164] (Figure 6 and 7a,b). However, the enzyme is also located in the so-called contact sites, where inner and outer membranes are in even closer apposition. It is conceivable that here the octamer partially integrates into the lipid bilayer. A controversial issue has been the exchange of MtCK substrates and products with the cytosol, in particular free ADP with its known very low intracellular concentration. The outer mitochondrial membrane was long considered to be freely permeable for metabolites, since it contains abundant amounts of VDAC that can form an unspecific channel with a pore diameter of 1.8 - 3.0 nm. However, studies with mitochondria, reconstituted systems, and chemically skinned muscle fibres suggest that VDAC permeability is indeed regulated and can constitute a rate-limiting diffusion barrier. Accordingly, the IMS would form a dynamic microcompartment for adenylates [41, 165-169]. The cristae space observed in electron microscopic tomography is generally large enough to accommodate MtCK octamers attached along the cristae membranes (Figure 7c). The cristae formed by invaginations of the inner membrane show multi-branched structures with many blind-ending cisternae and are linked to the peripheral IMS by narrow openings, the so-called cristae junctions or pendiculae that sometimes are not larger than 10-15 nm. Modeling studies on metabolite diffusion suggest that these cristae junctions could constitute a diffusion limitation at least for free ADP [164].

Figure 6 – See end of the section for colour presentation.

Figure 7 – See end of the section for colour presentation.

Mitochondrial Contact Sites Contain Multiple Proteolipid Complexes

Contact sites between outer and peripheral inner membrane of mitochondria were first observed in chemically fixed mitochondria, where the intermembrane space is enlarged [170], and also as jumps in the fracture planes of freeze-fractured mitochondria [171, 172]. They fulfill various functions [173], including the import of mitochondrial precursor proteins, channeling of "high-energy" phosphates from mitochondria to the cytosol, transport of cholesterol, translocation of phospholipids, and finally integration of pro- and antiapoptotic signals and the formation of the mitochondrial permeability transition pore [174-178]. Contact sites seem to be regulated, dynamic structures. Their frequency depends on the metabolic activity of the cell [171] and they are induced by mitochondrial precursor proteins or ADP [172, 179].

The multiple function and regulation of contact sites is reflected by a complex protein composition and topology. Proteolipid complexes containing ANT and VDAC have been isolated [131] and interactions between these contact site proteins have been studied *in vitro* [130, 132, 143, 180, 181]. Depending on their specific function, ANT/VDAC complexes can recruit a large number of additional proteins [174, 182]. These include kinases that preferentially use mitochondrial ATP, either in the intermembrane space like MtCK [130, 133] and mitochondrial nucleoside diphosphate kinase (NDPK-D) [183], or at the cytosolic side of VDAC, like hexokinase and glycerol kinase [130, 184, 185]. Interestingly, some of the interacting proteins are involved in apoptotic and necrotic cell death, like cyclophilin-D at the matrix side of ANT [181, 186], cytochrome c at the intermembrane space side of VDAC [187], and members of the Bcl-2 family [178, 188-190]. Although composition, topology and function of these contact site complexes are controversial and not yet characterized in detail, it is evident that they not only participate in the transfer of metabolites, but also play a role in regulating cellular energy utilization and apoptosis. Even more, they may allow a crosstalk between apoptotic signaling and metabolism, where apoptosis is controlled by metabolic state and *vice versa* [178, 191-197].

MtCK Proteolipid Complexes: Formation, Topology and Regulation

Formation, topology and regulation of MtCK-containing ANT/VDAC complexes are not yet completely understood. Upon mitochondrial import of nascent MtCK via TIM/TOM complexes and cleavage of the targeting sequence [198], folding of MtCK immediately leads to formation of stable dimers (Figure 6a). At the given high MtCK concentration, neutral pH and the large membrane surface in the intermembrane space, MtCK should rapidly assemble into membrane-bound octamers (Figure 6b). Only octameric MtCK is able to interact simultaneously with two opposing membranes [120]. The direct interaction partners would be cardiolipin in the inner and other anionic phospholipids as well as VDAC in the outer mitochondrial membrane (Figure 6c). In addition, the high affinity of MtCK and ANT for cardiolipin would allow for close co-localization of both proteins in cardiolipin membrane patches, resulting in complexes containing octameric MtCK, VDAC, ANT and cardiolipin, as those isolated from mitochondria [130, 199]. Similarly, MtCK in the cristae space would associate only with inner membrane and ANT, either with one (Figure 7c) or possibly also both of its two membrane binding faces. The stoichiometry of MtCK-containing proteolipid complexes is not known, but the four-fold symmetry faces of the cuboidal octamer each expose four cardiolipin binding motifs, which may limit the maximal number of possible interactions. In the most probable topology, one MtCK octamer could accommodate four ANT dimers, giving rise to a molar dimer ratio of about 1:1, consistent with earlier speculations [200]. However, similar to the dynamic appearance of contact sites [172], MtCK complexes may also be transient structures with a constant turnover of components.

The enzymatically active, octameric and membrane bound state of MtCK is essential for both, formation of the described mitochondrial complexes and full *in vivo* functionality [78, 130]. An octamerization-incompetent but fully enzymatically active sMtCK mutant expressed in MtCK-deficient neonatal rat cardiomyoctes not only showed impaired membrane binding, but also reduced rates of creatine-stimulated oxidative phosphorylation [78]. Similarly, protective functions of MtCK in mitochondrial permeability transition and ultrastructure are

only observed with the MtCK octamer [199, 201, 202]. These data indicate that octamer/dimer transitions that occur under pathological conditions can modulate MtCK function. Calcium is yet another signal that seems to regulate MtCK complexes, since physiological calcium concentrations strengthen the MtCK/VDAC interaction [136]. This may improve the metabolite channeling of MtCK complexes (see below) under conditions of cytosolic calcium overload, which occur at low cellular energy state. Possibly, the calcium signal is perceived through the interaction partner VDAC, where a specific Ca^{2+} binding site has been identified [203]. It should be noted that phylogenetically ancient MtCK may form different kinds of complexes, if any, and show different regulation, in particular concerning metabolite channeling with ANT (see below). Invertebrate MtCK lacks the vertebrate cardiolipin interaction motif and may at best exhibit a different, possibly hydrophobic membrane interaction [204]. This would indicate that cardiolipin-binding and metabolite channeling with ANT has been acquired by MtCK only later at the dawn of vertebrate evolution.

MtCK Proteolipid Complexes in Metabolite Channeling

A large body of evidence supports a specific metabolic function of proteolipid complexes containing MtCK and ANT [13, 18, 22, 51, 205]. In peripheral contact sites, co-localization of MtCK with ANT, direct interaction of MtCK with VDAC, and possibly also the diffusion limitations at the outer mitochondrial membrane [28, 166, 206] create a microcompartment that maintains a privileged exchange of substrates and products, also called metabolite channeling or functional coupling [42, 53, 207] (Figure 7a,b). Here, MtCK preferentially uses mitochondrial ATP that is exported via ANT to phosphorylate creatine. *Vice versa*, the locally produced ADP is immediately re-imported into the mitochondrial matrix space via ANT, an obligatory ATP/ADP antiporter, and phosphocreatine is released into the cytosol via VDAC. However, the degree of such metabolite channeling seems to vary among different tissues, species, and developmental states [15, 208]. Recent experimental evidence for direct functional coupling between MtCK and oxidative phosphorylation comes from *in vivo* ^{31}P-MRS studies showing phosphocreatine resynthesis coupled to respiration [209], as well as from respirometry measurements with skinned muscle fibres from wild-type and transgenic mice lacking MtCK [210] and phosphocreatine generation in isolated respiring mitochondria kept without external nucleotides [53]. The latter study indicates an internal mitochondrial pool of adenine nucleotides that is constantly recycled via MtCK complexes with only minor leakage into the cytosol. Metabolite channeling within MtCK complexes would also circumvent the low diffusibility of ADP and diffusion restrictions that may exist at the mitochondrial outer membrane, both relevant at the given low intracellular free ADP concentration [168]. Furthermore, mitochondrial metabolism would not be regulated by intracellular free ADP concentration *per se*, but rather by intramitochondrial ADP production triggered via cytosolic creatine levels (Figure 1) [18, 211, 212].

In cristae, MtCK associates with ANT alone, which would still allow metabolite channeling between these two proteins (Figure 7c). Creatine and phosphocreatine, however, have to diffuse along the cristae space through the cristae junctions to reach VDAC. If the cristae junctions indeed would limit diffusion for ADP [164], this would add to the limited

permeability of the outer mitochondrial membrane and could reinforce the dynamic compartmentation of a "mitochondrial pool" of adenylates.

MtCK Proteolipid Complexes in Apoptosis and Mitochondrial Ultrastructure

Proteolipid complexes containing MtCK and ANT also exhibit a direct protective effect on mitochondrial permeability transition (MPT). This reversible and cyclosporin-sensitive process is caused by a large pore in the inner mitochondrial membrane and triggered by multiple signals, including Ca^{2+} and reactive oxygen species [213]. MPT leads to dissipation of the membrane potential, mitochondrial swelling and permeabilization of the outer membrane. The latter may trigger apoptosis by the release of pro-apoptotic proteins like cytochrome c [178, 214], or lead to necrotic cell death due to energy depletion [182, 213]. Possibly, time course, degree and abundance of MPT are decisive whether a given cell undergoes apoptotic or the necrotic cell death. *In vitro* and *in vivo*, contact site complexes containing ANT and VDAC exhibit many properties of the MPT pore [130, 132, 199] and ANT has become widely accepted as the putative pore-forming channel [174, 175, 178]. However, ANT seems not to be the only pore-forming protein of the inner membrane, since mitochondria from ANT-knockout mice still show MPT, albeit at higher Ca^{2+} concentrations and insensitive to cyclosporin [215, 216]. An involvement of MtCK in MPT and mitochondrial ultrastructure was demonstrated with liver mitochondria that were isolated either from transgenic mice that express uMtCK in their liver [217] or from control animals lacking liver MtCK [53, 153]. They clearly show that MtCK, in complexes with ANT and together with its substrates, is able to delay or even prevent Ca^{2+}-induced MPT [218]. It is not the presence of MtCK *per se* that inhibits MPT, but more precisely its enzymatic activity and its correct localization in mitochondrial complexes. No effect on MPT is observed if the enzyme is not supplied with its appropriate substrates or only added externally to mitochondria that lack endogenous MtCK [53]. This mechanism may be explained by the functional coupling of octameric MtCK to ANT. If MtCK is provided with substrate, it will maintain a high ADP concentration in the mitochondrial matrix (Figure 7a,b), which in turn is known to effectively inhibit MPT pore opening. A similar mechanism may apply for mitochondrial hexokinases, which were also reported to inhibit apoptosis [191, 192, 195, 197]. Similar to MtCK, they form complexes with VDAC and ANT and preferentially use mitochondrial ATP. Although MtCK not just acts as a “plug” between porin and ANT to prevent MPT, as speculated earlier, its presence has still an influence on number and stability of contact sites. Liver mitochondria from transgenic mice expressing uMtCK in this organ showed a three-fold increase in contact sites and increased resistance against detergent-induced lysis as compared to controls [202]. Remarkably, these transgenic livers also become largely tolerant against tumor necrosis factor TNF-α-induced apoptosis [219].

MtCK IN HUMAN HEALTH AND DISEASE – FROM MOLECULAR DAMAGE TO PATHOLOGICAL STATES?

Under situations of compromised cellular energy state, which are often linked to oxidative stress and calcium overload [220], two characteristics of MtCK are particularly relevant: its exquisite susceptibility to oxidative modifications and the compensatory upregulation of its gene expression. Both may impair or reinforce, respectively, the functions of mitochondrial MtCK complexes in cellular energy supply and protection of MPT.

MtCK - A Prime Target of Oxidative and Radical-induced Molecular Damage

All CK isoenzymes are extremely susceptible to damage by reactive oxygen and nitrogen species (ROS, RNS), including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), nitrogen monoxide (NO) and peroxynitrite (PN, $ONOO^-$) (e.g. [221-225]). In particular MtCK is a principal target of such damage, not only because if its crucial sensitivity [226-228], but also due to its mitochondrial localization. Most of the reactive species originate directly or indirectly from the activity of the mitochondrial respiratory chain, in particular under conditions of increased oxidative stress like ischemia/reperfusion injury, neurodegenerative disease, or aging [229-231]. NO is generated from L-arginine by NO synthase (NOS) in the cytosol, but also directly in mitochondria [232-234]. Superoxide originates from leaks in the respiratory chain; it dismutates to form hydrogen peroxide and can further react to form the hydroxyl radical, a potent reactive species. Alternatively, an abundance of O_2^- may react with mitochondrial NO to form the potent oxidant PN even inside the organelle [235], and there may be a threshold of regulatory versus cytotoxic function of NO and its derivatives. Mitochondrial ROS can also be generated by pharmacological interventions, e.g. with anthracyclines. This prominent class of anti-cancer drugs accumulates at the cardiolipin-rich mitochondrial inner membrane [236] and oxidizes sulfhydryl groups by direct and indirect mechanisms [151]. Oxidative stress in tissues can further induce the release of Fe^{3+} from its storage proteins, making it an available catalyst for free radical reactions [221] and thus enhancing CK inactivation [237].

Ample *in vitro* and *in vivo* evidence has demonstrated that ROS and NOS affect CK isoenzymes by enzymatic inactivation [221, 238, 239]. However, reactive species also interfere with oligomeric state and membrane binding capacity of MtCK *in vitro* [151, 152, 226] and *in vivo* ([240] and Tokarska-Schlattner, unpublished results). CK inactivation is partially reversible by reducing agents, suggesting the involvement of the highly susceptible MtCK cysteines [241]. This is also true for MtCK inactivation observed with oxygen free radicals [227, 228], NO [242], superoxide and hydrogen peroxide [243], or anthracyclines [151, 244]. To a large degree, however, CK inactivation is irreversible, especially at higher ROS concentrations or if due to PN [222]. MtCK is highly susceptible to PN treatment *in vitro* and is inactivated already at μM PN concentrations [226], well within the physiological range [245]. A PN doses dependant drop in MtCK activity is found with isolated enzyme in solution or bound to membranes, as well as with intact respiring mitochondria. Since MtCK inactivation occurs at PN concentrations much below those affecting the respiratory chain

itself, this isoenzyme is indeed a prime target of PN-induced modification and inactivation [152, 226]. PN also leads to reduced octamerization of dimeric MtCK and dose dependent dissociation of octamers into dimers [152, 226]. Long-term incubation with anthracyclines *in vitro* has similar effects on MtCK activity and octamer stability [151]. Different protection assays suggest that deleterious effects of anthracyclines at low concentrations are due to reversible oxidative damage affecting sulfhydryl groups, followed by irreversible damage due to generation of free oxygen radicals at higher drug concentrations. In addition, anthracyclines impair membrane interaction of MtCK in a dose-, time-, and drug-dependent manner [151]. This rather fast effect is mainly due to their high affinity to cardiolipin and the resulting competition with MtCK for membrane binding sites. Octamer destabilization by PN and all deleterious effects of anthracyclines are much more pronounced for sMtCK than for the uMtCK isoenzyme, a fact with potentially important functional implications [151, 152].

Molecular Basis of MtCK Damage

Oxidative modification of MtCK by PN and ROS involves cysteines, tryptophans, and at higher concentrations, also tyrosines, as suggested by the protective effect of reducing agents and changes in endogenous fluorescence spectra [226, 227]. Inactivation of MtCK occurs directly in the active site, since it can be protected by CK substrates [151, 226, 243], while reduced octamer formation is due to modifications at the dimer/dimer interface. At low PN concentrations and with oxygen radicals, the latter modifications mainly occur with free MtCK dimers, thus inhibiting octamerization [228, 240]. At higher PN concentrations, however, also the dimer/dimer interfaces inside the MtCK octamer becomes modified, thus triggering dimerization of the octameric enzyme [152, 240]. Reduced affinity of MtCK to acidic phospholipids like cardiolipin can involve different mechanisms such as dimerization of the octameric enzyme or competitive inhibition of the binding process as in case of anthracyclines. Sulfhydryl-group reagents also inhibit membrane interaction, suggesting a contribution of cysteines [119, 144].

A study using site-directed mutagenesis and mass spectrometry [152] could unambiguously identify the residues that are altered by PN treatment *in vitro*. In the active site, the conserved active site cysteine Cys278 [113] is modified by reversible S-nitrosylation and irreversible single or double oxidation, thus explaining the loss of enzymatic activity in the oxidized enzyme. Another potentially susceptible residue in the active site however, Trp223, is not modified by PN. At the dimer/dimer interface, Met267, Trp268 and/or Trp 264 are modified, the latter being the most critical single residue for octamer formation [69, 70]. Finally, the C-terminal cysteine Cys358 was found to be oxidized and may contribute to a reduced membrane affinity of the C-terminal phospholipid binding motif [152].

Impairment of MtCK in Ischemia, Cardiomyopathy and Neurodegenerative Disorders

Mitochondrial dysfunction and the production of reactive oxygen species is a common denominator of ischemia/reperfusion damage, as well as of many neuro-muscular dystrophies and neurodegenerative or other age-related diseases [230, 246-250] [220]. Since so many

different pathologies involve excessive ROS production, the exquisite susceptibility of CK to reactive species is highly relevant for human health and disease. These pathological phenotypes are often characterized by oxidative damage, poor energetic state of the cells, as well as increased apoptotic elimination. Inactivation of CK isoenzymes, as well as membrane-detached and dimerized MtCK, would interrupt the CK/phosphocreatine-shuttle and contribute to impaired cellular energetics, similar to the phenotype of double-knockout mice lacking both mitochondrial and cytosolic CK [251]. In addition, MtCK could no longer protect against mitochondrial permeability transition [53] or preserve mitochondrial ultrastructure [202].

In muscle, as seen with CK knockouts, inactivation of mitochondrial and cytosolic CK under oxidative stress would primarily affect work performance and Ca^{2+}-homeostasis [29]. With respect to MtCK, this may become detrimental especially in oxidative tissues like heart [20]. Here, a perturbation of cellular prooxidant/antioxidant balance occurs during oxygen deficiency and reoxygenation in ischemia/reperfusion injury, leading to increased levels of O_2^-, NO and PN and impaired energetic state [229, 252, 253]. Enzymatic inactivation of myofibrilar MMCK [239, 254] and sMtCK [243], as well as dissociation of sMtCK octamers *in vivo* [240] and loss of functional coupling between sMtCK, ANT and oxidative phosphorylation [255, 256] have been observed that will lead to further energy-depletion. In turn, this will affect cytosolic ion pumps (Ca^{2+}-ATPase; Na^+/K^+-ATPase), which depend on phosphocreatine-buffered ATP levels, and finally impair the ion balance, especially calcium handling [8, 29, 30, 257-259]. This would result in chronic cytosolic Ca^{2+} overload and even more pronounced ROS generation, thus entering a vicious cycle with progressive inactivation of CK and sMtCK octamer decay [240]. All these factors would also favor mitochondrial permeability transition, apoptosis and/or necrosis. Several studies with perfused heart suggest that indeed global CK inactivation directly affects heart performance [260-262]. Interestingly, a major consequence of ischemic preconditioning, a cardioprotective treatment [263], is a higher flux through the CK system [264] and preservation of functional coupling between sMtCK and ANT [256, 265].

Decreased functionality of CK at sites of energy production and utilization may contribute to alterations in energy fluxes and calcium homeostasis in congestive heart failure [266, 267]. Work with MMCK/sMtCK double knock-out mice showed that below a certain level of CK activity, increases in cardiac work become energetically less efficient [268]. In animal models of dilated cardiomyopathy, hypertrophy and heart failure, impairment of the CK/phosphocreatine system preceded the development of contractile dysfunction and led to decreased energy reserve [269-271]. A decrease of the sMtCK/beta-actin ratio was linearly related to a reduction of CK flux rate in failing heart [272], and a 4-fold decrease of sMtCK activity and protein content was observed in an animal model using aortic banding [273]. Oxidative damage of sMtCK may also play a role in acute and chronic cardiotoxicity of anthracyclines, which severely limit the clinical use of these anti-cancer drugs [274]. Cardiac injury has been related to the impairment of mitochondrial functions, such as respiratory rate and generation of high-energy phosphates. In fact, anthracyclines like doxorubicin and idarubicin lead to oxidative damage and functional impairment of the cardiac sMtCK *in vitro* [151] and *in vivo* [247], while the uMtCK isoenzyme is much less affected.

The pathogenesis of many neurodegenerative and neuro-muscular diseases has been linked to mitochondrial dysfunction [248]. This is due to abnormalities in the respiratory chain or altered superoxide dismutase, leading to generation of ROS, a deteriorated energetic

state and finally to necrotic and apoptotic cell death [230]. Due to the exclusive susceptibility to ROS, CK inactivation and MtCK octamer destabilization are likely to occur and MtCK functions in energy buffering and possibly in the control of MPT will be compromised [275]. In fact, an aberrant cytosol/membrane partitioning of CK, as well as CK inactivation were observed in Alzheimer's disease [276, 277] and amyotrophic lateral sclerosis (ALS) [278]. In ALS, a mutation in Cu/Zn superoxide dismutase which stabilizes PN may lead to enhanced nitration and inactivation of important target enzymes, including uMtCK [279]. Supportive to a role of the CK system in these disorders is the protective effect of creatine supplementation that has been observed in several studies, including human Duchenne muscular dystrophy [280-282], mitochondrial cytopathies [283], myophosphorylase deficiency [284], and in animal models of amyotrophic lateral sclerosis [285-287], Huntington's disease [288, 289], Parkinsonism [290, 291], as well as in brain ischemia [292, 293]. However, uMtCK may not be essential for all of these effects, as shown with uMtCK knockout mice [294]. Creatine supplementation is now entering first clinical trials as a potentially useful adjuvant therapy to ameliorate the phenotype of many of these diseases [220, 295].

MtCK Overexpression in Mitochondrial Myopathies, Aging and Cancer

In contrast to a functional and structural impairment of MtCK by oxidative modification, overexpression of these isoenzymes may represent a mechanism to compensate for a low energy state. In patients with mitochondrial cytopathies, like MELAS, MERF or CPEO, the sMtCK isoenzyme is highly upregulated and forms regular, mostly multi-layer structures in the mitochondrial cristae [296]. These crystalline intramitochondrial inclusion are the well known hallmarks of this class of diseases, and they disappear in these patients after treatment with creatine [297]. Similar sMtCK enriched inclusion bodies have been observed in the mitochondria of creatine-depleted adult rat cardiomyocytes [298] and of rat skeletal muscle that was creatine-depleted by feeding the animals the creatine analogue guanidino propionic acid [299]. Apparently, the deteriorated energetic state of these cells triggers an unknown feedback-mechanism that leads to a compensatory upregulation of sMtCK expression to an extend that favors protein crystallization.

Upregulation of sMtCK in heart has been found in chronic restraint stressed rats, which show severe cardiac dysfunction, impaired mitochondrial respiration and apoptotic cell death [300]. Another, almost 4-fold upregulation of sMtCK has been detected in gastrocnemius of aged mice [301], together with mitochondrial dysfunction, reduced glycolysis, and the induction of genes involved in (oxidative) stress response. The low cellular energy state observed in these studies again suggest sMtCK upregulation as a compensatory mechanism that aims at improving oxidative energy metabolism. Interestingly, upregulation of sMtCK in aged mice was entirely inhibited by caloric restriction [301]. This nutritional regime delays the aging process by increasing protein turnover, reducing oxidative stress and thus decreasing macromolecular damage [250].

Overexpression of uMtCK has been reported for several tumors with poor prognosis [302-304]. Namely in a Hodgkin’s disease cell line, uMtCK, but not cytosolic BBCK, is one of the very few genes that is specifically upregulated [305]. Addition of cyclocreatine, that is known to inhibit CK-expressing tumors by yielding a dead-end phospho-compound, also inhibited growth of the Hodgkin’s cells, indicating some important function of uMtCK in

these tumors. Increased levels of uMtCK levels in malignant cells may be part of a metabolic adaptation of cancer cells [306] to perform high growth rates under oxygen and glucose restriction as typical for many tumors. Increased MtCK levels could help to sustain high energy turnover, but would be also protective against stress situations like hypoxia and possibly protect cells from apoptosis. Indeed, protection of cells from anoxia was reported after addition of creatine to hippocampal brain slices, which already express CK at high levels [307]. ATP levels and membrane potentials were sustained much longer in the creatine perfused slices compared to controls, and brain damage could thus be avoided [308]. Similarly, transgenic liver expressing CK and supplemented with creatine was protected from many deleterious metabolic insults, including ischemia, hypoxia or endotoxins perfusion [309, 310]. Tumor necrosis factor-challenged livers overexpressing uMtCK also showed a clear reduction of necrotic and apoptotic cell death [219]. If uMtCK would protect tumor cells from apoptotic elimination, this could explain why uMtCK overexpressing tumors are very aggressive and renowned for a poor prognosis. Creatine supplementation of patients with MtCK-bearing tumors could potentially promote the persistence of such cancers, provided they express creatine transporter. However, all available studies show a slight but significant inhibition of cancer cell growth with creatine supplementation *in vitro* [311] as well as *in vivo* [312, 313].

CONCLUSIONS AND OUTLOOK

Recent years have seen many achievements towards a molecular description of structure and function of MtCK and CK isoenzymes in general. However, despite or rather because of this progress, still some old questions remain unsolved and new avenues of research are open for the future. Progress in a given scientific field is often linked to either new methodological and technological achievements or pure serendipity. Heterologous expression of CK isoenzymes [63, 85, 314], improving the quality of protein crystals suitable for X-ray crystallography, as well as the development of phase extension methods to ameliorate resolution of the first CK structure [56] have contributed to obtain molecular structures for all CK isoenzymes [56, 61-64, 67]. Interestingly, it was a mitochondrial CK and an enzyme from chicken that had never been used before in crystallization trials, which was solved first. The transition state structures of homologous arginine kinase [57] and a dimeric CK [66], showing for the first time the "closed" conformation of the enzyme, have already provided some important insights and stimulating clues into the functioning of the enzyme, without however entirely explaining the catalytic mechanism. Further high resolution structures of the kinase in presence of different substrates would be helpful for our understanding of such bimolecular reactions. They may also be useful for the design of CK-specific inhibitors, likely to be of experimental and clinical relevance, considering the possible role of uMtCK in tumor progression and malignancy.

The CK X-ray structures have already inspired may new structure/function studies. Besides classical methods to determine molecular properties of CK, there has been a significant contribution by new approaches like surface plasmon resonance and fluorescence spectroscopy. Novel applications for these techniques allow to quantitatively determine interactions of a soluble protein like CK with other proteins, lipid membranes, and even

integral membrane proteins [76, 136]. This will help to understand the specific role of MtCK in the cellular regulatory and energy transducing network. In fact, MtCK phosphotransfer contributes to a cellular energy transfer network between ATP-generating mitochondria and ATP-dependent processes in sarcoplasmic reticulum, myofibrils, and nuclei [17, 36, 38, 44, 45, 315].

An especially exciting aspect for the molecular physiology of mitochondrial CK is the change of paradigms concerning the mitochondrial organelle. From a "simple" ATP-generating system, mitochondria are steadily evolving to a complex integrator of cellular signaling pathways, especially for apoptotic cell death and calcium homeostasis, as well as to a central factor in many different pathologies and aging [154, 155, 176, 182, 248]. By its localization in mitochondrial contact site complexes, MtCK interferes with some of these processes like regulation of oxidative phosphorylation and mitochondrial permeability transition [53, 130, 153, 210]. Similar to hexokinase at the cytosolic side of VDAC, MtCK may integrate metabolic and apoptotic signals to mount an appropriate cellular response [178, 191-197]. In this respect, the exquisite susceptibility of MtCK for oxidative damage and the concurrent impairment of the CK system may be of prime importance for human health and disease.

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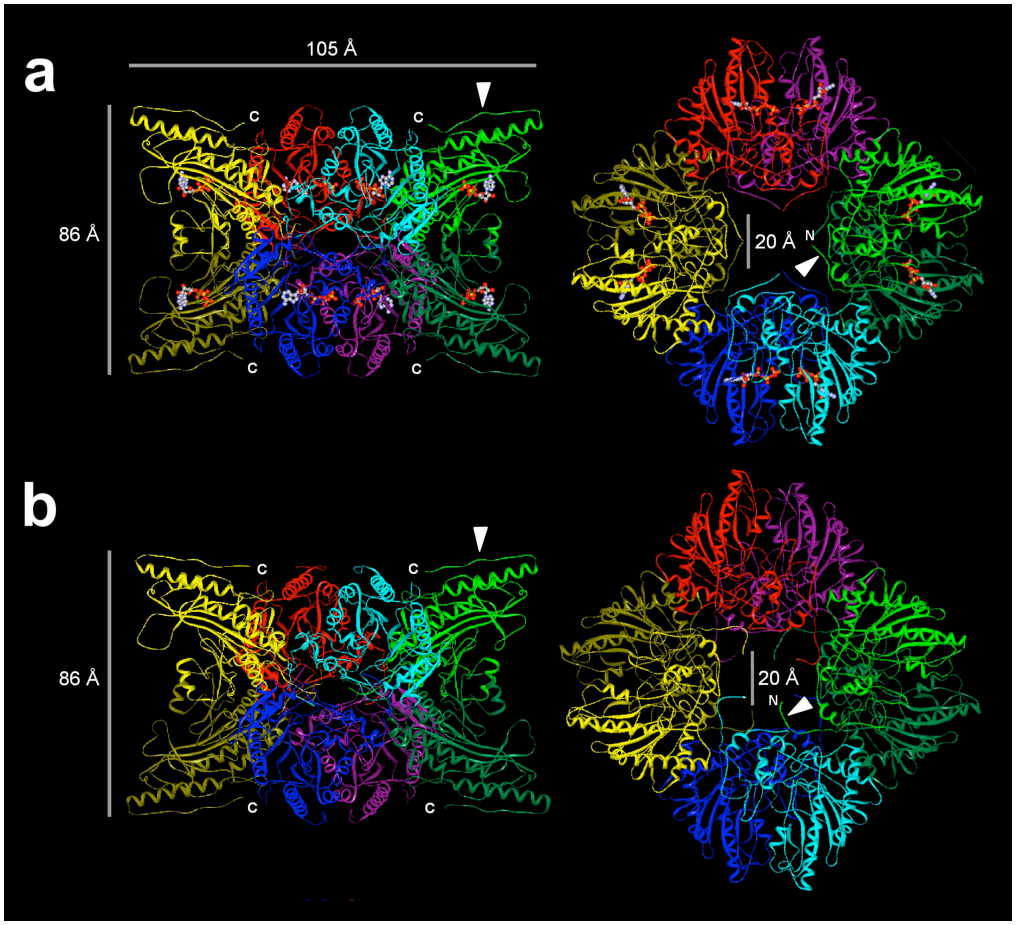

Figure 2: Molecular structure of MtCK. The octameric structure of **(a)** chicken sarcomeric sMtCK with ATP (solved at 3 Å; [56]) and **(b)** human ubiquitous uMtCK (solved at 2.7 Å; [67]). The side views (left) show a two-fold symmetry; the arrows indicate one of the eight C-terminal stretches that are involved in binding to anionic phospholipids. The top or bottom views (right) reveal a 4-fold rotational symmetry of the dimers arranged around a 20 Å large central channel. The arrows indicate one of the eight N-termini. Note back folding of the N-termini to the same dimer in sMtCK (a) and their contacts with neighboring dimers in uMtCK, thus enhancing dimer/dimer contact (b). All molecules are depicted in a schematic backbone representation with monomers in different colors.

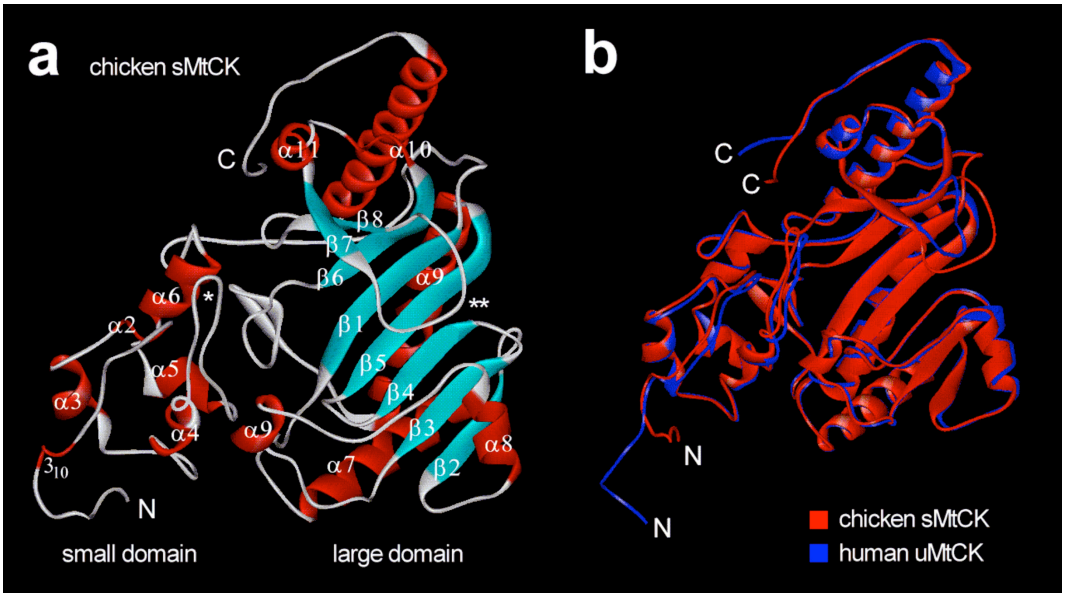

Figure 3: Chain fold of MtCK isoenzymes. (a) Schematic backbone representation of a chicken sMtCK monomer showing the general chain fold with all secondary structure elements as indicated (α-helices in red, β-sheets in cyan) and the flexible loops at positions 61-65 (*) and 316-326 (**); note: α1 in sMtCK is a 3_{10} helix. The general order of secondary elements in MtCK is: N - α1(3_{10}) - α2 - α3 - α4 - α5 - α6 - β1 - α7 - β2 - α8 - α9 - β3 - β4 - β5 - α10 - β6 - α11 - α12 - β7 - β8 - α13 - C. **(b)** Superposition of monomers of chicken sMtCK (red) and human uMtCK (blue) in schematic backbone representation indicate the extensive structural homology.

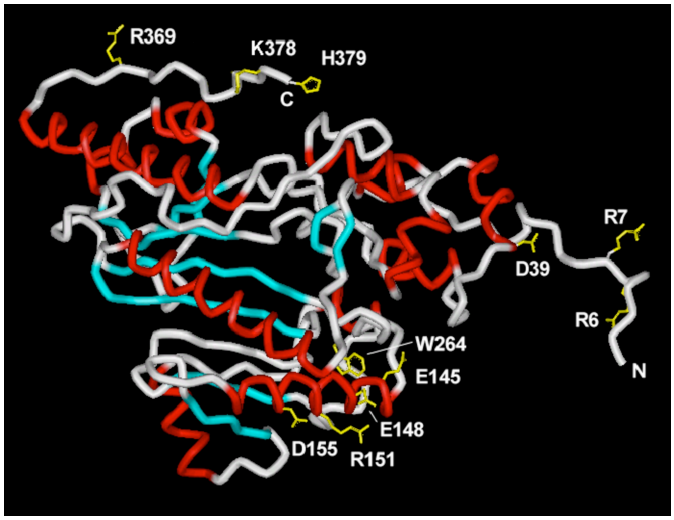

Figure 4: MtCK residues involved in octamer stability and cardiolipin binding. Backbone representation of a human uMtCK monomer colored according to secondary structure (α-helices in red, β-sheets in cyan) with side chains of key residues (yellow) involved in dimer/dimer interaction (bottom right, according to [67]) and cardiolipin-binding (C-terminal, in analogy to human sMtCK [143]).

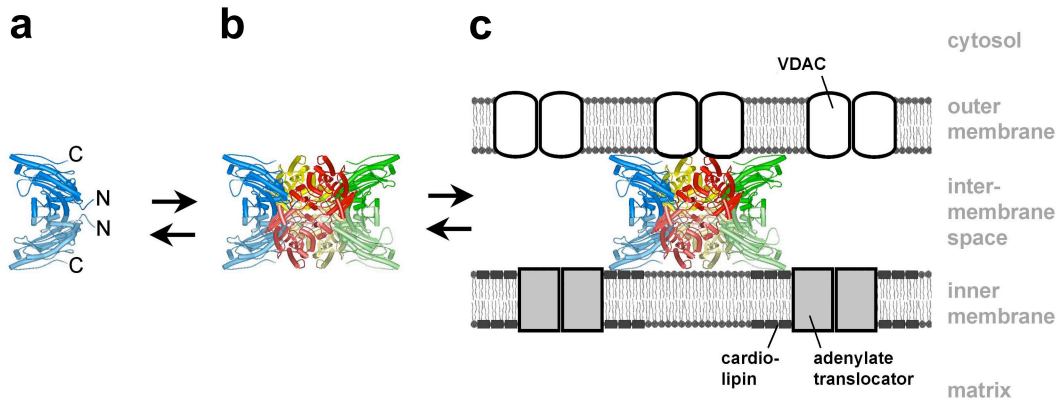

Figure 6: MtCK in the mitochondrial intermembrane space. The model shows a cartoon representation of chicken sMtCK. **(a)** After import of nascent MtCK into the mitochondrial intermembrane space and cleavage of the mitochondrial targeting sequence (prepeptide), MtCK assembles into dimers. **(b)** Dimers rapidly associate into octamers; this is a reversible reaction, but octamers are strongly favored at the pH and MtCK concentration present at this location. **(c)** Octameric MtCK binds to mitochondrial membranes and assembles into proteolipid complexes containing VDAC and ANT. Principal receptors of MtCK are cardiolipin in the inner and VDAC together with other anionic phospholipids in the outer membrane. MtCK can therefore mediate contacts between inner and outer membrane as evidenced by MtCK-induced crosslinking of phospholipid vesicles [120, 125]. Interaction of MtCK with ANT is most likely indirect and involves common cardiolipin patches (see dark rectangles). The membrane bound state of octameric MtCK is strongly favored by the large membrane surface and the high affinity of MtCK to cardiolipin and VDAC [76, 136]. Binding of dimers to phospholipid membranes occurs with much lower affinity and probably involves octamerization while bound to the membrane.

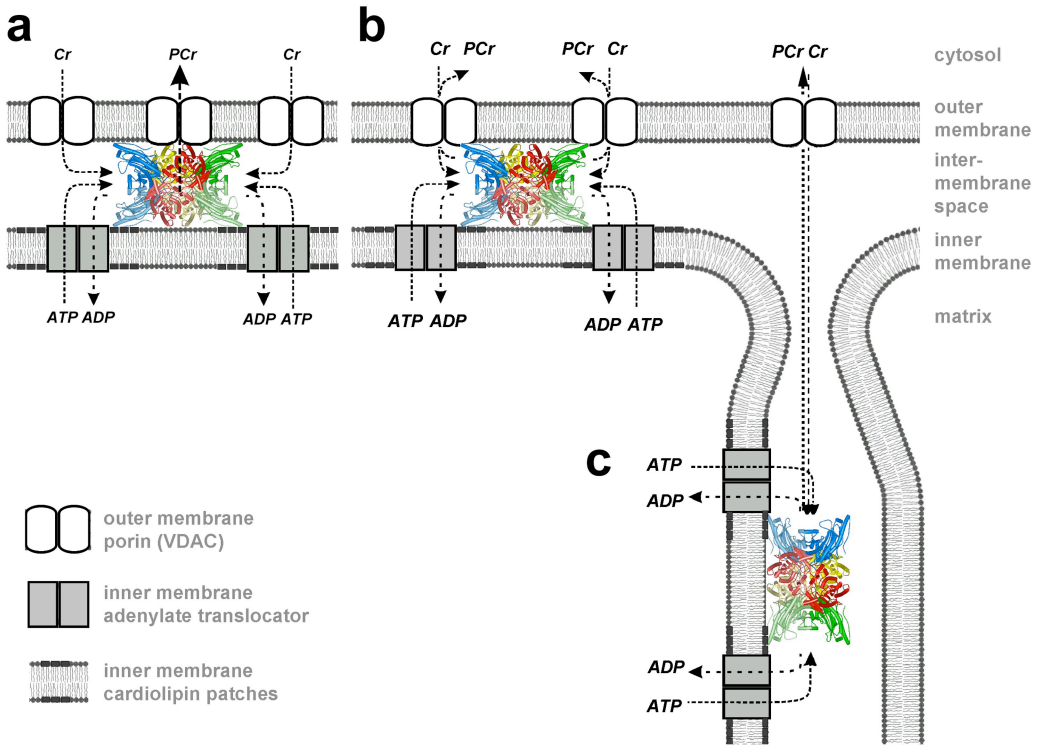

Figure 7: Dual localization and putative metabolite routes of MtCK microcompartments. MtCK is found in the intermembrane space, mainly in the so-called mitochondrial contact sites associated with ANT and VDAC **(a,b)**, as well as in the cristae space associated with ANT only **(c)**. This allows for a direct exchange of MtCK substrates and products which is depicted by arrows. **(a)** In contact sites, phosphocreatine may escape by a "backdoor" seen in the MtCK structure into the central channel linked to a VDAC molecule in the outer membrane [5]. This scheme would facilitate a directed, vectorial transport of "high energy" phosphate into the cytosol. **(b)** Alternatively, all metabolites may traverse the intermembrane space, which would involve a lower degree of metabolite channeling. **(c)** In cristae, only ATP/ADP exchange would occur as in contact sites, while creatine and phosphocreatine would have to diffuse along the cristae space and through the cristae junctions to reach VDAC.