Molecular and Cellular Biochemistry **184:** 125–140, 1998.

Functional aspects of the X-ray structure of mitochondrial creatine kinase: A molecular physiology approach

Uwe Schlattner,[1] Michael Forstner,[1] Michael Eder,[1] Olaf Stachowiak,[1] Karin Fritz-Wolf[2] and Theo Wallimann[1]

[1]*Swiss Federal Institute of Technology, Institute of Cell Biology, ETH Zürich, Switzerland;* [2]*Max-Planck Institut für medizinische Forschung, Abt. Biophysik, Jahnstr. 29, Heidelberg, Germany*

Abstract

Mitochondrial creatine kinase (Mi-CK) is a central enzyme in energy metabolism of tissues with high and fluctuating energy requirements. In this review, recent progress in the functional and structural characterization of Mi-CK is summarized with special emphasis on the solved X-ray structure of chicken Mi_b-CK octamer (Fritz-Wolf *et al*., Nature 381, 341–345, 1996). The new results are discussed in a historical context and related to the characteristics of CK isoforms as known from a large number of biophysical and biochemical studies. Finally, two hypothetical functional aspects of the Mi-CK structure are proposed: (i) putative membrane binding motifs at the top and bottom faces of the octamer and (ii) a possible functional role of the central 20 Å channel. (Mol Cell Biochem **184**: 125–140, 1998)

Key words: active site, catalytic mechanism, creatine kinase, high-energy phosphate transport, membrane binding, metabolic channeling, mitochondrial energetics, octamer/dimer equilibrium, X-ray structure

Abbreviations: ANT – adenine nucleotide translocator; Cr – creatine; cCr – cyclocreatine; CK – creatine kinase; Mi-CK – mitochondrial CK isoforms; Mi_b-CK – sarcomeric Mi-CK isoform; MM-CK, BB-CK – cytosolic CK isoforms; PCr – N-phosphoryl-creatine; SR – sarcoplasmic-endoplasmic reticulum; TSAC – transition state analogue complex

Introduction

The 'energy-rich' compound, N-phosphoryl creatine (PCr) was first identified in 1928 in muscle [1] and was initially thought to represent the direct chemical energy source for muscle contraction. However, the discovery of the enzyme ATP:creatine phosphotransferase or creatine kinase (CK, EC 2.7.3.2) in 1934 by Lohman [2] showed that the N-phosphoryl group of PCr is transphosphorylated onto ADP to regenerate ATP [3], the latter being the universal energy currency in all living systems [4]. CK was first purified in 1954 by Kuby *et al*. [5] from rabbit muscle and its properties were described by Kuby and Noltman [6]. At this time and for many years to come, CK was considered a strictly soluble enzyme simply fulfilling the role of a temporal ATP buffer or back-up system, as still depicted in current textbooks of biochemistry and cell biology.

However, after the discovery of (i) the cytosolic isoforms of CK [7], i.e. muscle-type MM-CK and brain-type BB-CK, (ii) the developmental transition of CK isoforms during muscle cell differentiation from ubiquitous BB-CK to sarcomeric muscle-specific MM-CK via the transitory MB-CK hybrid [8] and (iii) the identification of a novel mitochondrial CK isoform (Mi-CK), strictly confined to mitochondria [9], a different view of the enzyme's function emerged. Right after the discovery of Mi-CK, it was postulated that ATP, formed by oxidative phosphorylation in muscle, would be transphosphorylated to PCr which then

Address for offprints: T. Wallimann, Institute of Cell Biology, ETH Zürich-Hönggerberg HPM F42, CH - 8093 Zürich, Switzerland

would diffuse to the myofibrils and be converted into ATP again ([10], p.170 and [11, 12]). The proposal that creatine may stimulate mitochondrial respiration was born soon after [13] and this concept has been corroborated by numerous consecutive studies showing, for example, a functional coupling of Mi-CK with the adenine nucleotide translocator (ANT, for a review see [14]).

After the discovery of the isoenzyme-specific attachment of MM-CK to the myofibrillar M-band of sarcomeric muscle [15, 16], the idea gained support that the ATP-consuming contractile apparatus was connected to sites of energy production, e.g. mitochondria, via PCr and the CK isoforms located at these respective sites. Several models have since been proposed to describe the complex cellular energy supply system: The PCr-shuttle [17–23] or PCr-circuit [24–27] (for a collection of relevant publications and references see a special issue of Mol Cell Biol [28]). The idea behind the PCr-circuit model is that the PCr/CK system not only serves as a temporal energy buffer for ATP [29], but also as a spatial energy transport system connecting cellular sites of ATP-consumption, i.e. ATPases, to sites of energy production, i.e. glycolysis and/or oxidative phosphorylation. The latter function is especially important in highly polar cells such as photoreceptor cells or spermatozoa [30] where diffusion limitations of ATP and ADP can be overcome by PCr via the CK system [31].

In addition, the PCr-circuit represents a regulatory system for the physiologically required maintenance and adjustment of subcellular ATP/ADP ratios. For example, CK associated with the sarcoplasmic-endoplasmic reticulum (SR) [32] is thermodynamically important for efficient Ca^{2+}-pump activity, as shown both *in vitro* with isolated SR vesicles [33–35], as well as *in situ* with skinned muscle fibres [36]. Probably, the role of CK in the energetics of Ca^{2+}-homeostasis in excitable cells [30] represents the most crucial function of the CK system in general. This has recently become evident from analysis of the muscle phenotype of transgenic knock-out mice lacking the expression of both cytosolic MM-CK and sarcomeric Mi_b-CK. Skeletal muscles of these mice show problems with Ca^{2+}-release and uptake and display significantly prolonged relaxing times [37].

The temporal buffer function of the CK system, which seems generally accepted now (see above), has been corroborated elegantly by a transgenic approach with mice expressing BB-CK in the liver, where normally no or only very little CK activity is found. In the perfused livers of such transgenic animals, ATP levels and pH remain constant much longer after a fructose overload than in control livers [38]. In addition, the transgenic CK livers, if perfused with creatine, are protected by endogenously formed PCr from hypoxia and ischemia [39] and, due to a reinforced hepatic energy metabolism, regenerate significantly better, after 70% hepatectomy, compared to control livers [40].

The major point of discussion about the multiple functions of the CK system, at the present time, is related to the spatial buffer or transport function via PCr-shuttling. Results obtained by *in situ* ^{31}P-NMR saturation transfer measurements investigating the CK-mediated reaction flux at different work loads of muscle [41] or under creatine depletion [42] have been interpreted as proof against the PCr shuttle. However, the unexpectedly anomalous behaviour in ^{31}P-NMR terms of transgenic mice expressing graded CK levels [43] suggested that some of the CK-flux, measured *in situ* by saturation transfer ^{31}P-NMR, may evade detection by this method [44]. This problem seems to be exacerbated by the fact that, due to compartmentation of adenine nucleotides *in vivo*, some ATP may be invisible by NMR as well [45], indicating that a fresh look at the NMR data is needed and that this problem can only be solved by a multidisciplinary approach. Within this context, the dynamic interplay between the CK and the adenylate kinase system is also of relevance [46].

The recently solved X-ray structure of octameric Mi-CK [47] sheds new light on some important functional aspects of the CK system discussed above and seems to be consistent with the proposed energy channeling function of this enzyme [48]. The new insight into the atomic structure of the Mi-CK molecule allows now for a detailed structure/function analysis concerning the molecular physiology of this enzyme, its catalytic site and mechanism, its octamer/dimer equilibrium, as well as its interaction with mitochondrial membranes and the relevant proteins thereof. Some of these new and intriguing aspects of the Mi-CK structure are described here and certain prominent key features of the molecule are discussed below within the physiological framework of mitochondrial and cellular bioenergetics.

The first part will present a short overview of the chicken Mi_b-CK structure in the context of our present biochemical and biophysical knowledge about CK isoforms, including active site residues and catalytic mechanism. In the second part, models will be developed, based on the Mi-CK structure, to elucidate the unknown structural basis of some Mi-CK-specific functions, e.g. the strong binding to mitochondrial membranes and the functional coupling and metabolite channeling to ANT of the inner mitochondrial membrane and porin (or voltage-dependent anion channel, VDAC) of the outer mitochondrial membrane. Two structural features and their possible functional consequences will be discussed in detail: (i) the identical fourfold top and bottom faces of the octamer which contain putative membrane binding motifs likely to be involved in binding to mitochondrial membranes and, possibly, interaction with ANT or porin and (ii) the central, 20 Å wide channel which may be of significance for the exchange of energy metabolites between mitochondria and cytosol under the assumption that Mi-CK follows a ‘back door’ mechanism.

Crystal structure of chicken Mi_b-CK

CK is one of the enzymes with the longest history in crystallographic studies. Crystallization attempts date back to the 1950s, crystallization of cytosolic [5, 49–54] and mitochondrial [55, 56] isoenzymes have been reported by different groups. Despite of all these results, the first successful solution of the crystal structure of a guanidino kinase could only be reported recently [47], using chicken Mi_b-CK crystallized in our group [55].

The reason for the successful crystallization may have been the choice of the animal species, i.e. the chicken, with a body temperature of 42°C, as well as the choice of the mitochondrial CK isoform (Mi_b-CK), which had never before been used in crystallization trials. Octameric chicken Mi_b-CK from sarcomeric muscle turned out to be remarkably stable against denaturation and was surprisingly insensitive towards proteolysis, so that enzymatically active protein, mostly octameric Mi_b-CK, could be recovered from crystallization setups even after months of storage at 25°C.

Mi_b-CK was crystallized by the hanging drop approach using protein purified from chicken heart tissue [55, 56] or, later, from overexpressing *E. coli* bearing expression plasmid pRF23 [57]. Several tetragonal crystal forms were observed, two of which proved useful for crystallographic studies of the enzyme in the presence or absence of substrates (free enzyme and Mi_b-CK × ATP: space group $P4_2$, a=126.0 Å, c=144.7 Å; free enzyme: space group $P42_12$, a=171 Å, c=150 Å). The use of a single isomorphous derivative and a complex theoretical approach involving phase extension (W. Kabsch, unpublished) allowed the calculation of an electron density map to 3 Å resolution. It should be noted here that the intrinsic non-crystallographic symmetry of octameric Mi_b-CK was crucial for the solving the crystal structure. The polypeptide chain was fitted to the electron density map using the interactive program O [58] and the model refined in several steps using the X-PLOR software [59].

The course of the polypeptide chain of a monomer is shown in Fig. 1. The structures of Mi_b-CK-ATP and of the free enzyme were found to be very similar. Moreover, structural differences between the four crystallographically independent monomers in the octamer are rare, comprising residues 1–10, 60–66, 109–120 and 313–327. Uncertainties in the structure assignment comprise the five N-terminal residues and the loops 60–65 and 316–326 (see Fig. 1), which have a high temperature factor above 40 $Å^2$. This factor is a displacement parameter for an atom in a crystal, containing input from disorder in atomic positions as well as from thermal vibrations and thus indicates flexibility inside a structure. The overall dimensions of the monomer are approximately 36 Å × 42 Å × 69 Å. Assignment of the secondary structure by the procedure DSSP [60] is given in Fig. 2. Of the 380 residues, 33% are α-helical and only antiparallel β–strands were found. This is in good agreement with the results obtained by circular dichroism spectroscopy [61]. The monomer consists of a small (residues 1–112) N-terminal (domain I) and a large (residues 113–380) C-terminal domain (domain II) with the ATP binding site located in the cleft between the two domains (see Fig. 2). The small domain I is built from a short 3_{10}-helix and five α-helices of which helix α_3 is somewhat distorted. The large domain II contains an eightstranded antiparallel β-pleated sheet, flanked by seven α-helices. As seen in Fig. 2, some of the strands in the large β-sheet are unusually connected. Moreover, the Mi_b-CK fold is different from all other kinases for which a three-dimensional structure has hitherto been determined, although there are some similarities in the fold of domain II to glutathione S-transferase (E. Di Iorio, ETH-Zürich, personal communication). Figure 2 also shows the unusual arrangement of the β-strands in the eight-stranded β-pleated sheet of domain II, which can also be considered to consist of two nearly equally sized subdomains IIa and IIb, separated by β-strand 1.

As CK is known to be remarkably stable towards proteolysis, it is interesting to note that the main nicking site of CK susceptible to proteases, Asp323 [62] (Fig. 2), is located on the highly flexible loop 316–326. After partial unfolding of muscle-type cytosolic M-CK by denaturants, another region around amino acids 158–166 becomes sensitive to staphylococcal proteinase V8 and therefore was postulated to represent a domain/domain boundary [63]. In Mi_b-CK, this region forms an extended stretch between the α_6 and the β_2 regions as shown in Fig. 2 but cannot be considered a domain boundary. Recent experiments employing two genetically engineered fragments of Mi_b-CK [64], nevertheless, suggest that this region is probably defining the boundaries of folding entities within the Mi_b-CK structure since, more recently, a Mi_b-CK fragment consisting of residues 167–380 was shown to be an independent folding entity, whereas the fragment 1–166 could not fold to its native conformation (M. Forstner *et al*., unpublished).

The octamer is held together by numerous interactions of its monomers. Mi_b-CK octamers are quite stable and the dissociation into dimers upon dilution takes hours to weeks [65]. This process is greatly accelerated by the addition of a transition state analogue complex of the substrates (TSAC = creatine, Mg-ADP and nitrate), which leads to dissociation within minutes [66]. The dimers of all CK isoforrns are very stable and disintegrate into monomers only under chaotropic conditions. From this information it was possible to define the dimeric building blocks of the octamer from the number of interactions between the monomers, under the condition that the monomer-monomer interactions forming the dimer have to be more numerous and extensive than the interactions holding the dimers together to yield the octamer. The validity of this approach was supported by the finding that the Mi_b-CK dimer could be used to model the structure of dimeric M-

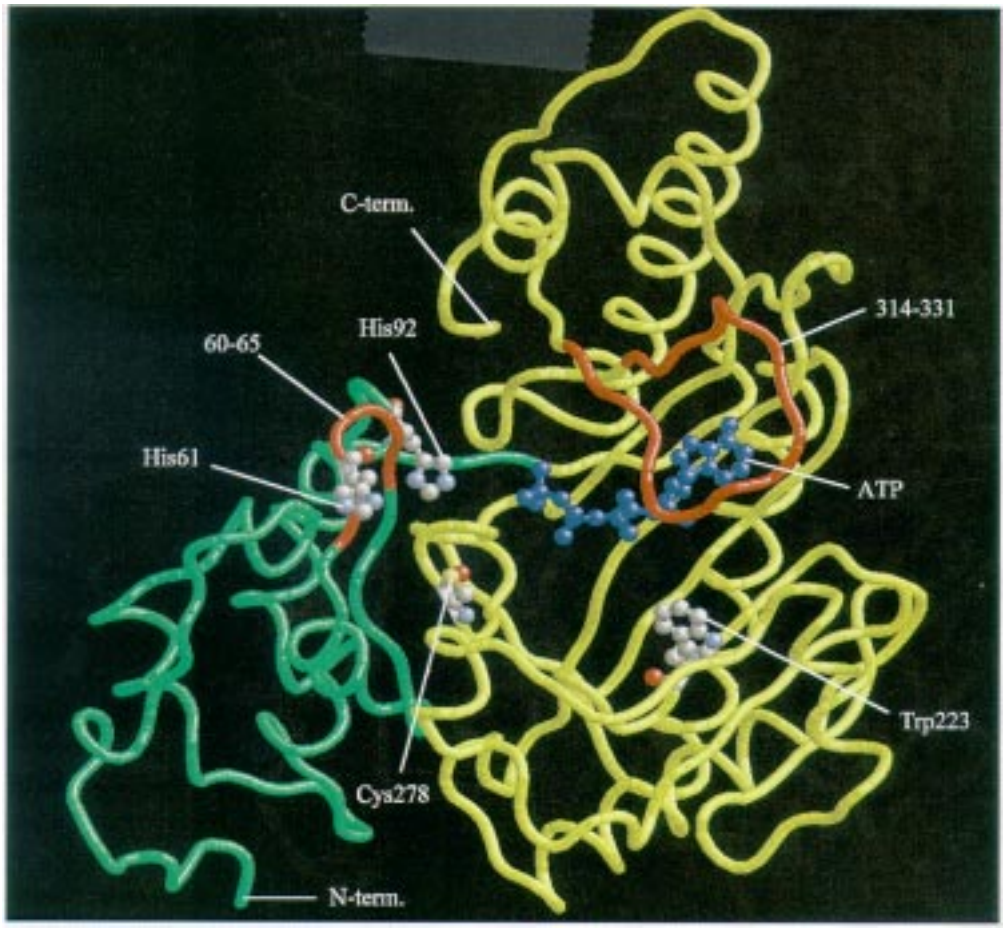

Fig. 1. Monomer structure and flexible regions of the Mi_b-CK monomer. The course of the polypeptide chain is shown in the 'backbone trace' representation as provided by the macromolecular rendering software RASMOL v. 2.6 [123]. Domain I is shown in green and domain II in yellow; the bound ATP is colored in blue. Flexible segments of the polypeptide chain with temperature factors above 40 $Å^2$ are colored in red. The position of the flexible loops (60–65 and 314–331, comprising the highly flexible stretch 316–326) and of the N-and C-termini is indicated. For His61, His92, Trp223 and Cys278, the side chains are drawn in a 'ball-and-stick' representation (C atoms in gray, N atoms in blue, O atoms in red, S atoms in yellow).

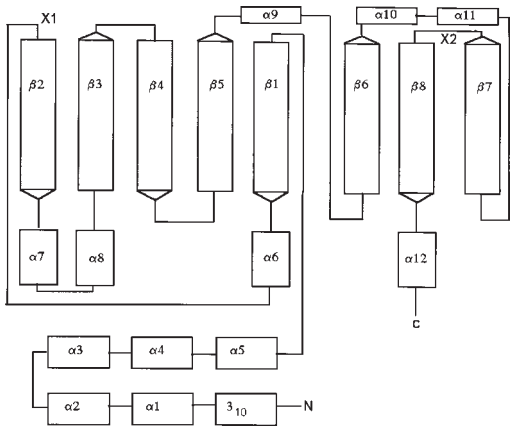

Fig. 2. Chain fold of the Mi_b-CK monomer. Schematic representation of secondary structure of Mi_b-CK. First and last amino acid residues in the helices and sheets were determined by the use of the automatic procedure DSSP [60]. The molecule can be partitioned into two domains. The small domain I consists of a 3_{10} helix [P11–D14] and five α-helices of which helix α3 is distorted [α1:C24-C28; α2:P31-L37; α3:L48-D57; α4:S76-V79; α5:A81–R91]. The large β-domain contains a central β-stranded antiparallel β-sheet [βl:V121–R130; β2:G166–S170; β3:G211–N215; β4:F220–I224; β5:T230–K237; β6:R287–K293; β7:L312–G316; β8:V328–N333] surrounded by seven α-helices [α6:R143–A158; α7:E176–D185; α8:P195–T198; α9: M241–R262; α10:P295–K299; α11:R302–L310; α12:E341–E363]. Two protease sensitive sites are indicated (X1: V8-protease sensitive site at amino acid residue 166; X2: multiple proteases-sensitive site on the flexible loop 316–326 at amino acid residue Asp323, see text for details).

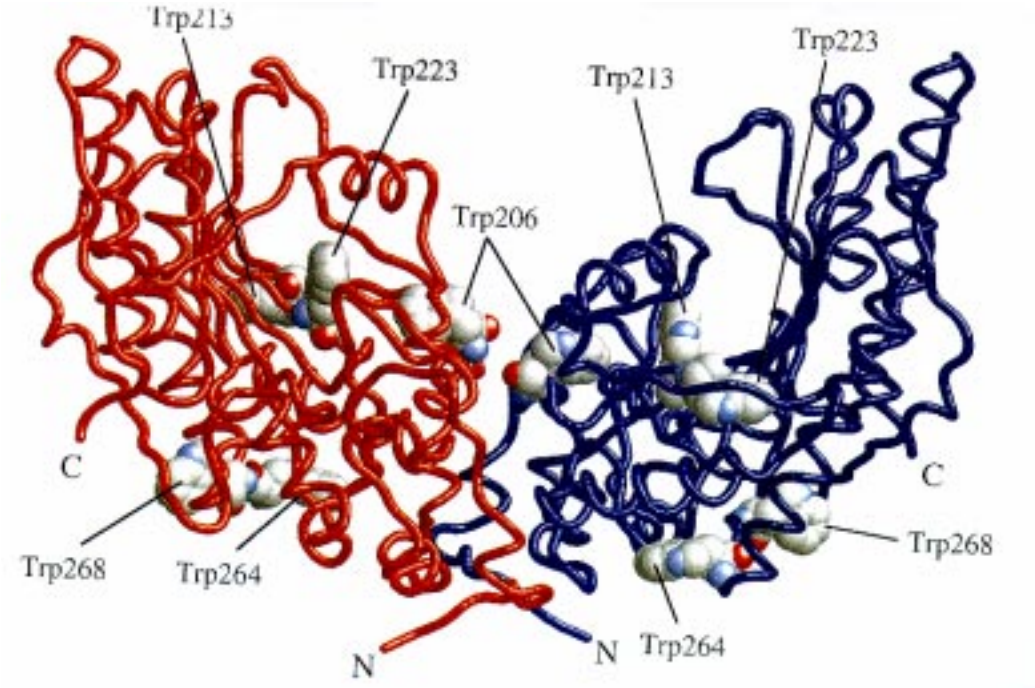

Fig. 3. The tryptophan residues of Mi_b-CK. A dimer of Mi_b-CK is shown in the 'backbone trace' representation as provided by the rendering program RASMOL v. 2.6 [123] with one monomer being colored red, the other blue. The side-chains of the tryptophan residues Trp206, Trp213, Trp223, Trp264, and Trp268 are shown in a 'spacefill' model representing the van der Waals-surface. The involvement of Trp206 in the monomer-monomer contact is evident from the figure, the position of Trp223 in the active site is also obvious and Trp264 is located at the dimer/dimer interphase (see Fig.3b in [47] and [67]). The N- and C-termini are indicated.

CK and compare the calculated scattering function of the model to the experimentally obtained small-angle scattering curve of M-CK in solution (Forstner *et al.*, submitted).

Moreover, the contribution of Trp206 (Fig. 3) in one of the monomer/monomer contact regions to the stability of the dimers has been proven by site-directed mutagenesis and

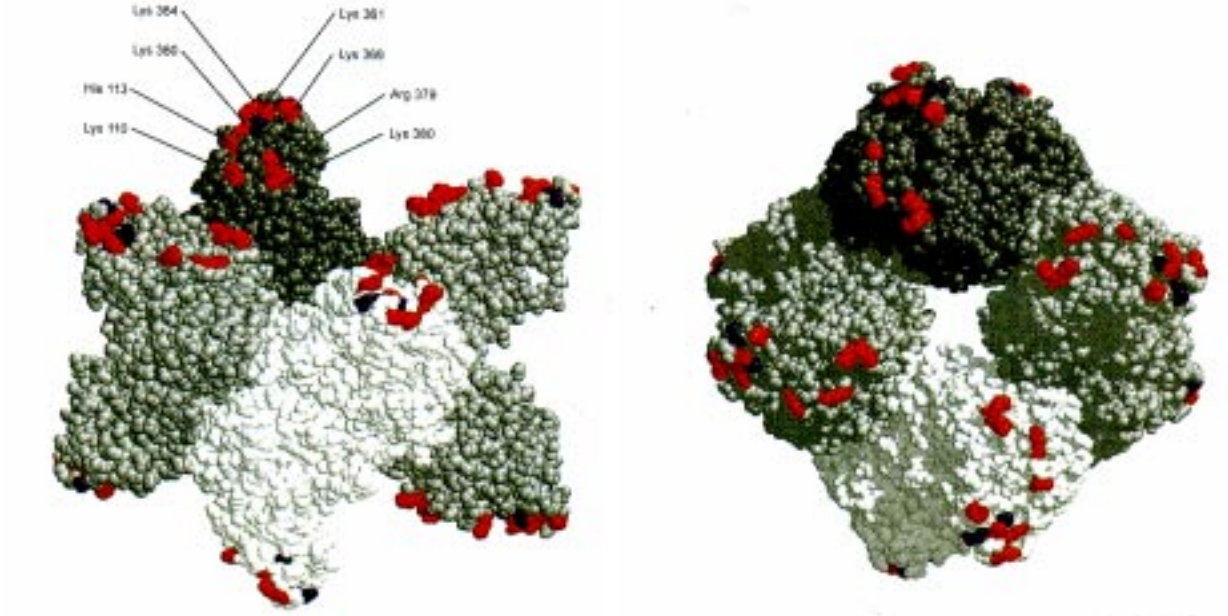

Fig. 4. Charged amino acids in the putative Mi-CK membrane binding motifs. The 'spacefill' model of the chicken Mi_b-CK octamer, with identical top and bottom faces for symmetry reasons, is shown (left) in a slightly tilted side view and (right) in a view onto the top (or bottom) membrane binding face (side length 96 Å) with the central 20 Å wide channel. Basic amino acids (positively charged) are depicted in red, acidic amino acids (negatively charged) are shown in blue. The four dimers forming the octameric structure are marked in different grey levels. The model representing the van der Waals-surface was prepared using the rendering software RASMOL v. 2.6 [123].

fluorescence spectroscopy [67] and the necessity for the presence of Trp264 [67], as well as of the intact N-terminus for octamer formulation has been demonstrated as well [68].

Active site residues and catalytic mechanism

Data analysis of the ATP containing co-crystals of Mi_b-CK reveals additional electron density for one nucleotide in each monomer which is not present in the native map. It is located in the cleft between the domains in the highly conserved region of all guanidino kineses. The adenine base fits into a pocket formed by the residues His186, Met235, Serl23, Argl25, His291, Gly289, and the pair Arg287, Asp330 which forms a salt bridge. The phosphate groups of ATP interact with four arginine residues at positions 125, 127, 287, and 315. Two additional residues, Arg91 and Arg336, are found at a distance of 5 Å from the γ-phosphate. This region, also including His92, leaves enough room to accommodate creatine in an orientation that would allow a direct in-line transfer of the phosphoryl group between the bound substrates.

By a number of experiments with chemical modification of SH-groups using different SH-reagents in the presence or absence of substrates, or the affinity label epoxycreatine [69], Cys278 was identified as the highly reactive 'essential' cysteine residue (see also Fig. 1). However, further studies of this type yielded contradictory results concerning the essentiality of Cys278 for enzyme catalysis (see [70]). Nevertheless, until recently, Cys278 has been suggested in the literature as an important residue for catalysis [71]. Thorough kinetic analysis of several replacement mutants of Cys278 has solved this long debated issue by providing clear-cut evidence that Cys278 is not essential for catalysis per se, but is important for the synergism of substrate binding [72].

NMR studies on the role of histidines at the active site have revealed four such residues at distances of 12, 12, 14 and >18 Å from the Cr^{3+} in the paramagnetic β,γ-bidentate Cr^{3+}-ATP complex bound to the enzyme [73]. One of these histidine residues, with an altered intrinsic pK near 7, was postulated to act as an acid-base catalyst [74]. We find His92, His291, His229, His61 and His186 at distances of 12, 12, 14, 17, and 18 Å, respectively, from the O7 atom of ATP (see Fig. 1). Recent studies by our [75] and other groups [76] have shown, however, that none of these residues can be considered essential for the functioning of CK, although some of them certainly are of importance for substrate binding in the active site of CK. However, replacement mutants of His61 had a strong negative effect on the enzyme activity, e.g. the respective His to Ala mutant showed approximately 1 and 10% of wild type activity in the forward and backward reaction, respectively [75]. The importance of His61 for the catalytic activity of CK is consistent with the assumption that the flexible loop formed by residues 60–65 and which is found rather distant from the ATP in the crystal structure might move towards the active site to promote catalysis [75].

Spectroscopic studies of cytosolic M-CK and its complexes with adenosine phosphates, as well as quenching studies, were suggestive for the occurrence of a tryptophan residue at or near the cosubstrate binding site [77]. Specific chemical modification of tryptophan residues revealed one of them as essential for enzymatic activity. These results are in agreement with an earlier ^{1}H-NMR study indicating an interaction between protons of the adenine ring of bound ADP and one or more aromatic side chains of the protein. Trp206 and Trp223 are conserved throughout the whole family of guanidino kinases [78] and replacement of Trp223 resulted in a complete inactivation of the enzyme, indicating that Trp223 is situated near the active site and is crucial for catalytic activity and, therefore, was termed the 'active site tryptophan' [67]. By NMR, the active site tryptophan was found to be in the vicinity (< 5 Å) of the nucleotide substrate [78a].

Incidentally, four of the five tryptophan residues of Mi_b-CK (Trp206, Trp213, Trp223, Trp264, see Fig. 3) are found rather closely clustered and oriented in such a way that energy transfer, indicated by fluorescence studies [67] may indeed be possible, whereas Trp268 is closer to the C-terminal membrane interaction face of the octamer. In the X-ray structure of the wildtype enzyme, Trp223 was found at a distance of about 10 Å from the adenine ring. We assume, however, that in the presence of all substrates or at the transition state, the nucleotide would move 'down' towards Trp223, thereby bringing the substrate closer to this residue, thus explaining the distance measured by NMR. In this context it is necessary to remember that the Mg-nucleotide rather than the free nucleotide is the substrate for CK and that only the Mg-nucleotide can induce structural changes essential for catalysis [79].

In comparison with other kinase structures, Mi_b-CK has acquired a different folding pattern for catalyzing phosphoryl transfer, a result which is of significance for the evolutionary positioning of CK. Furthermore, Mi-CK is the only isoform in the creatine kinase system which forms octamers, and the structure supports the hypothesis that Mi-CK has evolved to serve an additional function as a structural protein that mediates the adhesion between inner and outer mitochondrial membranes and between cristae membranes via electrostatic interactions due to charged residues located at its four-fold faces [25–27]. These properties of Mi-CK will be discussed in detail in the next chapters.

Membrane binding of Mi-CK

In view of the physiological function of CK, one of the most important properties of this enzyme is the distinct subcellular

localization of its different isoforms (for a review see [25, 26]). This isoenzyme-specific localization has been related to the functional coupling of CK to energy providing and energy consuming processes of distinct subcellular sites and is part of the PCr-shuttle or -circuit model of CK function (see above). Mitochondrial CK isoforms, sarcomeric Mi_b-CK and ubiquitous Mi_a-CK differ from their cytosolic counterparts not only by their organellar compartmentation, but also by their membrane-binding property. While cytosolic CKs are mainly soluble but certain fractions of the enzyme are also bound to particular subcellular structures (e.g. myofibrils, sarcoplasmic reticulum and plasma membrane), Mi-CKs are known to bind strongly to the outer surface of the inner mitochondrial membrane [80] and, in case of Mi_b-CK, to bridge mitochondrial membranes (for reviews see [81] and Stachowiak *et al*., this issue). Membrane binding of Mi-CK is considered the structural basis of functional coupling between Mi-CK and oxidative phosphorylation which occurs via the metabolic channeling between Mi-CK and the adenine nucleotide translocator (ANT) of the inner mitochondrial membrane (e.g. [82], see also below). Similarly, a functional interaction between the outer mitochondrial membrane porin and Mi-CK has been proposed [25].

It is generally accepted now that Mi-CK binds to membranes mainly by electrostatic interactions, although the participation of hydrophobic interactions cannot be ruled out [83]. As binding partners in the mitochondrial membranes, cardiolipin and other phospholipids were identified by different methods [83–87]. Highest affinity was determined for cardiolipin which contains a two-fold negatively charged headgroup and occurs mainly in the inner mitochondrial membrane. However, it is still debated whether the octamer only binds to the lipid surface [88] or penetrates partially into the membrane bilayer [87]; the latter interaction would be favored by cardiolipin which is capable to form non-bilayer structures [89, 90]. Such a 'dipping-in' could also explain the resistance of a certain Mi-CK fraction against detachment by high ionic strength or detergents and the arrangement of the bulky enzyme in the rather narrow mitochondrial intermembrane space.

Although an interaction between Mi-CK and ANT or porin has been shown on the functional level (for a review see [25]), direct evidence for a structural interaction *in vivo*, e.g. by crosslinking studies, has not been obtained so far [81, 91]. Furthermore, reconstituted ANT did not bind to Mi-CK [85] and electrostatic repulsion of the basic Mi-CK isoforms is suggested by the basic pI of ANT [84] and positive charges exposed to the intermembrane space by porin [92]. However, *in vitro* complex formation between Mi-CK, ANT and porin has clearly been demonstrated [93–95]. Since ANT is situated in a cardiolipin membrane patch [96, 97], the binding of Mi-CK to such patches is assumed to bring the ANT and Mi-CK in close vicinity to each other [25, 98, 99]. The observed interaction of Mi-CK with porin might occur by a similar complex formation via charged phospholipids [81] or by a different, hydrophobic binding mode [94].

Early studies on Mi-CK membrane binding domains

Mi-CK-membrane interactions have been studied mainly with respect to binding mode, binding constants and possible binding partners of Mi-CK; little is known about the structural domains of Mi-CK involved in membrane binding. In a first attempt to identify membrane binding domains, peptide fragments of rat sarcomeric Mi_b-CK, obtained by cyanogen bromide digestion, were analyzed in a binding assay with artificial cardiolipin vesicles [98]. Only one peptide, containing the 25 N-terminal amino acids, showed high affinity to cardiolipin. This approach, however, is prone to artifacts since an isolated peptide can behave differently compared to the same amino acid sequence in its native protein environment (see also below). To identify amino acids involved in membrane binding, the intact Mi-CK molecule was subjected to chemical modification of selected amino acids (Met, Cys, His, Lys and Arg) [98]. It occurred that, while a modification at Met, Cys, or His residues still allowed binding to cardiolipin, modification of Lys or Arg residues drastically reduced binding by over 90%. Although these results must be interpreted with caution, since chemical modifications could introduce overall structural changes, they corroborate evidence on electrostatic interaction between basic amino acids of Mi-CK with negatively charged cardiolipin.

A different early approach for the structural characterization of Mi-CK and its membrane interaction sites applied a variety of electron microscopic techniques for single molecule imaging [100–102]. These methods visualized for the first time the octamer structure of Mi-CK, conserved throughout different species from sea urchins to mammals [103], thus confirming ample biochemical and biophysical evidence on the octameric structure of membrane-bound Mi-CK *in vitro* (e.g. [65, 102]) and *in vivo* (e.g. [104]). In addition, these studies revealed the fourfold symmetry of the octamer with dimers arranged along the symmetry axis (compare to the atomic structure in Fig. 4). Most importantly, they showed that top and bottom faces of the octamer are identical and confer the 'sticky' character to the protein which is responsible for binding to artificial cardiolipin-rich membranes and other negatively charged surfaces [100–102]. Only under very particular conditions in solution, due to the absence of surfaces, top and bottom faces attach to each other and form Mi-CK filaments [101].

Putative membrane-binding motifs of Mi-CK

So far, information on the structural basis of Mi-CK membrane binding has been scarce. The recently solved octameric

structure of the sarcomeric Mi_b-CK from chicken (Fig. 4 and [47]) together with a number of new sequence data (reviewed in [78]) provide now the means for a closer analysis of the membrane binding face of Mi-CK. They allow to reconsider some of the earlier results quoted above and to designate new putative membrane-binding motifs. Those motifs should fulfill the following criteria: (i) since strong membrane-binding is specific for mitochondrial CK isoforms, the responsible sequence motif should be localized in Mi-CK specific, conserved sequence stretches; (ii) the primary structure should contain a positive net charge in order to interact with the negative charges of cardiolipin or other phospholipids and finally, (iii) the charged amino acids should be properly exposed at the top and bottom faces of the octameric Mi-CK structure known to interact with membranes [102].

A comparison of 28 known CK amino acid sequences [78] reveals the high degree of sequence conservation within the CK family, including six blocks of a near 100% sequence identity, separated by seven less conserved blocks. For their entire amino acid sequence, the four CK isoform classes (cytosolic B- and M-CK, sarcomeric and ubiquitous Mi-CK) show about 60% sequence identity, but this number increases to about 80% if mitochondrial and cytosolic isoforms are considered separately. Hence, as much as 20% of conserved Mi-CK sequences differ from cytosolic CK isoforms. Some of these conserved Mi-CK sequences are boxed in longer stretches and contain basic amino acids, among them the N-terminus (Lys5 - Cys24, sequence and numbering according to chicken Mi_b-CK), the C-terminus (Asp352 - Lys380) and some internal stretches, the longest being Asp96 - Arg119, Lys263 - Glu275 and Pro305 - Leu320. As mentioned above, the N-terminus (Thrl - Met25) was already proposed to be the cardiolipin-binding domain due to the binding properties of the corresponding peptide [98]. It contains the positively charged residues Arg19, Lys20 and His21 which are absolutely conserved in all Mi-CKs. However, the octameric CK X-ray-structure clearly shows that the N-terminus is not exposed at the surface but buried inside the Mi-CK octamer (see Fig. 2 in: Stachowiak *et al*., this issue) and therefore not able to interact with membranes. Kaldis *et al*. have shown the involvement of the N-terminus in another Mi-CK-specific property, that is the dimer-dimer-interaction inside the octamer [68].

A closer analysis of the octamer structure of chicken Mi_b-CK reveals two sequence stretches which are properly exposed at the top- and bottom faces of the octamer, carry a positive net charge and are also Mi-CK specific (see Fig. 2 in: Stachowiak *et al*., this issue, and Figs 4 and 5, herein). They encompass the C-terminus (Asp357 - Lys380) and a short internal stretch (Ala107 - Gln115). The C-terminal part of Mi-CK as defined by its location at the molecules surface consists of the distal part of an α-helix (Asp357 - Glu363) and a stretch without defined secondary structure. This sequence shows an accumulation of six or seven basic amino acids resulting in a positive net charge (Fig. 5). In chicken Mi_b-CK, the C-terminus contains six positive charges which are well exposed at the binding face (Fig. 5): five lysines

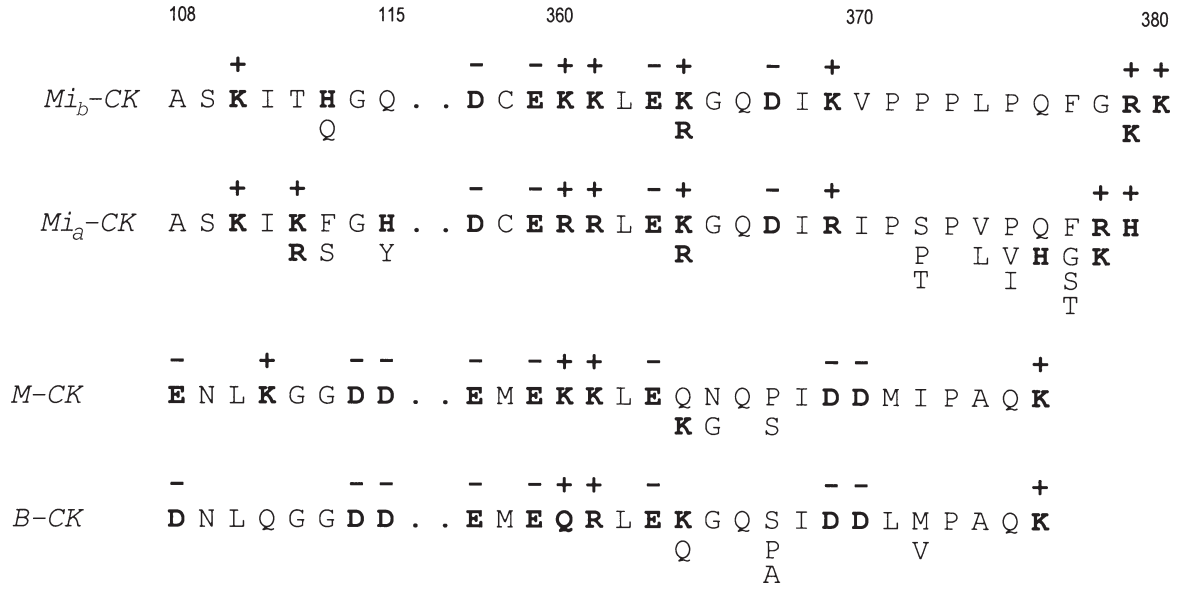

Fig. 5. Amino acid sequence of the putative membrane binding motifs. The homologous sequence stretches of the four CK isoforms, sarcomeric Mi_b-CK, ubiquitous Mi_a-CK, as well as cytosolic muscle-type M-CK and brain-type B-CK, are compared. For each CK isoform, the sequence from chicken is shown in the first line; differences occurring in other species are noted below. Charged amino acids are printed in bold letters and conserved charges are noted above the sequence. Sequence comparison was taken from [78], the amino acid numbering corresponds to the sarcomeric chicken Mi_b-CK.

(Lys360, Lys361, Lys364, Lys369, Lys380) and one arginine (Arg379). Out of the four negative charges (Asp357, Glu359, Glu363, Asp367), only the two asparagines (Asp357 and Asp367) are pointing to the binding face (marked in blue in Fig. 4). The homologous stretches of cytosolic CK isoforms (B-CK and M-CK) do not show such a density of positive charges. It has to be mentioned that the C-terminal sequence is not absolutely identical between sarcomeric and ubiquitous Mi-CK. While the number of basic amino acids is well conserved, their position and a sequence stretch at the very C-terminus show differences which could be responsible for divergent membrane binding properties of the two Mi-CK isoforms (Fig. 5). In both isoenzymes, charged residues at the C-terminus (Arg379 and Lys380) are separated from the other charges (Lys 360, Lys361, Lys364, Lys369) by a stretch of 8–9 residues containing 6–7 hydrophobic amino acids (Fig. 4). These stretches may possibly enter a phospholipid bilayer and thus contribute to the strengthening of the Mi-CK-membrane interaction.

The second sequence motif, a short internal amino acid stretch (Ala107 - Gln115) located at the interconnection between helices of the small and large Mi-CK domain [47] harbors one to three positive charges depending on the Mi-CK isoform and the species considered. In chicken Mi_b-CK, one lysine (Lys110) and one histidine (His113) are found (Fig. 4). By contrast, B-CK does not contain these positive charges and the single positive charge in M-CK is surrounded by several negative charges (Fig. 5). In all Mi-CKs, the cumulative positive net charge of both motifs is about four and is mainly due to lysines (Fig. 4). A similar result, i.e. four lysines involved in Mi-CK-membrane binding, was obtained by a pure modeling approach based on the pH-dependence of the binding process [105].

Although the basic amino acids mentioned are not contiguous on the Mi-CK primary structure, their arrangement on the octamer surface is such that they form clusters of two or four positive charges (Fig. 4). This could have a special significance, given the proximity of the two negatively charged phosphates of the cardiolipin headgroup. A similar 'collar' of lysines has been identified in ANT and proposed to mediate the contact to cardiolipin [106]. Another common feature of both putative membrane-binding motifs is the relatively high temperature factor compared to the main part of the Mi-CK structure [47]. The flexibility indicated by this fact may be useful for a 'docking' onto the negatively charged phospholipid heads.

Taken together, the properties of the two sequence motifs discussed fully support their putative function in membrane-binding, provided that the Mi-CK is in the octameric form and membrane binding occurs primarily due to electrostatic interactions with negatively charged membrane phospholipids and, possibly, a minor participation of hydrophobic forces. This binding model could explain the ability of the Mi-CK octamer to bridge two membranes *in vitro* by its top and bottom faces and to interact functionally with ANT and porin *in vivo* by a close co-location with these membrane proteins inside cardiolipin/phospholipid membrane patches. Formation of the proposed multienzyme complex for energy export (ANT, Mi-CK and porin) could be a transient and dynamic process, similar to the formation of contact sites. A detailed site-directed mutagenesis study of the putative membrane binding motifs of Mi-CK is underway and should provide a clue for defining the interaction sites at the Mi-CK-membrane interface.

Metabolic channeling - a mechanism for Mi-CK in mitochondrial contact-sites?

One of the most important requirements of a cell is to save metabolic energy and to control the flux of metabolites for the biosynthesis of compounds necessary to keep the plethora of biochemical reactions going. These requirements are of particular importance for reactions which directly influence the metabolic status of a cell and its future fate, e.g. energy-conversion reactions like the turnover of ATP, ADP, PCr and Cr catalyzed by CK isoforms. One of the mechanisms that evolved to reach a maximum of energetic efficiency and metabolic control is metabolic channeling: a precursor which is synthesized by enzyme (or subunit) A is passed to enzyme (or subunit) B without getting into contact with the bulk solution (for a review see [107]). Metabolic channeling has been well described for tryptophan synthase which possesses a 25 Å hydrophobic tunnel linking the active sites of two different subunits [108] and thus passing the substrate indole from the α- to the β-subunit. However, channeling can occur between huge covalently linked enzyme-complexes such as the fatty acid synthase, as well as between the structurally less tight coupled glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and aldolase [109].

The sequestering of intermediate products in a micro-compartment provides several advantages for the reaction sequence, but also for cellular metabolism in general. Since these intermediate products immediately serve as substrates for the subsequent reaction and do not equilibrate between microcompartment and bulk solution, which often would favor the backward-reaction, the difference in Gibbs' free energy for their formation is decreased. Competing side-reactions may be excluded and counteracting catabolic and anabolic pathways are separated thus preventing interference which would perturb metabolism. Furthermore, sequestered intermediates are present at high local concentrations and an apparently low K_m for these intermediates can be observed with the channeling complex compared to the non-channeling situation, e.g. *in vitro* measurement with isolated components. Finally, channeling intermediate products of a reaction

cascade in a microcompartment also allows to strictly control the whole process by feed-back regulatory mechanisms such as substrate activation, product inhibition and cooperativity and the over-all-flux of the reactions can be controlled very accurately, e.g. by a limiting step in the reaction sequence (for an overview and further information on the topic see 'Cell Architecture and Metabolic Channeling' by Ovàdi [107] and 'Channeling in Intermediary Metabolism' by Agius and Sherratt [110]).

As already mentioned before, there is functional as well as *in vitro* evidence for complexes containing Mi-CK, porin and ANT, located in the contact sites of the mitochondrial envelope. An important function of these ANT/Mi-CK/porin complexes could be metabolite channeling [48]. In contrast to tryptophan synthase, the function of a Mi-CK complex would not be a reaction cascade where an intermediate is transferred, but the transport of energy-rich phosphate compounds like ATP, ADP and PCr between subcellular compartments. This means that ATP reaching the mitochondrial intermembrane space via ANT is directly channeled to the Mi-CK octamer and used for synthesis of PCr which then is channeled out of the mitochondrion into the cytosol via Mi-CK and porin. Vice versa, ADP is back-channeled into the matrix via ANT.

A prerequisite for this mode of action is a physical interaction of Mi-CK and ANT or, at least, a close vicinity of both leading to 'leaky' channeling. The finding that externally added pyruvate kinase is not able to use the ADP produced by the Mi-CK reaction [82, 111] and the fact that about 50% of Mi-CK activity is resistant against iodo acetate treatment [93] strongly supports the model of Mi-CK in close vicinity to ANT and porin. Kottke and co-workers further showed that in mitoplasts supplied with ATP and creatine, the blockage of porin by a polyanion strikingly decreases Mi-CK activity [93]. In addition, creatine-stimulated mitochondrial respiration due to ADP synthesis via Mi-CK is not abolished by the addition of external ADP traps like PEP plus pyruvate kinase, showing that the ADP generated by Mi-CK is located in a microcompartment close to the inner mitochondrial membrane and does not equilibrate with the bulk ADP pool [82, 111]. An interaction of Mi-CK with porin is indicated by the fact that isolated porin is able to induce octamer formation of an N-terminal Mi-CK deletion mutant which is unable to build up octamers in free solution [94]. Finally, complex formation of octameric Mi-CK with porin and ANT has recently been demonstrated *in vitro* [94, 95].

There are several advantages of the porin/Mi-CK/ANT arrangement: (i) ATP originating from the matrix space does not equilibrate with the cytosolic ATP and is present at very high local concentrations which facilitate the formation of PCr from Cr; (ii) since ATP is directly turned over after being exported out of the matrix, it will generate high local concentrations of ADP in the intermembrane space which stimulates respiration and favors re-import of ADP into the matrix because of a steeper ADP gradient across the inner membrane [111]. This model also allows the ANT to act as an obligatory antiporter, since for every exported ATP there is an ADP molecule present which can be bound by the translocase. The thermodynamic advantage of such a complex is due to (i) the removal of the translocation product ATP, favoring further export of the latter via ANT, and (ii) the removal of once formed PCr from equilibrium by direct channeling into the cytosol via porin, favoring PCr synthesis by Mi-CK [111,112]. Possible transport and diffusion pathways involved of such a channeling mechanism are discussed in detail in the next chapter (see also Fig. 6).

Metabolite access to Mi-CK - a 'back door' hypothesis

As it has been outlined above, Mi-CK displays functional and possibly also structural coupling to ANT and porin, two membrane proteins involved in the transport of Mi-CK substrates and products. If this coupling involves metabolic channeling, a vectorial transport of substrates and products would be expected [48]. The X-ray structure of chicken Mi_b-CK [47] provides a new and suitable tool to develop models for metabolite transport in the multiprotein complex bridging the two mitochondrial membranes as suggested from *in vitro* studies [93–95]. In this respect, a specific structural feature of the octameric structure is of particular interest, i.e. the central pore or channel of approximately 20 Å in diameter penetrating the octamer. This channel has already been detected by electron microscopic studies of chicken Mi_b-CK [101] and has been proposed to connect the active site of Mi-CK with the surroundings, supposing that active sites were exposed towards the central channel. This assumption was plausible at a time where still no three-dimensional high-resolution structure of CK was available.

However, the X-ray structure of Mi_b-CK shows that the most obvious and direct metabolite access to the active site of Mi-CK is from the 'outside' of the molecule (see Fig. 3c in [47]) and not from the channel. The CK active sites, one for each monomer, are exposed on the concave side of the 'banana-shaped' dimers (Fig. 3), and four such dimers form an octamer by contacting each other via the convex 'backside' (involving an N-terminal stretch plus the region around Trp264, see Fig. 3 and also Fig. 3b in [47]). If the Mi-CK octamer binds to the inner and outer mitochondrial membranes by its identical top and bottom faces, as shown by Schnyder [102], the active sites face the intermembrane space surrounding the octamer and the channel is oriented orthogonally to the membranes and thus is excluded from direct interaction with the intermembrane space. For this kind of organization between Mi-CK and membranes, one could propose two different models which would explain a compartmentalized CK reaction as indicated by the functional coupling of Mi-CK to ANT and porin (see Fig. 6).

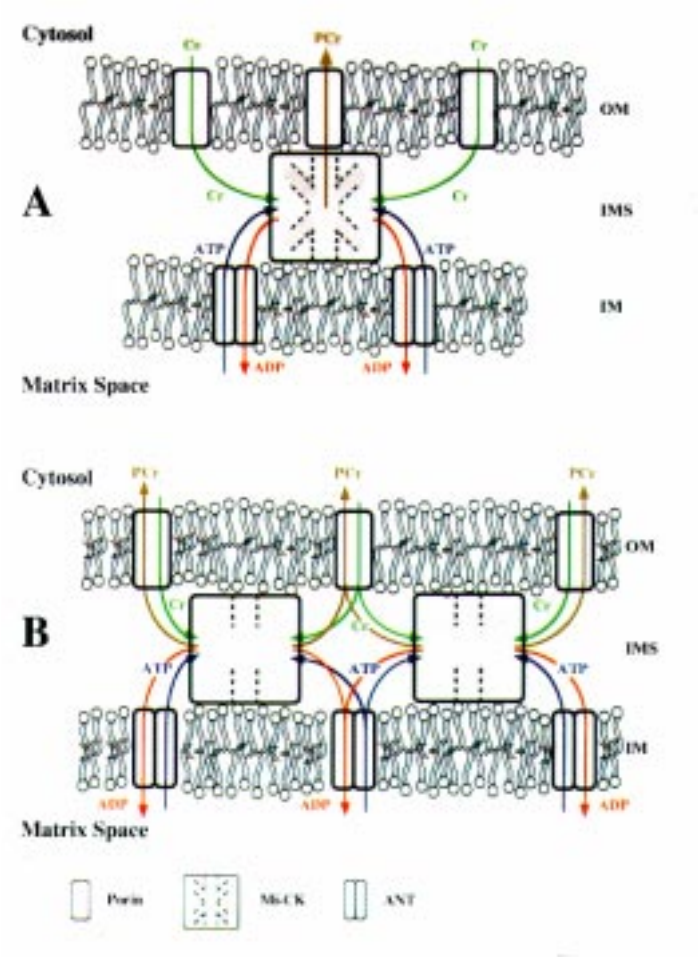

Fig. 6. Model considerations for microcompartmentation of the Mi-CK reaction. Two different models for microcompartmentation of substrates and products of the creatine kinase (CK) reaction, as well as for vectorial export of phosphocreatine out of the mitochondrion are shown. Both involve porin of the outer mitochondrial membrane (OM), adenine nucleotide translocator (ANT) of the inner membrane (IM) and Mi-CK octamers in the intermembrane space (IMS). The fluxes of the metabolites are depicted by arrows colored in red (ADP), blue (ATP), green (Cr) and brown (PCr). (A): Possible 'back door' mechanism. Cr and ATP enter the CK active sites from the IMS. After phosphoryltransfer, PCr leaves the active site via the suggested 'back door' of the enzyme, being guided into the central 20Å wide channel of the Mi-CK octamer. From there, it is exported into the cytosol via porin situated on top of the central channel (see also text). This model suggests that the central hole in the octamer and porin indeed serve as a channel connecting the CK active site with the cytosol. (B): Microcompartmentation without the need for a 'back door' mechanism. Reactants and products of the CK reaction enter and leave the enzyme from the 'front door'. Octamers situated close to each other are suggested to form a microcompartment including porin and ANT for the transport of the metabolites to and from the cytosol or mitochondrial matrix, respectively. In this model, the central hole of Mi-CK has no channeling function and would serve merely a structural role. The vectorial transport of PCr to the cytosol would be regulated by the accumulation of the product in the IMS, resulting in a change of ion selectivity of porin (see also text).

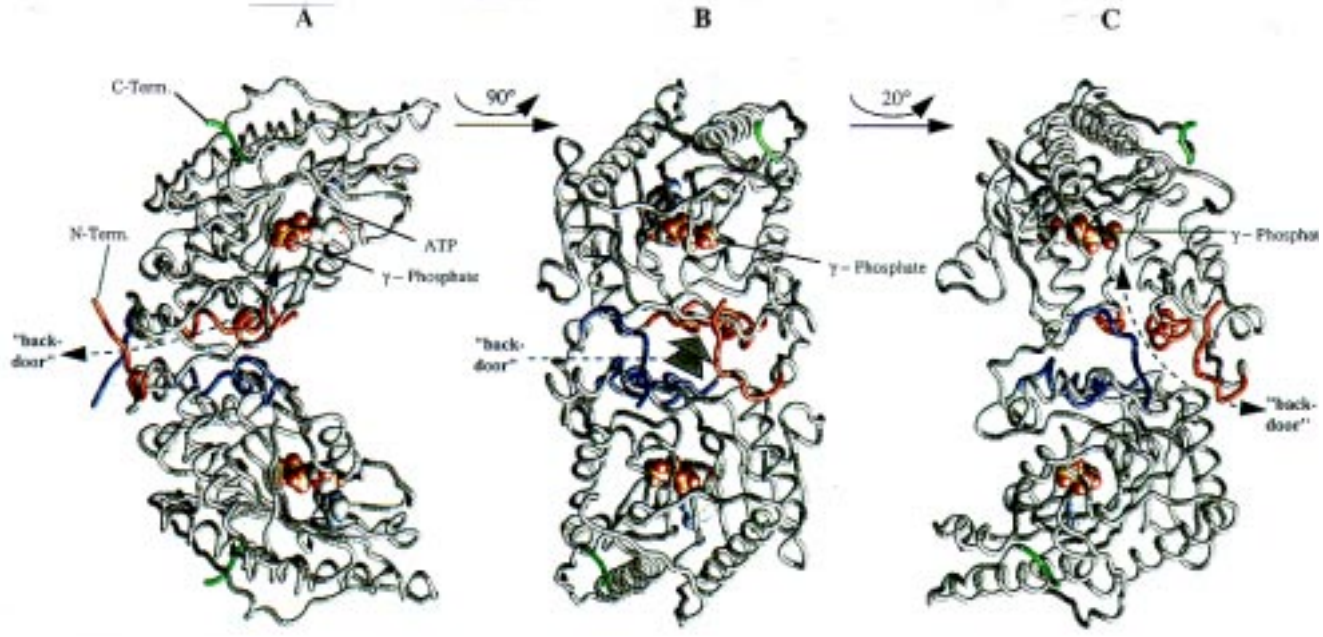

Fig. 7. Structural model of an alternative leaving-route for the phosphocreatine product through a 'back-door' exit. We propose a possible pathway of phosphocreatine (PCr) through an exit between two CK-monomers leading to the central main channel of the octamer. A 'backbone trace' representation of a 'banana-shaped' Mi-CK-dimer is shown with bound ATP (A). The dimer is rotated about 90° (B) and additional 20° (C) around its longitudinal axis to provide a better 3-dimensional visualization. The C-termini are marked in green, the N-termini and residues 47–59/194–202 are shown in red (monomer 1) and blue (monomer 2). The alternative leaving-route for PCr is depicted in each representation with a dashed arrow; the broad arrow in (B) indicates the 'back door'. Note that this 'back door' is deduced only from the X-ray structure of Mi-CK and is postulated on the basis of biochemical considerations (see text). The figure was prepared using the rendering software RASMOL v. 2.6 [123].

In the first model, depicted in Fig. 6A, access of substrates and products to the active site occurs from different sides of the enzyme, that is from the intermembrane space and from the central channel. In this case, a role of the channel in the transport of metabolites would require (i) direct structural interaction with porin (and/or ANT) and (ii) accessibility of the channel from the active sites of CK, positioned on the outside of the four side-faces of the MiCK octamer, by a so called 'back door' pathway. The existence of an alternative route for substrate, product or water ('back door' mechanism) has been proposed recently for myosin [113], acetylcholine esterase [114] and the tryptophan synthase $\alpha_2\beta_2$ multienzyme complex [115]. Such a 'back door' connecting the active site with the channel could, indeed, exist in Mi-CK, as shown in Fig. 7 for a Mi-CK dimer. Two helices in each of the monomers (residues 47–59 in domain I and 194–202 in domain II) and the N-termini form an opening face to the central channel and a small access route to the active site. However, this pathway would be probably too narrow for adenine nucleotides having a relatively large size and bulky conformation. Therefore, access of ATP and exit of ADP to and from the active site, respectively, is assumed to occur via the intermembrane space. It then follows that ANT of the inner mitochondrial membrane should be located in the vicinity of CK octamers without direct contact to the central channel, thus releasing ATP into the intermembrane space which would subsequently diffuse into the active site pocket of CK (Fig. 6A).

The described 'back door' pathway, however, would be best suited for PCr, since (i) the 'back door' directly points to a putative PCr/Cr binding site (facing the γ-phosphate of ATP) and (ii) the PCr molecule has a more elongated structure which could best fit through the 'back door' (due to the repulsion of charges, PCr becomes more elongated than Cr, which has a more or less cyclic structure). Thus, the model suggests that PCr is dissociating from the active site directly into the central channel of the octamer, thereby separating from ADP which cannot follow due to its larger size and also for electrostatic reasons. In addition, it is entirely conceivable that domain movement upon substrate binding leads to further opening of the 'back door', thus favoring PCr product release. In this model, the porin in the outer mitochondrial membrane would be in direct interaction with the central channel to transport synthesized PCr into the cytosol (Fig. 6A).

Although it is tempting to believe in the existence of such a 'back door' that would enable vectorial transport of PCr out of the mitochondrion or facilitate the proposed microcompartmentation of the CK reaction and its interaction with the transport proteins, alternative possibilities as the one depicted in Fig. 6B may be envisaged as well. In this model, several CK octamers arranged in close proximity (only two are drawn due to the limitation of the two-dimensional representation) enclose a part of the intermembrane space, thereby forming a microcompartment including ANT and porin. The central channel of the Mi-CK octamer would then have no other function than a mere structural role, stabilizing the convex 'backsides' (see Fig. 3) of the four dimers in the octamer and probably offering space for structural rearrangements of the monomers within the octamer during catalysis, as has bee shown to take place upon Mg^{2+}-nucleotide binding [79].

Several arguments, however, are in favor of a 'back door' mechanism (Fig. 6A). One of the strongest is the great thermodynamic advantage of such a mode of reaction due to the continuos withdrawing of one of the products from the active site environment. This mechanism would facilitate a non-equilibrium system, compared to the alternative model which releases all Mi-CK products into the microcompartment of the incoming substrates (Fig. 6B). Furthermore, it has been shown that product release is the rate limiting step of the CK reaction in the forward direction [74] and that PCr is the 'sticky' product. This would be in line with the certainly longer time for the diffusion of PCr out of the active site through a 'back door' compared to the fast release of ADP through the large open cleft in the CK structure. Another supportive argument comes from the inhibition mode of cyclocreatine (cCr). cCr, a competitive inhibitor of the CK reaction, has been shown to bind to the active site and to be phosphorylated [116]. The 'back door' model offers a simple explanation for this inhibitory mechanism. Although the cCr structure mimics the cyclic nature of Cr and cCr can be phosphorylated, the conformational change upon phosphorylation leading to a more elongated structure (see above) cannot take place like in the case of PCr. Therefore, phosphorylated cCr could be unable to use the release pathway of PCr through the 'back door' and thus would block the active site of Mi-CK.

The main argument against a 'back door' mechanism comes from the classical enzymological studies of the CK reaction. The forward reaction of CK was described to follow a random bi-bi mechanism [6, 74], while the proposed 'back door' pathway would rather imply a sequential scheme, involving binding of the substrates, domain closure induced by Mg-ATP [79] together with a possible further opening of the 'back door', PCr release via the 'back door' and, finally, Mg-ADP release into the intermembrane space. However, this mechanism is not necessarily in contradiction with the available kinetic data, since all of them have been obtained with dimeric MM-CK in free solution. Enzyme kinetics of Mi-CK might therefore be different, especially under *in vivo* conditions in the intermembrane space when attached to mitochondrial membranes. As of yet, there is no direct experimental proof for the proposed 'back door' mechanism in Mi-CK, and it should be noted once more that the appealing hypothesis presented herein is mere speculation based on structural data. Clearly, more studies addressing this intriguing question are necessary, including detailed enzyme kinetics of octameric Mi-CK, as well as a direct analysis of the 'back door' pathway, e.g. by introducing bulky residues with site directed mutagenesis.

Conclusions and outlook

It has taken more than 60 years since the first description of CK [2] until the first X-ray structure of a member of the CK isoform family could be solved [47]. This step forward has been recognized as a significant achievement and landmark in CK research [71, 117]. As outlined above, the octameric structure has already provided some important insights and stimulating clues into the functioning of the enzyme and, after all, is also rewarding from a purely aesthetical point of view. However, having the structure of the Mi-CK octamer at hand with many details becoming obvious now, we are brought back to real life again. Some difficult longstanding questions, e.g. about the kinetic mechanism of the enzyme and the amino acid residues involved in catalysis, have still to be answered in detail. Are there true catalytic residues or does CK belong to the category of 'conzymes' designed to merely bring the substrates into close alignment [71]?

To solve this puzzle, an X-ray structure of CK in the presence of reagents known to induce the enzymes' transition state, e.g. Mg-ADP, creatine and nitrate [118], will be of utmost importance. Such a structure of the 'closed configuration' of the enzyme should reveal not only the exact molecular outline of the active site pocket of CK caught in the act of catalysis, but should also shed some light on the expected hinge bending and domain movements taking place upon binding of Mg^{2+}-nucleotide [79]. After having learned that each subunit of CK has its own catalytic site, a further important task will be the elucidation of possible cooperativity between monomers in the dimer and, even more interestingly, in the octamer. The latter question, of course, will be most relevant to the issues of vectorial metabolite channeling and directed export of PCr, derived by Mi-CK from matrix generated ATP, out of the mitochondrion (see above).

The understanding of the molecular details of CK catalysis and the possible design of CK-specific inhibitors are likely to be of great clinical relevance, since an involvement of CK in signal transduction [119, 120], as well as in tumor growth and malignancy has been demonstrated and since certain creatine analogues were shown to display anti-cancer activity on explanted human tumors [121] or to enhance significantly the effect of chemotherapy on cancer cells [122].

The precise knowledge of CK structure and function at the molecular level may elucidate the *in vivo* function of this isoenzyme system in cells and organs with high and fluctuating energy requirements and thus may complement the work done with transgenic mice lacking the expression of one or several CK isoform *in vivo* [37]. Since every novel result produces a myriad of new questions, much of what has to be learned is still ahead of us.

Note added in proof

Recent ^{31}P-NMR studies comparing CK-mediated fluxes of inactive versus actively swimming sea-urchin sperm [124], as well as mathematical modeling of cardiomyocyte energy metabolism, including the known CK-'knock-out' models [125], are in support of a CK/PCr-shuttle in these cells.

With the help of the chicken Mi_b-CK coordinates, the X-ray structures of cytosolic B-CK (Rao JKM and Wlodawer A, personal communication) and the related lobster arginine kinase (Dumas C and Janin J, personal communication) have been solved. Arginine kinase from horseshoe crab has been crystallized in the presence of TSAC substrates [126] and may provide the first transition state structure of a guanidino kinase. A new study of Mi-CK membrane binding properties [127] suggested a partial disorganization of the used phospholipid bilayer upon interaction with Mi-CK and revealed a decreased Mi-CK binding after treatment with p-hydroxymercuribenzoat. The latter may be due to a modification of the C-terminal Cys358 (see Fig. 5), which could influence the binding capacity of the C-terminus.

Acknowledgments

Dr. W. Kabsch is gratefully acknowledged for discussion. This work was supported by the Swiss National Science Foundation (SNF grant No. 31-33907.92, to T.W.), by ETH graduate training grants for M.F., M.E. and O.S. and by financial support from the Swiss Society for Muscle Diseases, as well as by private sponsoring from Careal Holding AG and Synergen AG, Switzerland.

References

1. Eggleton P, Eggleton GP: Further observations of phosphagens. J Physiol 65: 15–24, 1928
2. Lohman K: Über die enzymatische Aufspaltung der Kreatinphosphorsaure; zugleich ein Beitrag zum Chemismus der Muskelkontraktion. Biochem Z 271: 264–277, 1934
3. Lehmann H: Über die Umesterung des Adenylsäuresystems mit Phosphagenen. Biochem Z 286: 336–343, 1936
4. Lipmann F: Metabolic generation and utilization of phosphate bond energy. Adv Enymol 1: 99–162, 1941
5. Kuby SA, Noda L, Lardy HA: Adenosinetriphosphate-creatine transphosphorylase. I. Isolation of the crystalline enyme from rabbit muscle. J Biol Chem 209: 191–201, 1954
6. Kuby SA, Noltman EA: ATP-Creatine Transphosphorylase. In: PD Boyer, H Lardy, K Myrback (eds). The Enymes, Vol. 6. Academic Press, NY, 1963, pp 515–521
7. Burger A, Eppenberger M, Wiesman U, Richterich R: Isoenzyme der Kreatinkinase. Helv Physiol Acta 21: C6–C10, 1963
8. Eppenberger HM, Eppenberger M, Richterich R, Aebi H: The ontogeny of creatine kinase isozymes. Dev Biol 10: 1–16, 1964

9. Jacobs H, Heldt HW, Klingenberg M: High activity of creatine kinase in mitochondria from muscle and brain. Evidence for a separate mitochondrial isoenzyme of creatine kinase. Biochem Biophys Res Commun 16: 516–521, 1964
10. Klingenberg M: Muskelmitochondrien. In: K Kramer, O Krayer, E Lehnartz, A von Muralt, HH Weber (eds). Ergebnisse der Physiologie, biologischen Chemie und experimentellen Pharmakologie. Springer, Berlin, 1964, pp 132–189
11. Gercken G, Schlette U: Metabolite status of the heart in acute insuffciency due to 1-fluoro-2,4 dinitrobenzene. Experientia 24: 17– 19, 1968
12. Naegle S: Die Bedeutung von Creatinphosphat und Adenosintriphosphat im Hinblick auf Energiebereitstellung, -transport und -verwertung in normalen und insuffzienten Herzmuskeln. Klin Wochenzeitschrift 48: 332–341, 1970
13. Bessman SP, Fonyo A: The possible role of the mitochondrial bound creatine kinase in regulation of mitochondrial respiration. Biochem Biophys Res Comm 22: 597–420, 1966
14. Saks VA, Khuchua ZA, Vasilyeva EV, Belikova OY, Kuznetsov AV: Metabolic compartmentation and substrate channelling in muscle cells. Role of coupled creatine kineses in *in vivo* regulation of cellular respiration - a synthesis. Mol Cell Biochem 133/134: 155–192, 1994
15. Turner D, Wallimann T, Eppenberger HM: A protein that binds specifically to the M-line of skeletal muscle is identified as the muscle form of creatine kinase. Proc Natl Acad Sci USA 70: 702–705, 1973
16. Wallimann T, Turner DC, Eppenberger HM: Localization of creatine kinase isoenzymes in myofibrils. I Chicken skeletal muscle. J Cell Biol 75: 297–317, 1977
17. Wallimann T: Creatinkinase-Isoenzyme und Myofibrillen-Struktur. Ph.D. Thesis ETH No. 5437, Swiss Federal Institute of Technology, Zürich, 1975
18. Saks VA, Rosenstraukh LV, Smirnov VN, Chazov EI: Role of creatine phosphokinase in cellular function and metabolism. Can J Physiol Pharmacol 56: 691–706, 1978
19. Bessman SP, Geiger PJ: Transport of energy in muscle: The phosphorylcreatine shuttle. Science 211: 449–452, 1981
20. McClellan G, Weisberg A, Winegrad S: Energy transport from mitochondria to myofibril by a creatine phosphate shuttle in cardiac cells. Am J Physiol 245: C423–C427, 1983
21. Bessmann SP, Carpenter CL: The creatine-creatine phosphate energy shuttle. Annu Rev Biochem 54: 831–862, 1985
22. Wallimann T, Eppenberger HM: Localization and function of M-line-bound creatine kinase. M-band model and creatine phosphate shuttle. In: JW Shay (ed). Cell and Muscle Motility Vol. 6, Plenum Publ, NY, 1985, pp239–285
23. Kammermeier H: Why do cells need phosphocreatine and a phosphocreatine shuttle. J Mol Cardiol 19: 115–118, 1987
24. Wallimann T, Schnyder T, Schlegel J, Wyss M, Wegmann G, Rossi AM, Hemmer W, Eppenberger HM, Quest AFG: Subcellular compartmentation of creatine kinase isoenzymes, regulation of CK and octameric structure of mitochondrial CK: Important aspects of the phosphoryl-creatine circuit. In: RJ Paul, G Elzinga, K Yamada (eds). Progress in Clinical and Biological Research. Vol. 315: 'Muscle Energetics', A.R. Liss, NY, 1989, pp 159–176
25. Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM: Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: The 'phosphocreatine circuit' for cellular energy homeostasis. Biochem J 281: 21–40, 1992
26. Wyss M, Smeitink J, Wevers RA, Wallimann T: Mitochondrial creatine kinase: A key enzyme of aerobic energy metabolism. Biochim Biophys Acta 1102: 119–166, 1992
27. Wallimann T: Dissecting the role of creatine kinase.The phenotype of 'gene knockout' mice deficient in a creatine kinase sheds new light on the physiological functions of the 'phosphocreatine circuit'. Current Biol 1: 42–46, 1994
28. Saks VA, Ventura-Clapier R: Cellular Bioenergetics: Role of Coupled Creatine Kinases. Mol Cell Biol 133/134, 1994
29. Meyer R, Sweeney HL, Kushmerick MJ: A simple analysis of the 'phosphocreatine shuttle'. Am J Physiol 246: C365–377, 1984
30. Wallimann T, Hemmer W: Creatine kinase in non-muscle tissues and cells. Mol Cell Biochem 133/134: 193–220, 1994
31. Kaldis P, Kamp G, Piendl T, Wallimann T: Functions of creatine kinase isoenzymes in spermatozoa. Adv Dev Biochem 5: 275–312, 1997
32. Baskin RJ, Deamer DW: A membrane-bound creatine phosphokinase in fragmented sarcoplasmic reticulum. J Biol Chem 245: 1345–1347, 1970
33. Levitsky DO, Levchenko TS, Saks VA, Sharov VG, Smirnov: The role of creatine phosphokinase in supplying energy for the calcium pump system of heart sarcoplasmic reticulum. Membrane Biochem 2: 81–96, 1978
34. Rossi AM, Eppenberger HM, Volpe P, Cotrufo R, Wallimann T: Muscle-type MM-creatine kinase is specifically bound to sarcoplasmic reticulum and can support Ca^{2+}-uptake and regulate local ATP/ADP ratios. J Biol Chem 265: 5258–5266, 1990
35. Korge P, Byrd SK, Campbell KB: Functional coupling between sarcoplasmic-reticulum-bound creatine kinase and Ca^{2+} -ATPase. Eur J Biochem 213: 973–980, 1993
36. Minajeva A, Ventura-Clapier R, Veksler V: Ca^{2+}-uptake by cardiac sarcoplasmic reticulum ATPase in situ strongly depends on bound creatine kinase. Pflügers Arch Eur J Physiol 432: 904–912, 1996
37. Steeghs K, Benders A, Oerlemans F, deHaan A, Heerschap A, Ruitenbeek W, Jost C, vanDeursen J, Perryman B, Pette D, Brückwilder M, Koudijs J, Jap P, Veerkamp J, Wieringa B: Altered Ca^{2+}-homeostasis in muscles with combined mitochondrial and cytosolic CK deficiencies. Cell 89: 93–103, 1997
38. Brosnan JM, Chen L, Wheeler CE, vanDyke TA, Koretzky AP: Phosphocreatine protects ATP from a fructose load in transgenic mouse liver expressing creatine kinase. Am J Physiol 260: C1191–C1200, 1991
39. Miller K, Halow J, Koretzky AP: Phosphocreatine protects transgenic mouse liver expressing creatine kinase from hypoxia and ischemia. Am J Physiol 265: C1544–C1551, 1993
40. Satoh S, Tanaka A, Hatano E, Inomoto T, Iwata S, Kitai T, Shinohara H, Tsunekawa S, Chance B, Yamaoka Y: Energy metabolism and regeneration in transgenic mouse livers expressing creatine kinase after major hepatectomy. Gastroenterol 110: 1166–1174, 1996
41. McFarland EW, Kushmerick MJ, Moerland T: Activity of creatine kinase in a contracting mammalian muscle of uniform fibre type. Biophys J 67: 1912–1924, 1994
42. Wiseman RW, Kushmerick M: Creatine kinase equilibrium follows solution thermodynamics in skeletal muscle: ^{31}P-NMR studies using creatine analogs. J Biol Chem 270: 12428–12438, 1995
43. VanDeursen J, Ruitenbeek W, Heerschap A, Jap P, Terlaak H, Wieringa B: Creatine kinase (CK) in skeleteal muscle energy metabolism: A study of mouse mutants with graded expression in muscle CK expression. Proc Natl Acad Sci USA 91: 9091–9095, 1994
44. Wallimann T: ^{31}P-NMR-measured creatine kinase reaction flux in muscle: A caveat! J Muscle Res Cell Motil 17: 177–181, 1996
45. Williams JP, Headrick JP: Differences in nucleotide compartmentation and energy state in isolated an in situ rat heart: Assessment by ^{31}P-NMR spectroscopy. Biochim Biophys Acta 1276: 71–79, 1996
46. Dzeja PP, Zeleznikar RJ, Goldberg ND: Suppression of creatine kinase-catalyzed phosphotransfer results in increased phosphoryl transfer by adenylate kinase in intact skeletal muscle. J Biol Chem 271:12847–12851, 1996
47. Fritz-Wolf K, Schnyder T, Wallimann T, Kabsch W: Structure of mitochondrial creatine kinase. Nature 381: 341–345, 1996
48. Wyss M, Wallimann T: Metabolite channelling in aerobic energy metabolism. J theor Biol 158: 129–132, 1992

49. Keutel HJ, Jacobs HK, Okabe K, Yue RH, Kuby SA: Studies on adenosine triphosphate transphosphorylases. VII. Isolation of the crystalline adenosine triphosphate - creatine transphosphorylase from calf brain. Biochemistry 7: 4283–4290, 1968
50. McPherson A: A preliminary crystallographic investigation of rabbit muscle creatine kinase. J Mol Biol 81: 83–86, 1973
51. Burgess AN, Liddell JM, Cook W, Tweedlie RM, Swan IDA: Creatine kinase. A new crystal form providing evidence of subunit structural homogeneity. J Mol Biol 123: 691–695, 1978
52. Takasawa T, Fukushi K, Shiokawa H: Crystallization and properties of creatine kinase from equine skeletal muscle. J Biochem 89: 1619– 1631, 1981
53. Gilliland GL, Sjolin L, Olsson GL: Crystallization and preliminary X-ray diffraction data of two crystal forms of bovine heart creatine kinase. J Mol Biol 170: 791–793, 1983
54. Hershenson S, Helmers N, Desmueles P, Stroud R: Purification and crystallization of creatine kinase from rabbit skeletal muscle. J Biol Chem 261: 3732–3736, 1986
55. Schnyder T, Sargent DF, Richmond TJ, Eppenberger HM, Wallimann T: Crystallization and preliminary X-ray analysis of two different forms of mitochondrial creatine kinase from chicken cardiac muscle. J Mol Biol 216: 809–812, 1990
56. Schnyder T, Winkler H, Gross H, Eppenberger HM, Wallimann T: Crystallization of mitochondrial creatine kinase. Growing of large protein crystals and electron microscopic investigation of microcrystals consisting of octamers. J Biol Chem 266: 5318–5322, 1991
57. Furter R, Kaldis P, Furter-Graves EM, Schnyder T, Eppenberger HM, Wallimann T: Expression of active octameric chicken cardiac mitochondrial creatine kinase in *Escherichia coli*. Biochem J 288: 771–775, 1992
58. Jones TA, Zou JY, Cowan SW, Kjelgaard M: Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Cryst A47: 110–119, 1991
59. Brünger AT: X-PLOR Version 3.1 Manual. Yale University Press, New Haven, USA, 1992
60. Kabsch W, Sander C: Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. Biopolymers 22: 2577–2637, 1983
61. Gross M: The tryptophan residues of mitochondrial creatine kinase: Roles in enzyme structure and function. Ph.D.Thesis ETH No. 10719, Swiss Federal Institute of Technology, Zürich, 1994
62. Wyss M, James P, Schlegel J, Wallimann T: Limited proteolysis of creatine kinase. Implications for the three-dimensional structure and for conformational substates. Biochemistry 32: 10727–10735, 1993
63. Morris GE, Jackson PJ: Identification by protein microsequencing of a proteinase-V8-cleavage site in a folding intermediate of chick muscle creatine kinase. Biochem J 280: 809–811, 1991
64. Gross M, Wyss M, Furter-Graves EM, Wallimann T, Furter R: Reconstitution of active octameric mitochondrial creatine kinase from two genetically engineered fragments. Protein Sci 5: 320–330, 1996
65. Schlegel J, Zurbriggen B, Wegmann G, Wyss M, Eppenberger HM, Wallimann T: Native mitochondrial creatine kinase (Mi-CK) forms octameric structures. I. Isolation of two interconvertible Mi-CK isoforms: Dimeric and octameric Mi-CK. J Biol Chem 263: 16942–16953, 1988
66. Gross M, Wallimann T: Kinetics of assembly and dissociation of the mitochondrial creatine kinase octamer. A fluorescence study. Biochemistry 32: 13933–13940, 1993
67. Gross M, Furter-Graves EM, Wallimann T, Eppenberger HM, Furter R: The tryptophan residues of mitochondrial creatine kinase: Roles of Trp-223, Trp-206, and Trp-264 in active-site and quaternary structure formation. Protein Sci 3: 1058–1068, 1994
68. Kaldis P, Furter R, Wallimann T: The N-terminal heptapeptide of mitochondrial creatine kinase is important for octamerization. Biochemistry 33: 952–959, 1994
69. Beuchter DD, Medzihradsky KF, Burlingame AL, Kenyon GL: The active site of creatine kinase. Affinity labeling of cysteine 282 with epoxycreatine. J Biol Chem 267: 2172–2178, 1992
70. Hou LX, Vollmer S: The activity of S-thiomethyl modified creatine kinase is due to the regeneration of free thiol at the active site. Biochim Biophys Acta 1205: 83–88, 1994
71. Stroud RM: Balancing ATP in the cell. Nature Struct Biol 3: 567–569, 1996
72. Furter R, Furter-Graves EM, Wallimann T: Creatine kinase: The reactive cysteine is required for synergism but is nonessential for catalysis. Biochemistry 32: 7022–7029, 1993
73. Rosevear PR, Desmeules P, Kenyon GL, Mildvan AS: Nuclear magnetic resonance studies of the role of histidine residues at the active site of rabbit muscle creatine kinase. Biochemistry 20: 6155–6164, 1981
74. Cook PF, Kenyon GL, Cleland WW: Use of pH studies to elucidate the catalytic mechanism of rabbit muscle creatine kinase. Biochemistry 20: 1204–1210, 1981
75. Forstner M, Müller A, Stolz M, Wallimann T: The active site histidines of creatine kinase. A critical role of His61 situated on a flexible loop. Prot Sci 6: 331–339, 1997
76. Chen LH, Borders CL, Vásquez JR, Kenyon GL: Rabbit muscle creatine kinase: Consequences of the mutagenesis of conserved histidine residues. Biochemistry 35: 7895–7902, 1996
77. Messmer CH, Kägi JHR: Tryptophan residues of creatine kinase: A fluorescence study. Biochemistry 24: 7172–7178, 1985
78. Mühlebach SM, Gross M, Wirz T, Wallimann T, Perriard JC, Wyss M: Sequence homology and structure predictions of the creatine kinase isozymes. Mol Cell Biochem 133/134: 245–262, 1994

78a. Vasàk M, Nagayama K, Wüthrich K, Mertens M, Kägi JHR: Creatine kinase. Nuclear magnetic resonance and fluorescence evidence for interaction of adenosine-5′-diphosphate with aromatic residue(s). Biochemistry 18, 5050–5055, 1979

79. Forstner M, Kriechbaum M, Laggner P, Wallimann T: Changes of creatine kinase structure upon ligand binding as seen by small-angle scattering. J Mol Struct 383: 217–222, 1996
80. Jacobus WE, Lehninger AL: Creatine kinase of rat heart mitochondria. Coupling of creatine phosphorylation to electron transport. J Biol Chem 248: 4803–4810, 1973
81. Schnyder T, Rojo M, Furter R, Wallimann T: The structure of mitochondrial creatine kinase and its membrane binding properties. Mol Cell Biochem 133/134: 115–123, 1994
82. Saks VA, Kuznetsov AV, Kupriyanov VV, Miceli MV, Jacobus WE: Creatine kinase of rat mitochondria: The demonstration of functional coupling to oxidative phosphorylation in an inner membrane matrix preparation. J Biol Chem 260: 7757–7764, 1985
83. Stachowiak O, Dolder M and Wallimann T: Membrane-binding and vesicle cross-linking kinetics of the mitochondrial creatine kinase octamer. Biochemistry 35: 15522–15528, 1996
84. Lipskaya TY, Templ VD, Belousova LV, Molokova EV, Rybina IV: Investigation of the interaction of mitochondrial creatine kinase with the membranes of the mitochondria. Biochimia USSR 45: 877–886, 1980
85. Müller M, Moser R, Cheneval D, Carafoli E: Cardiolipin is the membrane receptor for mitochondrial creatine phosphokinase. J Biol Chem 260: 3839–3843, 1985
86. Brooks SPJ, Suelter CH: Asociation of chicken mitochondrial creatine kinase with the inner mitochondrial membrane. Arch Biochem Biophys 253: 122–132, 1987
87. Rojo M, Hovius R, Demel RA, Wallimann T: Interaction of mitochondrial creatine kinase with model membranes. A monolayer study. FEBS Lett 281: 123–129, 1991
88. Cheneval D, Carafoli E, Powell GL, Marsh D: A spin-label electron spin resonance study of the binding of mitochondrial creatine kinase to

cardiolipin. Eur J Biochem 186: 415–419, 1989
89. De Kruijff B, Verkleij AJ, van Echteld CJA, Gerritsen WJ, Noordam PC, Mombers C, Rietveld A, de Gier J, Cullis PR, Hope MJ, Nayar R: Non-bilayer lipids and the inner mitochondrial membrane. In: HG Schweuger (ed). International Cell Biology. Springer-Verlag, Berlin, 1981, pp 559–671
90. Rietveld AG, Koorengevel MC, de Kruijff B: Non-bilayer lipids are required for efficient protein transport across the plasma membrane of *Escherichia coli*. EMBO J 22: 5506–5513, 1995
91. Font B, Eichenberger D, Goldschmidt D, Vial C: Interaction of creatine kinase and hexokinase with the mitochondrial membranes, and self-association of creatine kinase: crosslinking studies. Mol Cell Biochem 78: 131–140, 1987
92. De Pinto V, Palmieri F: Transmembrane arrangement of mitochondrial porin or voltage-depemdant anionchannel (VDAC). J Bioenerg Biomembr 24: 21–26, 1992
93. Kottke M, Adams V, Wallimann T, Nalam VK, Brdiczka D: Location and regulation of octameric mitochondrial creatine kinase in the contact sites. Biochem Biophys Acta 1061: 215–225, 1991
94. Brdiczka D, Kaldis P, Wallimann T: *In vitro* complex formation between the octamer of mitochondrial creatine kinase and porin. J Biol Chem 269: 27640–27644, 1994
95. Beutner G, Rück A, Riede B, Welte W, Brdiczka D: Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. FEBS Lett 396: 189–195, 1996
96. Beyer K, Klingenberg M: ADP/ATP carrier protein from beef heart mitochondria has high amounts of tightly bound cardiolipin, as revealed by ^{31}P nuclear magnetic resonance. Biochemistry 24: 8321–8326, 1985
97. Drees M and Beyer K: Interaction of phospholipids with the detergent-solubilized ADP/ATP carrier protein as studied by spin-label electron spin resonance. Biochemistry 27: 8584–8591, 1988
98. Cheneval D, Carafoli E: Identification and primary structure of the cardiolipin-binding domain of mitochondrial creatine kinase. Eur J Biochem 171: 1–9, 1988
99. Saks VA, Khuchua ZA, Kuznetsov AV: Specific inhibition of ATP-ADP translocase in cardiac mitoplasts by antibodies against mitochondrial creatine kinase. Biochim Biophys Acta 891: 138–144, 1987
100. Schnyder T, Engel A, Lustig A, Wallimann T: Native mitochondrial creatine kinase (Mi-CK) forms octameric structures. II. Characterization of dimers and octamers by ultracentrifugation, direct mass measurement by STEM and image analysis of single Mi-CK octamers. J Biol Chem 263: 16954–16962, 1988
101. Schnyder T, Gross H, Winkler H, Eppenberger HM, Wallimann T: Structure of the mitochondrial creatine kinase octamer: High resolution shadowing and image averaging of single molecules and formation of linear filaments under specific staining conditions. J Cell Biol 112: 95–101, 1991
102. Schnyder T, Cyrklaff M, Fuchs K, Wallimann T: Crystallization of mitochondrial creatine kinase on negatively charged lipid layers. J Struct Biol 112: 136–147, 1994
103. Wyss M, Maugham D, Wallimann T: Re-evaluation of the structure and physiological function of guanidino kinases in fruitfly (*Drosophila*), sea urchin (*Psamechinus miliaris*) and man. Biochem J 309: 255–261, 1995
104. Quemeneur E, Eichenberger D, Goldschmidt D, Vial C, Beauregard G, Potier M: The radiation inactivation method provides evidence that membrane-bound mitochondrial creatine kinase is an oligomer. Biochem Biophys Res Comm 153: 1310–1314, 1988
105. Fedosov SN, Belousova LV, Plesner IW: A model of mitochondrial creatine kinase binding to membranes: Adsorption constants, essential amino acids and the effect of ionic strength. Biochem Biophys Acta 1153: 322–330, 1993
106. Bogner W, Aquila H, Klingenberg M: The transmembrane arrangement of the ADP/ATP carrier as elucidated by the Iysine reagent pyridoxal 5-phosphate. Eur J Biochem 161: 611–620, 1986
107. Ovadi J: Cell Architecture and Metabolic Channeling. Springer-Verlag, Heidelberg, 1995
108. Anderson KS, Kim AY, Quillen JM, Sayers E, Yang XJ, Miles EW: Kinetic characterization of channel impaired mutants of tryptophan synthase. J Biol Chem 270: 29936–29944, 1995
109. Chumachenko YV: Some evidence in favour of the partnership between rabbit muscle aldolase and glyceraldehyde 3-phosphate dehydrogenase in the consecutive reactions. Ukr Biokhim Zh 66: 52–70, 1994
110. Agius L, Sherratt HSA (eds).: Channelling in Intermediary Metabolism. Portland Press, London and Miami, 1997
111. Gellerich FN, Schlame M, Bohnensack R, Kunz W: Dynamic compartmentation of adenine nucleotides in the mitochondrial intermembrane space of rat-heart mitochondria. Biochim Biophys Acta 890: 117–126, 1987
112. Gellerich FN, Kunz W: Cause and consequences of dynamic compartmentation of adenine nucleotides in the mitochondrial intermembrane space in respect to exchange of energy rich phosphates between cytosol and mitochondria. Biomed Biochim Acta 46: 545–548, 1987
113. Yount RG, Lawson D, Rayment I: Is Myosin a 'back door' enzyme? Biophys J 68: 44s–49s, 1995
114. Gilson MK, Straatsma TP, McCammon JA, Ripoll DR, Faerman CH, Axelsen PH, Silman I, Sussman JL: Open 'back door' in a molecular dynamics simulation of acetylcholinesterase. Science: 263, 1276–1278, 1994
115. Hyde CC, Ahmed SA, Padlan EA, Miles EW, Davies DR: Three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ multienzyme complex from *Salmonella typhimurium*. J Biol Chem 263: 17857–17871, 1988
116. Boehm EA, Radda GK, Tomlin H, Clark JF: The utilisation of creatine and its analogues by cytosolic and mitochondrial creatine kinase. Biochim. Biophys. Acta 1274: 119–128, 1996
117. Kenyon GL: Creatine kinase shapes up. Nature 381: 281–282, 1996
118. Milner-White EJ, Watts DC: Inhibition of adenosine 5′-triphosphate-creatine phosphotransferase by substrate-anion complexes. Evidence for the transition-state organization of the catalytic site. Biochem J 122: 717–740, 1971
119. Chida K, Tsunenaga M, Kasahara K, Kohno Y, Kuroki T: Regulation of creatine phosphokinase B activity by protein kinase C. Biochem Biophys Res Commun 173: 346–350, 1990
120. Hemmer W, Skarli M, Perriard JC, Wallimann T: Effect of okadoic acid on protein phosphorylation patterns of chicken myogenic cells with special reference to creatine kinase. FEBS Lett 327: 35–40, 1993
121. Martin KJ, Chen SF, Clark GM, Degen D, Wajima M, Von Hoff DD, Kaddurah-Daouk R: Evaluation of creatine analogues as a new class of anticancer agents using freshly explanted human tumor cells. J Natl Cancer Inst 86: 608–613, 1994
122. Teicher BA, Menon K, Northey D, Liu J, KuDe DW, Kaddurah-Daouk R: Cyclocreatine in cancer chemotherapy. Cancer Chemother Pharmacol 35: 411–416, 1995
123. Sayle RA, Milner-White EJ: RASMOL: Biomolecular graphics for all. Trends Biochem Sci 20: 374, 1995
124. Van Dorsten FA, Wyss M, Wallimann T, Nicolay K: Activation of sea-urchin sperm motility is accompanied by an increase in the creatine kinase exchange flux. Biochem J 325: 411–416, 1997
125. Aliev MK, Saks VA, Compartmentalized energy transfer in cardiomyocytes: Use of mathematical modeling for analysis of in vivo regulation of respiration. Biophys J 73: 428–445, 1997
126. Zhou G, Parthasarathy G, Somasundaram T, Ables A, Roy L, Strong SJ, Ellington WR, Chapman MS: Expression, purification from inclusion bodies, and crystal characterization of a transition state analog complex of arginin kinase: A model for studying phosphagen kinases. Prot Sci 6: 444–449, 1997
127. Vacheron MJ, Clottes E, Chautard C, Vial C: Mitochondrial creatine kinase interaction with phospholipid vesicles. Arch Biochem Biophys 344: 316–324, 1997