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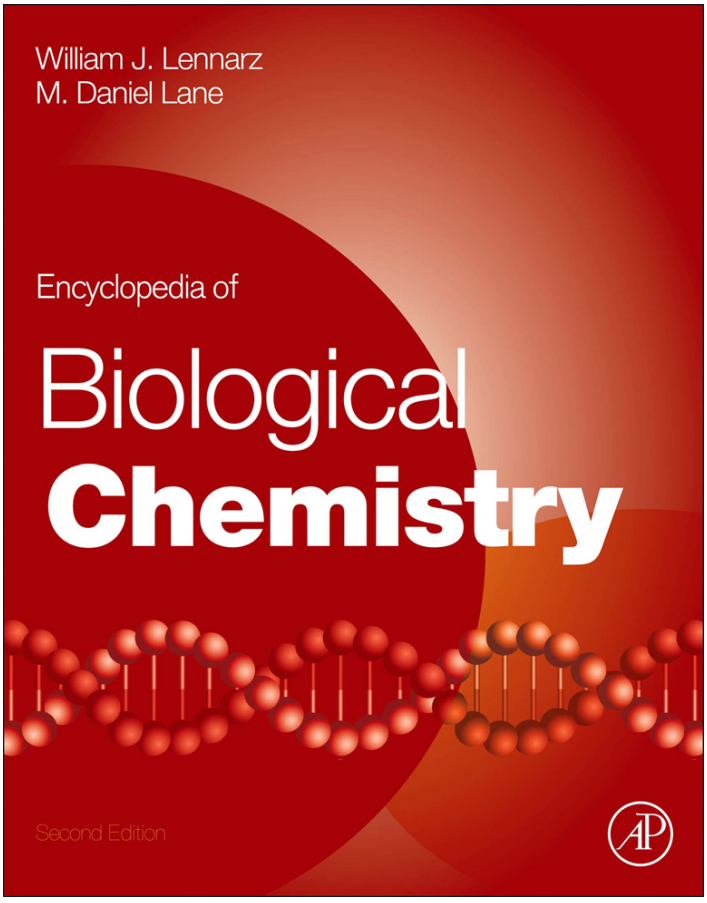

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Metabolite Channeling: Creatine Kinase Microcompartments

U Schlattner and M Tokarska-Schlattner, University Joseph Fourier and Inserm, Grenoble, France
T Wallimann, Institute of Cell Biology, ETH Zürich, Switzerland

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Glossary

Metabolic compartmentation Segregation of intermediates and enzymes of a metabolic pathway by membranes, binding to a specific surface or direct interaction in protein complexes allowing metabolite channeling.
Metabolite channeling Local transfer of metabolic intermediates between sequential enzymes or transport reactions without equilibration with bulk solution.
Microcompartment Structural unit allowing metabolic compartmentation, also called metabolon.
Mitochondrial contact sites Close adhesions of inner and outer mitochondrial membrane that can be observed by electron microscopy and can be isolated as a separate microcompartment. Contact sites consist of multilipid/protein complexes with variable composition and are involved in energy transduction (e.g., containing adenylate translocator (ANT)/voltage-dependent anion channel (VDAC) or ANT/mitochondrial creatine kinase (MtCK)/VDAC) or in protein import.
Mitochondrial permeability transition pore A multienzyme complex, probably composed of VDAC, ANT, Bax, cyclophilin, MtCK, and others, that is crucially involved in early events that trigger apoptosis-like release of cytochrome *c* and other apoptosis-inducing factors into the cytosol.

Subcellular microcompartments, consisting of multienzyme complexes embedded within the cellular, highly viscous matrix, associated with the cytoskeleton, or situated along membranes, are operating according to exclusion principles and favor preferred pathways of intermediates. This process, called metabolite or substrate channeling, is defined as transfer of intermediates between sequential enzymes without equilibration of these metabolites with the surrounding bulk solution. Such an association between two or more sequential enzyme or transport reactions in a microcompartment, forming a distinct functional pool of intermediates, is also called functional coupling. It can be considered as a general mechanism to increase efficiency of sequential reactions in a metabolic pathway. As metabolite channeling leads to segregation of a metabolic pathway from other cellular reactions, it represents a specific kind of metabolic compartmentation similar to that operating within membrane-separated organelles or by restricted two-dimensional diffusion at surface boundary layers. Here, metabolite channeling is described with special emphasis on high-energy phosphate channeling by creatine kinase (CK), the phosphocreatine circuit or shuttle, and the mitochondrial CK (MtCK) isoenzyme.

Subcellular Microcompartments and Mechanisms of Metabolite Channeling

Life most likely originated autotrophically *de novo* in metabolic complexes organized on FeS_2 (pyrite) mineral surfaces, the earliest form of microcompartments. Likewise, a cell is by no means represented best by a well-mixed bag of enzymes, behaving in complete equilibrium according to solution kinetics. Because of the intricate structural and functional organization of living cells, enzymes and metabolites do not behave as if they were freely diffusible in solution. Instead, they may form structurally, functionally, and temporally defined subcellular microcompartments, either via strong static or via fickle, dynamic interactions with other enzymes, proteins, or subcellular structures. Such a structural organization of pathway components is a general prerequisite for metabolite channeling. It may involve (1) huge covalently linked enzyme complexes (or multifunctional enzymes) such as fatty acid synthase (FAS), (2) kinetically stable multienzyme complexes such as pyruvate dehydrogenase (PDH) or bacterial and plant tryptophan synthase (TS), (3) more dynamic, reversibly associating enzymes such as glycolytic complexes containing glyceraldehyde phosphate dehydrogenase (GAPDH) or glycerol phosphate dehydrogenase (GPDH), or (4) colocalization on subcellular particles or biological membranes. These associations allow the transfer of intermediates between the channeling components by different mechanisms: (1) physical hindrance or electrostatic effects prevent mixing with bulk solution and drive a directed diffusion (e.g., TS and FAS), (2) sequential covalent binding to very close active sites in the reaction sequence (e.g., PDH), (3) transfer of noncovalently bound intermediates between active sites (e.g., nicotinamide adenine dinucleotide dehydrogenase), and (4) transfer in dynamic multienzyme complexes (GAPDH and GPDH) or in an unstirred membrane surface layer (MtCK). These mechanisms can be fulfilled in both, static and dynamic enzyme associations. However, although static associations allow for an almost perfect or tight channeling of metabolites, dynamic channeling is often only partial or leaky.

Advantages of Metabolite Channeling

Sequestering of intermediates in a microcompartment through channeling provides kinetic and regulatory advantages not only for the reaction sequence itself (see Figure 1) but also for cellular metabolism. In general: (1) enzyme reaction rates

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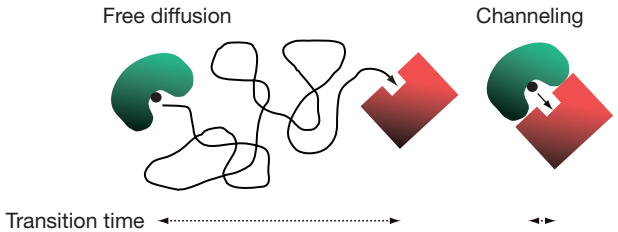

Figure 1 Free diffusion vs. metabolite channeling. Compartmentation of a reaction sequence without equilibration with bulk solution leads to shorter transition times and further advantages (see text).

and equilibria are controlled by local and enzyme-bound substrates, rather than bulk-phase substrate concentrations; (2) for a readily reversible reaction, local supply of substrate and removal of product may drive the reaction in the desired direction; (3) 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 nonchanneling situation measured with isolated components; (4) metabolites are isolated from competing reactions, for example, between anabolic and catabolic pathways; (5) the lifetime of the intermediate in the solvent phase is shortened relative to free diffusion, which may be essential in the case of unstable intermediates; (6) in certain cases, the unfavorable energetics of desolvating the substrate that precedes binding to the enzyme is avoided; (7) channeling components can be regulated by modulators that affect enzyme associations; and (8) a larger degree of metabolic control of the overall flux of the reactions can be achieved, for example, via feedback regulatory mechanisms such as substrate activation, product inhibition, and cooperativity.

Subcellular Targeting of Glycolytic Multienzyme Complexes

In muscle, glycolytic enzymes are targeted to the actin-containing thin filaments at the sarcomeric I-band region where they form highly complex metabolons. The I-band in *Drosophila* flight muscle contains a multienzyme complex consisting of GDPH-1, aldolase, and GAPDH. By elegant experiments with transgenic *Drosophila* expressing GDPH-3 instead of GDPH-1, it could be shown that all three glycolytic enzymes no longer colocalize in the I-band to form a microcompartment. Even though the full complement of active glycolytic enzymes was still present, their failure to colocalize in the sarcomer resulted in the inability to fly. Thus, correct targeting and formation of multienzyme complexes that lead to functionally coupled microcompartments and substrate/product channeling seem to be a prerequisite for proper function of glycolysis and ultimately for correct muscle function. In mammalian cells, CK is also participating in the glycolytic metabolon.

More recently, metabolomic studies have envisaged a comprehensive analysis of cellular metabolites, their changes in time, as well as the modeling of these processes. Such systems biology approaches have the potential to reveal many more cases of dynamic and time-resolved metabolite-channeling events within the cellular metabolic network.

Compartmentation of CK Isoenzymes and Channeling of High-Energy Phosphates

The CK/Phosphocreatine Circuit or Shuttle

One fundamental requirement of life is energy supply. Cellular energy demand and supply are balanced, and tightly regulated for economy and efficiency of energy use. CK is a major enzyme of higher eukaryotes that copes with high and fluctuating energy demands to maintain cellular-energy homeostasis, in general, and to guarantee stable, locally buffered adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratios, in particular.

The enzyme catalyzes the reversible phosphoryl transfer from ATP to creatine (Cr) to generate ADP and phosphocreatine (PCr). Thus, CK is able to conserve energy in the form of metabolically inert PCr and vice versa, to use PCr to replenish global as well as local cellular ATP pools. As PCr can accumulate to much higher cellular concentrations than ATP, the CK/PCr system constitutes an efficient and immediately available cellular energy buffer. In addition, tissue-specific CK isoenzymes are located in the cytosol (dimeric muscle-type MM-CK and brain-type BB-CK) and within the mitochondrial intermembrane space (sarcomeric MtCK and ubiquitous MtCK, both forming octamers and dimers). CK isoenzymes are often associated with sites of ATP supply, where they generate PCr, or with sites of ATP consumption, where they regenerate ATP by using PCr. Thus, together with the faster diffusion rate of PCr as compared to ATP, the CK/PCr system also supports an intrinsic energy-transfer system (CK/PCr circuit or shuttle), coupling sites of energy generation (oxidative phosphorylation or glycolysis) with sites of energy consumption (Figure 2). This circuit is particularly important in large and/or polar cells, such as spermatozoa where diffusional limitations of adenine nucleotides, especially ADP, along the sperm tail become especially apparent.

Channeling with Cytosolic CK

Cytosolic CK is only partially soluble. A significant fraction is structurally and functionally associated or colocalized with different, structurally bound ATPases or ATP-regulated processes. These include (1) different ion pumps in the plasma membrane, (2) the sarcomeric M-band of the myofibrils in muscle, (3) the calcium pump of the muscular sarcoplasmic reticulum, and (4) the ATP-gated K^+-channel. In all these cases, PCr is used for the local regeneration of ATP, which is directly channeled from CK to the ATP-consuming ATPase without major dilution by the surrounding bulk solution. Only in some cases, the physical basis for the metabolite channeling is known. For example, MM-CK uses a charge clamp consisting of four lysine residues to specifically bind to partner molecules, myomesin and M-protein in the M-band. This allows for an isoenzyme-specific association of MM-CK with the sarcomeric M-band of the myofibrillar apparatus. There, MM-CK is ideally positioned in the middle of the acto-myosin overlap zones, situated symmetrically on both sides of the M-band, to regenerate *in situ* the ATP that is hydrolyzed during muscle contraction.

Cytosolic CK is structurally associated with the key regulatory enzyme of glycolysis, phosphofructokinase, which itself

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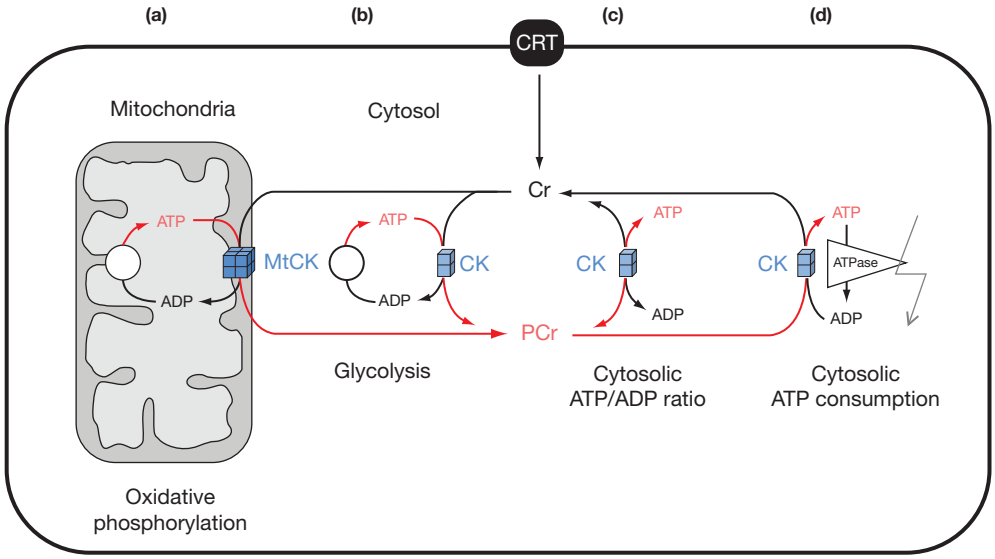

Figure 2 The creatine kinase (CK)/phosphocreatine (PCr) system. Isoenzymes of CK (blue) are found in different compartments such as mitochondria (octameric MtCK, (a)) and cytosol (dimeric CK, (b)–(d)) in soluble form (c) or associated to a different degree to ATP-delivering ((a) and (b)) or -consuming processes (d). A large cytosolic PCr pool up to 30 mM is built up by CK using ATP from oxidative phosphorylation like in heart (a) or glycolysis like in fast-twitch glycolytic muscle (b). PCr is then used to buffer global (c) and local (d) ATP/ADP ratios. In cells that are polarized and/or have very high or localized ATP consumption, these CK isoenzymes, together with easily diffusible PCr, also maintain an energy shuttle between ATP-providing or -consuming processes ((a) and (d)). Metabolite channeling occurs where CK is associated with ATP-providing or -consuming transporters, pumps, or enzymes ((a), (b), and (d)). Creatine (Cr) is synthesized in only few cell types (e.g., liver and kidney) and has to be taken up from the blood stream by a specific Cr transporter (CRT) that is highly expressed by Cr-containing target cells.

is regulated by ATP. Likewise, structural and functional interactions of cytosolic CK with the PAR-1 receptor of the thrombin-signaling pathway and with the ATP-gated K^+-channel, respectively, have been demonstrated. A tight functional coupling of CK to ATPases, for example, actomyosin ATPase and ion pumps, such as the K^+/Na^+-ATPase or the Ca^{2+}-ATPase, has the advantage (1) that product inhibition of the ATPase by ADP and H^+ is avoided, because the latter are both substrates of the CK reaction (PCr + ADP + $H^+ \leftrightarrow$ Cr + ATP) and (2) that the high free energy of ATP hydrolysis (ΔG_{ATP}) at sites of ATP hydrolysis is preserved by keeping locally very high ATP/ADP ratios due to coupling of CK with those ATPases *in situ* and, thus, preventing energy dissipation that would otherwise be caused by transport of ATP and mixing it with the bulk surrounding. Interestingly, the strongest phenotype of CK double knockout mice, which no longer express cytosolic MM-CK and mitochondrial MtCK in muscle, is characterized by significant difficulties with intracellular calcium handling and muscle relaxation, emphasizing the physiological importance of the CK system for the energetics of intracellular calcium homeostasis, in general, and the delivery of ATP to the energetically demanding Ca^{2+}-ATPase, in particular.

Channeling in Energy-Transducing Mitochondrial Microcompartments

MtCK forms mainly large, cuboidal octamers (Figure 3) that are present (1) between the outer and inner mitochondrial membrane (the so-called intermembrane space of mitochondria) and preferentially localized at the so-called mitochondrial contact sites between outer and inner mitochondrial membrane, as well as (2) in the cristae space (see Figure 3 inset). The kinase catalyzes the direct transphosphorylation of intramitochondrial-produced ATP and Cr from the cytosol into ADP and PCr. ADP then enters the matrix space to stimulate oxidative phosphorylation, giving rise to mitochondrial recycling of a specific pool of ATP and ADP, while PCr is the primary high-energy phosphoryl compound that leaves mitochondria into the cytosol. The molecular basis for such directed metabolite flux is channeling between the large, cuboidal MtCK octamer and two transmembrane proteins, adenylate translocator (ANT) and mitochondrial porin or voltage-dependent anion channel (VDAC). ANT is an obligatory antiporter for ATP/ADP exchange across the inner mitochondrial membrane, while VDAC is a nonspecific, potential-dependent pore in the outer mitochondrial membrane. The MtCK-linked metabolite channeling is based on (1) colocalization, (2) direct interactions, and (3) diffusion barriers. MtCK tightly binds to cardiolipin, an acidic phospholipid that is specific for the mitochondrial inner membrane. As ANT molecules are situated in a cardiolipin patches, this leads to colocalization and metabolite channeling between both proteins, MtCK and ANT, in the cristae as well as in the intermembrane space (Figure 3). MtCK in the intermembrane space further interacts with outer membrane phospholipids and VDAC, thus virtually cross-linking inner and outer membrane and contributing to the mitochondrial contact sites. Increasing the external calcium concentrations strengthens the interaction of MtCK with VDAC, which may improve channeling under cytosolic calcium overload as occurring at

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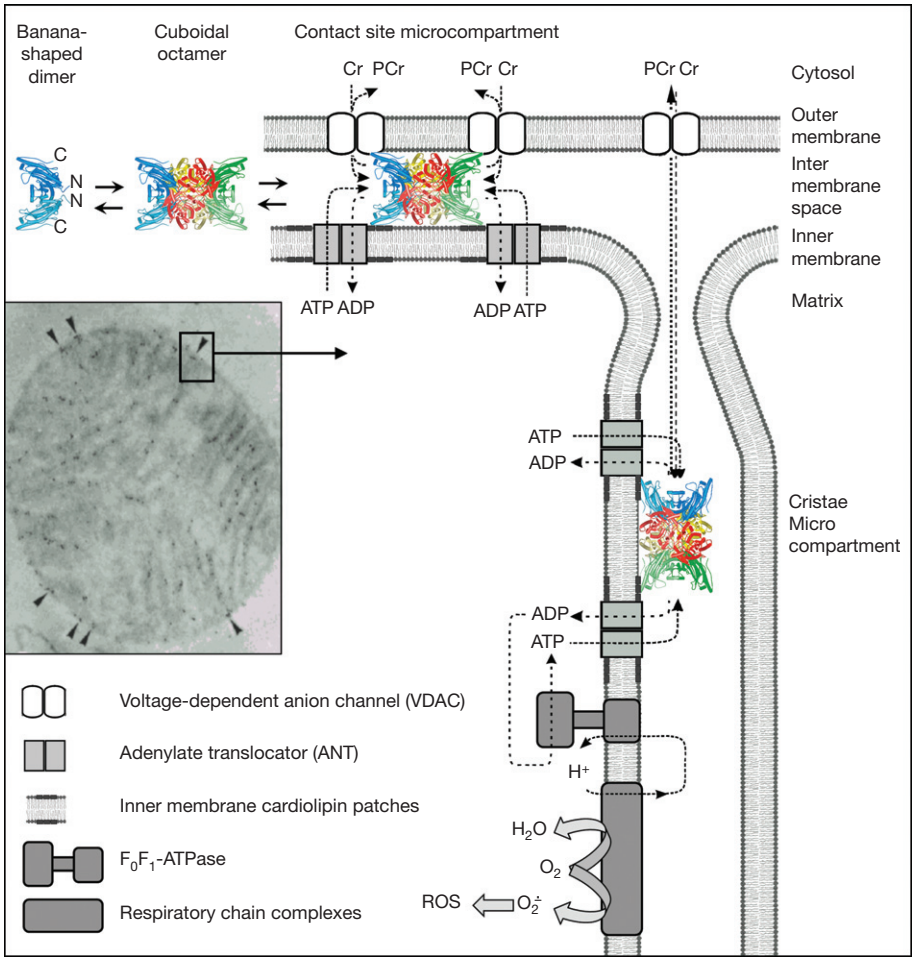

Figure 3 (Inset) Dual localization of mitochondrial CK by electron microscopy. Post-embedding immuno-gold labeling of MtCK in a mitochondrion from photoreceptor cells of chicken retina, showing localization of MtCK in the peripheral intermembrane space (arrows) and along the cristae membranes. Scheme: microcompartments and metabolite channeling of mitochondrial CK. After the import of nascent MtCK over the mitochondrial outer membrane and cleavage of the targeting sequence, MtCK first assembles into dimers. Dimers rapidly associate into octamers (top left), and although this reaction is reversible, octamer formation is strongly favored by high MtCK concentrations and MtCK binding to acidic phospholipids. MtCK is then found in two locations: (1) in the so-called mitochondrial contact sites associated with ANT and VDAC (top center) and (2) in the cristae associated with ANT only (at right). In contact sites, MtCK simultaneously binds to inner and outer membrane due to the identical top and bottom faces of the octamer. Binding partner in the inner membrane is the twofold negatively charged cardiolipin, which allows a functional interaction with ANT. This carrier is situated in cardiolipin membrane patches that can be induced by MtCK itself. In the outer membrane, MtCK interacts with other acidic phospholipids and, in a calcium-dependent manner, directly with VDAC. Substrate and product fluxes between MtCK and the associated proteins are depicted as arrows. In contact sites, this substrate channeling allows for a constant supply of substrates and removal of products at the active sites of MtCK. In cristae, only ATP/ADP exchange is facilitated through direct channeling to the MtCK active site, while Cr and PCr have to diffuse along the cristae space to reach VDAC. Further channeling may take place within mitochondrial supercomplexes that include carriers, ATPase and respiratory chain complexes (lower part). The active ATP/ADP exchange maintained by coupled MtCK favors ATP generation by the F_0F_1-ATPases and, thus, proper functioning of the respiratory chain, which could otherwise generate elevated levels of superoxide and reactive oxygen species (ROS).

low-cellular-energy state. Some studies suggest that only the membrane-bound, octameric form of MtCK is able to maintain the metabolite channeling described above. Finally, the limited permeability of VDAC and, thus, of the entire outer membrane creates a dynamic microcompartmentation of metabolites in the intermembrane space that contributes to MtCK-linked channeling and separate mitochondrial ATP and ADP pools. Similar to MtCK, hexokinase is able to use intramitochondrially produced ATP by binding to VDAC from the cytosolic mitochondrial surface at contact sites containing only ANT and VDAC. The direct functional coupling of MtCK to oxidative phosphorylation can be demonstrated with oxygraph respirometry on skinned muscle fibers from normal and transgenic mice lacking MtCK. Although in normal muscle fibers Cr stimulates mitochondrial respiration, this phenomenon is missing in fibers of MtCK knockout mice.

By favoring ATP/ADP exchange through ANT in the mitochondrial inner membrane, cytosolic Cr and the MtCK reaction not only stimulate the rate of mitochondrial respiration, but also lead to improved coupling between respiration, ATP synthesis, and mitochondrial energy channeling, which finally reduces formation of potentially deleterious reactive

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oxygen species (ROS; Figure 3, lower part). Both effects of MtCK-related metabolite channeling, stimulation of mitochondrial respiration and lowering of ROS production, may contribute to the remarkable cell- and specifically neuroprotective action of Cr supplementation *in vitro* and *in vivo*. This is an instructive example of how efficient, multiple metabolite channeling events can be beneficial for human health.

Oxidative damage of MtCK, induced by reactive oxygen and nitrogen species, generated under cellular stress situations, for example, in infarcted heart or under chemotherapeutic intervention by anthracyclines, leads to inactivation and dimerization of MtCK, as well as to dissociation of the enzyme from the mitochondrial inner membrane. Thus, important prerequisites for efficient channeling of high-energy phosphates by MtCK are negatively affected. These events contribute to cardiac energy failure and specific anthracycline cardiotoxicity.

Mt CK, Intramitochondrial Inclusions, and Low-Cellular-Energy State

MtCK is an indicator for cellular low-energy stress, that is, the expression of this enzyme is highly upregulated in patients with mitochondrial myopathies in the so-called ragged-red skeletal muscle fibers, where mitochondrial volume and size are markedly increased and where characteristic intramitochondrial railway-track inclusions are observed as a hallmark of pathology. The latter were shown to consist of crystalline sheets of MtCK (see Figure 4). Similar MtCK inclusions can also be induced in animals by chronic Cr depletion, leading to cellular low-energy state. Even more generally, cellular low-energy stress, be it induced by chronic endurance training, fasting, Cr depletion, or pathologies in ATP generation, such as mitochondrial dysfunction seen in patients with mitochondrial cytopathies, induces a coordinated induction of energy gene expression to compensate for impaired energy supply and transport. In the case of MtCK, one of the most prominent among these genes, overexpression is such that it leads to crystallization of the enzyme in a pathological state as described above.

Figure 4 Intramitochondrial inclusions in patients with mitochondrial myopathies consist of mitochondrial MtCK. Immunoelectron histochemistry of human intramitochondrial crystalline 'railway-track' inclusions seen in patients with mitochondrial myopathies showing 'ragged-red' skeletal fibers as a hallmark of the disease. Note the grossly enlarged mitochondrion, here from a patient with Kearns–Sayre syndrome, displaying the typical regularly spaced intracristae inclusions (dark) surrounded by mitochondrial inner cristae membranes. The section has been stained by rabbit anti-MtCK antibodies, followed by colloidal gold-conjugated second antibody (in collaboration with Dr. AM Stadhouders, Nijmegen, The Netherlands). Note that the dark inclusion bodies are heavily and specifically stained by the small 10-nm gold particles, indicating a high propensity of MtCK at these locations. Isolation of such inclusions plus image processing of sections through them revealed that they consist mainly of crystalline MtCK octamers, which crystallize in sheet-like structures. Modified from Stadhouders AM, Jap PHK, Winklert H-P, Eppenberger HM, and Wallimann T (1994) Mitochondrial creatine kinase: A major constituent of pathological inclusions seen in mitochondrial myopathies. *Proceedings of the National Academy of Sciences of the United States of America* 91(11): 5089–5093.

Mt CK and the Mitochondrial Permeability Transition Pore

MtCK, together with its substrate Cr, has been implicated in regulation of the mitochondrial permeability transition pore that is crucially involved in triggering apoptosis. This seems to be due to metabolite channeling in the MtCK/ANT microcompartment also, which maintains high ADP concentrations in the matrix space that are inhibitory for permeability transition. Thus, the CK system and its substrates seem to exert additional effects that are not necessarily directly coupled to improving cellular energetics. This may explain the remarkable cell- and neuroprotective effects of Cr supplementation that have been reported.

See also: **Bioenergetics:** Mitochondria: Source and Target of Free Radicals; Mitochondrial Channels; Mitochondrial Membranes, Structural Organization; Mitochondrial Metabolite Carrier Protein Family; Mitochondrial Outer Membrane and the VDAC Channel; P-Type Pumps: Na^+,K^+-ATPase.

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