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Mitochondrial Creatine Kinase in Contact Sites: Interaction with Porin and Adenine Nucleotide Translocase, Role in Permeability Transition and Sensitivity to Oxidative Damage

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Key Words

Creatine kinase · Creatine supplementation · Creatine-stimulated respiration · Mitochondria · Porin · Adenine nucleotide translocase · Permeability transition · Oxidative damage

Abstract

The creatine/phosphocreatine circuit provides an efficient energy buffering and transport system in a variety of cells with high and fluctuating energy requirements. It connects sites of energy production (mitochondria, glycolysis) with sites of energy consumption (various cellular ATPases). The cellular creatine/phosphocreatine pool is linked to the ATP/ADP pool by the action of different isoforms of creatine kinase located at distinct subcellular compartments. Octameric mitochondrial creatine kinase (MtCK), together with porin and adenine nucleotide translocase, forms a microcompartment at contact sites between inner and outer mitochondrial membranes and facilitates the production and export into the cytosol of phosphocreatine. MtCK is probably in direct protein-protein contact with outer membrane porin, whereas interaction with inner membrane adenine nucleotide translocase is rather mediated by acidic phopholipids (like cardiolipin) present in significant amounts in the inner membrane. Octamer-dimer transitions of MtCK as well as different creatine kinase substrates have a profound influence on controlling mitochondrial permeability transition (MPT). Inactivation by reactive oxygen species of MtCK and destabilization of its octameric structure are factors that contribute to impairment of energy homeostasis and facilitated opening of the MPT pore, which eventually lead to tissue damage during periods of ischemia/reperfusion.

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Introduction

Creatine (Cr) supplementation has been reported to have beneficial effects in several animal models of neurodegenerative diseases. It improves motor performance, extends the life span of transgenic mice and protects from loss of motor neurons in a model of amyotrophic lateral sclerosis [1]. Attenuation by creatine of N-methyl-*D*-aspartate excitotoxicity-mediated striatal lesions has also been observed [2]. Moreover, in cultures of rat hippocampal neurons, Cr protects against toxicity of glutamate and β-amyloid [3]. These positive effects of Cr can be attributed to its role in supporting energy supply and homeostasis via the creatine/phosphocreatine (Cr/PCr) circuit facilitated by the interplay between cytosolic and mitochondrial creatine kinases (CK) [reviewed in 4]. In this article, after a concise general introduction into the properties of the Cr/PCr circuit, we mainly focus on several structural and functional characteristics of mitochondrial creatine kinase (MtCK) that are relevant to its role in contact site formation and metabolite channelling. This includes a discussion of the current knowledge about the structural and functional interactions of MtCK with the voltage-dependent anion channel (VDAC), or porin of the outer, and adenine nucleotide translocase (ANT) of the inner mitochondrial membrane. In this context, a possible function of MtCK in controlling the mitochondrial permeability transition pore (MPT pore) is presented. The latter has been implied by some researchers to depend on the ANT and, therefore, we shall also briefly review some experimental pieces of evidence supporting the fact that ANT is able to switch from a specific adenine nucleotide carrier to an unspecific pore. Although the exact molecular identity of the MPT is still a matter of debate [5, 6], ANT in its state of an unspecific pore displays some remarkable characteristics of the MPT pore [7–9]. Impairment of MtCK activity and changes of the MtCK octamer/dimer equilibrium by the action of reactive oxygen species (ROS) that take place during ischemia/reperfusion events, leading to tissue damage, will be discussed in terms of energy production and permeability transition, without emphasizing the general role of mitochondria and MPT in cell death, a topic that has been exhaustively reviewed in recent years [10–14a]. Here, we presuppose that MPT pore opening can indeed lead to cell death.

The Cr/PCr Circuit

In cells with high and fluctuating energy demands, sites of energy production and energy consumption are connected by a Cr/PCr circuit [for reviews, see 4, 15, 16]. The reversible transphosphorylation of ATP to PCr is mediated by different isoforms of CK located in distinct subcellular compartments. Cells usually coexpress a pair of cytosolic and mitochondrial CK isoforms. The former are found mainly at sites of energy consumption, where they are functionally coupled to different cellular ATPases. Well-documented examples are the sarcoplasmic reticulum Ca^{2+}-ATPase [17–19], myosin ATPase [20–23], and plasma membrane Na,K-ATPase [24]. The main function of CK at these subcellular sites of high energy demand is to keep the local ATP/ADP ratio high and constant during periods of high cellular activity (e.g. during muscle contraction) by catalyzing the reverse CK reaction to locally produce in situ ATP from PCr. This function of the CK system has been designated as 'temporal energy buffering'. It could be shown that local ATP/ADP pools are maintained by CK and that they do not easily equilibrate with the global adenine nucleotide pool [19], a strong indication for microcom-

partmentation with locally restricted fluxes of ATP and ADP.

MtCK is strictly localized to the peripheral space between mitochondrial inner and outer membranes as well as to the space along the cristae [25]. It has preferential access to mitochondrial ATP produced by oxidative phosphorylation to generate PCr which is exported to the cytosol via the outer mitochondrial membrane protein porin [26–29]. Utilization of PCr by cytosolic CKs regenerates Cr which diffuses back to mitochondria for rephosphorylation, thereby closing the Cr/PCr circuit (or PCr shuttle). The benefits of such an energy shuttling system are particularly evident in elongated or polarized cells like photoreceptors [30] or sperms [31]. In rooster sperms, 25–30 mitochondria are strictly localized to the midpiece only, between the sperm's head and the almost 100-μm-long tail, and these mitochondria produce all the energy required for cell motility and fertilization. Estimation of diffusional fluxes of energy carrier metabolites between mitochondria and distally located dynein ATPases in the flagellum revealed that the diffusion of adenine nucleotides, especially ADP, is very limited over long distances, compared to that of Cr and PCr [32]. By the Cr/PCr circuit, energy flux is accelerated by several orders of magnitude and enables energy production to meet the rate of energy utilization [33]. Consequently, targetted inactivation of CK by dinitrofluorobenzene in sea urchin sperms led to progressive cessation of flagellar wave bending from the distal to the proximal parts of the sperm cell [34]. It depends largely on the cell type, whether the energy buffering or shuttling function of the CK system predominates [4, 35].

MtCK and Contact Sites

Structural Properties of MtCK Relevant to Contact Site Formation

In solution, MtCK isolated from tissues or after bacterial expression exists in an equilibrium of octamers and dimers [36, 37], while cytosolic CK forms exclusively dimers [38]. Electron-microscopic imaging of single MtCK particles and two-dimensional crystals gave the first indications of highly symmetrical cube-like octameric molecules with the four dimers arranged around a 4-fold axis, giving rise to a central channel of 20 Å in diameter [39–41]. This suggestive picture of overall MtCK structure was later confirmed by the high-resolution X-ray structure of chicken sarcomeric [42] and human ubiquitous MtCK [43]. The octameric molecule consists of two identical 'top' and 'bottom' faces with the C-terminal ends exposed on both faces. These positively charged C-terminal stretches are believed to be responsible for the high affinity of octameric MtCK to negatively charged phospholipid surfaces [44–46]. With model membranes and liposomes, it could be shown that the binding affinity of MtCK increases with increasing content of acidic phospholipids [47–49]. The symmetry of the MtCK octamer also facilitates cross-linking of two membranes with MtCK 'sandwiched' in between. This has been demonstrated in vitro by surface pressure measurements of lipid monolayers spread at an air-water interface [50]. Injection of octameric MtCK in the subphase resulted in an increase of the surface pressure due to binding of MtCK particles to the monolayer. Subsequent injection of radioactively labelled liposomes resulted in a significant rise in surface radioactivity indicating binding of vesicles to the surface monolayer mediated by MtCK octamers [50]. Similar measurements with monolayers and liposomes composed of natural phospholipid

mixtures derived from outer and inner mitochondrial membranes gave unequivocal evidence that MtCK can indeed cross-link the two mitochondrial membranes. This is in strong support for the ability of octameric MtCK to mediate contact site formation in mitochondria. In contrast, dimeric MtCK as well as dimeric cytosolic muscle or brain type CK failed to cross-link two membranes, although these species showed weak membrane binding affinities [49, 50]. It is interesting to note that rebinding of dimeric MtCK to previously depleted mitoplasts resulted in partial reoctamerization of the enzyme, most likely by lateral diffusion of dimers weakly bound to the membranes [51].

Interaction of MtCK with Outer and Inner Membrane Components in Mitochondrial Contact Sites

The Physiological Significance of Contact Sites: Cr-Stimulated Respiration. There is a general agreement now from membrane binding measurements (see above) that the major interaction forces between MtCK and phospholipids are of electrostatic nature. Therefore, it is obvious to speculate that the same forces are responsible for MtCK-mediated contact site formation in mitochondria. The inner mitochondrial membrane contains 13–16% of the acidic phospholipid diphosphatidylglycerol (cardiolipin) [52], which seems to be concentrated in contact sites [53]. The outer membrane contains only trace amounts of diphosphatidylglycerol, but other negatively charged phospholipids like phosphatidylinositol are present in significant amounts [52, 54]. Thus, the actual in situ conditions at the contact site regions for the binding of MtCK and for cross-linking of mitochondrial membranes at these sites seem favorable. However, the question of whether protein-protein interactions also play a role in MtCK-mediated contact site formation still remains open. To get a possible answer to this, we first have to consider the physiological significance of contact sites. It has to be stressed that contact sites are dynamic structures of variable composition and serve different functions, such as protein import into mitochondria [55–57], translocation of phospholipids and phospholipid precursors [58–61], transport of cholesterol for steroidogenesis [62], and oxidation of cytosolic NADH [63]. Here, mitochondrial contact sites are discussed only in the context of metabolite channelling and permeability transition, where in both cases, MtCK serves a functional as well as structural role.

Comparative measurements of the rates of oxidative phosphorylation in situ and in isolated mitochondria in the presence and absence of Cr led to the suggestion that MtCK together with the ANT of the inner and porin (VDAC) of the outer membrane form a functional microcompartment [27, 64–67]. Within this concept, ATP produced by oxidative phosphorylation inside the mitochondria is exported by the ANT and channelled to the active site of MtCK for transphosphorylation to PCr. PCr is then funnelled to VDAC to be expelled to the cytosol, while ADP is channelled back to the mitochondrial matrix via the ANT to further support oxidative phosphorylation. These observations were summarized in a model where MtCK, VDAC and ANT are in close proximity to form a ternary complex and a microcompartment at the contact sites to facilitate the production and export into the cytosol of PCr and the regeneration of ADP in the intermembrane space. Within the complex, MtCK is flanked by a still unknown number of VDAC and ANT molecules at opposite faces of the MtCK cube [see e.g. fig. 1 in 35]. Together with the limited permeability of the outer membrane for adenine nucleotides [66, 68], this model assures that cytosolic and intermemembrane space

ATP/ADP pools are separated and prevented from intermixing, and that MtCK exerts a major control over oxidative phosphorylation. This has been convincingly demonstrated in a recent study with skinned muscle fibers from wild-type and MtCK-deficient transgenic mice [26]. The stimulatory effect of creatine on oxidative phosphorylation in the presence of small amounts of ADP is mainly observed with octameric MtCK, but not with mutant enzymes which do not form octamers [69]. Thus, only the octameric form of the enzyme, capable of mediating contact site formation, seems to be relevant for metabolite channelling. This fact seems to bear some clinical relevance since in animal models of heart disease, where cellular energy is compromised, a significant proportion of MtCK dimers were found which supposedly cannot support mitochondrial energy channelling [70].

Solubilization of the contact site fraction by mild detergent extraction of rat brain homogenates and subsequent purification by ion exchange chromatography and gel filtration resulted in coelution of octameric MtCK together with VDAC and ANT [71]. Reconstitution of these complexes into ATP-loaded phospholipid vesicles resulted in atractyloside-sensitive production of PCr after addition of Cr to the outside of the liposomes [71]. Thus, complexes of MtCK, VDAC and ANT can be reconstituted as functional units displaying exactly those properties that one expects from their function as metabolite channelling complex for high-energy phosphates in intact mitochondria.

Structural Interactions between MtCK, ANT and Porin. The close functional coupling of MtCK, VDAC and ANT in contact sites suggests direct pairwise structural interactions between MtCK and VDAC and between MtCK and ANT. In the latter case, however, apart from the experiments with reconstituted complexes which clearly demonstrate functional coupling of MtCK and ANT (see above), attempts to demonstrate physical and structural interactions between MtCK and ANT have failed so far. This may reflect the dynamic nature of contact sites, since their frequency is highly depending on the metabolic state of mitochondria. That is, the number of contact sites increases under conditions of active oxidative phosphorylation, i.e. in the presence of ADP [72], whereas in resting or uncoupled mitochondria, contact sites are much less frequent [66]. Their number is also modulated by the ANT inhibitor carboxyatractylate (CAT), a finding that suggested regulation of contact sites by the ANT [72]. Considering this rather complex and dynamic, ANT-ligand-dependent regulation pattern of contact sites, it is not entirely surprising that formation of MtCK-ANT complexes from isolated components could never be demonstrated so far in vitro. Isolated ANT, solubilized in Triton X-100, contains 6–8 tightly bound cardiolipin molecules per carrier molecule [73, 74], which are indispensable for its adenine nucleotide transport activity [75]. Therefore, MtCK-ANT interactions may be mediated indirectly by cardiolipin rather than through direct protein-protein contacts. The putative MtCK-ANT interaction will be discussed further in the next chapter, where data on the control by MtCK and its substrates of mitochondrial permeability transition are described.

A substantial body of experimental evidence, however, points to the existence of structural complexes between MtCK and VDAC. When isolated from osmotically disrupted mitochondria, a considerable part of the total MtCK activity was found in the contact site fraction cosedimenting with hexokinase (HK) [76]. Similarly, disruption of the outer membrane with digitonin did not liberate all of the MtCK and HK activity. A signifi-

cant fraction of octameric MtCK still remained bound to the thus isolated mitoplasts [77, 78]. Interestingly, in mitoplasts, MtCK was shown to be regulated by the outer membrane porin, i.e. addition of polyanion to inhibit adenine nucleotide transport through VDAC also inhibited MtCK activity. Moreover, ADP produced by MtCK in these mitoplasts was not available for external pyruvate kinase. Direct evidence for a structural MtCK-VDAC interaction came from in vitro experiments where both, wild-type MtCK as well as an N-terminally truncated mutant of MtCK, which is unable to reoctamerize [79], were shown to form complexes with VDAC [80]. The mutant MtCK-porin complex had the same apparent molecular mass of 400 kD as the complex formed between VDAC and wild-type MtCK. From these observations it was suggested that MtCK, upon complexation with VDAC, is stabilized in its octameric form. From in vitro experiments with purified proteins, it can be concluded that formation of complexes between VDAC and MtCK does not seem to require phospholipids, in contrast to what may be the case for the MtCK-ANT interaction (see above). Reconstitution of in vitro formed MtCK-VDAC complexes into planar lipid membranes revealed low ion conductivities of VDAC, similar to those found in the presence of polyanions, like dextran sulfate or polyaspartic acid [80]. Polyanions reduce the gating potential where VDAC switches from the high conductance to a low conductance state [81, 82]. This agrees with the observation in intact mitochondria, where only about 50% of the total MtCK activity present in mitochondria could be measured upon addition of external PCr [78]. If VDAC in complex with MtCK is in the low conductance, cation-selective state, diffusion of anionic PCr through VDAC should be restricted, whereas diffusion of the uncharged Cr should be unhindered. A preference for cytosolic Cr over PCr of VDAC would be advantageous to drive the MtCK reaction in the intermembrane compartment into the direction of PCr production, consistent with the properties of the PCr shuttle, by which Cr enters and PCr leaves the mitochondria.

Recently, a topological model of VDAC from *Neurospora crassa* was deduced from biotin modification of cysteine residues, introduced by site-directed mutagenesis, to create double mutants [83]. The relative position of biotinylated cysteine pairs with respect to a planar lipid membrane was assessed by binding streptavidin from either side (*cis* and/or *trans*) to reconstituted mutant VDAC channels. Channel properties were analyzed in terms of voltage gating and conductance. The resulting folding pattern shows that VDAC consists of one N-terminal α-helix and 13 transmembrane β-strands. In combination with data obtained with peptide-specific anti-VDAC antibodies and analysis of the accessibility to their epitopes in intact mitochondria and after lysis of the outer membrane [84], the model also proposes which protein domains are facing the aqueous compartment on either side of the membrane. Accordingly, two major loops protrude out of the cytosolic surface of the membrane, whereas only one larger and two minor loops are exposed to the intermembrane space. Thus, the possibilities for MtCK to form protein-protein contacts from the intermembrane space with VDAC seem to be rather limited according to this static model. However, VDAC seems to be a highly dynamic structure, in particular during voltage gating. Transition from the high to the low conductance state is associated with major domain movements out of the pore, including the N-terminal α-helix and three adjacent β-strands [85, 86], and a reduction of the pore diameter from 30 to 18 Å [85]. As mentioned above, in a complex with MtCK, VDAC likely

adopts the low conductance state. This is reminiscent of what has been described for the VDAC modulator, a homodimeric mitochondrial protein of 100 kD, which significantly reduces the voltage dependence of VDAC [87, 88]. Although located in the intermembrane space, addition of the purified, putative VDAC-regulatory protein to intact mitochondria reduced mitochondrial metabolic activities by decreasing the permeability of the outer membrane to metabolites [89]. It is conceivable that MtCK, like the VDAC modulator [86], can interact with parts of these mobile domains, thereby locking VDAC into the closed state. We recently imaged the surface structure of VDAC at low (15 Å) resolution by metal-shadowing and cryo-electron microscopy of porin crystals [90]. Crystals were grown in the presence of phospholipids to create a lipid bilayer environment for the insertion of VDAC channels. Within each unit cell, two VDAC molecules were incorporated in opposite orientations with respect to the bilayer. Major protein domains extruding from the bilayer surface were clearly seen mainly around one of the two channels (fig. 1). One may assume that these molecules are inserted with the cytosolic surface directed to the viewer, whereas adjacent channels are oriented so as to be viewed from the intermembrane space. This interpretation would be in line with the structural model discussed above. In accordance with this, substrate-dependent binding of HK to porin crystals could be measured, but not binding of MtCK (unpubl. observations). Binding of the latter is presumably prevented by steric hindrance due to the close packing of VDAC molecules in the crystals, preventing access of MtCK to its binding sites.

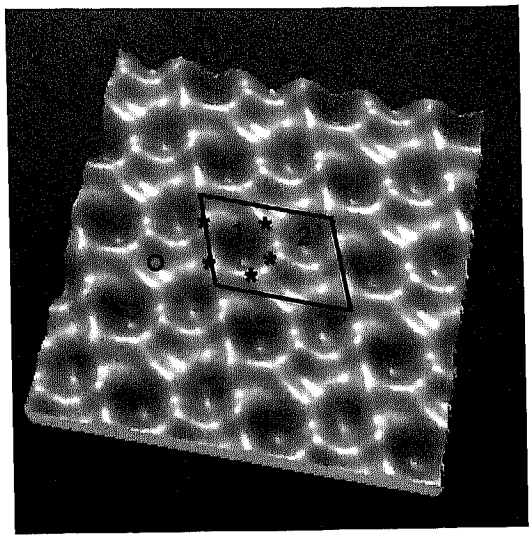

Fig. 1. Structure of mitochondrial porin (VDAC) in two-dimensional crystals. Surface relief reconstruction (perspective view) of VDAC crystals. The parallelogram-shaped unit cell (outlined) has dimensions of 81 × 57 Å and accommodates two porin channels (labelled 1 and 2) which are incorporated in opposite orientations. Major protein protrusions are indicated by asterisks. The circle indicates the level of the bilayer surface. Porin channel 1 is assumed to be viewed from the cytoplasmic side and channel 2 from the intermembrane space [adapted from 90, with permission].

MtCK and the Permeability Transition

MPT-Like Properties of ANT

In the presence of Cr, oxidative phosphorylation is highly stimulated at low ADP concentrations due to metabolite channelling between MtCK and ANT [26]. Given this functional coupling and the notion that the ANT can form an unspecific pore under certain conditions [8, 9], this suggests a regulatory role for MtCK in MPT. Evidence for an involvement of ANT in MPT was first derived from experiments with intact mitochondria. Since the fundamental analysis of the

mitochondrial Ca^{2+}-induced membrane transition [91, 92], it is known that ANT ligands influence the open-closed transition of this unselective and multiply regulated channel of the inner membrane [for reviews, see 6, 93, 94]. Two inhibitors of ATP/ADP exchange, CAT and bongkrekic acid (BA), operate in opposite ways, the former increasing the open probability of the MPT, the latter decreasing it. These compounds lock the ANT either in the 'c' (CAT) or the 'm' (BA) conformation. Only the 'c' conformation, where the adenine nucleotide binding site is facing the intermembrane space, seems to be relevant for MPT, but not the matrix-facing 'm' conformation [95–97]. However, results from experiments with intact mitochondria should still be interpreted with some care if conclusions about the molecular identity of the MPT pore are drawn. In case of the ANT ligands, it has to be stressed that the 'm' to 'c' conformational change is accompanied by major structural rearrangements [98, 99]. In addition, the adenine nucleotide binding site of ANT contains 3 positive countercharges [100, 101] which are cotranslocated during ADP/ATP exchange and, thus, during the 'm' to 'c' conversion. These charge movements may indirectly influence the MPT by the membrane potential which is a known modulator of the open-closed probability of the MPT [102, 103]. Furthermore, a mitochondrial multiconductance channel, measured by patch clamping of mitoplasts and considered to be the electrophysiological counterpart of the MPT [104], was also detected in an ANT-deficient yeast strain [105]. Finally, it has been shown that the MPT is highly influenced by electron flux through respiratory complex I. MPT inhibition occurs if the electron flux is suppressed by quinones [106, 107]. This led to the suggestion that the MPT may reside within complex I [6]. Thus, there are some serious arguments against ANT being the sole MPT pore-forming channel. However, we can imagine that long-term pore opening in the mitochondrial inner membrane, with short circuiting of the proton current, will have the same deleterious consequences on cellular energy homeostasis, irrespective of the molecular species providing an unspecific diffusion channel. Therefore, it is worth noticing the pore properties of the isolated and reconstituted ANT. Coreconstitution of purified ANT and bacteriorhodopsin into proteoliposomes led to a much faster dissipation of the ΔpH induced by a short light flash than in liposomes containing the light-driven proton pump as the sole component [108]. Treatment with the SH-modifying compound mersalyl gave a further increase of the rate of ΔpH dissipation. This was attributed to modification of thiol groups on the ANT, confirming the pore-forming capacity of ANT (and other members of the mitochondrial carrier familiy like the aspartate/glutamate carrier) upon treatment with SH reagents [109, 110]. Single-channel current measurements on excised patches with reconstituted ANT revealed a large Ca^{2+}-dependent channel with different conductance sublevels similar to those of the multiconductance channel [9]. The channel also responded in the same manner (open-closed) to the ANT ligands CAT, BA and ADP, and, most interestingly, channel conductance was abolished upon washing out the Ca^{2+} as is the case for the MPT [111]. The observed Ca^{2+} dependence of ANT pore formation was tentatively interpreted by an indirect influence of Ca^{2+} on ANT. In this model, Ca^{2+} binds to the phosphate groups of cardiolipin which is bound tightly to the ANT, thereby releasing positive charges at the interface of ANT dimers [see fig. 7 in 9]. The resulting electrostatic repulsion of ANT subunits would then give rise to a large central channel. This rough model can, however, not account for the specific dependence of the MPT on Ca^{2+} in intact mitochon-

dria which is antagonized by Mg^{2+} (and other divalent cations) [112]. These findings were corroborated by another approach with highly purified ANT reconstituted into malate-loaded liposomes [8]. Malate was released after treatment with Ca^{2+}, atractyloside and $HgCl_2$ but not with ADP. The effects of these compounds on the reconstituted ANT are the same as for the MPT. In all cases reported above, channel activity was not sensitive against the MPT inhibitor cyclosporin A (CsA) as the preparations did not contain matrix cyclophilin D which is recruited to the inner membrane under MPT-promoting conditions and provides the intramitochondrial receptor for CsA [97, 113, 114]. If, however, ANT is coreconstituted with cyclophilin D, pore activity becomes sensitive to CsA. This has been shown with a VDAC-ANT complex [7], in which the CsA/cyclophilin-sensitivity-mediating component within the complex seems to be the ANT [7, 115]. The electrophysiological channel properties of ANT are also modulated by the proapoptotic protein Bax. Atractyloside-treated ANT cooperates with Bax to form a composite channel with qualitative and quantitative different characteristics compared to channels formed by the respective components alone. In contrast, in complex with the cell death antagonist Bcl-2, channel activities of ANT are completely abolished [116]. These findings may be relevant to the function of Bax and Bcl-2 in mitochondria, both having been reported to modulate MPT directly or indirectly in the direction expected from their pro- (Bax) and anti-apoptotic (Bcl-2) action in cells [117, 118].

Taken together, the pore-forming properties of ANT are experimentally well supported and these characteristics may be of physiological significance under conditions promoting in situ MPT pore opening. Although atractyloside and BA are nonphysiological but specific ligands of the ANT, they are widely used in cell cultures [119–125], and in some studies their action seems to be related to MPT. Thus, it is likely that ANT is a key player of MPT, at least in some cells and under a certain set of conditions.

Control of MPT by MtCK and the CK Substrates, Cr and Cyclocreatine

In the section The Physiological Significance of Contact Sites: Cr-Stimulated Respiration, we reported on the isolation of MtCK-VDAC-ANT complexes from rat brains and their functional reconstitution [71]. An interesting feature of these reconstituted complexes is also related to MPT. Addition of Ca^{2+} to malate-loaded vesicles gave no detectable release of the entrapped malate. However, after treatment of the vesicles with a transition state analog complex to promote dissociation of octameric MtCK into dimers [126] and subsequent addition of Ca^{2+}, malate was liberated in a dose-dependent way [127]. As malate is not a substrate of the ANT, it was concluded that under these conditions the ANT adopts a pore-like conformation allowing an unspecific flux of nontransportable substrates [127, 128]. In this model, octameric MtCK, which is squeezed in between ANT and VDAC, would restrict ANT in its flexibility, preventing conformational changes other than those required for ATP/ADP exchange. Upon dissociation and probably release of MtCK from its binding sites within the reconstituted complexes, ANT is liberated from conformational constraints and can switch to the pore-like conformation [see fig. 2 in 128]. Further evidence for a control of MPT by MtCK came from experiments with intact mitochondria from transgenic mice expressing MtCK in their livers. These mitochondria responded by MPT pore opening upon treatment with Ca^{2+} and atractyloside in the same way as liver mitochondria from control mice lacking MtCK [127]. In the presence of MtCK

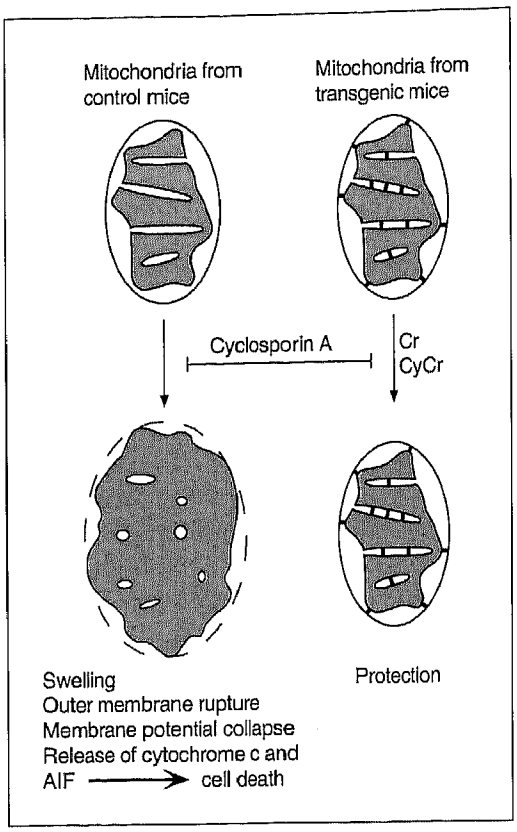

Fig. 2. Protection of MtCK containing mitochondria by Cr and CyCr against mitochondrial permeability transition. Schematic representation of mitochondria from control (left) and transgenic mice expressing MtCK (shown as black squares) in their liver (right). CsA-sensitive swelling (triggered by Ca^{2+} and atractyloside [127]) due to MPT pore opening is observed only in mitochondria from control animals, if Cr or CyCr are present, eventually leading to outer membrane rupture, membrane potential collapse and release of cytochrome c and apoptosis-inducing factor. Under the same conditions, mitochondria from transgenic mice containing MtCK are protected.

substrates, however, a dramatic difference between control and transgenic liver mitochondria was observed (fig. 2). Whereas mitochondria from control animals showed CsA-inhibited MPT pore opening (measured by uncoupled respiration with succinate as substrate, and recently also by mitochondrial swelling experiments (unpubl. data), those from transgenic animals were resistant and remained closed if Cr or cyclocreatine (CyCr) were present. The inhibitory effects of these CK substrates were comparable to those of CsA. These effects of CK substrates were seen only with Cr or CyCr, an analog of Cr which can be phosphorylated by MtCK. On the other hand, PCr and β-guanidinopropionic acid (β-GPA) were inefficient in preventing pore opening in transgenic liver mitochondria. β-GPA is another substrate analog of Cr, but not effective in stimulating oxidative phosphorylation [129]. These results were interpreted in terms of a substrate-dependent association (promoted by Cr or CyCr but not by PCr or β-GPA) of MtCK to the inner membrane and in particular to ANT [127]. This effect may not depend on conformational changes of MtCK induced by substrate binding because the radii of gyration (measured by small-angle X-ray scattering) do not significantly differ between octameric MtCK with and without bound Cr [130]. By comparing the results obtained with intact mitochondria with those of the reconstituted MtCK-VDAC-ANT complexes discussed above, one might be confused about the different observations made in the absence of CK substrates. In the isolated complexes, the mere presence of MtCK is sufficient to prevent the ANT from adopting a pore conformation and substrates do not seem to be required. In contrast, in intact mitochondria, CK substrates must be present to prevent MPT pore opening. This apparent discrepancy may, however, again reflect the dynamic nature of contact sites, which were frozen in a fixed state during isolation of the complexes.

At this point it is opportune to briefly discuss some MPT-related properties of complexes composed of HK, VDAC and ANT as

well. Like the MtCK containing complexes, these complexes also exhibit MPT-like behavior in reconstituted systems [71, 131, 132]. In particular, they are much more sensitive to Ca^{2+}-induced pore formation. This can be understood by comparing the topology of the HK with the MtCK complexes. HK has no structural contact with the ANT, but is bound to its receptor VDAC [128]. Interestingly, in the presence of the HK substrates glucose and ATP (either alone or both in combination), pore opening is drastically reduced in reconstituted complexes and the combination of HK substrates is most efficient [133]. Under these latter conditions, ADP is produced from glucose and ATP, and ADP is known to be a very potent inhibitor of MPT [5, 91, 134]. The analogy to the observation that Cr inhibits MPT in transgenic liver mitochondria is striking and it is tempting to speculate that a similar mechanism of MPT protection is operating, even in the absence of exogeneous ATP. From the above discussion, we may conclude that inhibition of MPT by MtCK and Cr is the consequence of both, a structural interaction between MtCK and ANT (as observed with the reconstituted complexes), and their close functional coupling (observed in mitochondria). Thus, factors impairing MtCK activity and/or destabilizing its octameric structure are expected to sensitize mitochondria to MPT pore opening and lead a cell into apoptosis or necrosis.

Impairing the Functional and Structural Integrity of MtCK

Sensitivity of MtCK to ROS

Mitochondria are the main sources of ROS, in particular during periods of ischemia/reperfusion where reoxygenation leads to enhanced oxidative stress by inducing increased levels of superoxide anion O_2^-, nitric oxide (NO) and peroxynitrite (PN) [135, 136]. After local reperfusion of isolated ischemic rat hearts, significantly elevated levels of NO, superoxide and PN were detected only in the affected part of the tissue [137]. MtCK, located in the mitochondrial compartment and therefore in close proximity of the production sites of ROS, is permenantly exposed to damage by these compounds. Several studies show that CK activity is very sensitive to H_2O_2, O_2^-, NO and PN. For example mitochondrial membranes were incubated with a xanthine/xanthine oxidase system producing O_2^- which reduced MtCK activity in a time- and dose-dependent manner. Xanthine (0.133 m*M*) and xanthine oxidase (0.002 U/ml) decreased CK activity by 60%, whereas 1 m*M* DTT or 10 m*M* cysteine could completely block this inhibition by O_2^- [138], suggesting that oxidation of sulfhydryl groups is involved in the inactivation of the enzyme. Inactivation of purified muscle type CK was shown to be enhanced by adding Fe^{2+} in micromolar concentrations [139], but CK activity could also be protected by reduced glutathione, corroborating the above conclusions.

It has been shown that different NO donors, like SNAP or SNAC, can inactivate cytosolic rabbit muscle CK in a time- and concentration-dependent manner. This inactivation was reversible by the addition of 10 m*M* DTT [140, 141], reducing the S-nitrosation which is responsible for the inactivation of CK. A similar effect of NO on CK was observed in an isolated rat heart perfused with SNAC [141], in adult rat ventricular myocytes [140], in CK preparations solubilized from mitochondria, in isolated mitochondria and in saponin-skinned muscle fibers [142]. In all these cases CK activity was decreased and could be recovered by adding DTT.

In contrast to these findings, inactivation due to PN is irreversible which is the case for cytosolic muscle type CK [143] as well as for

MtCK [144]. Mitochondria are productive sources of PN during the reperfusion period due to a mitochondrially located NO synthase [145]. PN modifies mostly irreversibly metalloproteins like respiratory chain complexes I, II and III [146] and aconitase [147]. PN is a powerful oxidant which can modify chemically the side chains of several amino acids, including oxidation of thiols [148, 149] as well as nitration of tryptophan [150] and tyrosine residues [151].

A 350 μM addition of PN (which is well within the physiological range [152]) to purified MtCK reduced the enzyme activity to 14% of control. With a full set of substrates of the forward reaction (Cr and MgATP) the remaining activity was increased to 28%, whereas only one substrate (either Cr or MgATP) showed no or little protection. There was an even more pronounced protective effect with the addition of the substrates of the reverse reaction (MgADP and PCr) resulting in a residual activity of 50%. At the level of mitochondria, inhibition of MtCK occurred at doses of PN where other components of the respiratory chain and oxidative phosphorylation system were not yet affected, showing that MtCK is extremely sensitive to PN-induced damage [144]. The most sensitive residue in MtCK to PN modification also seems to be the active site cysteine 278 [144, 153, 154].

Due to different behaviors in reversibility of CK activity, the cause of inactivation can be distinguished: inactivation by NO is due to S-nitrosation of Cys 278 (MtCK numbering) and is reversible by DTT, inactivation by PN is probably due to oxidation of the same active site cysteine and is irreversible. It remains an open question whether reversible inhibition of cytosolic and mitochondrial CK by NO may be of physiological significance in regulation of energy metabolism in vivo and whether this adds to the multitude of cellular processes controlled by NO. On the other hand, it is to be expected that irreversible inhibition of the CK system severely impairs cellular energy homeostasis.

Another target of PN modification is Trp 264 which is situated at the dimer-dimer interface of octameric MtCK [154]. Modification of this residue may be responsible for the observed destabilization of MtCK octamers and failure of reoctamerization of MtCK dimers [70].

Consequences of MtCK Inhibition and Destabilization during Ischemia/Reperfusion

The primary consequences of ischemia are loss of ATP (and ADP due to degradation [155]), a significant rise in intracellular phosphate [156], and a fall in pH due to lactic acid production [157]. These are followed by elevated cytosolic Ca^{2+} levels [158] which can lead to stimulation of mitochondrial NO synthase and generation of ROS, including PN [145, 159]. Upon reperfusion, additional oxidative stress is imposed resulting in a vicious cycle of further increase of Ca^{2+}, mitochondrial Ca^{2+} overload, and energy depletion due to MPT opening [160–162]. Under these conditions, one would expect that MtCK activity and oligomeric state are also affected. Indeed, a significant decrease of the octamer/dimer ratio of MtCK has been observed in ischemic rat hearts [70], which does not seem to be changed further during reperfusion, despite additional ROS production within this period. As outlined in the section Control of MPT by MtCK and the CK Substrates, Cr and Cyclocreatine, dimerization of MtCK probably leads to dissociation of the enzyme from its binding sites at the inner membrane and may contribute to MPT pore opening.

The apparent paradox that the most serious cellular damage does not occur during the anoxic phase, but after reoxygenation [163]

may also be a consequence of the impaired production of high-energy phosphates by MtCK which is a prime target for oxidative damage in mitochondria. This in turn would further disturb Ca^{2+} homeostasis and, consequently, prevent full recovery of the affected tissue. The observation that Cr protects mitochondria from MPT pore opening [127] and also partially protects MtCK from inactivation by ROS may be of clinical relevance, e.g. during organ transplantation or cardiac surgery. Supplementation of reperfusion media by creatine may help to keep tissue damage at a minimum.

It looks like an old enzyme, CK, discovered in the 1930s [for review, see 164], is entering new avenues concerning clinical applications. Cr supplementation, now widely used by athletes [165], will become a clinically important adjuvant therapy for a number of pathological conditions [166], including neuromuscular and neurodegenerative diseases [1, 167, 168].

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