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II-4 The structure of mitochondrial creatine kinase and its membrane binding properties

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Abstract

The biochemical and biophysical characterization of the mitochondrial creatine kinase (Mi-CK) from chicken cardiac muscle is reviewed with emphasis on the structure of the octameric oligomer by electron microscopy and on its membrane binding properties. Information about shape, molecular symmetry and dimensions of the Mi-CK octamer, as obtained by different sample preparation techniques in combination with image processing methods, are compared. The organization of the four dimeric subunits into the Mi-CK complex as apparent in the end-on projections is discussed and the consistently observed high binding affinity of the four-fold symmetric end-on faces towards many support films and towards each other is outlined. A study on the oligomeric state of the enzyme in solution and in intact mitochondria, using chemical crosslinking reagents, is presented together with the results of a search for a possible linkage of Mi-CK with the adenine nucleotide translocator (ANT). The nature of Mi-CK binding to model membranes, demonstrating that rather the octameric than the dimeric subspecies is involved in lipid interaction and membrane contact formation, is resumed and put into relation to our structural observations. The findings are discussed in light of a possible *in vivo* function of the Mi-CK octamer bridging the gap between outer and inner mitochondrial membranes at the contact sites. (Mol Cell Biochem **133/134:** 115–123, 1994)

Key words: mitochondrial creatine kinase, electron microscopy, crosslinking, membrane binding

Introduction

The isoenzymes of creatine kinases (CK) catalyze the reversible transfer of a phosphoryl group from ATP to creatine and are expressed in a temporarily regulated manner in tissues specified by high energy turnover, e.g., brain, retina, spermatozoa, heart and skeletal muscle (reviewed in [1]). Sequence comparison and circular dichroism studies of the four CK isoforms identified so far show high sequence homology and secondary structure conservation [2]. In order to understand the role of CK isoenzymes *in vivo*, their isoform-specific subcellular localization is of particular interest; the cytosolic M- and B-isoforms are soluble and partly associated with structures at sites of energy consumption, such as myofibrils, sarcoplasmic reticulum and plasma membranes. In contrast, the two mitochondrial isoforms, the sarcomeric Mi_b-CK and the ubiquitous Mi_a-CK, are restricted to the mitochondria, the site of energy production through oxidative phosphorylation.

The spatial sequestering of cytosolic and mitochondrial CK isoenzymes led to the postulation of a phospho-

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creatine/creatine circuit, proposing that creatine (Cr) is phosphorylated to phosphocreatine (PCr) in the mitochondrial intermembrane space by Mi-CK which then is transported across the outer membrane. Liberated into the cytosol, PCr diffuses to sites of energy consumption where the cytosolic isoenzymes make use of it to regenerate *in situ* ATP for cellular processes. The creatine so generated diffuses back to the mitochondria where it is transported across the outer membrane to serve again as a substrate for Mi-CK.

Several lines of evidence concerning the intracellular compartmentation and functional coupling of CK isoenzymes to ATP-producing and ATP-requiring processes support the existence of such a PCr/Cr circuit (reviewed in [1]). This article reviews and discusses findings on the unique structure and membrane binding property of the Mi-CK isoenzymes which suggest to play a crucial role in the PCr/Cr circuit.

Localization, isolation and biochemical characterization of chicken Mi-CK

Mi-CK [3] is restricted to the mitochondrial intermembrane compartment, where it binds to the inner mitochondrial membrane [4]. In respiring mitochondria, Mi-CK preferentially utilizes ATP, that is synthesized in the mitochondrial matrix for the phosphorylation of extramitochondrial Cr [5]. Mi-CK activity is therefore functionally coupled to oxidative phosphorylation. Such a coupling involves two additional components, the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane and the outer mitochondrial membrane porin VDAC (reviewed in [6]). The mechanism underlying the functional coupling for the transport of CK substrates and products remains to be established.

Isolation of chicken mitochondrial creatine kinase from heart tissue yielded enzyme preparations consisting of two oligomeric species [7]. Gel permeation chromatography [7], hydrodynamic measurements by ultracentrifugation, electron optical investigations by scanning transmission electron microscopy [8] and radiation inactivation measurements [9] showed that the majority of Mi-CK exist as octameric species while a minor species is dimeric. This oligomerization pattern has also been shown to occur for Mi-CK purified from chicken brain [10] and was later corroborated in several tissues of many vertebrates (reviewed in [11]).

The octamer to dimer conversion can be reversibly modulated; dimerization is favored at low protein concentration [7], high pH and by the presence of substrates [12]. Interestingly, Mi-CK isoenzymes isolated from different organisms and tissues show different octamer stability [10]. Monomerization of Mi-CK only occurred in high concentrations of guanidine hydrochloride as judged from ultra-centrifugation experiments [8]. Hybridization experiments with the two Mi-CK isoforms (Mi_b-CK and Mi_a-CK) revealed hetero-octamers consisting of intact Mi_a-CK and Mi_b-CK homo-dimers, but no hetero-dimers could be detected [11]. Therefore, the dimer can be considered as the basic building block of the octamer, which is the oligomeric form of Mi-CK that is energetically favored in solution. This is also indicated by the observation that Mi_b-CK heterologously expressed in *E. coli* formed octameric molecules as well [13]. For octamer assembly, ionic interactions of the very *N*-terminal sequence [14] as well as hydrophobic interactions involving a Mi-CK-specific tryptophan residue are relevant [15]. The findings that extraction of Mi-CK from mitochondria yielded mostly octameric molecules, that rebinding of dimeric Mi-CK to mitoplasts lead to partial re-octamerization [16] and that the dimeric molecule was only favored in a very dilute aqueous solution of pure Mi-CK, argue for the octameric species to be predominant *in vivo*. However, whether the octamer-dimer conversion observed *in vitro* may have a regulatory role of Mi-CK function and energy supply is still a matter of debate.

In order to better understand the structure-function relationship of the mitochondrial creatine kinase, its role in energy metabolism and in the postulated PCr/Cr circuit, over the past years the Mi-CK octamer has become a subject of extensive electron optical investigation.

Structure of the Mi-CK octamer by electron microscopy

A broad spectrum of sample preparation techniques for electron microscopy was applied in combination with methods for image reconstruction to study the Mi-CK octamer [8, 17]. Negatively stained octamers are square-shaped projections of about 10 nm in side-length with peripheral and central stain accumulation. The laterally accumulated stain protrudes less densely packed protein regions, giving rise to the characteristic pinwheel-like appearance of the octamers top/bottom projection (Fig. 1A). The central stain spot with a diameter of

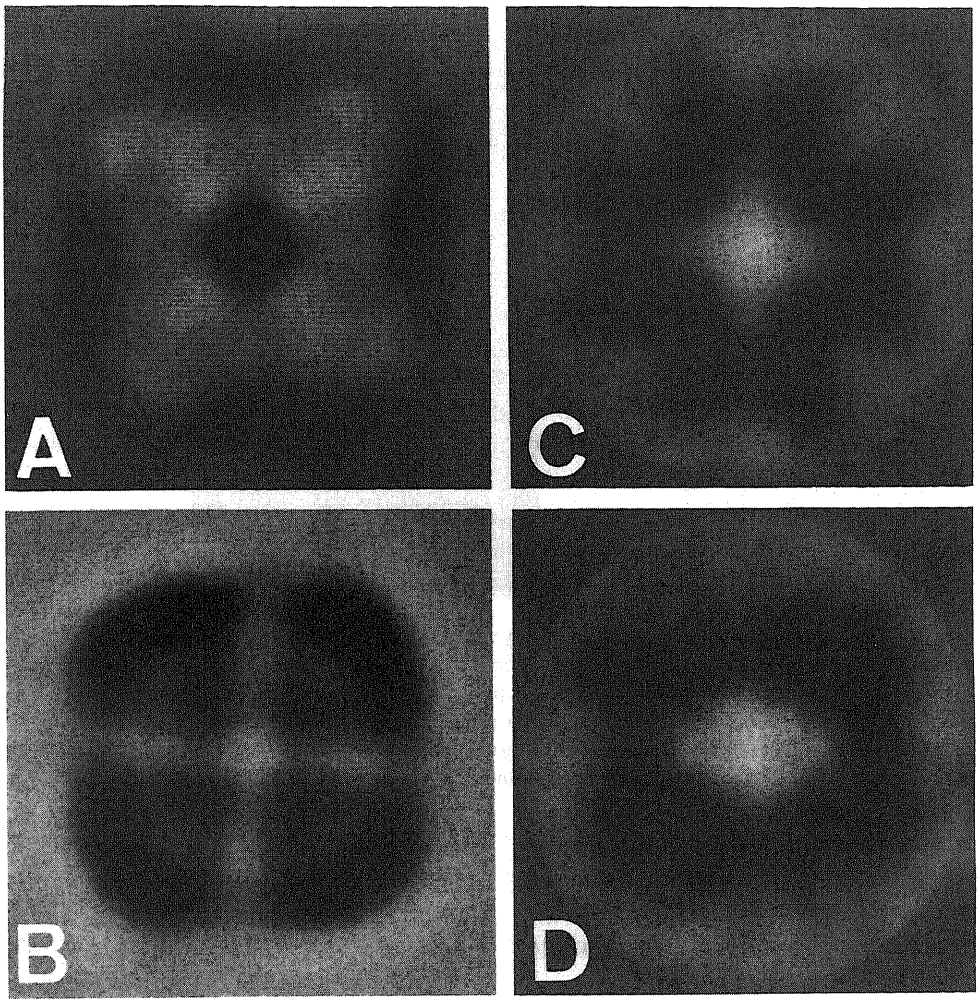

Fig. 1. Comparison of averaged images of Mi-CK octamers obtained by different sample preparation techniques: end-on views of negatively stained (A), rotary shadowed (B), and ice-embedded Mi-CK octamers (C) [22]. The most obvious feature in common is the four-fold symmetry axis through the center of the molecule. Using the bare-grid technique for cryo-electron microscopy the side-on view could be visualized (D). The octamer with the large water filled central cavity has molecule dimensions of about $10 \times 10 \times 8.4$ nm.

2.5 nm is indicative of a cavity or depression and is the origin of the octamer's four-fold symmetry axis.

To circumvent problems with the interpretation of images often caused by the negative stain method, the octamer's surface topography was replicated by evaporation of heavy metal at low object temperature [18]. Unidirectional shadowing led to the perspective impression of the surface of the octamer with its four hill-like quadrants. From the length of the molecules' shadow a quasi cubic three-dimensional shape of the octamer could be deduced [17]. The averaged image of rotary shadowed particles (Fig. 1B) shows strong contrast at the periphery due to metal accumulation at the vertical faces of the molecule. Symmetry and dimensions are in correspondence to negatively stained molecules. The cross of low contrast in the center of the octamer surface, *viz.* crevices that are shielded from heavy metal deposition, subdivides the molecule into four quadrants. Per quadrant two maxima of contrast are observed, one located near the center and another at the corner of the molecule. These findings strongly suggest an arrangement of the long dimer axis being parallel to the four-fold axis of the octamer. This would imply that the molecule exhibits vertical or side-faces which are different from the top/bottom faces visualized so far (Fig. 1A and B). However, visual inspection of well-preserved particles and multivariate statistical analysis [19] applied to more than thousand particles failed to reveal other views of the molecule [20].

Only in accidentally created linear Mi-CK filaments the octamer's side-view could be finally visualized [17]. In this case, top and bottom faces became associated in

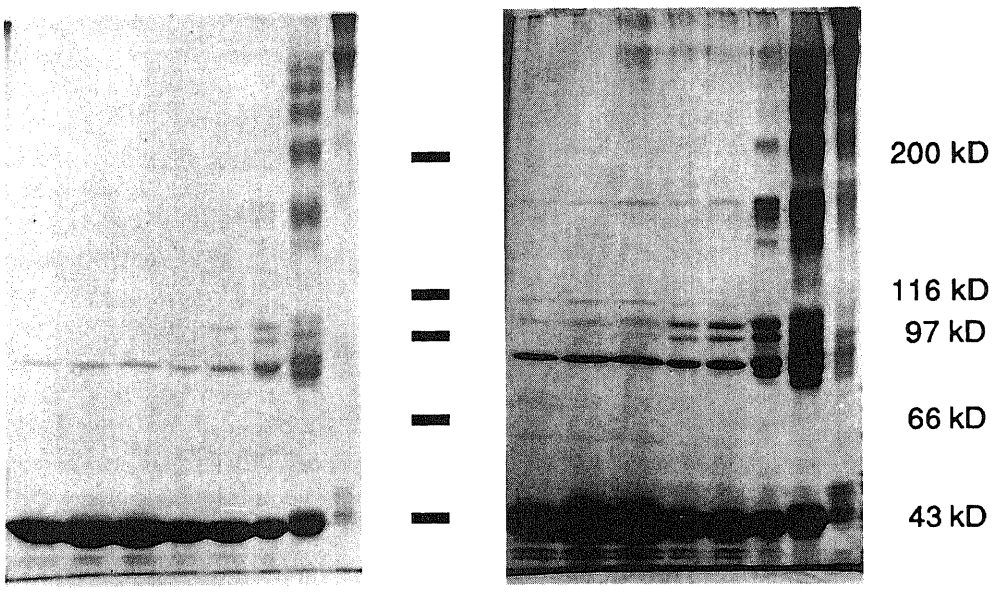

Fig. 2. Mi-CK treated with a lysine-specific crosslinking agent and analyzed by SDS-PAGE with Coomassie Blue (left) and silver staining (right). About 1 mg/ml soluble Mi-CK octamers were incubated with dimethyl suberimidate (DMS) in a concentration range from 1 μM to 50 mM (from left to right) and run on a gradient gel [24]. At 25 mM DMS about 18 crosslink products differing in their molecular weight are seen to be clustered in eight categories representing the oligomeric states of Mi-CK from monomer to octamer (second lane from the right in both pictures). At a concentration of 50 mM DMS, intermolecular crosslinkages (collision complexes) were formed, which were to large to enter the stacking gel (first lane from the right in both pictures). Molecular weight standards (middle) are defined on the right.

solution by forming an alternate stacking of the octamers. The two-fold symmetric side-view of the octamer in the filament, showed the banana-shaped dimers arranged with their long axes (8.4 nm) parallel to the filament axis [17].

The fact that octamers can form linear filaments in solution and that only one view of the molecule (top/bottom projection) could be observed, suggest that the top and bottom faces of Mi-CK must have a strong tendency to interact with supporting films and, under certain conditions, with each other.

To avoid such adsorption constraints, Mi-CK octamers were vitrified in a thin ice film [21] and imaged in an unstained frozen-hydrated state. With the cryo-technique a projection of the molecule's volume is observed similar to negative staining, but specimen flattening and stain accumulation artefacts are circumvented. Although the top/bottom projection ('end-on view') again was predominant, several views of randomly oriented molecules also could be distinguished. The averaged motif of the end-on view (Fig. 1C) shows the expected molecular dimensions and symmetry [22]. The four protein densities organized around the water filled hole are bi-lobed, each lobe most probably representing a part of the Mi-CK monomer.

The averaged side-view obtained from frozen hydrated octamers (Fig. 1D) must be treated as a preliminary result since the number of molecules taken for image processing was limited by the low and random yield of molecules differing from the end-view orientation and the low signal-to-noise ratio of ice-embedded molecules. Nevertheless, molecular dimensions (8.4 × 10 nm) and the expected two-fold symmetry correspond to the motif found in filaments. This infers that in Mi-CK octamers top and bottom faces are structurally identical.

Chemical crosslinking of Mi-CK octamers

In vivo, Mi-CK molecules are restricted to the narrow mitochondrial intermembrane compartment and are bound to the inner mitochondrial membrane [23]. In order to investigate the oligomeric nature of the membrane bound enzyme, the technique of chemical crosslinking was applied. In a first step, different types of crosslinking agents were tested with purified Mi-CK octamers in solution, and the crosslink products were analyzed by SDS-PAGE. Incubation with a lysine-specific crosslinking agent revealed around 18 high molecular weight bands clustered into eight categories (Fig. 2) representing intramolecular crosslinking products of the

monomeric up to the octameric subspecies [24]. These results corroborate the octameric structure of Mi-CK and are in agreement with similar crosslinking studies from other authors [25, 26].

In a second step, the reactivity of crosslinkers was tested in buffer compositions where Mi-CK was shown to be bound to the inner mitochondrial membrane [24]. After having found suitable buffer milieu and concentration range for several types of crosslinker, they were directly applied to mitochondria and mitoplasts and the crosslink products analyzed. In another experiment, pure Mi-CK was first labelled by photo-activable heterobifunctional crosslinkers and rebound to mitoplasts, that have been depleted of Mi-CK before. After removal of excess labelling agents, the solution of mitoplasts with bound, modified Mi-CK was illuminated by UV-light to induce photoactivable crosslinking. A comparison of all experiments revealed SDS-gel banding patterns of Mi-CK crosslinking products which corresponded to that of crosslinked pure Mi-CK octamers. This infers that rather the octameric than the dimeric species of Mi-CK is located at the mitochondrial inner membrane, which is in correspondence to the results obtained by radiation inactivation [9] and gel chromatography [16].

Search for heterologous crosslinking products

Based on the experimental demonstration of a functional coupling of Mi-CK activity to oxidative phosphorylation [4, 27], a direct physical interaction of membrane bound Mi-CK with the adenine nucleotide translocator (ANT) (see e.g. [28]) as well as with the mitochondrial outer membrane pore [29] has been postulated. In order to investigate the putative physical interaction between Mi-CK and ANT, mitochondria were treated with crosslinking agents. The crosslinking products were separated on SDS-PAGE and analyzed with specific antibody staining. Under reaction conditions where intramolecular crosslinking of Mi-CK was observed, no heterologous crosslinking product between Mi-CK and ANT could be demonstrated by immuno-blotting with anti-Mi-CK and anti-ANT antibodies [24]. With increase of the crosslinking reagent concentration, the ANT-specific immuno-signal became broader and weaker, shifted progressively toward higher apparent molecular weights, without forming any discrete crosslinking products [24].

In another series of experiments, freshly prepared mitochondria were treated with several types of crosslinking agents and were then differentially extracted. After the crosslinking reaction, mitochondria were hypotonically shocked and mitochondrial proteins extracted, first by phosphate and then by urea treatment. All fractions, including the residual membrane pellets after urea extraction, were separated on SDS-PAGE for immunochemical detection. Mi-CK could be found in the phosphate extract only. Depending on the type of chemical agent, different discrete high molecular weight bands of Mi-CK were observed. The crosslinking products co-migrated with those obtained from crosslinking of soluble, pure Mi-CK octamers. Immuno staining against ANT could be exclusively found in the membrane pellet fraction; at lower crosslinker concentration a signal corresponding in size to monomeric ANT and at higher concentration a high molecular smear was usually found. These results show that all defined Mi-CK crosslinking products were of intramolecular character. Neither an oligomeric state of ANT nor a linkage between Mi-CK and ANT or another protein could be demonstrated under the conditions chosen. These findings have been discussed in light of the difficulties to crosslink a soluble protein to a membrane protein elsewhere [24].

The interaction of Mi-CK with membranes

The inability to demonstrate a physical interaction of Mi-CK to membrane proteins suggests that lipids of the bilayer might act as the binding targets for Mi-CK on mitochondrial membranes. Therefore, the interaction of Mi-CK with membranes and lipid monolayers spread at the air/water interface was studied [34]. It was shown that Mi-CK induced an increase in surface pressure when interacting with lipid films, a property shared by many lipid binding proteins. The increase in surface pressure was dependent on the initial pressure and on the protein concentration in the subphase. For octameric Mi-CK, the induction of pressure increase ceased at an initial surface pressure of 30–32 mN/m (i.e. critical surface pressure). Since this is the equivalent surface pressure of lipid bilayers [31], this suggests that the cationic Mi-CK molecules may mainly interact with the polar head groups of the lipids. Dimeric Mi-CK showed a lower affinity to lipids and a lower critical surface pressure than the octameric species, indicating that the octamer of Mi-CK might be the species that preferentially interacts with mitochondrial membranes *in vivo* as well.

Mi-CK interacted in a similar manner with spread inner and outer mitochondrial membranes, as well as with those formed with lipids from microsomal membranes [34]. These results favour the hypothesis that the binding of Mi-CK to membranes is mediated by lipids. This explains the observation that Mi-CK-depleted heart mitoplasts rebind exogenous Mi-CK in a two to 16-fold excess of the endogenous amount. The same was found for liver mitoplasts, which are free of endogenous Mi-CK, but have a lipid composition similar to heart mitoplasts [32].

The interaction of Mi-CK with pure phosphatidylcholine monolayers resulted in a lower surface pressure increase than with monolayers of cellular lipid extracts. The increase in surface pressure induced by Mi-CK augmented with increasing content of anionic phospholipid in the phosphatidylcholine monolayer with the effect being more prominent for cardiolipin than for phosphatidyl-serine or -inositol. These findings sustain the notion, that the cationic Mi-CK interact solely with the polar head groups of lipid molecules in an electrostatic way. This is also in agreement with earlier suggestions based on the fact that Mi-CK desorption from the mitoplasts is strongly dependent on both, the ionic strength [33] and the pH of the extraction buffer [16].

The ability of Mi-CK to mediate intermembrane adhesion

Since Mi-CK was shown to be able to interact in a similar manner with inner and outer mitochondrial membranes, the question was raised, whether Mi-CK can bind two membrane interfaces simultaneously [30]. For this, Mi-CK was bound to a monolayer and the subphase was washed with buffer in order to remove unbound protein. Then, radioactive labelled vesicles were injected in the subphase. A subsequent raise in surface radioactivity showed that Mi-CK was able to interact simultaneously with inner and outer mitochondrial membranes. Mi-CK mediated intermembrane adhesion of whole and pure lipid membranes, whereas other cationic enzymes of the intermembrane space (cytochrome c and adenylate kinase) or the cytosolic isoenzymes of CK failed to induce contact formation. The two oligomeric forms of Mi-CK differed substantially in their ability to mediate membrane contact formation, the octamer being about three fold more potent. Regarding the basic nature of Mi-CK, control experiments with poly-L-lysine peptides showed an induction of intermembrane binding as well as membrane fusion. Interestingly, the extent of contact formation mediated by poly-L-lysines was lower than that of octameric Mi-CK [30].

Significance for *in vivo* function of Mi-CK

Cube-like Mi-CK octamers visualized by electron microscope have indistinguishable top and bottom faces, which were able to adsorb to several kinds of support films [17]. In contrast to the sticky top/bottom faces (end-on views), the four side faces could not be visualized as long as an adsorption matrix was present. They became, however, manifest when top/bottom faces of the octamers got associated to form linear filaments [17]. Here, we could directly demonstrate the second view (side-on view) of the Mi-CK octamer by vitrifying single molecules in ice.

Mi-CK octamers have been shown to interact with model as well as purified mitochondrial membranes [34] and to be involved in intermembrane adhesion [30]. Since the interacting faces of the octamer must have identical binding properties, the top and bottom faces are suggested to mediate the membrane contact formation *in vitro*. It is tentative that *in vivo* Mi-CK interacts in a similar way with opposed mitochondrial membranes. This view is supported by the narrow gap observed between all membranes in mitochondria [35]. Therefore, the Mi-CK octamer is expected to simultaneously interact by its top and bottom faces with inner and outer membrane and so functioning as a mitochondrial membrane 'gap junction'.

Even though we could not show a direct interaction between Mi-CK and any other protein, the above hypothesis is in line with those studies which have described a functional coupling of Mi-CK to the ANT (see e.g. [28]) and to the mitochondrial porin [29]. Together with the presented low resolution structure, the membrane properties and the ultrastructural location of Mi-CK, we suggest that Mi-CK is able to form a multienzyme complex mediated by phospholipid interactions. This complex would consist of the ANT in the inner membrane, Mi-CK as the gap bridging protein, and porin in the outer membrane (Fig. 3).

A reversible formation of a multienzyme complex would be an appropriate mechanism for coupling Mi-CK catalysis to oxidative phosphorylation and the delivery of PCr to the cytosol (Fig. 3). Possible ways to achieve the assembly and disassembly of the multien-

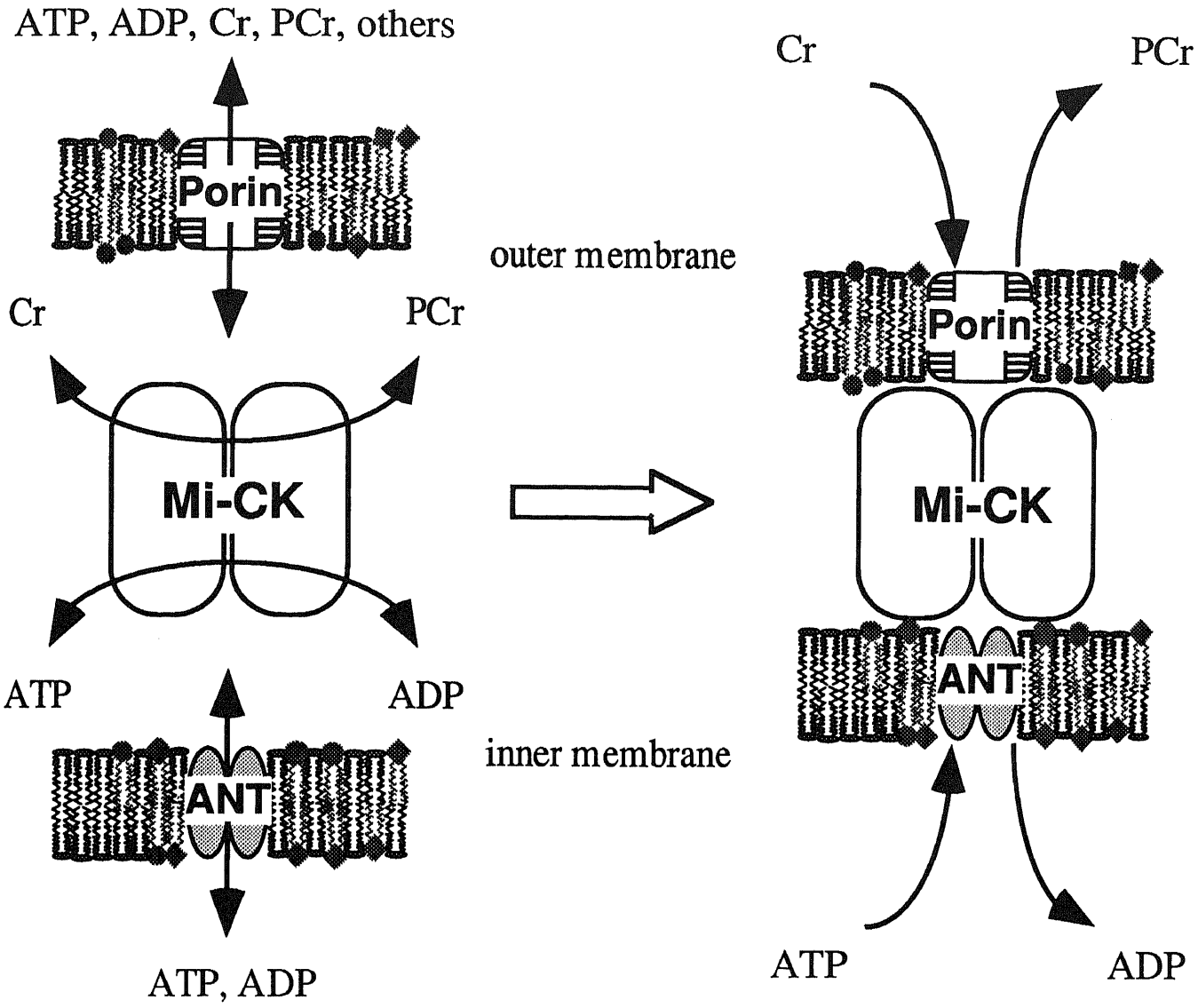

Fig. 3. Model for a multienzyme complex where the creatine kinase reaction is coupled to oxidative phosphorylation (according to [37]). The enzymes involved in this functional coupling, Mi-CK, ANT and porin, all catalyze reversible reactions. The creatine kinase reaction utilizes ATP synthesized by oxidative phosphorylation in the mitochondrial matrix and creatine (Cr) from the cytosolic compartment and shuttles phosphocreatine (PCr) back to the cytosol, whereas ADP gets imported into the matrix (left drawing). An intimate coupling is maintained by a simultaneous interaction of the Mi-CK octamer with the mitochondrial inner and outer membrane bringing the enzyme in close contact to ANT and porin. The postulated multienzyme complex (right drawing) would ensure a synchronous and vectorial action of the enzymes involved and would increase the thermodynamic efficiency of mitochondrial high energy phosphate synthesis and transport.

zyme complex could be a shift in the dimer/octamer ratio of Mi-CK [16] resulting in altered membrane binding properties (see above), or changes in membrane potential or membrane structure in dependence of the metabolic state, which were demonstrated to occur in mitochondria (see e.g. [36]). Experimental approaches to detect short-lived protein-lipid and protein-protein interactions will be useful to address questions concerning the postulated multienzyme complex.

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