

Review

Mitochondrial creatine kinase in human health and disease

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Abstract

Mitochondrial creatine kinase (MtCK), together with cytosolic creatine kinase isoenzymes and the highly diffusible CK reaction product, phosphocreatine, provide a temporal and spatial energy buffer to maintain cellular energy homeostasis. Mitochondrial proteolipid complexes containing MtCK form microcompartments that are involved in channeling energy in form of phosphocreatine rather than ATP into the cytosol. Under situations of compromised cellular energy state, which are often linked to ischemia, oxidative stress and calcium overload, two characteristics of mitochondrial creatine kinase are particularly relevant: its exquisite susceptibility to oxidative modifications and the compensatory up-regulation of its gene expression, in some cases leading to accumulation of crystalline MtCK inclusion bodies in mitochondria that are the clinical hallmarks for mitochondrial cytopathies. Both of these events may either impair or reinforce, respectively, the functions of mitochondrial MtCK complexes in cellular energy supply and protection of mitochondria from the so-called permeability transition leading to apoptosis or necrosis.

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1. Introduction

Creatine kinase (CK) is a central controller of cellular energy homeostasis. By reversible interconversion of creatine into phosphocreatine, CK builds up a large pool of rapidly diffusing phosphocreatine for temporal and spatial buffering of ATP levels. Thus, CK plays a particularly important role in tissues with large and fluctuating energy demands like muscle and brain, with the mitochondrial isoenzyme of CK (MtCK) being important for the energetics of oxidative tissue (see Section 2.). Pathological situations often alter MtCK functioning, either directly impairing MtCK by oxidative and radical damage (see Section 3), or up-regulating MtCK expression as a compensatory measure of impaired energy state (see Section 4). These changes associate CK and MtCK dysfunction with pathological changes in cellular energy state, and give an estimate on the importance of CK for cellular energetics in general. This overview will focus on (patho-)physiological aspects of MtCK, but also refers to a number of recent reviews on other aspects of CK structure and physiology [1–10].

2. Mitochondrial creatine kinase—an enzyme central to cellular energetics

2.1. Interplay of mitochondrial and cytosolic creatine kinase isoenzymes

The existence of tissue and compartment-specific isoenzymes of creatine kinase (CK) is an important property of this long-known enzyme [11] and a key to its functions in cellular energy metabolism. Most vertebrate tissues express two CK isoenzymes, a dimeric cytosolic and a mostly octameric MtCK that is localized in cristae and intermembrane space [1,12,13] (Fig. 1). Mitochondrial and cytosolic CK have diverged at least 670 million years ago, [4], suggesting that compartmentalized CK isoenzymes have evolved very early to fulfill specific functions. By contrast, the occurrence of two tissue-specific MtCK isoenzymes [14] 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 and sperm [15–17].

The interplay between cytosolic and mitochondrial CK isoenzymes fulfills multiple roles in cellular energy homeo-

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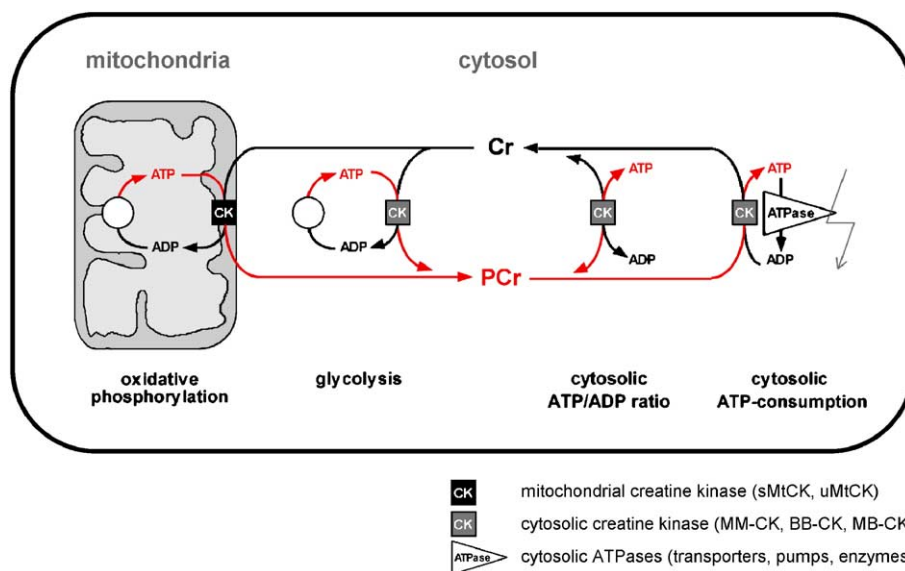


Fig. 1. The creatine kinase/phosphocreatine system. Compartment-specific isoenzymes of creatine kinase (CK) are found in mitochondria (sMtCK, uMtCK, left) and cytosol (MM-CK, BB-CK, MB-CK, right). They are either associated with ATP-delivering processes (oxidative phosphorylation or glycolysis, left) and ATP-consuming processes (ATPases, to maintain local ATP/ADP ratios, rightmost), or occur in soluble form (to maintain global cytosolic ATP/ADP ratios, center right). A large cytosolic phosphocreatine (PCr) pool of up to 30 mM is built up by CK from creatine (Cr), using ATP from oxidative phosphorylation (e.g., in heart) or glycolysis (e.g., in fast-twitch glycolytic muscle). The large phosphocreatine pool is then used as a temporal energy buffer to maintain constant global and local ATP/ADP ratios over a wide range of workloads. The higher diffusibility of phosphocreatine, as compared to ATP, together with localized CK isoenzymes, is used for spatial energy buffering, i.e., for an energy shuttle between ATP-providing or-consuming processes. The latter seems to be most important for cells that are polarized and/or have very high or localized ATP consumption.

stasis [3,5,18–23] (Fig. 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 concentrations. 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 [18]. 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, forming microcompartments which often facilitate a direct exchange of ADP and ATP between the association partners without mixing with bulk solution, a process termed metabolite channeling or functional coupling [24,25]. This differential microcompartmentation of CK isoenzymes 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 (see Section 2.4). Due to 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” [13,26,27] or “energy circuit” [18], bridging sites of energy generation with sites of energy consumption (Fig. 1). Although some of these functions have been a matter of debate [28–30], there is growing evidence for CK and creatine microcompartments, as well as for the presence of a CK/phosphocreatine shuttle at least in large and polar cells. 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 [5,31–36]. The most convincing evidence comes from Ca^{2+} -handling deficiencies in CK knockout mice [37,38], in vivo analysis of polar cells like spermatozoa [39,40], direct in vivo ^{18}O labeling of phosphoryl moieties in intact muscle [41,42], ^{31}P - and ^1H -NMR in perfused heart [34,43,44], experiments with chemically skinned muscle fibers [7,36], as well as in silico modeling approaches [45–47].

2.2. Dual localization of creatine kinase microcompartments in mitochondria

Mitochondria are no longer only the cellular “powerhouse”, but they are increasingly recognized as key-players in cellular regulatory systems like Ca^{2+} -handling and apoptosis [48,49]. 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 their structure and localization, while a more physiological look at these aspects is given in the contribution of Brdiczka et al. in this issue.

MtCK is localized in both, the peripheral intermembrane space (IMS) and the cristae space, as observed with immunogold electron microscopy (Fig. 2) [50,51]. Recent evidence with rapid freezing techniques and electron microscopic tomography confirmed that the IMS is much narrower than

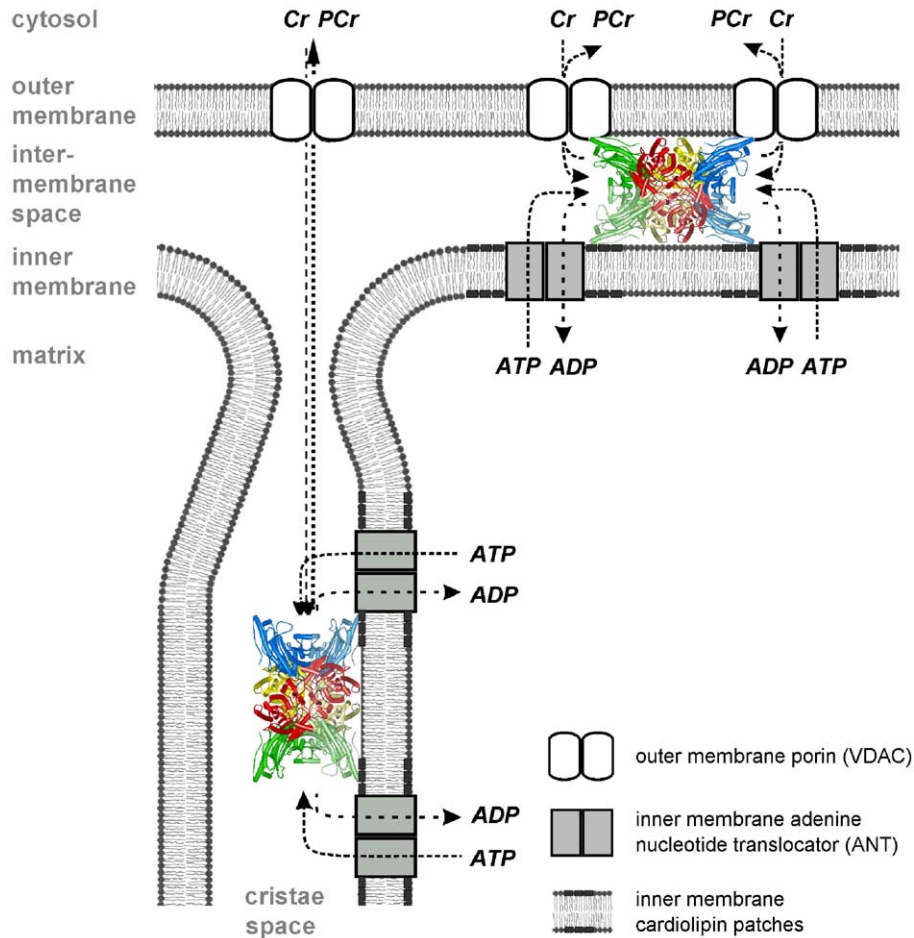


Fig. 2. MtCK microcompartments: dual localization and putative metabolite routes. MtCK associates into octamers that bind to mitochondrial membranes and form proteolipid complexes with VDAC and ANT (“contact site complexes”, top right) or with ANT alone (“cristae complexes”, bottom left). Interaction of MtCK with ANT is most likely indirect and involves common cardiolipin patches (see dark rectangles), while interaction with VDAC is direct and regulated by Ca^{2+} [83]. The membrane bound state of MtCK is strongly favored by the large membrane surface and the high affinity of octameric MtCK to cardiolipin and VDAC [81,83]. The proteolipid complexes allow for a direct exchange of MtCK substrates and products (substrate or metabolite channeling, depicted by arrows). In contact site complexes (top right), this substrate channeling allows for a constant supply of substrates and removal of products at the active sites of MtCK. In cristae complexes (bottom left), only ATP/ADP exchange is facilitated through direct channeling to the MtCK active site, while creatine (Cr) and phosphocreatine (PCr) have to diffuse along the cristae space to reach VDAC. (MtCK is shown as a cartoon representation of chicken sMtCK, PDB 1CRK [53], with dimers in different colors and monomers of the same dimer in different color intensities.)

generally assumed [52]. With a height of about 9 nm [53], the MtCK octamer would just fit in-between the two mitochondrial membranes (Fig. 2) [52,54]. A controversial issue has been the exchange of MtCK substrates and products with the cytosol, in particular free ADP with its known very low μM intracellular concentration. The outer mitochondrial membrane was long considered to be freely permeable for metabolites, since it contains abundant amounts of voltage-dependent anion channel (VDAC or porin) 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 fibers 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 [46,55–58]. The cristae space observed in electron microscopic tomography is generally large enough to accommodate MtCK octamers attached along the cristae membranes (Fig. 2). 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 pediculi 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 [54].

2.3. MtCK proteolipid complexes in contact sites

Contact sites, where outer and peripheral inner membrane of mitochondria seem to be connected, were first observed in chemically fixed mitochondria that show an enlarged intermembrane space [59], and also as jumps in the fracture planes of freeze-fractured mitochondria [60,61]. Such contact sites are formed by proteolipid complexes that fulfill various functions [62], including the import of mitochondrial precursor proteins and the channeling of “high-energy” phosphates from mito-

chondria to the cytosol. Contact sites seem to be regulated, dynamic structures, since their frequency depends on the metabolic activity of the cell [60] and they are induced by the presence of mitochondrial precursor proteins or ADP [61,63]. Proteolipid complexes containing adenine translocator (ANT) in the inner membrane and VDAC in the outer membrane have been isolated [64] and interactions between these contact site proteins have been studied *in vitro* [65–68]. Such ANT/VDAC complexes can recruit a large number of additional proteins [69], like kinases that preferentially use mitochondrial ATP in the intermembrane space (e.g., MtCK or mitochondrial NDP kinase-D [70]) or at the cytosolic side of VDAC (e.g., hexokinase and glycerol kinase [65]). Some other interacting proteins are involved in apoptotic and necrotic cell death, like cyclophilin-D at the matrix side of ANT [67,71], cytochrome *c* at the intermembrane space side of VDAC [72,73], and members of the Bcl-2 family [74]. Although composition and function of these contact site complexes are controversial, 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 cross-talk between apoptotic signaling and metabolism, where apoptosis is controlled by metabolic state and vice versa [73–79].

Formation, topology, and regulation of MtCK-containing complexes are not yet completely understood. Upon mitochondrial import of nascent MtCK via TIM/TOM complexes and cleavage of the targeting sequence [80], folding of MtCK immediately leads to formation of stable dimers. 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 [81]. Only octameric MtCK shows a high affinity to cardiolipin and other anionic phospholipids and is therefore able to bind to membranes and to cross-link the two peripheral mitochondrial membranes (Fig. 2) [82]. The direct interaction partners would be cardiolipin in the inner and VDAC in the outer mitochondrial membrane. 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 [1,81,83]. Indeed, such complexes have been isolated from mitochondria [65,84]. Similarly, MtCK in the cristae space would associate only with inner membrane and ANT, either with one (Fig. 2) or possibly also both of its two membrane binding faces. According to the dynamic appearance of contact sites in general [61], MtCK complexes may be transient structures with a constant turnover of components. For example, dimerization of octameric MtCK would detach it from membranes and thus disintegrate the complexes. However, the enzymatically active, octameric and membrane bound state of MtCK is essential for full *in vivo* functionality [65,85]. An octamerization-incompetent but fully enzymatically active sMtCK mutant expressed in MtCK-deficient neonatal rat cardiomyocytes not only showed impaired membrane binding, but also reduced rates of creatine-stimulated oxidative phosphorylation [85]. Similarly, protective functions of MtCK in mitochondrial permeability transition and ultrastructure are only observed with the MtCK octamer

[8,84,86]. These data indicate that octamer/dimer transitions that occur at least under pathological conditions (see Section 3) can modulate MtCK function. Calcium is yet another signal that seems to regulate MtCK complexes, since physiological calcium concentrations strengthen the MtCK/VDAC interaction [83]. This may improve the metabolite channeling of MtCK complexes (see Section 2.4) 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 [87].

2.4. Function of MtCK proteolipid complexes in metabolite channeling and apoptosis

A large body of evidence supports a specific metabolic function of proteolipid complexes containing MtCK and ANT [18,21,25,88]. 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 [56] create a microcompartment that maintains a privileged exchange of substrates and products, also called metabolite channeling or functional coupling [6,47,89] (Fig. 2). 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 then released into the cytosol via VDAC. However, the degree of such metabolite channeling seems to vary among different tissues, species, and developmental states [3,90]. 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 [91], as well as from respirometry measurements with skinned muscle fibers from wild-type and transgenic mice lacking MtCK [92], and phosphocreatine generation in isolated respiring mitochondria kept without external nucleotides [6]. 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 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 [57]. Furthermore, mitochondrial metabolism would not be regulated by intracellular free ADP concentration per se, but rather by intra-mitochondrial ADP production triggered via cytosolic creatine [21,93,94].

In cristae, MtCK associates with ANT alone, which would still allow metabolite channeling between these two proteins (Fig. 2). 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 [54], this would add to the limited permeability of the outer mitochondrial membrane and could reinforce the dynamic compartmentation of a mitochondrial pool of adenylates. 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 [95]. 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* or lead to necrotic cell death due to energy depletion [69,74,95]. 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 [65,66,84] and ANT has become widely accepted as a pore-forming channel [69,74,96]. While outer membrane VDAC seems to participate in MPT [97], the molecular nature of the inner membrane MPT pore is still disputed [98–100]. 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 [101,102].

An involvement of MtCK in MPT and mitochondrial ultrastructure has been demonstrated with liver mitochondria that were isolated either from transgenic mice that express uMtCK in their liver [103] or from control animals lacking liver MtCK [6,104]. These experiments clearly show that MtCK, in complexes with ANT and together with its substrates, is able to delay or even prevent Ca^{2+} -induced MPT [105]. 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 if it is only added externally to mitochondria that lack endogenous MtCK [6]. This can 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 (Fig. 2), which in turn is known to effectively inhibit MPT pore opening. Thus, MtCK could play the role of an energy sensor, coupling cellular energy state to programmed cell death. A similar mechanism may apply for the anti-apoptotic effect of hexokinase [75–79], which is located at the cytosolic side of VDAC or VDAC/ANT complexes and also preferentially uses mitochondrial ATP.

Finally, the presence of MtCK seems to have 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 [86]. Remarkably, these transgenic livers also become largely tolerant against tumor necrosis factor TNF- α -induced apoptosis [106].

3. Mitochondrial creatine kinase—from molecular damage to pathological states?

Mitochondrial dysfunction and the production of reactive oxygen and nitrogen species (ROS, RNS) are common

denominators of ischemia/reperfusion damage, as well as of many neuro-muscular dystrophies and neurodegenerative or other age-related disorders [107–110]. Their pathological phenotypes are often characterized by oxidative damage, poor energetic state of the cells, Ca^{2+} overload, as well as increased apoptotic elimination. The exquisite susceptibility of CK to reactive species is therefore highly relevant for the etiology of these diseases. Inactivation of CK isoenzymes, as well as membrane detachment and dimerization of 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 [111], and could no longer protect against mitochondrial permeability transition [6].

3.1. MtCK—a prime target of oxidative and radical-induced molecular damage

All CK isoenzymes are extremely susceptible to damage by ROS and RNS, including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\bullet), nitrogen monoxide (NO) and peroxynitrite (PN, ONOO^-) (e.g., [112–116]). MtCK in particular is a principal target of such damage, not only because of its crucial sensitivity [117–119], 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 [108,120,121]. NO is generated from L-arginine by NO synthase in the cytosol and possibly also in mitochondria [122]. 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 [123], 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, a prominent class of anti-cancer drugs [124] (see Section 3.3). 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 [112] and thus enhancing CK inactivation [125].

Ample in vitro and in vivo evidence has demonstrated that ROS and RNS affect CK isoenzymes by enzymatic inactivation [112,126,127]. However, these reactive species also interfere with oligomeric state and membrane binding capacity of MtCK in vitro [117,124,128] and in vivo [129,130]. MtCK inactivation observed with oxygen free radicals [118,119], NO [131], superoxide and hydrogen peroxide [132], or anthracyclines [124,133] is partially reversible by reducing agents. To a large degree, however, CK inactivation is irreversible, especially at higher ROS concentrations or if caused by to PN [113]. MtCK is particularly susceptible to PN treatment in vitro and is inactivated already at μM PN concentrations [117], well within the physiological range [134]. A PN dose-dependant drop in

MtCK activity is found with isolated enzyme in solution or bound to membranes, as well as with intact respiring mitochondria [117]. 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 [117,128]. PN also leads to reduced octamerization of dimeric MtCK and dose dependent dissociation of octamers into dimers [117,128]. The sMtCK octamers are more susceptible to PN than those of uMtCK [128], indicating that deleterious PN effects may be more pronounced in sMtCK-expressing heart and skeletal muscles.

Oxidative modification of MtCK by ROS and RNS involves cysteines, tryptophans, and at higher concentrations, also tyrosines, as suggested by the protective effect of reducing agents and changes in endogenous fluorescence spectra [117,118]. Inactivation of MtCK occurs directly in the active site, since it can be protected by CK substrates [117,124,132], 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 [119,129]. At higher PN concentrations, however, also the dimer/dimer interfaces inside the MtCK octamer becomes modified, thus triggering dimerization of the octameric enzyme [128,129]. Reduced affinity of MtCK to acidic phospholipids, like cardiolipin, caused by free radical damage, 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 [68,135,136].

In a recent study using site-directed mutagenesis and mass spectrometry [128], one could unambiguously identify the MtCK residues that are altered by PN treatment in vitro (Fig. 3). In the active site, the conserved active site cysteine Cys278 [137] is modified by reversible S-nitrosylation and irreversible single or double oxidation, thus explaining the entire loss of enzymatic activity in the oxidized enzyme. At the dimer/dimer interface, however, Met267, Trp268 and/or Trp264 are modified, the latter being the most critical single residue for octamer formation [138,139]. Finally, the C-terminal cysteine Cys358 was also found to be oxidized and may contribute to a reduced membrane affinity of the C-terminal phospholipid binding motif [68,128].

3.2. Impairment of MtCK in ischemia and cardiomyopathy

Heart displays an amazingly high metabolic homeostasis despite large fluctuations in work load, a phenomenon that Peter Hochachka has called “stability paradox” [140]. CK isoenzymes provide a temporal and spacial “energy shuttle” between different cellular compartments and thus play an important role in cardiac energy homeostasis [21] (see Section 2.1). In particular, a functional MtCK isoenzyme seems to be important for optimal cardiac function and coupling between energy generating and energy consuming processes [34,92,141]. Heart

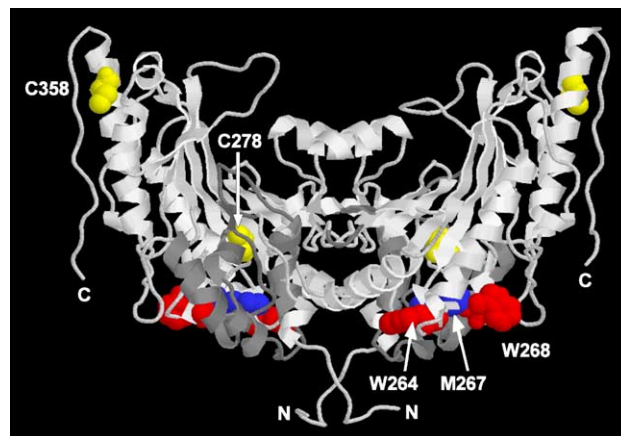


Fig. 3. Oxidative molecular damage of MtCK. Spatial localization of peroxynitrite-modified residues in the molecular structure of a dimer of human uMtCK (backbone representation, PDB 1QK1 [186]). The dimer/dimer interface that would connect to neighboring dimers in the octameric structure (not shown) is situated at the lower, convex side of the banana-shaped dimer. Trp264 (red), Met267 (blue) and to minor degree Trp268 (red) are located at the dimer/dimer interface, Cys278 (yellow) identifies the active site, and Cys358 (yellow) is near the octamer face that binds to mitochondrial membranes. Note also the far-reaching N-termini of uMtCK that are mainly responsible for higher octamer stability of uMtCK by providing additional polar interactions between the dimers [81,186].

contains up to 40% of cellular volume in form of mitochondria and mainly relies on oxidative phosphorylation for ATP production. The sMtCK isoenzyme can make up to 25% of total CK activity in rat heart, the highest value among all tissues [130,142]. Thus, it is not astonishing that CK knockout mice show a cardiac phenotype [143–145] and that impaired sMtCK function is relevant for different myocardial diseases [146–148]. In addition, transgenic mice lacking guanidinoacetate-N-methyltransferase, one of the two enzymes needed for endogenous creatine synthesis, also show an obvious phenotype, e.g., reduced inotropic reserve and increased susceptibility to cardiac ischemia/reperfusion injury, due to creatine- and phosphocreatine-deficiency [149].

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 [37]. With respect to MtCK, this may become detrimental especially in oxidative tissues like heart [23]. Here, a perturbation of cellular pro-oxidant/anti-oxidant 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 [120,150,151]. Enzymatic inactivation of myofibrillar MM-CK [127,152] and sMtCK [132], as well as dissociation of sMtCK octamers in vivo [129] and loss of functional coupling between sMtCK, ANT, and oxidative phosphorylation [153,154] 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 [15,37,38,155–157]. This would result in chronic cytosolic Ca^{2+} overload and even more pronounced ROS and NOS generation, thus entering a vicious cycle with progressive inactivation of CK and sMtCK

octamer decay [129]. 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 [141,144,158]. Interestingly, a major consequence of ischemic preconditioning, a cardioprotective treatment [159], is a higher flux through the CK system [160] and preservation of functional coupling between sMtCK and ANT [154,161].

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 [162,163]. Work with MM-CK/sMtCK double knock-out mice showed that below a certain level of CK activity, increases in cardiac work become energetically less efficient [164]. 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 [146,165,166]. A decrease of the sMtCK/beta-actin ratio was linearly related to a reduction of CK flux rate in failing heart [167], and a 4-fold decrease of sMtCK activity and protein content was observed in an animal model using aortic banding [168]. Thus, an intact CK system seems indispensable for normal heart function.

3.3. *Involvement of MtCK in anthracycline-induced cardiotoxicity*

Chemotherapeutics of the anthracycline group, with doxorubicin (Adriamycin™) as the leading drug, have been used for over 30 years for efficiently treating various malignancies. However, severe acute and chronic cardiotoxicity still represent serious complications of anthracycline therapy [169–171]. Cardiac injury has been related to the impairment of mitochondrial functions, such as respiratory rate and generation of high-energy phosphates [172,173]. Several more recent lines of evidence suggest an involvement of cardiac sMtCK and other kinases regulating energy supply in the development of energy deficits and anthracycline cardiotoxicity (for a review, see [174]).

In oxidative muscles like heart, which have a high density of mitochondria and heavily rely on oxidative ATP generation, sMtCK plays an especially important role in the control of mitochondrial respiration [92]. If mitochondria are exposed to anthracyclines, MtCK as well as ANT are preferentially hit by the drug, since both of these proteins and the drug share cardiolipin as the high affinity binding partner in the inner mitochondrial membrane [175,176]. Besides nuclei, mitochondrial membranes have been identified as the most important intracellular sites of drug accumulation [177], with local concentrations much higher than in plasma [178]. Recent studies have shown that anthracyclines like doxorubicin, daunorubicin and idarubicin indeed lead to oxidative damage and functional impairment of MtCK in a dose-, time-, and drug-dependent manner *in vitro* [124] and *in vivo* [130]. Anthracyclines not only lead to inactivation, which occurs with all CK isoenzymes, but also to specific damage of the mitochondrial isoform, namely dissociation of MtCK octamers

into dimers and inhibition of MtCK membrane (cardiolipin) interaction [124,175,179]. Inhibition of MtCK/membrane interaction was rather fast and mainly due to the high affinity of the drug to cardiolipin and the resulting competition between drug and MtCK for membrane binding sites. In contrast, enzymatic inactivation and dissociation of MtCK octamers into dimers occur in solution only after hours to days. Different protection assays and site-directed mutagenesis of sMtCK suggest that deleterious effects of anthracyclines at low concentrations (<100 μM) are due to reversible oxidative damage affecting sulfhydryl groups, mainly affecting the highly reactive active site cysteine, followed at higher drug concentrations (>100 μM) by irreversible damage due to generation of free oxygen radicals like the superoxide anion. In the cellular metabolic network, different intermediate processes would enhance anthracycline action. The presence of intercellular iron and higher number of sites available for anthraquinone redox cycling likely favor *in situ* production of ROS and RNS [171,180,181], and anthracycline metabolites generated in the cells might also potentiate the toxic effects [169,170,178]. For doxorubicin, complexation with iron has been shown to accelerate inactivation of sMtCK. In isolated heart mitochondria, 10–30 μM doxorubicin complexed with iron induced sMtCK inactivation within several tens of minutes [133]. Dimerization of sMtCK and inhibition of membrane binding was also observed in perfused heart as a model for acute anthracycline toxicity [130]. Effects were detectable after 1 h of perfusion with 2 μM doxorubicin, corresponding to peak plasma concentration in patients after bolus injection [182], and became significant at 20 μM together with the appearance of important changes in heart performance.

Long-term sMtCK damage due to anthracycline firmly bound to the cardiolipin-rich inner mitochondria membrane may be at the origin of numerous deleterious processes that promote chronic forms of anthracycline-induced cardiac dysfunction. Compromised sMtCK functions, together with anthracycline-induced damage to other components of sMtCK microcompartments such as ANT [183,184] would impair energy transfer between mitochondria and cytosol [1,25]. This would lead to lowered phosphocreatine and phosphocreatine/ATP levels, which are typical for animal models of anthracycline cardiotoxicity [172], as well as for patients. These deleterious effects on the heart may persist several years after completion of anthracycline treatment [173]. Dimerization and inhibition of sMtCK membrane binding could also destabilize mitochondrial contact sites [88], as it has been observed in doxorubicin-treated rat mitochondria [185]. In the view of a postulated role of MtCK in prevention of mitochondrial permeability transition, sMtCK defects would sensitize cardiac cells to apoptosis [6,104], which is indeed frequently observed in anthracycline-treated cardiomyocytes [169].

As in case of PN-induced damage, the cardiac sMtCK isoenzyme occurred to be more sensitive to anthracyclines than the ubiquitous uMtCK [124], probably due to the slight differences in molecular properties and structures [53,81,186]. The higher susceptibility of sMtCK may be an important factor contributing to the cardioselective toxicity of anthra-

cyclines. Organs that lack MtCK (liver) or express high levels of uMtCK (e.g., kidney and brain) do not show severe anthracycline toxicity [187]. In addition, yet another study revealed sMtCK to be more sensitive to inhibition by anthracyclines than the respiratory chain complexes, with a significant 15% decrease in sMtCK activity in mitochondria from doxorubicin-treated mice [188], suggesting sMtCK as a prime target of doxorubicin damage in mitochondria. Thus, impaired sMtCK functions may play a central role in drug cardiotoxicity.

3.4. Impairment of MtCK in neurodegenerative disorders

The pathogenesis of many neurodegenerative and neuromuscular diseases has been linked to mitochondrial dysfunction [107]. 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 [108]. Due to the exclusive susceptibility to ROS, CK inactivation and MtCK octamer destabilization are likely to occur and thus to compromise brain uMtCK functions in energy buffering and possibly in the control of MPT [189]. In fact, an aberrant cytosol/membrane partitioning of CK, as well as CK inactivation were observed in Alzheimer's disease [190,191] and amyotrophic lateral sclerosis (ALS) [192]. 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 [193]. 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 [194–196], mitochondrial cytopathies [197], myophosphorylase deficiency [198], and in animal models of amyotrophic lateral sclerosis [199–201], Huntington's disease [202,203], Parkinsonism [204,205], as well as in brain ischemia [206,207]. However, uMtCK may not be essential for all of these effects, as shown with uMtCK knockout mice [208]. Creatine supplementation is now entering first clinical trials as a potentially useful adjuvant therapy to ameliorate the phenotype of many of these diseases [110].

4. Up-regulation of mitochondrial creatine kinase in human health and disease

4.1. Up-regulation of MtCK expression—a compensatory metabolic measure?

Adaptive changes in the CK isoenzyme expression pattern take place not only during development of skeletal muscle [209], but also during chronic electro-stimulation of muscle in animal models [210] and as a consequence of long-endurance marathon training in human subjects [211]. In all these cases, the relative expression level of sMtCK versus cytosolic MMCK, as well as the total amount of sMtCK, are significantly increased. These data indicate (i) that sMtCK expression follows the simultaneous induction of mitochondria, since

mitochondrial volume is also increased by the above treatments, and (ii) that in situations of high aerobic workload, the energy transport function of sMtCK is important.

A significant increase in specific MtCK activity is also observed in mitochondria isolated from muscle, brain, and heart of creatine-depleted animals [212] fed with the creatine analog β -guanidino propionic acid (β -GPA, see Section 4.2). This treatment is effective, in a tissue-specific manner, in lowering the cellular phosphocreatine/ATP energy ratio and also leads to compensatory over-expression of MtCK by mostly unknown mechanisms. One of the regulatory factors involved in sMtCK up-regulation is the mitochondrial transcription factor A (mtTFA), the mRNA of which was highly up-regulated upon β -GPA administration [213]. Similar over-expression of the sMtCK isoenzyme has been reported for patients with mitochondrial cytopathies, like MELAS, MERF, or CPEO (see Section 4.3) [214].

Up-regulation of sMtCK in heart has been also found in rats stressed by chronic restraint, showing severe cardiac dysfunction, impaired mitochondrial respiration and apoptotic cell death [215]. Another, almost 4-fold up-regulation of sMtCK has been detected in a genomics approach in gastrocnemius of aged mice [216], 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 up-regulation as a compensatory mechanism that aims at improving oxidative energy metabolism. Interestingly, up-regulation of sMtCK in aged mice was entirely inhibited by caloric restriction [216]. This nutritional regime delays the aging process by increasing protein turnover, reducing oxidative stress and thus decreasing macromolecular damage [109]. Up-regulation of uMtCK expression is known for some aggressive tumors, where conditions of low energy state, hypoxia and nutrient restriction may benefit from MtCK functions (see Section 4.4).

Taken together, the above data suggest that MtCK expression is regulated in conjunction with the metabolic energy state of the cell. 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.

4.2. Crystalline intra-mitochondrial inclusions in creatine-depleted muscle

Induction of energy stress in skeletal muscle, e.g., by ischemia [217] or creatine depletion by treatment with β -GPA, [218–220], or omission of creatine supplementation to adult rat cardiomyocytes in culture [221], all lead to a time dependent appearance of elongated giant mitochondria with rod-like, crystalline mitochondrial inclusion bodies (MIBs), as observed by electron microscopy. By immuno-gold staining, it was shown for the first time that the inclusions in cardiomyocytes are very strongly and specifically labeled by specific anti-MtCK antibodies, indicating that these MIBs must contain a significant proportion of MtCK [221].

Creatine depletion by β -GPA feeding is achieved through competitive inhibition of the creatine transporter responsible for cellular creatine uptake, thus leading to a decrease in intracellular total creatine, including the phosphocreatine content, in skeletal muscle, brain and heart. β -GPA is also a competitive inhibitor of the CK reaction. Cytosolic, but not mitochondrial CK slowly reacts with β -GPA to yield phospho- β -GPA [222,223], which in the long term replaces cellular phosphocreatine since it is a very poor substrate for the reverse reaction [224,225]. Upon feeding rats with β -GPA, MIBs first and prominently appear in soleus muscle (at 55 days), then in quadriceps and diaphragm (at 71 days) and at a later stage also in heart, albeit few in numbers (after 126 days) [226]. In soleus muscle and diaphragm, aesthetically highly rewarding MIBs are very frequently observed preferentially in elongated giant subsarcolemmal mitochondria. It was possible to isolate these structures from such mitochondria and to show by Western blot, as well as by structural analysis, that they consist mostly of sMtCK and actually represent crystalline arrays of octameric sMtCK, respectively. This finding was corroborated by immuno-gold staining of MIBs in situ with specific anti-sMtCK antibodies [226]. Detailed structural analysis of isolated MIB crystals reveal typical square-shaped motives corresponding to regularly packed sMtCK octamers in crystalline sheets that are sandwiched in situ between mitochondrial membranes. In early nascent MIBs analyzed by electron microscopy, when the crystal sheets are cut perpendicularly, one can observe regularly lined-up sMtCK octamers that are sandwiched in and linking two mitochondrial cristae membranes (Type 1, Fig. 4) or, alterna-

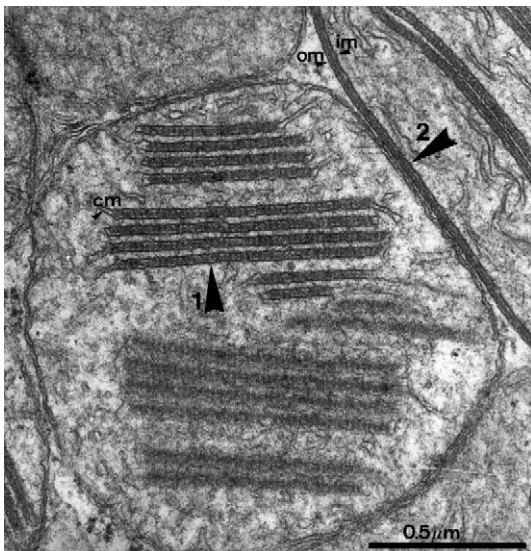


Fig. 4. sMtCK overexpression leads to crystalline mitochondrial inclusion bodies. High magnification electron micrograph of the two different types of inclusion bodies found in separate neighboring mitochondria in diaphragm muscle of GPA fed rats (i.e., Types 1 and 2 as indicated with the large arrowheads and numbers 1 and 2, respectively). Note the regular periodicity of the electron dense structures. Both types of inclusion bodies are surrounded by mitochondrial membranes: Type 1 by cristae membranes (cm) and Type 2 by the inner and outer membranes (im and om, respectively). Similar intramitochondrial inclusions consisting of crystalline MtCK are found as histological hallmarks in mitochondria of patients with mitochondrial cytopathies [214].

tively, are placed between an outer and an inner mitochondrial membrane in situ (Type 2, Fig. 4) [226]. This is supporting evidence for the double localization of MtCK in mitochondria, (i) between inner and outer mitochondrial membrane at the so-called contact sites [50,86,88] and (ii) along cristae membranes [50] (see Fig. 2 and Section 2.2). In addition, these data support the notion that MtCK octamers are able to interact with mitochondrial membranes in situ. This, according to in vitro data with isolated MtCK and phospholipid membranes, is achieved via a specific C-terminal domain in MtCK that mediates an interaction of the molecule with phospholipid-, preferentially cardiolipin-containing membranes [68]. In addition, as shown earlier, MtCK octamers unlike the dimers are able to cross-link phospholipid membranes also in vitro [81,227].

4.3. Crystalline intra-mitochondrial inclusions in patients with mitochondrial cytopathies

Human patients with mitochondrial cytopathies (for reviews see [228,229]) that lead to impairment of mitochondrial function and eventually to a pathological energy deficiency (for a review, see [230]) show a coordinated induction of genes that are important for cellular energetics, including sMtCK mRNA [231]. In fact, one of the prominent up-regulated proteins in skeletal muscle of these patients was indeed sMtCK [214,232]. Interestingly, patients with mitochondrial cytopathies, such as chronic progressive external ophthalmoplegia (CPEO), Kearns–Sayre syndrome, MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) or MERF syndrome (myoclonus epilepsy with ragged-red fibers), show in their muscles often highly enlarged mitochondria with MIBs, also called “parking lot inclusions”, due to their regular arrangement (for a review see [233]). Recently, similar MIBs were identified in patients with CADASIL, that is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy [234].

All these MIBs that are found in patients are very reminiscent in their structural appearance to those observed in the animal models described above. Like in these animal models, the crystals are found in muscle mitochondria of mitochondrial cytopathy patients either in the intra-cristae space sandwiched between two cristae membranes or in the inter-membrane space between outer and inner mitochondrial membranes. Crystallographic analysis of very thin sections of patient’s MIBs by electron microscopy and laser diffraction revealed two types of crystals, type-I and type-II, existing in type 1 and type 2 muscle fibers [235]. Both types of crystals show crystalline arrangements of square shaped structures of 10 nm side width with a central cavity. These dimensions and appearance, respectively, correspond very well with the correct dimension of the atomic structure of the MtCK octamer, as derived earlier by X-ray crystallography of MtCK protein crystals [1,53].

In support of this, both types of MIBs were indeed labeled very heavily with anti-sMtCK antibodies [214], such that one can conclude that the main component of both types of MIBs in

human patients is also represented by sMtCK. This finding solved a long-standing enigma, for these “parking lot” MIBs in muscle biopsies of patients with mitochondrial cytopathies, most prominently in those showing ragged-red fibers, had been known for decades, but their composition remained elusive until recently. Whether similar, albeit only rarely found paracrystalline inclusions in skeletal muscle biopsies of zidovudine-treated AIDS patients [236] are also composed of sMtCK octamers has not yet been determined, but the morphological similarity of the latter to genuine MIBs found in mitochondrial cytopathies is striking.

A current interpretation for the physiological function of MIBs consisting of crystalline sheets of sMtCK is that due to cellular energy stress, as mentioned above, sMtCK is up-regulated as a compensation for an impaired energy state to increase sMtCK-mediated energy channeling. However, as the amount of sMtCK inside mitochondria is increasing, a critical concentration may be reached where the enzyme starts to crystallize along mitochondrial membranes. In parallel, the formation of sMtCK crystals could also be favored by lipid oxidation and/or free radical damage to MtCK itself (see Section 3). In line with this finding is the fact that isolated muscle mitochondria from β -GPA fed animals displaying a large number of MIBs do no longer show creatine-stimulated respiration [237]. Therefore, one has to conclude that sMtCK within the crystals is enzymatically non-functional and thus is no longer able to fulfill its normal energy channeling task [18,238] and its regulatory function for the mitochondrial permeability transition [88]. Thus, the presence of MIBs in muscle biopsies is a clear indication for cell pathology.

In conclusion, the appearance of sMtCK-containing intra-mitochondrial “parking-lot” like inclusion bodies are a clinical hallmark for mitochondrial cytopathies and represent the common denominator between human mitochondrial cytopathies and impairment of cellular energetics, e.g., caused by gene mutations affecting mitochondrial function or initiated upon depletion of the energy precursor creatine [237]. The very important fact that these inclusions vanish upon creatine supplementation not only in β -GPA-treated, creatine depleted animals, but also disappeared in a human athlete patient with a novel cytochrome b mutation [239], is of clinical relevance and stresses the physiological importance of the phosphocreatine/CK system. In the latter case, free radical production, which is a general problem in mitochondrial cytopathies, was attenuated as well by creatine. This may be explained by the fact that creatine, and even more so creatine plus ADP and nitrate forming a transition-state complex in the active site of CK, protect MtCK from inactivation by peroxynitrite [117]. Some clinical studies [197,240], but not all [241], have shown that creatine supplementation may enhance function in patients with mitochondrial cytopathies.

4.4. Overexpression of MtCK in cancer

Overexpression of uMtCK has been reported for several tumors with poor prognosis [242–244]. Namely in a Hodgkin’s

disease cell line, uMtCK, but not cytosolic BB-CK, is one of the very few genes that is specifically up-regulated [245]. 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 [246] 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 [247]. ATP levels and membrane potentials were sustained much longer in the creatine perfused slices compared to controls, and brain damage could thus be avoided [248]. Similarly, transgenic liver expressing CK and supplemented with creatine was protected from many deleterious metabolic insults, including ischemia, hypoxia or endotoxins perfusion [249,250]. Tumor necrosis factor-challenged livers overexpressing uMtCK also showed a clear reduction of necrotic and apoptotic cell death [106]. 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. Resistance to apoptosis is still the principal reason for resistance of cancers to chemotherapy or radiation. Creatine supplementation of patients with uMtCK-bearing tumors could potentially promote the persistence of such cancers, provided they express creatine transporter. However, all available studies rather show a slight but significant inhibition of cancer cell growth with creatine supplementation *in vitro* [251] as well as *in vivo* [252,253].

5. Conclusions

An especially exciting aspect of 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 complex integrators 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 [48,49,69,107,254]. By its localization in mitochondrial contact site complexes, MtCK interferes with some of these processes like regulation of oxidative phosphorylation, as well as mitochondrial permeability transition [6,65,92,104]. Similar to hexokinase at the cytosolic side of VDAC, MtCK may integrate metabolic and apoptotic signals to mount an appropriate cellular response [73–79]. 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, while up-regulation of MtCK expression under different forms of energy stress can be regarded as a compensatory measure to maintain MtCK levels for proper cell functioning.

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