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Oligomeric state and membrane binding behaviour of creatine kinase isoenzymes: Implications for cellular function and mitochondrial structure

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Abstract

The membrane binding properties of cytosolic and mitochondrial creatine kinase isoenzymes are reviewed in this article. Differences between both dimeric and octameric mitochondrial creatine kinase (Mi-CK) attached to membranes and the unbound form are elaborated with respect to possible biological function. The formation of crystalline mitochondrial inclusions under pathological conditions and its possible origin in the membrane attachment capabilities of Mi-CK are discussed. Finally, the implications of these results on mitochondrial energy transduction and structure are presented. (Mol Cell Biochem **184**: 141–151, 1998)

Key words: Mi-CK, dimer/octamer equilibrium, membrane binding domains, lipid vesicle cross-linking, VDAC (porin), ANT

Introduction

Cells with high and fluctuating energy metabolism usually co-express a cytosolic and a mitochondrial creatine kinase isoenzyme (CK, EC 2.7.3.2) to transphosphorylate the high-energy phosphoguanidino compound phosphocreatine (PCr), which is used as an energy-storage and -transport metabolite, to ADP, thereby restoring the ATP-pool of the cell (for review see [1–4]). Since the concentrations of ATP and ADP and their local ratio in the cell control many metabolic processes (by allosteric interactions and phosphorylation potential), these parameters have to be held relatively constant within their physiological range. At rest, ATP and ADP are present in the 3–5 mM range and 10–30 μM range, respectively. At times of high workload, the cell can immediately regenerate ATP as it is consumed from the PCr pool (30–40 mM) via the action of creatine kinase. This is called temporal energy buffering. A further advantage of creatine kinase function is providing metabolic capacity [5, 6] which means that using the CK system, the cell is able to provide facilitated diffusion of ATP and ADP to sites of demand thereby creating a spatial buffering effect. This concept is especially important for polar cells, e.g. spermatozoa and photoreceptor cells where diffusion distances from mitochondria to sites of energy consumption are long (for review see [2, 7, 8]).

The CK system can be found in vertebrates, whereas in invertebrates other guanidinokinases are expressed, such as arginine, glycocyamine, lumbricine and taurocyamine kinase [9–11].

To exert its function in an optimized manner, the CK system is compartmentalized, e.g. there are dimeric CK isoforms which are exclusively found in the cytosol compartment, like MM-CK (muscle), BB-CK (brain) and MB-CK, the only known heterodimeric *in vivo* CK-isoform which is expressed during muscle development and to some extent also in adult cardiac muscle, whereas mitochondrial CK (Mi-CK) isoforms are strictly localized within the intermembrane space of mitochondria [3, 12]. In contrast to the cytosolic isoforms, Mi-CKs can occur as dimers and octamers, the latter being built up by association of two dimers to instable tetramers, which then react in a fast step to form octamers [13]. Whereas the cytosolic CK isoforms mainly use PCr to reproduce ATP at sites of high energy consumption, such as the myofibrillar actomyosin ATPase in muscle [14, 15], the Ca^{2+}-ATPase of the sarcoplasmic reticulum [16, 17] or the rods of photoreceptor cells [18], the mitochondrial isoform

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is mainly responsible for the turnover of ATP which is exported out of the mitochondrial matrix by the adenine nucleotide translocase (ANT, synonymes ADT1 or AAC; for review see [19, 20]), thereby forming PCr which is exported into the cytosol via porin of the outer mitochondrial membrane and then used at sites of high energy demand by the reverse CK reaction yielding ATP and Cr. This postulated exchange of PCr/Cr and ATP/ADP between the cytosol and the mitochondrion is referred to as 'creatine phosphate shuttle' or 'phosphocreatine circuit' [1, 2, 21]. In the above mentioned photoreceptor cells, Mi-CK is localised in the mitochondria clustered exclusively in the inner segment of the cell body [22], whereas BB-CK is localised within the outer cell-segments which do not provide energy by oxidative phosphorylation [18]. For there are several energy-consuming conversions taking place in the outer segments, such as the photic process, CK is supposed to provide energy-buffering capacity by regenerating ATP being an important prerequisite for the reformation of GTP [18]. Hence, this is an example for a complete PCr circuit within polarized cells. A similar function of the CK system with respect to facilitated diffusion of ATP and transport of high-energy phosphate from the mitochondria to the dynein-ATPase is postulated to take place in spermatozoa of certain species, e.g. sea urchin (for review see [8]).

To understand the role of octameric Mi_b-CK between the mitochondrial membranes, it has to be taken into consideration that the mitochondrial isoenzyme forms highly symmetrical cube-like octamers (P422 symmetry) possessing identical top and bottom faces [23–26]. This particular arrangement is supposed to be one of the Mi-CK properties facilitating membrane cross-linking at mitochondrial contact-sites [27].

The interconvertible oligomeric states of Mi-CK

For both types of mitochondrial creatine kinase isoenzymes (Mi_a-CK, mainly expressed in non-muscle tissues and in brain, and sarcomeric Mi_b-CK mainly expressed in skeletal muscle and in heart) the oligomeric state of the enzyme is supposed to be important with respect to the formation of membrane contact-sites, e.g. a close approximation of (i) inner and outer mitochondrial membrane and (ii) adjacent inner membranes only (inter-cristae contacts). In the following chapters, we will concentrate on chicken Mi_b-CK which has a more basic pI than Mi_a-CK [28, 29]. For this isoform the dimer/octamer interconversion has been extensively studied *in vitro* by fluorescence spectroscopy [13]. The decay of the octamer in the presence of the transition-state analog complex (TSAC) consisting of Mg-ADP, Cr and nitrate leads to a fluorescence decrease of about 25%, caused by the quenching of the dimer/dimer interface tryptophan residue (Trp264) which upon dimerisation becomes exposed to the aequous solution [30, 31]. With the rate constant of the decay being 0.19 min^{-1} and the remaining octamer-content at equilibrium being about 5%, TSAC is very efficient at quantitatively dissociating the octamer (see Fig. 1). The reason for this effect is the trigonal-planar nitrate ion which mimicks the transition state of the terminal phosphate of ATP when being transferred to creatine (see planar transition-state in SN_2 reactions) thereby locking the enzyme in a conformation which destabilizes the octamer. The treatment of octameric Mi-CK with radicals like peroxynitrite completely abolishes its activity and also the TSAC effect, showing that an intact acitve-site is a prerequisite for the substrate analog-induced decay [31a]. Although a number of parameters like pH, ionic strength and substrates have been shown to affect the dimer/octamer equilibrium [29, 32], the *in vivo* signal for the decay of the octameric isoform into dimers has not yet been identified. *In*

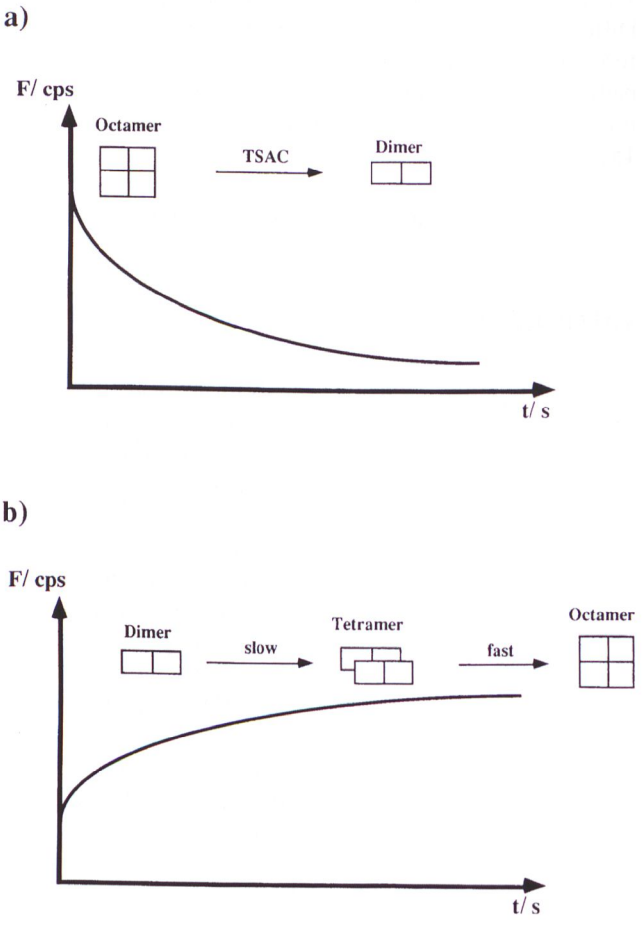

Fig. 1. Schematic representation of the TSAC-induced octamer decay of Mi-CK in free solution monitored by fluorescence spectroscopy (a) and reoligomerisation of the dimers upon removal of the TSAC complex (by addition of EDTA, to complex TSAC-magnesium, (b) The bimolecular association rate constant was determined to 318 M^{-1} s^{-1}, the dissociation rate constant was shown to be 0.19 min^{-1} [13].

vitro measurements have proven the destabilizing effect of high pH, high salt concentration, dilution of the protein and low temperature on the octamer, the latter being a consequence of the hydrophobic contribution of the dimer/dimer interfaces [33]. It has to be pointed out that the assembly of the octamer could be an almost irreversible reaction *in vivo*. Whereas the disassembly of the octamer happens in an all-or-nothing fashion, the reformation of the latter takes place via the tetramer, which is a short-lifetime intermediate in solution and therefore cannot be detected by gel-permeation chromatography [13]. It may be speculated that the octameric and/or tetrameric isoform is stabilized by mitochondrial membranes, which have a high affinity for Mi-CK. Rebinding experiments with dimeric CK [32] suggest a role of the membrane in reoligomerisation of dimeric Mi_b-CK. As far as the kinetic parameters are concerned, there are only subtle differences in K_m and V_{max} between octameric and dimeric isoforms of Mi_b-CK [34] (with three-fold higher K_m(Cr) for the octamer) showing that the formation of the octamer has structural rather than catalytic advantages. One of the effects which could be relevant may be of cooperative nature. Nevertheless, experiments elucidating cooperative substrate binding kinetics within the octamer are still missing. They could be a crucial step towards a better understanding of Mi-CK regulation between the mitochondrial inner and outer as well as the cristae membranes.

On treating Mi_b-CK with chaotropic agents, such as guanidinium hydrochloride, the monomer is yielded. However, as soon as the enzyme is solved under non-denaturing conditions, an active dimer is reformed again, showing that the monomer is functionally irrelevant under *in vivo* conditions and may only be found as an intermediate state towards the dimer after the TOM (translocase of outer membrane)-mediated import of CK pre-protein sequence into the intermembrane space and the cleavage of the signal-peptide [35] (for review on mitochondrial protein import see [36]).

Membrane binding and functional coupling of cytosolic CK isoenzymes

The interaction of *cytosolic* CK isoforms with membranes plays a particularly important role in the energetic supply of ion-pumping systems. An important feature of these (dimeric) isoenzymes is their functional coupling to ATPases like the Ca^{2+}-ATPase in the SR membrane [16, 17, 37–39] as well as the ouabain-sensitive Na^+/K^+-ATPase in kidney [40] and in the postsynaptic membrane of electrocytes in the electric organ of *Torpedo*, an electric fish [41]. Originally derived from myogenic cells, the electric organ is composed of large (each 0.1 mm of height), disc-shaped electrocytes which are aligned on top of each other to form the columns of the organ. The ventral postsynaptic membrane of the electrocyte contains a large number of nicotinic acetylcholine receptors (receptor-gated ion channels) which allow sodium influx into the cell on binding acetylcholine. This happens when the fish is producing an electric discharge for the purpose of killing its prey or defending itself. To restore the intracellular resting conditions after the discharge, the sodium ions are extruded from the cell by Na^+/K^+-ATPase action [41]. The ATPase is located within the tube-shaped, invaginated parts of the dorsal electrocyte membrane face (called canniculi) and is driven by the hydrolysis of ATP generated by the CK-catalysed reaction of ADP and PCr [42]. Accordingly, the role of CK in electrocytes is to keep the local ATP/ADP ratios high and thus make ion pump function near its maximal efficiency by co-localisation of CK with the ATPase [2, 7, 42, 43]. Since the drop of PCr concentration on rechargement of the electrocyte can be abolished by specifically blocking the Na^+/K^+-ATPase with ouabain, it can be concluded that the high intracellular PCr concentration in electrocytes is mainly used for the fueling of sodium extrusion out of the cell [43, 45]. This is corroborated by the immunohistochemical localisation of CK along the ventral postsynaptic membrane as well as on the dorsal membrane system of electrocytes where high concentrations of Na^+/K^+ ATPase are found [44]. Direct evidence for the tight coupling between the CK reaction and the Na^+/K^+-ATPase was found by *in vivo* ^{31}P-NMR saturation transfer measurements [45] which showed a highly increased flux through the creatine kinase reaction on recharging the electric organ.

Confusion arose as antibody labeling experiments with heterologous anti-chicken B-CK antibodies indicated that CK in the electric organ was brain-type B-CK, but later the same protein was identified as genuine M-CK [44]. Sequence comparison with 20 other known CK-sequences then showed that electric organ CK, first called 'acetylcholine receptor-rich membrane-associated peripheral v_2 -protein', contained indeed both B-and M-CK sequence motifs [46].

In muscle, MM-CK has been proven to be specifically associated with the SR membrane [37, 38, 39] and the T-tubule system by immuno-gold labeling of SR membrane vesicles as well as *in situ* immuno-gold labeling of permeabilized muscle [39]. Treatment of isolated SR vesicles with high-salt and low-salt/EDTA did not cause a striking release of CK activity [39], leading to the conclusion that CK is either tightly associated with the membrane or bound to an SR protein component. Regarding Ca^{2+}-ATPase function in the sarcoplasmic reticulum, M-CK is able to maitain a high local ATP/ADP ratio necessary for ATP-dependent calcium pumping [39, 47] and provides much more efficient energy supply than external systems like pyruvate kinase [17] due to its colocalisation and coupling with the pump [44, 47]. This effect is of special importance in 'emergency situations' where a high cytoplasmic Ca^{2+} concentration has to be diminished by sequestering Ca^{2+} as rapidly as possible into

the SR [17, 48]. Additionally, the role of MM-CK in the SR may not be exclusively restricted to energy-supply for calcium pumping. As shown by Ferris and co-workers, external ATP can allosterically regulate the inositol 1,4,5-trisphosphate receptor-mediated efflux of Ca^{2+} from the SR [49] which would suggest a regulatory impact of the CK reaction in the SR. Furthermore, the phosphate-induced hampering of calcium uptake was shown to be minimized by addition of PCr also proving a regulatory role of the CK system [50]. Very recent investigations which were carried out with intact SR-structures in skinned muscle-fibres also support the finding that CK-produced ATP, probably being formed in a microenvironment close to the ATP binding-site of the calcium pump, is much more efficient in driving calcium uptake into the SR than externally generated ATP [17].

Unlike the CK isoforms mentioned above, sea-urchin sperm-tail CK (TCK) from *Strongylocentrotus purpuratus* is able to insert into lipid bilayers via the rare C_{14}-lipid compound myristic acid [51] which is attached to the amino-terminus of the enzyme and seems to play a role in targeting the enzyme to the flagellum during spermiogenesis. Two co-existing pools of TCK (TCKI and TCKII), both with a molecular weight of approximately 145 kD have been identified [51]. Whereas TCKII turned out to be much more efficient in liposome binding *in vitro* due to its myristoylation, non-myristoylated TCKI was shown to be convertible to TCKII *in vitro* by administration of N-myristoyl transferase [52]. Hence, the rate of myristoylation turns out to be a crucial factor controlling the membrane association behaviour of the entire TCK pool in the sperm cell. These data indicate that a certain proportion, varying in quantitative terms within different cell types, of dimeric 'cytosolic' CK can indeed be specifically bound to cellular membranes.

Membranes as receptors for Mi-CK

In contrast to the cytosolic CK isoforms, the mitochondrial CK isoenzymes (Mi_a-CK and Mi_b-CK) are the only known

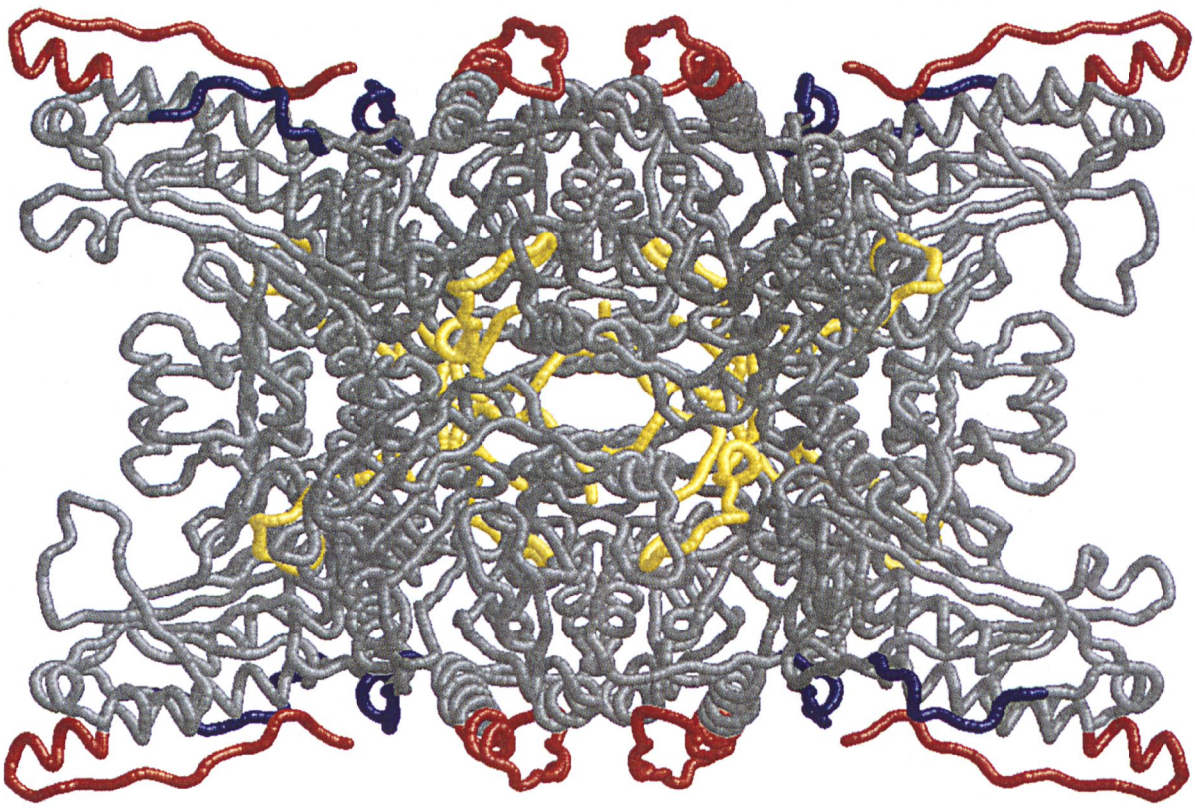

Fig. 2. Putative membrane binding motifs of sarcomeric Mi-CK. Side view of the chicken sarcomeric Mi-CK (Mib-CK) octamer; the polypeptide chain is shown in the backbone trace representation provided by the rendering software RASMOL v. 2.6. Putative membrane binding motifs which are symmetrically exposed at the top and bottom faces of the octamer are indicated in red and blue: The C-terminal domain (red, Asp 357 - Lys 380, including 5 Lys and 1 Arg) and a short internal stretch (blue, Ala 107 - Gln 115, including 1 Lys and 1 His). The N-terminal sequence (Tier 1 - Met 25) which is buried inside the octamer is shown in yellow (for further details see [23, 89]).

members of the guanidino kinase family which can also be found in the octameric state. The following considerations will be restricted to chicken Mi_b-CK, the basic mitochondrial isoform (pI > 9) from sarcomeric muscle because most of the experiments done so far have been performed with this isoform. Mi-CKs are known to bind strongly to the outer surface of the inner mitochondrial membrane [12] and, in the case of sarcomeric Mi-CK (Mi_b-CK), to bridge inner and outer mitochondrial membranes [25, 27]. One characteristic feature of octameric Mib-CK is its high affinity for both the inner and outer mitochondrial membrane which was measured by membrane surface-pressure determination [27, 53] and rebinding experiments with Mi-CK-depleted mitoplasts [32]. Furthermore, acidic phospholipids such as cardiolipin facilitate the 2D-crystallization of the Mi_b-CK octamer as shown by Schnyder *et al.* [24, 26]. In contrast to flagellar sperm TCK, where myristoylation provides a membrane anchor that inserts into the membrane (see above), such evidence is missing for Mi_b-CK. Most probably, the C-termini of Mi-CK, which contain clusters of basic amino acids (Lys and Arg, see Fig. 2), are responsible for the tight binding of octameric Mi_b-CK to mitochondrial membranes, or at least play an important role in membrane docking of the enzyme [89]. As a membrane receptor for Mi_b-CK, cardiolipin (CL), a characteristic (acidic) phospholipid of the inner mitochondrial membrane, has first been identified [54].

Interaction of Mi-CK with outer mitochondrial membranes and porin (VDAC)

The mitochondrial envelope can form contact sites, a close superposition of the two mitochondrial boundary membranes which can be visualized by freeze-fracturing (for review see [55]). Contact sites are dynamic structures and their formation correlates with activity of oxidative phosphorylation [56] and is regulated by [ADP] [57]. Mitochondrial contact sites are enriched in Mi-CK and also contain ANT and a third protein, the outer membrane porin or voltage dependent anion channel (VDAC) [58, 59]. VDAC is responsible for the permeability of the outer mitochondrial membrane (for reviews see [60, 61]) but can adopt a low conductance cation-selective state which could account for a metabolic compartmentation of nucleotides in the intermembrane space, especially at the contact sites (for review see [62]). Extramitochondrial creatine, which could permeate cation-selective VDAC, effectively stimulates the mitochondrial phosphocreatine synthesis via Mi-CK and finally oxidative phosphorylation [63], suggesting a functional coupling between VDAC and Mi-CK [2].

It was proposed that contact sites operate as micro-compartments or multienzyme complexes for energy export (for reviews see [55, 64]). Such a compartment would confer a thermodynamic advantage to the Mi-CK reaction, since products of the Mi-CK reaction, phosphocreatine and ADP, are constantly removed via VDAC and ANT [65]. The microcompartment model suggests that Mi-CK, which is in general only attached to the inner mitochondrial membrane, binds in contact sites to the outer mitochondrial membrane as well. It should be noted, however, that Mi-CK is not a prerequisite for contact site formation which also occurs in tissues exempt from Mi-CK, e.g. the liver.

The micro-compartment-model of Mi-CK is strongly supported by *in situ* localization of Mi-CK showing Mi-CK along the cristae membranes and between inner and outer membranes [22] as well as by *in vitro* studies demonstrating that Mi-CK interacts not only with inner but also with outer mitochondrial membrane preparations [53] and, most importantly, is able to bridge two membranes [27]. By this bridging capability, Mi-CK is well suited for contact site localization. However, despite the body of evidence supporting an interaction of Mi-CK with the outer membrane and functional interaction with VDAC, it is still debated how these interactions occur and what their structural basis is. A spatial association of Mi-CK with VDAC has been shown in digitonin-treated mitochondria [59] and *in vitro* complex formation of Mi-CK with VDAC [66] and ANT [59, 67] has been demonstrated. In the complexed state, VDAC induces octamerization of a polymerization-deficient Mi-CK mutant [66].

Although these results suggest a rather direct physical interaction between Mi-CK and VDAC, this could not be confirmed by crosslinking experiments so far [25]. However, since top and bottom faces of the Mi-CK octamer are identical, it can be assumed that Mi-CK binding to the outer membrane can also occur by electrostatic interaction of Mi-CK with negative charges of outer membrane phospholipid headgroups. According to current transmembrane models of the mammalian VDAC structure [68], only three negatively charged amino acids together with several positive charges of VDAC are facing the intermembrane space. In addition, the N-terminal negative charges of VDAC are not accessible in VDAC-containing Triton micelles [68]. Therefore, it has been suggested that the association between Mi-CK and VADC could be due to additional hydrophobic interactions [66].

In an alternative model, maintaining the well known electrostatic binding behaviour of Mi-CK, a close co-localization between Mi-CK and VDAC could be mediated via charged phospholipids, in analogy to the presumed Mi-CK-ANT interaction [25]. Information on the lipid composition of mitochondrial outer membranes is scarce but its cardiolipin content is assumed to be low at least in liver mitochondria [69]. Thus, for the interaction of the enzyme with outer membrane, binding of Mi-CK to different other phospholipids [53] could become important. Certainly further research is needed to characterize the *in vivo* interaction between Mi-CK and VDAC in the outer mitochondrial membrane.

Interaction of Mi-CK with inner mitochondrial membranes and ANT

Binding of Mi-CK to membranes involves low and high affinity binding sites [70]. While reconstituted ANT did not increase Mi-CK binding [54] and other membrane proteins inhibited binding [71] quite efficiently, several studies uncovered an interaction of Mi-CK with membrane phospholipids [54, 71, 72]. However, it is still debated which phospholipids are involved and how the Mi-CK/phospholipid interface is organized.

Out of different phospholipids examined in artificial membrane systems, only cardiolipin was found to convey high affinity binding sites for Mi-CK [54]. Mi-CK-binding to mitochondria could be drastically inhibited by a prior modification of cardiolipin by (i) the cardiolipin-specific drug adriamycin [54] or (ii) treatment with phospholipase A2, but not with phospholipase C which is not acting on cardiolipin [70]. Preferential binding of Mi-CK to cardiolipin could explain the specificity of Mi-CK for mitochondrial membranes, since no other cellular membrane contains this phospholipid in significant amounts [69]. It was concluded that cardiolipin is the only membrane receptor for Mi-CK [54]. However, when Mi-CK-membrane interactions were determined by their influence on the surface pressure of model membranes, a pressure increase was not only detectable with cardiolipin membranes but also, to a minor degree, with other charged phospholipids (e.g. phosphatidylserine or phosphatidylinositol), preparations of inner or outer mitochondrial membranes and even with microsomal membranes known to be devoid of cardiolipin [53]. Obviously, not only cardiolipin but also several other negatively charged phospholipids are capable to interact with Mi-CK *in vitro*. Accordingly, the binding of Mi-CK to cardiolipin cannot be called absolutely specific, but, because of its high abundance in the inner membrane and its two negative charges, it nevertheless seems to be the most important interacting lipid. This is especially relevant in the presence of divalent cations such as magnesium or calcium which have been shown to induce a clustering of acidic phospholipids such as cardiolipin [73], thereby causing phase separation in lipid model membranes where fatty acid chains of different length and degree of saturation are present in the acidic and non-acidic lipid species.

It has to be pointed out that *in vitro* also the cytosolic isoforms BB- and MM-CK bind to membrane preparations from the inner mitochondrial membrane [53].

Further membrane binding properties of Mi-CK were studied and quantified by different biophysical methods in artificial cardiolipin membranes. Among the most important results is the fact that these membranes favor the assembly of sarcomeric Mi-CK dimers into octamers [32]. It remains, however, an open question in which way the Mi-CK octamer interacts structurally with the membrane lipid bilayer since conflicting results were obtained by different methods. Measurements of membrane surface pressure showed a pressure increase upon Mi-CK binding, indicating a partial penetration of MiCK into the membrane bilayer [53]. Insertion of Mi-CK into the membrane could have a shielding effect and would explain why neither high ionic strength nor detergents were able to detach Mi-CK quantitatively from mitochondrial membranes. These results are not in accordance with ESR studies performed with cardiolipin-bound Mi-CK [74], which indicate that the lipid chains of cardiolipin are not strikingly restricted in their mobility on binding of Mi-CK. This would exclude an insertion of Mi-CK into the bilayer.

Functional coupling of Mi-CK to oxidative phosphorylation occurs via ANT, the only but abundant transport system for ATP and ADP in the inner mitochondrial membrane (for a review see [19, 20]). This suggested a close arrangement between ANT and Mi-CK [75–78]. So far, the large body of evidence for a functional interaction between Mi-CK and ANT has been accompanied by experiments showing a lack of direct structural interaction, e.g. reconstituted ANT did not bind to Mi-CK [54], ANT could not be found unequivocally in crosslinking products with Mi-CK [25, 79] and finally, the basic overall pI of ANT and Mi-CK suggested an electrostatic repulsion between both proteins. However, the presence of ANT in the close vicinity of Mi-CK has been demonstrated by the effect of Mi-CK antibodes on Mi-CK and ANT activity in mitoplasts. Certain Mi-CK antibodies which bind to Mi-CK without affecting its enzymatic activity were able to inhibit adenylate transport by ANT [78].

The apparent discrepancy between functional and structural evidence can be reconciled by the fact that ANT tightly binds about 6 moles of cardiolipin [80] and that the transporter may even be located inside a much larger cardiolipin membrane patch [81]. Such a cluster of cardiolipin could in turn bind Mi-CK [70, 82] and this would result in a close co-localisation of Mi-CK and ANT [78, 83]. Mi-CK and ANT could thus interact by frequent collision within the cardiolipin domain in the inner mitochondrial membrane [78].

Factors influencing Mi-CK membrane attachment

Dissociation and binding studies of Mi-CK with mitoplasts have shown a crucial influence of ionic compounds on the interaction of Mi-CK with the inner mitochondrial membrane (e.g. [71, 72, 84, 85]). Dissociation of Mi-CK from mitoplasts is determined by ionic strength of the medium [72] and by ionic composition [84, 85]. The effect of most ions was reported to correlate with their ionic strength, indicating a competition for charges in the membrane. One of the most powerful and rather specific Mi-CK-dissociating ions is phosphate, which most effectively removes the enzyme from mitochondrial inner membranes, most probably by competing

with the negatively charged cardiolipin headgroups [71, 85–87]. Monovalent and especially divalent cations (e.g. Ca^{2+} and Mg^{2+}) are less effective [54]. Similar ion-effects were obtained when the functional coupling of Mi-CK to oxidative phosphorylation was monitored in mitochondria *in vitro*. Coupling was reduced by high phosphate buffer [87], probably due to the dissociation of Mi-CK from inner mitochondrial membranes, but not in a physiological salt solution with higher ionic strength [84]. Such a lack of correlation between ionic strength and the dissociating effect could be due to binding of these ions to the active center or other sites of Mi-CK rather than to the membrane binding sites, thus inducing structural changes and/or dissociation of Mi-CK octamers into dimers and finally leading to a loss of membrane binding. The CK substrates MgADP and MgATP, chloride ions and negatively charged organic mercurials may also act in this way.

The vast majority of results is in line with a predominantly electrostatic interaction of MiCK-with mitochondrial membranes. Only a few binding studies pointed to a limited influence of non-ionic interactions [53, 72], although hydrophobic interaction chromatography revealed that Mi-CK is more hydrophobic than the cytosolic isoforms [3]. A major role of hydrophobic interactions is disproved by the effect of Triton X-100 alone, which was not able to release more than one half of Mi-CK from mitoplasts at concentrations known to solubilize most membrane proteins [70]. Binding of Mi-CK to cardiolipin may be purely ionic [32, 74], because application of basic pH and high ionic strength is sufficient for Mi-CK release from the membrane. Moreover, the cardiolipin-content is a factor that governs dissociation of bound octameric Mi-CK from the membrane [88], but does not alter the association properties of the octamer. This effect can be sufficiently explained by ionic interactions which, having a short range, only come into play when Mi-CK and the acidic phospholipid are already in close vicinity (Debye length less then 1.5 nm at 50 mM NaCl), e.g. after membrane binding of Mi-CK. These considerations, however, do not exclude a hydrophobic component being relevant in the attraction and binding process of Mi-CK.

Quantitation of Mi-CK membrane binding and cross-linking

The membrane binding and dissociation kinetics of octameric Mi_b-CK have recently been extensively studied in a novel approach using immobilised model membranes and plasmon resonance as detection method [88]. The existence of two independent binding sites for the octamer with strikingly different affinity to CL-containing vesicles was shown. The data obtained were corroborated by earlier experiments with mitoplasts which also proved the existence of two kinds of binding sites [70]. For vesicles with two different CL-contents (16% and 100%) no major differences in the fast association (high affinity) rate constants were seen (pseudo first-order, both approximately 0.11 s^{-1}, see Fig. 3), whereas dissociation was much slower in the case of membranes made of pure CL. In this case, more Mi-CK octamer (about 84%) stayed bound to the vesicles under nonequilibrium conditions, whereas vesicles with a lower CL content only retained 66% of the once bound CK, showing the very high affinity of Mi-CK to membranes. These data also indicate that the membrane binding behaviour of octameric Mi-CK is controlled by the rate of dissociation at different CL-contents rather than by the rate of association. The thereof calculated equilibrium constants (a high- and a low-affinity constant for each CL-content) strongly indicate that under *in vivo* steady-state conditions most of the octamer must be membrane associated. Furthermore, high- and low-affinity constants differed by four orders of magnitude, thus the low affinity equilibrium constant represents very loosely associated CK molecules.

Similar results were obtained with mitoplasts, which had been depleted of Mi-CK [32], showing that even under conditions favouring the dissociation (e.g. dilution) most of the octamer stayed bound to the membrane and with chemical cross-linking studies using glutaraldehyde [71], which indicated the presence of two distinct binding sites for Mi-CK. In free solution, the injection of octameric Mi-CK leads to lipid vesicle cross-linking resulting in the formation of bridged Mi-CK/vesicle aggregates [88]. The kinetics of the cross-linking reaction can be followed using a constant-angle

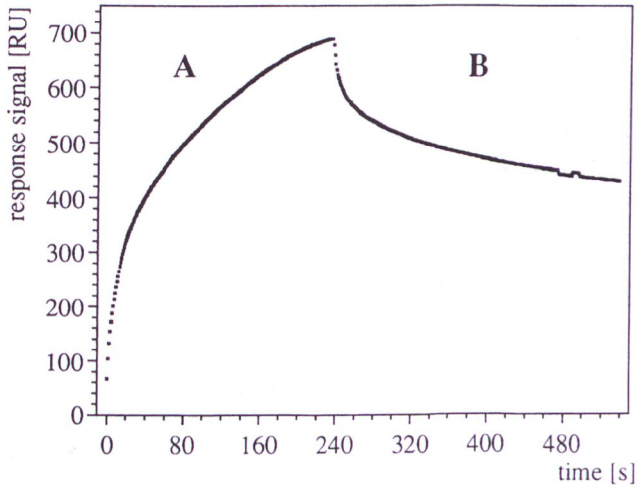

Fig. 3. Membrane binding and dissociation kinetics of octameric Mi_b-CK and phosphatidylcholine vesicles containing 16% cardiolipin in a buffer (pH 7.0) containing 50 mM NaCl and 10 mM TES. Association (a) and dissociation kinetics (b) of the Mi-CK octamer reacting with CL vesicles of 180 nm diameter are shown. About 66% of the associated octameric Mi-CK stays bound in the dissociation phase (b). For experimental details see [88].

(90°) light-scattering approach indicating an extensive formation of enzyme cross-linked vesicle aggregates (Fig. 4). The rate constant for the cross-linking reaction was two orders of magnitude slower than the fast association rate constant for the reaction of Mi-CK with immobilised vesicles and was determined to 2.55 * 10^{-3} s^{-1} for membranes made of pure CL. Therefore, the cross-linking reaction is significantly slower than the fast association of Mi-CK to the membrane and has to be regarded as the second step in the vesicle/CK reaction, with the first step being mainly association of the octamer with *one* membrane. As this reaction is merely reversible by dilution, the stability of the Mi-CK-induced membrane contact sites must be very high under steady-state conditions, e.g. as long as the octamer does not decay. This could have major importance for the formation of energy transduction contact sites *in vivo*, which are the structural basis of complexes involved in metabolic channeling [59, 89].

Possible functions of the dimer/octamer equilibrium in vivo

The above considerations raise the question of the particular importance of the dimer/octamer equilibrium, because dimeric Mi-CK has a weaker affinity to the membrane and interacts less strongly at high pH conditions [32] than the octamer. Most probably, pH and phosphate concentration are two signals also triggering the binding kinetics of the two oligomers of Mi_b-CK *in vivo*: Whereas a high pH still enables the octamer to stay bound, it also induces its decay into dimers in free solution. Therefore, a pH of about 8 which is supposed to be relevant in respiring mitochondria, combined with high phosphate concentrations could control the selective re-binding of the octamer to the mitochondrial membranes, where it is stabilised and resistant against dimerisation. Dimers would stay in the intermembrane space where they could diffuse more easily and assemble octamers at sites of more acidic pH. The major structural difference between dimer and octamer is that the octamer has a cube-like shape with identical top and bottom faces, each exposing four C-termini which is sufficient for membrane binding at two adjacent sides of the cube [89]. Thus, octameric Mi-CK can form stable cross-links between membranes (see also Fig. 5) thereby inducing membrane contact sites *in vitro* [27], whereas dimeric Mi-CK and the (dimeric) cytosolic CK-isoforms fail to induce bridged membrane structures [27, 88], the reason probably being that there is more than one C-terminus necessary at each side of the molecule to stabilize an intermembrane contact, or that a different membrane binding mode of dimeric Mi-CK has to be considered, e.g. both C-termini of the dimeric molecule are bound to the same membrane (Fig. 5). These different binding modes of dimeric and octameric Mi-CK still remain a matter of speculation since there are hitherto no detailed experimental membrane binding data available for dimeric CK.

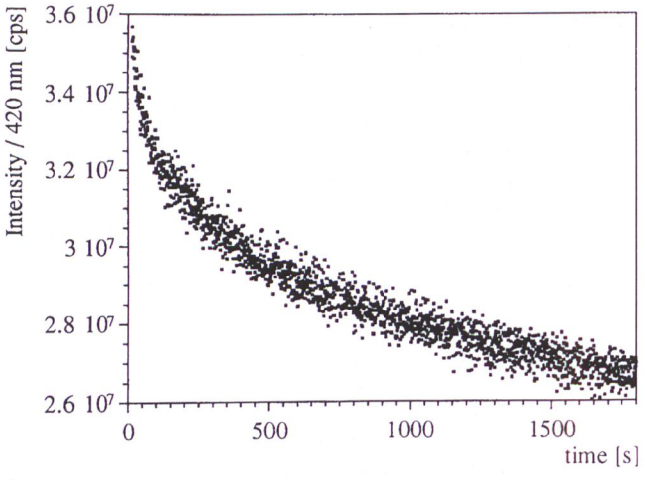

Fig. 4. Mi-CK induced vesicle cross-linking. Fixed angle (90°) light scattering can monitor the cross-linking reaction of CL vesicles (100% CL) with Mi-CK octamers in free solution. The time constant at 25°C for this association (pseudo first-order reaction) was determined to 2.55 10^{-3} s^{-1} [88].

Conclusion

Although significant new insight into the structure/function relationship of MiCK/membrane interaction has been gained by quantitative biophysical approaches [88, 89], the so far existing data on Mi-CK membrane binding capabilities still leave some important questions unanswered. One major point of discussion is related to mitochondrial ultrastructure: EM-pictures of mitochondria shown in textbooks display separated inner and outer membranes, where Mi-CK octamers would

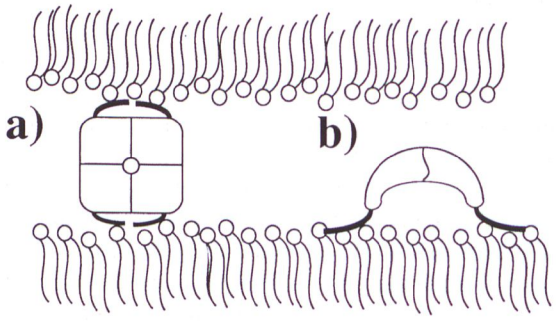

Fig. 5. Schematic model of possible binding modes of Mi-CK. Whereas the octamer can use its four C-termini exposed at each face of the molecule for lipid interaction, thereby connecting two opposing membranes (a), the dimer only posseses two C-termini which are supposed to interact with the same membrane surface, thus lacking the crosslinking effect (b).

easily fit in between. However, high-pressure frozen samples which are supposed to contain less artefacts display an intermembrane space of 4–5 nm only [90], but the height of the MiCK octamer in the protein crystals is 8.6 nm. Hence, there are problems to fit the bulky enzyme into the small compartment between the mitochondrial membranes. There are two possible explanations for that phenomenon, one being (i) that these EM-pictures show an artefact-stricken intermembrane space which is too narrow due to shrinking effects, or (ii) that octameric Mi-CK partially inserts into the membrane or at least forms a kind of membrane 'hole' or indentation which harbour the enzyme. The latter hypothesis is supported by the cardiolipin-inherent property to form non-bilayer phases [91] and by the fact that Mi-CK causes a surface pressure increase upon binding to membranes [27]. Another unsolved problem is the kind and stoichiometry of possible complexes formed between Mi-CK and proteins of the inner and outer mitochondrial membrane, as a direct biophysical evidence for the postulated (and functionally shown) ANT/Mi-CK interaction is still missing. These considerations are of special importance for the formation and regulation of the permeability transition pore [67, 92] and the establishment of energy transduction contact sites. Furthermore, the binding mode for dimeric Mi-CK may differ from the octameric oligomer inasmuch as the dimer is not able to induce bridged membrane structures (e.g. contact sites, [88]).

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Note added in proof

Recent experiments studying the binding characteristics of Mi-CK with liposomes [93] fully confirmed that Mi-CK/phospholipid interaction is largely of electrostatic nature. Interestingly, while the nucleotide substrates ATP and ADP had no influence on Mi-CK binding to liposomes, para-hydroxymercuri-benzoate, a negatively charged organomercurial, partially decreased Mi-CK binding and the anti-cancer agent adriamycin efficiently prevented Mi-CK binding. This latter finding may explain some of the cardiotoxic side-effects often observed with this drug. Most interestingly, differential scanning calorimetry performed by the same authors suggests a partial disorganisation of the phospholipid bilayer upon interaction with Mi-CK, which is fully in line with our earlier experiments where binding of Mi-CK to a monolayer of outer mitochondrial phospholipids was shown to result in a significant increase in surface pressure [27, 53], also indicating that Mi-CK is locally disturbing the topology of the phospholipids or is inserting to a certain extent into the monolayer.

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