7 The Phosphocreatine Circuit: Molecular and Cellular Physiology of Creatine Kinases, Sensitivity to Free Radicals, and Enhancement by Creatine Supplementation*

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Abstract

Evidence for the important physiological role of the creatine kinase (CK)phosphocreatine (PCr) system in energy homeostasis in sarcomeric muscle, brain, and other organs of high and fluctuating energy requirements is presented in the context of defined subcellular compartments of CK isoenzymes with processes of energy production, i.e., glycolysis and mitochondrial oxidative phosphorylation, and those of energy utilization, i.e., ATPases, ATP-gated ion channels, and ATP-dependent signaling events. The concepts of functional coupling and metabolite channeling are discussed, and the CK subcompartments that have been characterized in great detail are described. Mitochondrial CK (MtCK) plays a central role (1) in high-energy phosphoryl transfer and channeling, (2) in metabolic feedback regulation of mitochondrial respiration in vivo, (3) in stabilizing the contact sites of inner and outer mitochondrial membranes, (4) in delaying and preventing mitochondrial permeability pore opening, an early event in apoptosis, and (5) in preventing, by efficient ADP recycling inside mitochondria and by optimal coupling of respiration with ATP synthesis, excessive free oxygen radical (ROS) formation. Additional new and exciting findings indicate that octameric MtCK, a highly symmetrical cube-like molecule that is able to cross-link two membranes, is also involved in lipid transfer between mitochondrial membranes and in clustering of cardiolipin (CL) and the formation of membrane patches. Because of the high reactivity of the active-site cysteine of CK isoenzymes, this enzyme is highly susceptible to oxidation that leads to inactivation. Oxidation of other residues at the dimer-dimer interface of MtCK induces dimerization of the MtCK octamer, the functional entity in the mitochondrial intermembrane space, and dissociation of MtCK from the mitochondrial membranes.

* A list of abbreviations used can be found at the end of this chapter.

Under oxidative stress, as observed in mitochondrial myopathies and neurodegenerative diseases, these negative effects on MtCK function lead to further deterioration of cellular energy status, chronic calcium overload, and exacerbation of oxidative stress by generation of more ROS, events that eventually lead into apoptosis. On the other hand, the very same exquisite sensitivity of CK to free radical damage renders this enzyme vulnerable to anthracyclines. Sarcomeric sMtCK in the heart is especially susceptible to ROS damage caused by anthracyclines and more sensitive than ubiquitous uMtCK this fact can explain at least in part the specific cardiotoxicity of this potent class of anticancer drugs. After the importance of the CK-PCr system was recognized, transgenic mice lacking expression of the various CK isoforms were created. The most severe phenotype is seen after knockout of both brain CK isoforms (BB-CK and uMtCK), and this is fully in line with the recently discovered creatine deficiency syndrome in mentally retarded patients. Based on genetic deficiencies either in endogenous creatine (Cr) synthesis or in the Cr transporter (CRT) gene, these patients are basically devoid of Cr in their brains. These data support the immanent physiological importance of the CK-PCr system for normal function of brain, muscle, and other cells and organs with high and fluctuating energy demands. Alternatively, enhancing Cr content by Cr supplementation, now widely and successfully used by athletes, also revealed astonishing neuroprotective effects in cases of acute traumatic brain and spinal cord injury as well as in brain ischemia. In addition, as supported by clinical trials, Cr supplementation positively affects skeletomuscular health, i.e., muscle strength and bone mineral density. Cr also improves mental performance and short-term memory. Cr supplementation may become an adjuvant therapeutic measure for chronic neuromuscular and neurodegenerative diseases, such as muscular dystrophy, ALS, MS, and Huntington's, Parkinson's, and Alzheimer's diseases.

7.1

Phosphotransfer Enzymes: The Creatine Kinase System

7.1.1

Microcompartments: A Principle of Life

Life most likely originated autotrophically *de novo* in metabolic complexes organized on FeS₂ (pyrite) mineral surfaces, the earliest form of microcompartments [1]. Thus, because subcellular compartments were already formed at the origin of life, it is hard to understand why the notion was conceived, and still persists, that a living cell, which evolved by this principle, should behave as a well-mixed bag filled with enzymes, substrates, and products [2]. For example, using ³¹P-NMR magnetization transfer measurements with muscle *in vivo* on a global level, involving a volume of approximately 1 cm³ of tissue, it was assumed that creatine kinase (CK) (PCr^{2–} + MgADP[–] + H⁺ \leftarrow CK \rightarrow MgATP^{2–} + Cr), as well as the adenylate kinase (AdK) (2MgADP[–] \leftarrow AdK \rightarrow MgATP^{2–} + MgAMP) were in chemical equilibrium [3]. For this, it was concluded (i) that in muscle cells, CK, with its cytosolic and mitochondrial isoforms already known at that time [4], were in equilibrium, (ii) that the CK metabolites ATP and ADP, as well as phosphocreatine (PCr) and creatine (Cr), were freely mixing in these cells as in solution, and thus (iii) that the CK system would behave according to solution kinetics [2, 5]. However, based on our data showing subcellular compartmentation of CK isoenzymes, this equilibrium concept must be seriously questioned.

7.1.2

Subcellular Compartments and Microcompartments of CK

In fact, recent developments in modern cell biology show that even so-called soluble cytosolic enzymes, such as the cytosolic isoforms of CK, are organized by specific subcellular localizations into metabolically coupled subcompartments, microcompartments, or specialized "metabolons," where they may work far from chemical equilibrium, in the direction of either entirely ATP regeneration or PCr production [4, 6-9] (see also Chapters 3 and 11). In parallel, compartmentalized AdK [10] is at work and, depending on the workload, operates more or less independently or in tandem with the CK system [11-13]. Microcompartments and other subcellular compartments, consisting of multi-enzyme complexes that may be embedded within the cellular "ground substance," associated with the cytoskeleton, or situated in an unstirred layer along cellular membranes, operate according to exclusion principles and favor preferred pathways of intermediates [14]. 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" (for a detailed explanation, see Chapter 11). This greatly facilitates metabolite channeling in such a way that those substrates and products of sequential reactions do not mix with the bulk cytosolic phase [15]. These general mechanisms are thought to increase the efficiency of sequential reactions in a metabolic pathway by helping to avoid a potentially chaotic situation due to macromolecular crowding (see also Chapter 3) [16]. Such mechanisms also may play a significant role in cell signaling where, upon cell activation or stress, a host of different signaling cascades are activated simultaneously, proceeding either independently, or restricted to subcellular microcompartments, or jointly by crosstalk with each other. If these mechanisms were random in a cell, precise spatial and temporal signaling would be impossible.

This idea is supported by the fact that, as recently discovered, even small metabolites – such as calcium [17] or cyclic AMP [18], which work as second messengers – are diffusion restricted in living cells and thus form subcellular microdomains that can be visualized, for example, as calcium sparks or propagating calcium waves [19]. Thus, they do not behave as if they were freely diffusible in solution [9, 20]. This also holds true for the rather bulky and charged adenosine nucleotides ATP, ADP, and AMP. As a matter of fact, there are abundant data available in the literature showing compartmentation of adenine nucleotides, particularly in muscle (see Chapters 3 and 11). Such a concept is attractive especially for AMP, which in resting cells is present at submicromolar concentrations. Upon activation, [AMP] may rise by several orders of magnitude to

stimulate AMP-stimulated protein kinase (AMPK), a metabolic master switch that is also compartmentalized (see Chapter 9).

Thus, enzymes and their corresponding metabolites, substrates, and products, e.g., working as part of a metabolic or cellular signaling cascade, may form structurally, functionally, and temporally defined four-dimensional microcompartments and other subcellular compartments [21], either via strong, static interactions or via fickle, dynamic interactions with other enzymes, proteins, or subcellular structures, the cytoskeleton representing a principle basis for such interactions.

There are interesting data concerning compartmentation of ATP coming from NMR studies *in vivo* [22–24]. For example, dipolar coupling effects, due to anisotropic motional averaging, have been observed for ¹H proton resonances of creatine (Cr), taurine, and lactate in muscle [25]. The most likely explanation for these effects is that even small compounds such as Cr and PCr, together with their hydration spheres, are large enough to be hindered by isotropic tumbling in the

Fig. 7.1 The CK-phosphocreatine circuit. Creatine (Cr), either synthesized in the body or taken up from alimentary sources, e.g., meat and fish, is transported into muscle and other cells with high and fluctuating energy requirements by a specific creatine transporter (CRT). Imported Cr is charged to the high-energy compound phosphocreatine (PCr) by the action of strictly soluble cytosolic CK (CK_c, 3), by CK coupled to glycolysis (CKg, 2), or by mitochondrial CK coupled to oxidative phosphorylation (MtCK, 1). According to the CK equilibrium reaction, in a resting cell, this results at equilibrium in approximately two-thirds [PCr] and one-third [Cr] and in a very high ATP:ADP ratio (≥100:1). Isoenzymes of CK not only are cytosolic (CK_c, 3) but also are associated (CK_a, 4) in compartments to ATP-consuming processes (ATPase), such as the myofibrillar actomyosin ATPase, the SR Ca²⁺-ATPase, the plasma membrane Na^+/K^+ -ATPase, the ATP-gated K⁺ channel, or ATP-requiring cell signaling (4), where CK regenerates in situ the ATP utilized by these processes, drawing from the large PCr pool. This represents the ATP-consuming end of the CK-PCr-Cr circuit (4). In addition, CK is also associated to glycolytic enzyme complexes (CK_{σ} , **2**), where glycolytically generated ATP is transphosphorylated into PCr that is fed into the large PCr pool. This represents the first of the two ATPproducing sites of the CK-PCr-Cr circuit (2). Mitochondrial MtCK is specifically located in

the intermembrane space of mitochondria (MtCK [1]), and by functional coupling to the adenine nucleotide translocator (ANT) of the mitochondrial inner membrane, this MtCK accepts mitochondrially generated ATP and transphosphorylates it into PCr, which then leaves the mitochondria. This represents the second ATP-producing site of the CK-PCr-Cr circuit (1). A large cytosolic PCr pool of up to 30 mM is built up by CK using ATP from oxidative phosphorylation (1), as in the heart, or from glycolysis (2), as in fast-twitch glycolytic skeletal muscle. PCr is then used to buffer global cytosolic (3) and local (4) ATP:ADP ratios. This represents the temporal buffer function of the system. In cells that are polarized and/or have very high or localized ATP consumption (4), the differentially localized CK isoenzymes, together with easily diffusible PCr and Cr, can also maintain a high-energy PCr circuit or shuttle between ATP-providing (1, 2) and ATP-consuming processes (3, 4). Thus, the energy-producing and -consuming terminals of the shuttle are connected via PCr and Cr, with no need for ATP to diffuse, e.g., from mitochondria (1) to the sites of ATPases (4). Metabolite channeling occurs where CK is associated with ATPproviding (1, 2) or ATP-consuming processes (4), such as ATPases, ATP-gated ion channels, ion pumps and transporters, metabolic enzymes, and protein kinases for cell signaling.



Fig. 7.1 (legend see p. 198)

elongated spaces between the thick and thin filaments in muscle, which in the actomyosin overlap zone are extremely crowded [26] and thus lead to anisotropic ordering of Cr and PCr along the myofilament axis [27]. This orientationdependent dipolar splitting of the methyl group protons of Cr and PCr in muscle is no longer observable two hours postmortem [28]. Thus, PCr and Cr, and even more so the adenosine nucleotides ATP and ADP, may form distinct subcellular pools that are more or less movable or movement restricted, and some but not others, due to additional subcellular compartmentation, may be immediately available for CK-mediated phosphoryl transfer reactions [29]. These data are fully in line with considerations made by the late Peter Hochachka, who surmised that intracellular ordering of metabolic pathways, together with intracellular circulation or convection, is a fundamental principle for homeostasis and metabolic regulation that may explain the cellular energy stability paradox [6, 30-32]. These considerations are also in line with the philosophical negentropy principle of Schrödinger, according to which cellular metabolism is always linked to a high degree of intracellular organization (see Chapters 3 and 11).

7.1.3 Tissue-specific Expression of Creatine Kinase Isoenzymes

The existence of tissue- and compartment-specific isoenzymes of creatine kinase (CK) is an important property of this long-known enzyme [33] and is a key to its

functions in cellular energy metabolism. Most vertebrate tissues express two CK isoenzyme combinations: either dimeric, cytosolic, muscle-type MM-CK together with mostly octameric muscle-type mitochondrial sMtCK or, alternatively, ubiquitous brain-type BB-CK together with ubiquitous uMtCK [4]. The first CK isoenzyme combination, MM-CK with sarcomeric sMtCK, is expressed in differentiated sarcomeric muscle, cardiac muscle [34], and skeletal muscle [26], whereas the second combination, BB-CK with uMtCK, is prominently expressed in brain [35], neuronal cells [36], retina photoreceptor cells [37, 38], hair bundles of the inner ear [39], smooth muscle [40], kidney [41], endothelial cells [42], spermatozoa [43], and skin [44], while CK expression levels are generally very low or absent in liver [45]. Hybrid cytosolic MB-CK, on the other hand, is expressed only transiently during muscle differentiation but persists in adult cardiac muscle (for reviews, see [4, 46, 47]).

Octameric MtCK is localized in the cristae as well as in the intermembrane space of mitochondria, preferentially at the contact sites between the inner and outer mitochondrial membrane (Figs. 7.1 and 7.4) [7, 8, 38, 47–49]. Mitochondrial and cytosolic CK diverged at least 670 million years ago [50], suggesting that compartmentalized CK isoenzymes evolved very early during evolution in the context of functional coupling between MtCK and oxidative phosphorylation [51–53] and metabolite channeling [7, 8, 54].

7.1.4

Temporal and Spatial Buffering Functions of Creatine Kinases: The CK–Phosphocreatine Circuit

Life is based on the conversion of free energy by cells that are open thermodynamic systems, exchanging both mass and energy with the surrounding medium (see Chapter 3). Cellular energy demand and supply are balanced and tightly regulated for economy and efficiency of energy use. Tissues and cells with high and fluctuating energy requirements may increase the rate of ATP hydrolysis within seconds by several orders of magnitude, but intracellular ATP levels remain amazingly constant. This stability paradox [30, 31] can be explained by the action of immediately available, fast, and efficiently working energy-supporting and -backup systems, such as CK and AdK that connect sites of energy consumption with those of energy production via phosphoryl transfer networks [4, 6, 11, 12]. 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 ATP:ADP ratios in particular [4, 47, 53, 55–59].

"Cytosolic" CK isoenzymes are not strictly soluble, but a certain variable fraction thereof is bound permanently to or associated transiently with subcellular structures (e.g., the myofibrillar M-band [60], the myofibrillar I-band in conjunction with a glycolytic enzyme metabolon [61]) or cellular membranes, such as the plasma membrane, the sarcoplasmic reticulum (SR), or the Golgi apparatus, where bound CK is functionally coupled to the Na⁺/K⁺-ATPase [41, 62], the Ca²⁺-ATPase [63], or the Golgi matrix protein GM-130 [64]. On the other hand, MtCK is exclusively found in mitochondria [48]. This compartmentation of CK involves direct or indirect association of CK with ATP-providing (mitochondria and glycolysis) and ATP-consuming processes (ATPases and ATP-dependent cell functions), forming distinct compartments that facilitate a direct exchange of ADP and ATP between associated CK and its substrates (PCr and Cr) and the respective association partners of CK, without mixing with the bulk cytosol (Fig. 7.1). The interplay between cytosolic and mitochondrial CK isoenzymes fulfils multiple roles in cellular energy homeostasis (see also Chapters 3 and 11) [4, 6–8, 29, 65, 66].

First, both isoenzymes contribute to the buildup of a large intracellular pool of phosphocreatine (Fig. 7.1) that represents an efficient temporal energy buffer and prevents a rapid decrease in global ATP concentrations upon cell activation or sudden stress conditions (Fig. 7.1, cytosolic CK_c equilibrates the cytosolic overall ATP:ADP ratio) [56]. This buffering function of CK also could be demonstrated by an inverse genetics approach. Introducing the genes for phosphagen kinases, either arginine kinase (AK) or CK, into bacteria or yeast cells leads to functional expression of CK or AK and, together with creatine or arginine supplementation, renders these cells resistant to transient metabolic stresses by stabilizing intracellular ATP concentrations [67, 68]. A clear advantage of the PCr–Cr system is that the compounds are "metabolically inert." Unlike the adenosine nucleotides, which interact with and regulate a plethora of cellular proteins or enzymes, respectively, no other enzyme seems to exist that binds and metabolizes PCr or Cr except for CK. This allows an accumulation of this guanidino compound in the cell basically without interfering with basic metabolism.

Secondly, a very important additional function of CK is based on the concepts of subcellular compartmentation of CK isoenzymes and diffusion limitations of ATP and especially ADP. The so-called PCr–Cr circuit or shuttle theory postulates that not ATP and ADP, but rather PCr and Cr, diffuse from subcellular sites of ATP generation (mitochondria or glycolysis) to distant ATPases, e.g., the myofibrillar calcium-activated MgATPase, the SR Ca²⁺ pump ATPase, the Na⁺/K⁺ pump ATPase, or ATP-gated processes such as the K+ATP channel or ATPdependent cell signaling (Fig. 7.1) [4, 47, 55, 57, 59, 65, 66, 69, 70]. In fact, the advantage of PCr and Cr as metabolically inert "energy transport compounds" over ATP and especially ADP has been experimentally confirmed in an in vivo model system with spermatozoa. These are elongated, highly polar cells, where energy production by mitochondria in the midpiece, near the sperm head, is distant from energy utilization in the long sperm tail [43, 71]. However, this principle is also likely to hold true for skeletal and cardiac muscle as well as for brain and retina, where cells are operated by a very high-energy flux that puts enormous strain on subcellular energy delivery and regulation of these processes (see also Chapters 3 and 11) [6].

Third, subcellularly compartmented CK isoenzymes allow maintenance of high local ATP:ADP ratios in the vicinity of cellular ATPases. Thus, they main-

tain a maximal change in the Gibbs free energy of ATP hydrolysis, $\Delta G_{ATP} =$ $\Delta G^{\circ}_{obs} - RT \times \ln([ATP]/[ADP] \times [P_i])$, which is proportional to the [ATP]:[ADP] ratio ΔG°_{obs} corresponds to the standard tree energy change at 25°C and pH 7.0). This energetic aspect is especially important for thermodynamically unfavorable reactions, i.e., for ATPases that need a high free energy change for optimal function, such as the sarcoplasmic reticulum Ca^{2+} pump (see below). On the other hand, localization of MtCK at the outer surface of the mitochondrial inner membrane in conjunction with the adenine nucleotide transporter (ANT) allows, by a direct transfer of substrates, for immediate transphosphorylation of matrix-generated ATP into PCr and also for a rapid recycling of ADP into the mitochondrial matrix [72]. This results in a strong stimulation of oxidative phosphorylation by MtCK (for a detailed explanation, see Chapters 3 and 11 and Figs. 7.1 and 7.4) [51]. Because of the specific localization of mitochondrial and cytosolic CK isoenzymes, the much faster diffusion rate of PCr as compared to ATP (the latter being decreased in some subcellular compartments by orders of magnitude [70, 73]), and the very much faster diffusion rate of Cr compared with ADP [43], the CK-PCr system provides for a spatial "energy shuttle" or "energy circuit" (Fig. 7.1) that bridges sites of ATP generation with sites of ATP consumption. Thus, MtCK and the CK-PCr system contribute to an intricate metabolic energy transfer network in the cell, connecting mitochondria with myofibrils, the sarcoplasmic reticulum, and nuclei [11, 24, 65, 74-76].

A tight functional coupling of CK to ATPases has the advantage (1) that product inhibition of the ATPase by ADP and H⁺ is avoided, since the latter are both substrates of the CK reaction that are continuously removed if the enzyme is active (PCr + ADP + H⁺ \leftrightarrow Cr + ATP) and (2) that the high Gibbs free energy change of ATP hydrolysis (ΔG_{ATP}) at sites of ATP consumption is preserved by keeping very high local ATP:ADP ratios due to coupling of CK with said ATPases *in situ* and thus preventing energy dissipation caused by transport of ATP and avoiding mixing it with the adenine nucleotides of the surrounding bulk solution [4, 11, 46].

Another important function of CK is the release of P_i due to PCr hydrolysis by the CK reaction with its manifold regulatory consequences for energy metabolism [4]. Finally, via the CK reaction, the inhibitory actions of ADP on ATPases are minimized by keeping [ADP] very low as long as possible. In this way, the buildup of AMP and the subsequent degradation of AMP and IMP into inosine, which would ultimately leave the cell, are delayed, and thus a loss of valuable nucleotides is largely prevented (see Chapters 3, 8 and 9) [4].

To summarize, convincing evidence for the importance of the compartmentalized CK system and the *in vivo* operation of a CK-mediated PCr–Cr circuit as well as the existence of phosphotransfer networks in tissues and cells with high and fluctuating energy requirements comes from

- 1. in situ immuno-localization studies of CK isoenzymes in tissues [4, 46, 66];
- 2. kinetic and thermodynamic analysis of the MtCK reaction coupled to oxidative

phosphorylation via ANT in both isolated mitochondria and permeabilized cells (see also Chapters 3, 11, 12, and 15) [48, 75, 77];

- careful analysis of the phenotype of CK transgenic knockout mice, e.g., showing obvious deficiency in skeletal and cardiac muscle Ca²⁺ handling [78–80]; and
- direct *in vivo* ¹⁸O labeling of phosphoryl moieties in intact muscle (see also Chapters 3, 8, and 11) [12, 81].

Fig. 7.2 The function of muscle-type MM-CK at the sarcomeric M-band. This model combines biochemical data on the molecular interactions of the myomesin with the thick filament, titin, and MM-CK with the information provided by immunoelectron microscopy.

(A) Model of myomesin arrangement in the M-band (modified from [98]). Myosin and titin filaments are represented extremely schematically (A, B). The myomesin molecule is drawn according to structural predictions. The unique N-terminal domain My1 (A, 1), which is predicted to have a disordered conformation and possibly to wrap around the thick filament to provide the essential strength for a myomesin-myosin interface [88], is followed by 12 structural modules with strong homology to either fibronectin (Fn) or Ig-like domains (shown as ellipses). Myomesin molecules, forming antiparallel dimers via their last C-terminal domain My13 (A, 2) [98], are shown to associate with their My4-6 domains with the m4 titin domain (A, 3, black ellipse) and run along the antiparallel titin molecules that overlap with their C-terminal ends (titin N and C). (B) Superimposed onto the basic structural scaffold (A), binding of MM-CK dimers [437] to myomesin domains My7-My8 is shown [103] (B). Note that the interaction site of MM-CK with myomesin corresponds to the symmetrically off-center M4 and M4' mbridge positions (indicated by black M4' and M4 boxes in A and B), which is fully in line with immunoelectron microscopic labeling by anti-MM-CK antibodies [95]. Thus, the presence of MM-CK together with myomesin at these locations explains the appearance of the electron-dense M4' and M4 lines on EM pictures of the sarcomere. For reasons of

simplicity, and because the exact position of the M-protein is not known, it is omitted from the scheme. However, there is good reason to believe that M-protein, also likely to form antiparallel dimers, is largely responsible for the electron density of the central M1 m-bridges [91], the positions of which are indicated in A (black M1 box on top). Because MM-CK was also shown to specifically interact with M-protein at domains M6-8 [103], MM-CK binding to M-protein thus also may contribute to the electron density of this M1 line [102]. The MM-CK integrated into the M-band scaffold regenerates ATP that is hydrolyzed in the actomyosin overlap zone of the sarcomere on both sides of the central M-band by tapping from the large pool of more or less freely diffusible PCr. The microcompartmentation and functional coupling of the actomyosin ATPase cycle in situ with the CK reaction $(MgADP^{-} + H^{+} \leftarrow CK \rightarrow Mg ATP^{2-} + Cr)$ (symbolized by a central ellipsoid) lead to maintenance of locally high ATP:ADP ratios by CK and efficient local ATP regeneration on both sides of the central M-band, with adenosine nucleotides cycling along the filament axes within the crowded myofibrils [26, 108]. Because ADP and protons are substrates of the CK reaction, this prevents inhibition by protons and ADP of the actomyosin kinetic cycle [4, 105, 108]. Convection by the action of myosin crossbridges [30, 31] and piston-like movement of the actin filament during contraction and relaxation would direct a forward flow of ADP towards the M-band for regeneration by CK and PCr and redirect a backward flow of ATP towards the actomyosin overlap regions, respectively [26].

The energy-shuttling function of the system has been experimentally demonstrated in a convincing fashion by the *in vivo* analysis of activated sea urchin spermatozoa by ³¹P-NMR CK flux measurements in combination with oxygraphy [43, 82]. Recently, evidence of the importance of the CK–PCr circuit for organ function, as well as of the existence of distinct non-equilibrating pools of ATP, has come from ³¹P- and ¹H-NMR studies with intact *ex vivo* perfused heart [23, 24, 79, 83–85]. The outcomes of these experimental approaches have also been substantiated by results obtained from computer modeling approaches (see Chapters 12 and 13) [72, 86, 87].

7.1.5

A Closer Look at CK (Micro)Compartments and High-energy Phosphate Channeling

In the following sections, we will discuss subcellular CK compartments in more detail (see Sections 7.1.6 to 7.1.13), because they represent important puzzle pieces for understanding the architecture and functioning of the CK–PCr circuit (Fig. 7.1). A significant fraction of "cytosolic" CK is structurally and functionally associated or co-localized with different structurally bound ATPases: (1) different ion pumps in the plasma membrane, (2) the sarcomeric M-band and I-band of



Fig. 7.2 (legend see p. 203)



Fig. 7.3 The function of muscle-type MM-CK at the sarcoplasmic reticulum. The propinquity of MM-CK with the SR Ca²⁺ pump, the most abundant SR protein, allows for the formation of a shielded subcompartment between the two entities and for functional coupling between CK and the Ca²⁺-ATPase. Thus, ATP generated in situ by the CK reaction at the expense of PCr has preferential access to the pump and facilitates maximal Ca²⁺ uptake into isolated SR vesicles [63]. This is corroborated by the fact that neither externally added ATPregenerating systems (PEP and pyruvate kinase) nor ATP traps [128] significantly affect CK-mediated Ca²⁺ uptake [127], indicating that CK, due to its high affinity for ADP (Km ADP of MM-CK = $15-35 \mu M$ [437]), is able to

maintain a very high local ATP:ADP ratio in the vicinity of the SR Ca²⁺ pump and to channel the ATP directly to the Ca²⁺-ATPase, thus maintaining the thermodynamic driving force of this energetically demanding ion pump [4, 132, 134, 438]. This illustration may be taken as representative of other subcellular CK compartments with ion pumps, ATP-gated ion channels, membrane proteins, or membrane-associated signaling by protein kinases, such the Na^+/K^+ pump ATPase [41], the ATP-gated K⁺ channel [146], the Golgi matrix protein GM130 [64], and insulin [155] or thrombin [156] signaling events, respectively, where CK was shown to be associated and functionally coupled with these processes.

the myofibrils in muscle, and (3) the calcium pump of the muscular sarcoplasmic reticulum. In all these cases, PCr is used for local regeneration of ATP, which is directly channeled from CK to the consuming ATPase without major dilution by the surrounding bulk solution. On the ATP-generating side, part of the cytosolic CK is associated with glycolytic enzymes, and, more importantly, the mitochondrial proteolipid complexes containing MtCK are coupled to oxidative ATP production. In particular, three well-characterized functionally coupled subcellular CK compartments will be presented in more detail. Two are at the receiving end of the CK–PCr circuit at ATP-utilizing sites: muscle-type MM-CK at the sarcomeric M-band of myofibrils, regenerating ATP for the actomyosin ATPase (Fig. 7.2; see Section 7.1.6), and at the sarcoplasmic reticulum (SR), regenerating ATP for the Ca^{2+} pump ATPase (Fig. 7.3; see Section 7.1.7). The third well-characterized example is concerned with high-energy phosphate channeling by

MtCK in mitochondria, constituting an energy-producing terminus of the circuit (Fig. 7.4; see Section 7.1.13).

Prerequisites for a functionally coupled CK compartment at ATP-utilizing sites include (1) a co-localization and association of CK to such processes, (2) a confirmation that PCr would preferentially drive such ATPases via associated CK, and

Fig. 7.4 Functions of mitochondrial MtCK compartments and metabolite channeling by MtCK. After the import of nascent MtCK over the mitochondrial outer membrane and cleavage of the N-terminal targeting sequence, MtCK first assembles into dimers. Dimers rapidly associate into octamers (not shown); although this reaction is reversible, octamer formation is strongly favored by the high MtCK concentration in the intermembrane space. The symmetrical and cube-like MtCK octamers (top left) then directly bind to acidic phospholipids of the outer mitochondrial membrane (OM) and preferentially to cardiolipin (CL) of the inner mitochondrial membrane (IM). MtCK is found in two locations: in the so-called mitochondrial contact sites associated with ANT and VDAC (shown here) and in the cristae associated with ANT only (not shown). In contact sites, MtCK simultaneously binds to the IM, as well as to the OM, due to the identical top and bottom faces of the MtCK octamer. The binding partner in the IM is the twofold negatively charged CL (marked in dark grey), which allows a functional interaction with the adenine nucleotide translocator (ANT) that is situated in CL membrane patches (marked in dark grey). In the OM, MtCK interacts with other acidic phospholipids and, in a calcium-dependent manner, directly with VDAC. The main substrate and product fluxes are indicated by dark arrows. Minor or alternative fluxes are shown in light gray. It should be noted that for simplification and guidance of the reader, the main fluxes are shown to flow through two different VDAC and ANT molecules, although ANT is a true antiporter that stoichiometrically exchanges one ATP for one ADP. In this scheme, ATP generated by oxidative phosphorylation via the F_1 -ATPase (1) is transported through the

IM by ANT (2) in exchange for ADP. This ATP may either leave the mitochondrion directly via outer membrane VDAC or is preferentially accepted and transphosphorylated into PCr by octameric MtCK in the intermembrane space (3). PCr then preferentially leaves the mitochondrion via VDAC and feeds into the large cytosolic PCr pool (4). ADP generated from the MtCK transphosphorylation reaction is accepted by ANT and immediately transported back into the matrix to be recharged (5). 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 the VDAC (not shown; for details, see Refs. [7] and [8]). The tight functional coupling of MtCK to the ANT leads to saturation of the ANT on the outer side of the IM with ADP, which is transported back into the matrix to be recharged by the F1-ATPase, thus efficiently coupling electron transport to ATP generation and thus lowering the production of free oxygen radicals (ROS) [192]. On the other hand, the tight functional coupling of ANT to MtCK leads to a saturation of MtCK with ANTdelivered ATP and a locally high ATP:ADP ratio in the vicinity of MtCK, and in combination with cytosolic Cr, entering the intermembrane space via VDAC (6), it drives the synthesis by MtCK of PCr from ATP without a loss of its energy content, thus maintaining maximal thermodynamic efficiency for high-energy phosphate synthesis in the form of PCr (4), which then is exported into the cytosol. This represents an instructive example of functional coupling and metabolite channeling. (Adapted from [4, 192, 294]).



Fig. 7.4 (legend see p. 206)

(3) experimental evidence that PCr-driven ATPase function is not significantly affected by externally added ATP or soluble ATP-regenerating systems, such as phosphoenolpyruvate (PEP) and pyruvate kinase, nor by added ATP traps. If these three conditions were fulfilled, this would indicate that CK-generated ATP (from PCr) has preferential access to the ATPase due to the presence of a shielded microcompartment. As a consequence of such functional coupling of CK with the ATPase, the apparent K_m for ATP of the ATPase is lowered. Thus, for maintaining full ATPase activity, a lower adenine nucleotide concentration is necessary in the presence of PCr compared with that for ATP alone. Such data are available both for M-band–associated and SR-associated CK, as discussed in some detail below (see also Figs. 7.2 and 7.3), and the same principles apply for MtCK-generated ADP that is preferentially recycled in mitochondria to stimulate oxidative phosphorylation (see Fig. 7.4).

7.1.6

Subcellular Compartmentation of CK at the Myofibrillar M-band: ATP Regeneration for Muscle Contraction

The M-band of the striated muscle sarcomere represents the most thoroughly characterized CK microcompartment. The M-band is visible as a substriated electron-dense structure at the central region of individual sarcomeres (for review, see [59, 88]). There, the so-called m-bridges, running perpendicular to the thick

filament axis, connect individual thick filaments at their bare zones that are free of myosin heads [89]. So far, besides the giant titin molecules that extend from both sides of the adjacent sarcomeric Z-disks into the M-band [90] and overlap there with their C-termini [91] (Fig. 7.2A), three additional major constituents of the M-band have been identified: the 165-kDa M-protein [92, 93], the 185-kDa myomesin [94], and the 84-kDa dimeric muscle-type MM-CK [59, 60, 95] (for review, see [4]) (Fig. 7.2B). Additionally, other minor proteins such as enolase, AMPK, calpain, and DRAL/FHL-2 have been identified at this location [96, 97], suggesting a general role of the M-band as a targeting site for metabolic enzymes. Myomesin and M-protein are highly modular proteins composed mainly from the conserved sequence of Ig- and Fn-like domains. The antiparallel dimers of myomesin molecules [98] might form the elastic connection between the neighboring thick filaments (Fig. 7.2A). This cross-link was suggested to play an important mechanical role and promote sarcomere stability in the contracting muscle [88, 99]. We have found that some 5-10% of total cytosolic MM-CK is located in an isoenzyme-specific manner at this sarcomeric structure [60, 100, 101]. Based on the fact that a significant proportion of the electron density of the M-band is lost upon incubation of muscle fibers with an excess of specific, high-affinity monovalent Fab antibodies against MM-CK, it was concluded that the MM-CK is responsible for the electron-dense appearance of the M-band on electron micrographs [102]. Accordingly, direct immunolabeling with anti-MM-CK IgG antibodies resulted in a double-striped pattern corresponding to the off-center m-bridge stripes [95]. This pattern indicated that MM-CK bound to the M-band mainly follows the location of the so-called M4 m-bridges that connect thick filaments in the bare zone (Fig. 7.2B). Localization at the M-band is specific for the MM-CK isoform and is not found for the highly homologous BB-CK. It results from two MM-CK-specific lysine "charge clamps" at positions K8 and K24 and K104 and K115 [100]. Binding of MM-CK via these lysine charge clamps leads to a strong but pHdependent interaction of this CK isoform with the two central fibronectin-like Fn7-8 domains of myomesin, as well as with the homologous Fn6-8 domains of M-protein [103]. According to the current model, the MM-CK interacting Fn7-8 domains of myomesin are localized at the level of M4/M4' bridges (see Fig. 7.2B), in agreement with the higher electron density found at the M4 m-bridge level. Because M-protein is likely to be located in the vicinity of the central M1bridge [91], it is most likely that MM-CK attached to the M-protein is also responsible for the electron density of this structure. This may explain the correlation between the M1 line appearance and M-protein expression [89]. Another exciting fact results from reengineering the above lysine charge clamps of MM-CK into the corresponding amino acid positions of BB-CK. This results in a gain of function of the mutant BB-CK, which is now able to bind specifically to the myofibrillar M-band [100] and to interact with myomesin, just like MM-CK [103]. Thus, the specific interaction sites of MM-CK with the two M-band proteins, myomesin and M-protein are now well defined.

The amount of MM-CK bound to the M-band is sufficient to regenerate the ATP hydrolyzed by the actomyosin ATPase, running at maximal velocity, in

isolated myofibrils [57, 104, 105]. The apparent $K_{\rm m}$ of ATP for the actomyosin ATPase was reduced by endogenous MM-CK because of its specific location at the M-band, an effect that could not be mimicked by external addition of an alternative ATP regeneration system [105, 106]. This is consistent with tight functional coupling between CK and the actomyosin ATPase. This in situ role of MM-CK was corroborated by contraction and rigor experiments with chemically skinned intact muscle fibers [107, 108]. The functional development of this role of CK could be followed during perinatal maturation of the heart [109]. Ablation of the MM-CK gene leads to a distinct but not very severe phenotype affecting muscle contraction, insofar as muscles of these mice lack burst activity [110] and show less peak muscle force that cannot be maintained [111]. Alterations in myofibrillar functions were obvious as well [112]. Later on, it became clear that in transgenic knockout mice, lacking either cytosolic MM-CK or mitochondrial MtCK, these isoforms can to some extent compensate for each other [113] and, together with a host of metabolic and structural adaptations that take place in the muscles of these transgenic animals [114], lead to a relatively mild muscle phenotype [115]. This muscle phenotype, however, becomes more severe if both MM-CK and MtCK genes are ablated simultaneously [80, 115]. This complex situation argues for the importance of the CK system in muscle but at the same time reveals that muscle cells, with their potential for plasticity, can undergo remarkable structural and functional adaptations. For example, adenylate kinase or glycolysis as an ATP provider may take over parts of the functions that are otherwise guaranteed by CK flux reactions [12, 116-118]. In addition, amazing structural remodeling of cell architecture to compensate for the loss of PCr shuttling can be observed. These adaptations can be considered safeguard mechanisms to sustain important functions of energy provision in muscle. For example, in the double-knockout mouse lacking both CK isoforms (MM-CK and sMtCK) in muscle [80], the mitochondrial density and location are increased drastically in muscle, and the organelles are placed in rows between individual myofibrils [116-118]. By this adaptive strategy, the diffusion distance for ATP from mitochondria to myofibrils is shortened because PCr shuttling is no longer possible [119]. These compensatory adaptations in CK double-knockout mice seem to be even more prominent in the heart muscle, to safeguard the performance of this organ that is essential for life [120]. The most clear-cut phenotype of these double-knockout CK animals was again a muscle phenotype affecting contraction [121] and leading to a significant disturbance of Ca^{2+} homeostasis (see below) [80, 117].

7.1.7

Subcellular Localization of CK at the Sarcoplasmic Reticulum and Plasma Membrane

CK activity in crude sarcoplasmic reticulum (SR) preparations was first measured in 1970 [122]. This finding was extended by functional assays demonstrating that the fraction of CK, which is specifically bound to highly purified SR membrane

vesicles, is able to fully support, at the expense of PCr, Ca²⁺ uptake into the SR vesicles by the SR Ca²⁺-ATPase pump [63, 123]. Inhibition of CK bound to SR vesicles by N-nitroso-glutathione (a nitric oxide donor) or DNFB (an inhibitor) leads to a significant decrease in [124] or complete abolition of Ca²⁺ uptake [63] if ATP derived from PCr and catalyzed by SR-bound CK drives the reaction. CK has been localized along SR membranes in situ by immunogold labeling of ultra-thin cryosections of muscle [26, 63], as well as on isolated SR vesicles in vitro [63]. Functional coupling of this membrane-bound CK with the Ca²⁺-ATPase pump, representing the most prominent polypeptide in isolated SR preparations, also can be shown directly [125]. By overlay blot binding assays, a direct pH-dependent association of MM-CK to a number of SR proteins was demonstrated, and two domains on MM-CK could be identified that are responsible for this interaction [126]. Binding of CK to the SR Ca²⁺-ATPase results in a close propinquity of the two molecules (Fig. 7.3). Functional coupling of SRbound CK to the Ca²⁺-ATPase also has been shown in situ with permeabilized intact muscle fibers, where it could be demonstrated that ATP generated by SRbound CK is more effective than externally added ATP at similar concentrations [127]. Similarly, ATP generated by a soluble ATP-regenerating system, such as PEP and pyruvate kinase [127], or by addition of an ATP trap to the system did not significantly interfere with PCr-supported Ca²⁺ uptake by the SR [128]. This indicates that the compartment is shielded against outside influences and that ATP generated by CK is preferentially channeled to the Ca²⁺-ATPase. Thus, coupling of CK to the Ca²⁺-ATPase results in preferential delivery of CK-PCr-derived ATP to this ion pump (Fig. 7.3) as well as in promotion of a disengagement of the MgADP-bound state of the Ca²⁺-ATPase. Both lead to a significant decrease in the apparent K_m for ATP of the Ca²⁺-ATPase. The importance of CK for Ca²⁺ handling has been corroborated with skinned fibers from normal muscle [129] and with muscle from CK double-deficient transgenic mice [130], as well as with intact single cardiomyocytes [131]. The SR Ca^{2+} uptake operates with a $\Delta G_{Ca2+-transport}$ of approximately +51 kJ mol⁻¹, and the Gibbs free energy change of ATP hydrolysis (ΔG_{ATP}) at physiological concentrations of ATP (5–8 mM), free ADP (10-50 μ M), and P_i (5-10 mM) in resting muscle may be estimated to be approximately $-55 \text{ kJ} \text{ mol}^{-1}$ (for review, see Ref. [46]). The latter is only slightly higher than the Gibbs free energy change $(\Delta G_{Ca^{2+}-transport})$ needed to support the thermodynamically unfavorable Ca²⁺ uptake and indicates that the SR Ca²⁺-ATPase is working close to thermodynamic equilibrium and therefore greatly depends on a high local ATP:ADP ratio for efficient sequestration of Ca²⁺ into the SR lumen [46, 63, 132]. Inhibition of SR-bound CK or CK associated with other ion pumps (e.g., by oxidative damage, as seen in many neuromuscular and neurodegenerative diseases, including mitochondrial myopathies [133]) therefore would decrease ΔG_{ATP} , limit the thermodynamic driving force of the SR Ca²⁺ pump or other ion pumps, and thus cause a decrease in contractile reserve [134]. This lowered cellular energy status, under chronic conditions, would lead to pathological Ca²⁺ overload and generation of even more ROS damage, which is typical for such diseases [135]. Because CK, including MtCK, is extremely susceptible to oxidative damage (see Section 7.4), it is obvious that compromising this enzyme by ROS damage under pathological conditions will hamper the function of the Ca^{2+} pump, thus acerbating any preexisting Ca^{2+} overload situation and eventually leading to cell death.

Muscle MM-CK and sMtCK double-knockout mice revealed that CK failure profoundly affects the ability of these mice to engage in chronic bouts of endurance running exercise and that this decrease in performance is associated with muscle wasting [136]. As a main phenotype, these mice display significant difficulties with intracellular Ca²⁺ handling and muscle relaxation [80], again emphasizing the physiological importance of the CK system for the energetics of intracellular Ca²⁺ homeostasis, the delivery of ATP to the energetically demanding Ca²⁺ pump, and specifically for optimal refilling of the SR Ca²⁺ store [78]. It has been suggested that Ca²⁺ uptake into the SR is supported not only by CK but also by glycolysis, via glycolytic enzymes bound to the SR triads and SR vesicles [26, 137, 138]. Indeed, in the above CK double-knockout mice, the absence of MM-CK at the SR could be compensated at least in part by glycolysis, which took over, as an adaptational measure, some energetic support by delivering glycolytic ATP to drive calcium uptake into the SR [116].

7.1.8

Subcellular Compartments of CK at the Plasma Membrane and Functional Coupling with Na $^+/K^+$ -ATPase

A certain fraction of total CK is associated with plasma membranes [139], where the enzyme is functionally coupled to the Na⁺/K⁺-ATPase or sodium pump [41, 140, 141]. As a nice example to support this notion, significant amounts of CK are associated to postsynaptic membranes of the *Torpedo* electric organ [142]. After discharge of this organ, the levels of PCr fall rapidly due to the almost exclusive utilization by CK of PCr for fueling the fully activated Na⁺/K⁺-ATPase to recharge the electrocytes. Indeed, an intimate functional coupling between CK and this Na⁺/K⁺ pump could directly be demonstrated by *in vivo* saturation transfer ³¹P-NMR measurement [143].

7.1.9 Structural and Functional Coupling of Cytosolic CK with ATP-sensitive K⁺ Channels and KCC2

Sarcolemmal ATP-sensitive K⁺ channels (K⁺_{ATP}) belong to the group of intracellular energy sensors, coupling the metabolic status of the cell with membrane excitability. These channels are selectively permeable to K⁺ ions and are closed at normal millimolar concentrations of intracellular ATP. In other words, if this channel saw normal bulk cytosolic [ATP], it would stay closed all the time, because bulk [ATP] is kept homeostatically constant in a cell as long as possible [31]. However, active membrane ATPases, such as Na⁺/K⁺-ATPase, reduce the local ATP concentrations, thereby setting the ATP:ADP ratio near the plasma

membrane distinct from the bulk cytosol [20, 70] and allowing opening of the K⁺_{ATP} even if bulk cytosolic [ATP] remains high. The direct physical association of cytosolic CK with the K⁺_{ATP} channel [144], as well as functional coupling of CK with vicinal Na^+/K^+ pumps (see above), together with the dynamics of highenergy phosphoryl transfer through the cytoplasm via the CK reaction, permit a high-fidelity transmission of energetic signals into the sub-membrane compartment, thus synchronizing cell metabolism with K⁺_{ATP} electrical activity [145, 146]. In transgenic mice carrying a germ line deletion of the MM-CK gene, regulation of the K⁺_{ATP} by PCr and concomitant signal delivery to K⁺_{ATP} channels is disrupted, and a cellular phenotype with increased electrical vulnerability of the cardiomyocytes is generated [145]. In addition, CK can functionally couple with K⁺_{ATP} through direct CK-dependent regulation of the ATPase catalytic cycle within SUR, the K+ATP regulatory subunit, promoting disengagement of the MgADP-bound state and thus channel closure [147]. In pancreatic β -cells, K⁺_{ATP} helps to control insulin secretion, and thus functional coupling of the CK system to this channel is clinically relevant. Strategies of structural compartmentation of CK and functional coupling similar to those shown to take place with the K⁺_{ATP} channel [145] could also apply to other ATP-gated processes in the cell. In fact, a specific association has been shown between CK and the neuron-specific K⁺-Cl⁻ co-transporter (KCC2) that may be ATP induced [148]. Furthermore, it could be shown that CK activates KCC2 in its subcellular microenvironment at the plasma membrane [149]. This complex membrane sensor of the cellular energy state is responsible for decreasing the entry of Ca²⁺ into the cells under conditions of energy deficiency as manifested by a decrease in PCr content. Disturbance of the CK system and loss of functional coupling of CK to the KCC2 ion transporter could contribute to acute ischemic contractile failure of the heart, a condition in which CK is impaired (see Chapter 11).

7.1.10

CK Interaction with the Golgi GM130 Protein

Recently, brain-type BB-CK was shown to transiently associate specifically and colocalize with the *cis*-Golgi matrix protein (GM130) during early prophase of mitosis [64]. This is exactly the time point when energy is needed by signaling pathways to initiate the fragmentation of the Golgi apparatus [150–152]. Because high-energy phosphates are needed for the various phosphorylation processes, it is likely that the interaction between GM130 and BB-CK facilitates GM130 phosphorylation by ATP-requiring protein kinases. In support of this notion, recent results of a proteomic approach revealed a co-purification of BB-CK in a complex with Golgi casein kinase as well as with GM130 [153]. Taken together, these data strongly suggest that BB-CK is structurally and functionally coupled in a dynamic and cell cycle–dependent manner to the Golgi apparatus. There, BB-CK is most likely a member of a large protein complex that also comprises several protein kinases. Thus, as a novel function, CK family members may be linked to signaling cascades regulating the integrity of the Golgi apparatus and thus may be involved in the control of the cell cycle [64]. Interestingly, BB-CK was recently shown to be in a complex together with the chloride intracellular channel (CLIC), dynamin I, α -tubulin and β -actin, and isoforms of the 14-3-3-protein [154]. This may implicate BB-CK in further cellular processes different from those mentioned above.

7.1.11

Specific Compartments of Cytosolic CK with Insulin and Thrombin Signaling Pathways

Insulin signaling requires autophosphorylation of the insulin receptor kinase domain, and this process is strongly inhibited by ADP. Thus, CK associated at the plasma membrane locally provides the ATP needed for autophosphorylation and, at the same time, effectively removes ADP from this microcompartment and thus relieves the inhibition mentioned [155]. This also holds true for other signaling pathways, e.g., thrombin signaling, where a structural and functional interaction of cytosolic CK with the protease-activated receptor-1 (PAR-1) has been convincingly shown *in vitro* as well as *in vivo*. CK bound to PAR-1 is poised to provide bursts of site-specific, high-energy phosphate necessary for efficient thrombin receptor signal transduction during cytoskeleton reorganization [156]. Hence, we consider it likely that CK is also involved in similar functionally coupled compartments with a multitude of other receptors and protein kinases belonging to other signaling pathways. A connection between CK and AMPK has been suggested (see Chapter 9).

7.1.12

Subcellular Compartments of CK with Glycolytic Enzymes and Targeting of Glycolytic Multi-enzyme Complexes

In muscle, glycolytic enzymes are targeted to the actin-containing thin filaments at the sarcomeric I-band region, where they form highly complex glycolytic metabolons. By elegant experiments with transgenic *Drosophila*, which express the glycerol-3-phosphate dehydrogenase isoform (GDPH-1) but not the GDPH-3 isoform in their flight muscle, it could be shown that neither glyceraldehyde-3-phosphate dehydrogenase (GAPDH) nor aldolase would co-localize at the I-band in flight muscle of transgenic flies where GDPH-1 had been replaced by GDPH-3. Even though the full complement of active glycolytic enzymes was present in these transgenic flight muscles, with the exception of an isoenzyme switch from GDPH-1 to GDPH-3, the failure of the glycolytic enzymes to co-localize in the sarcomere resulted in the inability to fly [157]. Thus, formation of functionally coupled and correctly targeted multi-enzyme complexes with substrate/ product channeling seems to be paramount for proper functioning of glycolysis and ultimately for correct muscle function.

Interestingly, CK also seems to participate in the glycolytic metabolon, because in muscle MM-CK was also localized specifically in the sarcomeric I-band to-

gether with other glycolytic enzymes, such as aldolase and phosphofructokinase (PFK) [26, 61]. Close functional coupling between CK and pyruvate kinase [158] and glycogen phosphorylase [132], both forming so-called diazyme complexes with CK, has been shown and discussed in theoretical terms, respectively. Recently, structural coupling of cytosolic CK to the key regulatory enzyme of glycolysis, PFK-1, which itself is strongly regulated by ATP, has also been demonstrated [61]. It thus is entirely reasonable to assume that CK co-recruited together with PFK and aldolase [61] into the glycolytic metabolon could be directly coupled to glycolysis and transfer the glycolytically produced ATP immediately into PCr (Fig. 7.1, CK_g). By this strategy, glycolytic ATP would not accumulate, which otherwise would result in inhibition of PFK and shutting off of glycolysis, especially during high-intensity performance of fast-twitch glycolytic muscle [61]. Thus, CK seems not only to be involved in removing glycolytically generated, metabolically highly active ATP into the PCr pool but also, by adjusting ATP:ADP ratios in situ within the glycolytic metabolon and by tight functional coupling with PFK-1, to regulate glycolytic flux [61] and glycogenolysis [132]. Finally, functional coupling of glycolysis and PCr utilization has been directly shown by ³¹P-NMR experiments in vivo with anoxic muscle of a fish species that can survive under completely anaerobic conditions, deriving its total energy from glycogenolysis [159] (for review, see [4, 46, 61]).

7.1.13

High-energy Phosphate Channeling by MtCK in Energy-transducing Mitochondrial Compartments

Mitochondrial CK (MtCK) forms mainly large, cube-like octamers that are present (1) between the outer and inner mitochondrial membrane (the so-called intermembrane space of mitochondria) (Fig. 7.4) and preferentially localized at the socalled mitochondrial contact sites [38, 160] as well as (2) in the cristae space (for details, see [7, 8, 38]). The kinase catalyzes the direct transphosphorylation of ATP, produced inside mitochondria, using Cr from the cytosol to give PCr and ADP. Subsequently, the ADP thus generated 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 the mitochondria and moves into the cytosol [48]. The molecular basis for such directed metabolite flux is channeling between the large, cube-like MtCK octamer [49, 161, 162] and two transmembrane proteins: the adenine translocator (ANT) and the mitochondrial porin or voltage-dependent anion channel (VDAC). ANT is an obligatory antiporter for ATP/ADP exchange across the inner mitochondrial membrane [163, 164], while VDAC is a nonspecific, potentialdependent pore in the outer mitochondrial membrane [165]. MtCK-linked metabolite channeling is based on co-localization, direct interactions, and diffusion barriers. MtCK tightly binds to cardiolipin that is specific for the mitochondrial inner membrane (see below). Because ANT is situated in a cardiolipin patch (see [166] and Section 7.3), this leads to co-localization and metabolite channeling between both proteins, MtCK and ANT, in the cristae and intermembrane space

[160]. MtCK in the intermembrane space further interacts with outer membrane phospholipids and, in a Ca²⁺-dependent manner, with VDAC, thus virtually cross-linking the inner and outer membrane and contributing to the mitochondrial contact sites (Fig. 7.4). Increasing external Ca²⁺ concentrations strengthen the interaction of MtCK with VDAC [167], which may improve high energy phosphate channeling under cytosolic calcium overload that occurs at low cellular energy states. Some studies show that only the membrane-bound, octameric form of MtCK is able to maintain the metabolite channeling described above [168]. Finally, the limited permeability of VDAC and thus of the entire outer mitochondrial membrane in the cells in vivo creates a dynamic compartmentation of metabolites in the intermembrane space [169–171] that contributes to MtCK-linked channeling and separate mitochondrial ATP and ADP pools [77] (see Chapter 6 and Fig. 7.4). 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 [160]. The direct functional coupling of MtCK to oxidative phosphorylation can be demonstrated with respirometric oxygraphy on skinned muscle fibers from normal and transgenic mice lacking MtCK [51]. Oxidative damage of MtCK, induced by free reactive oxygen (ROS) and nitrogen species (RNS), such as peroxynitrite (PN), generated under cellular stress situations (e.g., in infarcted heart or under chemotherapeutic intervention by anthracyclines) leads to inactivation and dimerization of MtCK octamers as well as to dissociation of the enzyme from the mitochondrial inner membrane [172], thus negatively interfering with important prerequisites for efficient channeling of high-energy phosphates by MtCK (for details, see Section 7.4). These events contribute to cardiac energy failure [173] and specific cardiotoxicity [172], respectively.

7.2 Creatine Kinases and Cell Pathology

7.2.1

Mitochondrial MtCK, the Mitochondrial Permeability Transition Pore, and Apoptosis

Proteolipid complexes containing MtCK and ANT exhibit a direct protective effect on mitochondrial permeability transition (MPT). This reversible and cyclosporine-sensitive process is caused by a large pore in the inner mitochondrial membrane and is triggered by multiple signals, including Ca²⁺ and ROS [174]. MPT leads to dissipation of the membrane potential, mitochondrial swelling, and permeabilization of the outer membrane. The latter may trigger apoptosis by the release of proapoptotic proteins such as cytochrome *c* [175] or lead to necrotic cell death due to energy depletion [176]. Both *in vitro* and *in vivo*, contact site complexes containing ANT and VDAC exhibit many properties of the MPT pore [160, 177], and the ANT has become widely accepted as the putative poreforming channel [175, 178]. However, ANT does not seem to be the only poreforming protein of the inner membrane, since mitochondria from ANT-knockout

mice still show MPT, although this occurs only at higher Ca²⁺ concentrations and the MPT is insensitive to cyclosporine [179, 180]. An involvement of MtCK in MPT and mitochondrial ultrastructure was demonstrated with liver mitochondria that were isolated either from transgenic mice expressing ubiquitous uMtCK in their liver [181] or from control animals lacking liver MtCK [182-184]. These experiments clearly show that MtCK, in complexes with ANT and together with its substrates creatine and ADP, is able to delay or even prevent Ca²⁺-induced MPT pore opening [182, 183]. It is not the presence of MtCK per se that inhibits MPT but more precisely its enzymatic activity and its correct localization in mitochondrial complexes. No effect on MPT is observed if the enzyme is not supplied with its appropriate substrate, Cr, or if MtCK is only added externally to liver mitochondria that lack endogenous MtCK [182]. This mechanism may be explained by functional coupling of octameric MtCK to ANT. If MtCK is provided with substrate, it will maintain a high ADP concentration in the mitochondrial matrix (Fig. 7.4), which in turn is known to effectively inhibit MPT pore opening. MtCK does not merely act as a "plug" between porin and ANT to prevent MPT, as speculated earlier; rather, its presence has a clear influence on the number and stability of contact sites. Liver mitochondria from transgenic mice, expressing uMtCK in this organ, showed a threefold increase in the number of recognizable contact sites by electron microscopy and an increased resistance to detergentinduced lysis as compared with controls [185]. Remarkably, these transgenic CK livers, including those that express cytosolic BB-CK, become largely tolerant to liver toxins as well as to tumor necrosis factor α (TNF- α)-induced apoptosis [186]. The decisive involvement of MtCK, together with its substrate Cr, in the regulation of the mitochondrial permeability transition pore [182, 184] may explain at least in part the remarkable cell- and neuroprotective effects of creatine that have been reported lately (see Section 7.5). Thus, the CK system and its substrates seem to exert additional effects that are not necessarily directly coupled to an improved cellular energy status.

7.2.2

Mitochondrial MtCK and Intramitochondrial Inclusions in Mitochondrial Myopathy

Interestingly, MtCK expression levels are an indicator of 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 hall-mark of pathology. The latter have been shown to basically consist of crystalline sheets of MtCK (see Fig. 7.4) [133]. Similar MtCK inclusions can also be induced in animals by chronic Cr depletion, leading to cellular low-energy stress [187]. These data indicate that cellular low-energy stress, be it by chronic endurance training, fasting [188], Cr depletion, or pathologies in ATP generation (e.g., mitochondrial dysfunction, as seen in patients with mitochondrial cytopathies), induces a coordinated induction of the expression of genes related to energy

metabolism, the most prominent among them being represented by MtCK [189]. Presumably, this is to compensate for a lack of energy supply and transport, which leads to overexpression and crystallization of MtCK under pathological conditions [133]. Interestingly, Cr supplementation of a patient with a novel cytochrome *b* mutation resulted in attenuation of free radical production and concomitant disappearance of the intramitochondrial MtCK inclusions in his muscles [190]. This could indicate that the para-crystalline MtCK inclusions form as a result of oxidative damage to MtCK.

Interestingly enough, sMtCK is also significantly upregulated in aged mice, but this can be entirely inhibited by caloric restriction [188]. Such a nutritional regime delays the aging process by increasing protein turnover, reducing oxidative stress, and thus decreasing macromolecular damage [191]. The compensatory upregulation of MtCK expression would be protective, since it was shown recently that MtCK can reduce mitochondrial free radical generation, based on its efficient recycling of ADP in mitochondria. If MtCK is fully functional, respiration is optimally coupled to ATP synthesis, thus reducing mitochondrial damage by ROS and RNS [192]. This is important also for MtCK function itself, as the enzyme is extremely susceptible to oxidation (see Section 7.4).

7.2.3

Overexpression of MtCK in Certain Malignancies

In normal liver, only very low levels of mostly cytosolic BB-CK are found, the origin of which may be vascular smooth muscle and endothelial cells. Normal hepatocytes do not seem to express CK at all. However, MtCK expression has been observed in regenerating and malignant liver [193], specifically in hepatocellular carcinomas [45], and in mammary carcinoma cell lines [194], where strong induction of BB-CK and uMtCK, respectively, was noted. Generally, it was found that in those tumors that start to overexpress MtCK, mostly adenomas and lymphomas [195], the mortality rate was significantly higher compared with patients with similar malignomas that did not express MtCK [196]. It also seems that leakage of MtCK into the bloodstream of patients can be used as a tumor-associated serum marker: 85% of a patient group with metastatic or infiltrating malignancy, mostly hepatic and gastric carcinomas, showed MtCK in the serum [197]. Considering the fact that MtCK stabilizes mitochondrial membranes and protects these organelles from undergoing permeability transition, the resistance of MtCKexpressing cancer cells to apoptosis-inducing chemotherapy may at least in part be due to the presence of elevated levels of MtCK in these cancer cells (see above).

7.3 Novel Membrane-related Functions of MtCK

Two additional and novel functions of the symmetrical, cube-like octameric MtCK protein [161, 162] were discovered very recently. They deal with the basic func-

tions of lipid transfer from one mitochondrial membrane to another [198] as well as with MtCK-induced clustering of cardiolipin [199].

7.3.1

Transfer of Lipids by Proteins That Bridge the Inner and Outer Mitochondrial Membrane

Two mitochondrial proteins localized in contact sites, i.e., mitochondrial creatine kinase (MtCK) [38] and the mitochondrial isoform of nucleoside diphosphate kinase (NDPK-D), are both basic peripheral membrane proteins [200]. An important component of the interaction of MtCK and NDPK-D with membranes is through the cationic segments in these proteins that preferentially interact with anionic lipids to form cardiolipin-rich domains [199, 201]. In addition to forming membrane domains, these proteins can cross-bridge bilayers because of their symmetrical homo-oligomeric structure [202]. This feature gives them the ability to facilitate the transfer of lipid from one bilayer to another (Fig. 7.5) [198].



Fig. 7.5 Octameric MtCK cross-links two membrane vesicles and facilitates lipid transfer between these vesicles. (A) A population of large (200 nm in diameter) unilamellar DOPC:DOPE:CL (1:1:1) phospholipid vesicles (large acceptor vesicles) were mixed with a population of smaller vesicles (50 nm in diameter) having the same lipid composition and also containing some fluorescent probe (small donor vesicle with a fluorescent lipid having a black-dotted head group). (B) At t = 0, octameric MtCK (actual side

view of the X-ray structure of octameric MtCK [161, 162] is shown) is added to the vesicles, and within minutes MtCK binds to and crosslinks two vesicles based on its symmetrical top and bottom faces exposing membraneinteraction domains [54].

(C) MtCK sandwiched in between these vesicles facilitates, also within minutes, the transfer of fluorescently labeled phospholipid from the small donor to the large acceptor vesicle, as measured in a fluorometer cuvette by dequenching of the fluorescent probe and visualized here as black-dotted fluorescent phospholipid molecules present in the large acceptor vesicle population. Protein bridging of vesicles by MtCK causes probe transfer as observed by probe dequenching, with no fusion or leakage taking place (measured independently; for experimental details and original data, see [198]).

Mitochondrial Creatine Kinase Both uMtCK and sMtCK have several C-terminal lysine residues that are important for binding to anionic lipids [54]. It is known that uMtCK binds only to liposomes containing anionic lipids [203]. One of the important anionic lipids for binding of MtCK to the mitochondrial membrane is cardiolipin (CL) [202, 204–206]. MtCK undergoes a concentration-dependent, reversible oligomerization between the octameric and dimeric forms [207], with octameric uMtCK dissociating 23–24 times slower compared with sMtCK [208]. The crystal structures of both octameric MtCKs are known [161, 162]. The octameric form binds rapidly to anionic phospholipids, while the dimeric forms binds much more slowly, possibly requiring prior octamerization [209]. This may simply be a thermodynamic consequence of the equilibration of the dimeric and octameric forms in solution, with the small amount of octameric form that is present, even in dilute solution, preferentially binding to the membrane.

Mitochondrial Nucleoside Diphosphate Kinase The nucleoside diphosphate kinase (NDPK) is a large family of hexameric isoenzymes that are encoded by different nm23 genes that exert multiple functions in cellular energetics, signaling, proliferation, and differentiation. Although NDPK activity was reported in mitochondria with different localization depending on the species or organ [210], only NDPK-D has a mitochondrial targeting sequence and has been unambiguously localized as bound to the inner mitochondrial membrane (IM), in particular to contact sites [211, 212]. NDPK-D plays an important role in mitochondrial iron homeostasis [213]. NDPK-D also shows properties similar to uMtCK, insofar as it also binds to anionic phospholipids such as CL. This protein has a cationic cluster that is thought to be responsible for binding to anionic lipids. NDPK-D is also capable of cross-linking two lipid bilayers, probably due to the symmetry of its hexameric structure ([198] and Tokarska-Schlattner, Schlattner, and Lacombe, unpublished data). However, unlike uMtCK, NDPK-D remains hexameric at any protein concentration. There is particular interest in the products of the nm23 gene family because they are involved in tumor progression and metastasis [214]. NDPK-D was found to be overexpressed in a majority of gastric and colon cancers [215], possibly linking NDPK-D to the development of tumors.

7.3.2 Lipid Transfer at Contact Sites

Connections between the outer and peripheral inner membranes of mitochondria, termed contact sites, are revealed by morphological analysis (e.g., in chemically fixed mitochondria where the intermembrane space is enlarged [216]), as jumps in the fracture planes of freeze-fractured mitochondria [217], or in threedimensional electron tomography [218]. At contact sites it is suggested that uMtCK binds to CL on the inner mitochondrial membrane (IM), stabilizing a CL-rich domain and binding to the voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane (OM) to bridge the IM and OM [219, 220]. VDAC is part of the permeability transition pore and the sustained opening of

this pore is important for necrosis [221], having important implications for energy flux processes as well as for apoptosis [6–8, 170, 182]. The function of contact sites is modulated by the presence of CL [166, 222, 223]. In general, mitochondrial lipids play an important and specific biological role [224].

Contact sites are considered a domain within the mitochondrial membrane [225]. They are regulated, dynamic structures, as their number depends on the metabolic activity of the cell [226]. Because of the close approach of the two membranes, these contact sites could favor interbilayer transfer of lipids. The transfer of phospholipids from one membrane to another is in general a very slow process. Therefore, specific pathways have to be postulated for this process to occur in apoptotic mitochondria. Likely candidates that could facilitate this process are the proteins MtCK and NDPK-D, which are able to bridge the IM and OM [29, 49, 212, 227] and, in the case of MtCK, interfere with apoptotic signaling [7, 8, 182].

7.3.3

Cardiolipin Transfer and Apoptosis

Cardiolipin is synthesized on the IM [228, 229], and the major fraction of CL in the cells is present on the inner leaflet of the IM [230]. CL moves to the surface of mitochondria early in apoptosis [231-233]. Monolysocardiolipin (MCL) is an important CL metabolite, naturally present in mitochondria, that is involved as an intermediate in the remodeling of the acyl-chain composition of CL [234]. Like CL, MCL also binds to truncated Bid (t-Bid), and its concentration in the mitochondria increases during FAS-induced apoptosis [235]. Thus, the movement and domain formation of MCL, as well as CL itself, are important to mitochondrial function and apoptosis. The process of movement of CL or MCL from the IM to the exterior of mitochondria involves both interbilayer transfer from the IM to OM and flip-flop across the OM membrane. Several proteins are involved in this lipid movement, including the phospholipid transferase PLS3 [236] and an unidentified flipase that promotes transfer of phospholipids into the mitochondria [237]. In addition, MtCK and NDPK-D also facilitate the transfer of lipids between these membranes [198]. Transfer of lipids between bilayers is more commonly facilitated by specific lipid transfer proteins [238-240]. There is a report indicating that t-Bid also has lipid transfer activity [241], but this may be a result of its structural similarity to other lipid transfer proteins [242]. Thus, the observation that uMtCK and NDPK-D have such lipid transfer activity is novel. The biological importance of this lipid transfer is shown by the fact that a promyelocytic leukemia cell line that exhibits less exposed CL is resistant to apoptosis [232]. In contrast, a lipid transfer protein from maize produces proapoptotic effects on mammalian mitochondria [243]. In addition, tumor cells that are poorly differentiated have fewer contact sites [244]. Such cells are also more resistant to apoptosis [245], again suggesting a relationship between apoptosis and the presence of contact sites where these proteins are known to be located. NDPK-D and MtCK also differ in their expression levels in different cancer cell lines. This may have consequences on the susceptibility of these cells to chemotherapeutic agents,

since a major drawback of such therapies is the resistance of tumor cells to apoptosis. It is known that the Bcl-2 proteins t-Bid and Bax bind more readily to membranes with exposed CL [246–249]. Hence, there is a clear association between apoptosis and the exposure of CL on the surface of mitochondria.

7.3.4

Cardiolipin Domains and Mitochondrial Kinases

It would be expected that the lateral distribution of lipids in the membrane would affect their rate of interbilayer transfer, as well as affecting the stability of the protein bridges between the IM and OM. As shown recently, uMtCK promotes the segregation of anionic lipids (Fig. 7.6) [199]. The formation of CL-rich domains by the mitochondrial protein uMtCK was initially assessed by differential scanning calorimetry (DSC) using mixtures of tetraoleoyl cardiolipin (CL) and phosphatidylethanolamine (PE). These are two of the major lipid components of mitochondrial contact sites [201]. The mixture has a sufficiently small number of components that it will exhibit cooperative phase transitions detectable by DSC. One of two different species of PE was used: either 1-palmitoyl-2-oleoyl PE



Fig. 7.6 MtCK binding to cardiolipin-containing phospholipid membranes leads to clustering of cardiolipin around the MtCK octamer. Cardiolipin (CL) clustering was measured by differential scanning calorimetry (DSC). Multilamellar vesicles composed of POPE:CL (0.75:0.25) are heated in a DSC cell, yielding a broad phase transition with no differentiated peaks for the mixture. When heating is performed in the presence of uMtCK, a partial separation of the components of the lipid mixture is observed, with sharpening of the peaks, as a result of the preferential association of the protein with CL (for original data, see [199]). This preferential association of octameric MtCK with CL and the clustering of CL molecules is shown here schematically in a diagram depicting a membrane leaflet containing a mixture of POPE (gray) and CL (with black head groups), with the CL uniformly distributed before MtCK protein is added (A). After the addition of MtCK (an MtCK octamer shown as an X-ray structure from its side view is depicted [161, 162]), the protein binds to the membrane by its symmetrical top or bottom face, and subsequently CL molecules (black head groups) begin to preferentially interact with and cluster around the MtCK octamer (B).

(POPE) or dipalmitoyl PE (DPPE). POPE is similar to forms of PE found in biological membranes, but mixtures of CL with DPPE accentuate the effects of the proteins, allowing a comparison of the relative potency of different proteins relative to domain formation in the absence of proteins. The CL does not exhibit any phase transition above 0 °C, while POPE and DPPE have gel-to-liquid crystalline transitions at 25 and 65 °C, respectively. In a lipid mixture of CL with one of the forms of PE, a broad phase transition can be observed over a range of temperatures between that of the two pure-lipid components. If a protein preferentially binds to CL, forming a domain of this lipid, the remainder of the membrane becomes enriched in the PE component. As a result, the phase transition in the presence of such a protein will exhibit a component with higher transition temperature and higher transition enthalpy, approaching the characteristics of a pure PE domain. In addition, the potency of the dimeric and octameric forms of uMtCK to induce the formation of domains was compared. This was done at the same concentrations of uMtCK for both forms, since a freshly diluted solution of the octameric form dissociates slowly, over a period of hours, to the dimeric form. Replacing CL with a structurally related anionic lipid (phosphatidylglycerol) abolishes protein-induced domain formation, pointing to the specificity of this protein-lipid interaction. Our results demonstrate that the octameric form of uMtCK is effective in preferentially interacting with CL, leaving a PE-enriched component that exhibits characteristic phase transition behavior [199]. This property is specific and is not seen with either the dimeric form of uMtCK or this protein after it has undergone irreversible thermal denaturation. Furthermore, the ability of octameric uMtCK to form CL-rich domains is much greater than that of other cationic proteins found in the mitochondria, and the formation of these domains is expected to affect the rates of movement of CL between monolayers in a bilayer. The presence of exposed CL-rich domains at contact sites in mitochondria is important for the binding of the proapoptotic protein t-Bid, which results in the promotion of apoptosis [250, 251].

7.3.5

Perspectives: Relationship Between the Surface Exposure of Cardiolipin and Expression Levels of Mitochondrial Kinases

Exposure of CL on the surface of mitochondria entails two processes. Because in mitochondria most of the CL is on the IM, CL must be transferred from the IM to the OM and undergo transbilayer diffusion (flip-flop) from one side of the OM to the other [252]. The rates of both types of lipid transfer determine how quickly CL becomes exposed on the surface of mitochondria, resulting in apoptosis.

One might expect that the interaction of CL with a protein at the membrane interface would inhibit its translocation across the bilayer. It is known that in many cancers, particularly those with poor prognosis, there is an overexpression of uMtCK [196, 197]. The cells in these tumors are also resistant to apoptosis. On the basis of the observation that uMtCK promotes interbilayer transfer, one would expect these tumors to be more sensitive to apoptosis. It is possible that the ten-

dency of uMtCK to interact with CL and form membrane domains actually slows the rate of flip-flop. As a result, although there would be transfer of CL from the IM to the OM, there would be no exposure of this lipid on the surface of the mitochondria. Thus, one may suggest that the relationship between MtCK and NDPK-D expression and CL exposure on the mitochondrial surface is biphasic. At very low levels of expression of the kinases, there are fewer contact sites and hence few domains with a closely spaced IM and OM where interbilayer lipid transfer could occur. At very high expression levels, there is rapid transfer from the IM to the OM. However, CL domain formation could reduce the movement of CL across the OM. Thus, there would be less CL exposed on the surface of the mitochondria, despite the increased rate of interbilayer transfer. Only intermediate levels of expression lead to interbilayer CL transfer, but because of limited CL domain formation, flip-flop could occur more rapidly, resulting in exposure of CL and apoptosis. This hypothesis provides an ad hoc explanation for the apparent anomaly of both low and high levels of MtCK expression resulting in resistance to apoptosis. The entire scheme of apoptosis and its control are more complex, but CL transfer to the outside of the mitochondria is likely an important contributing factor.

7.4 Exquisite Sensitivity of the Creatine Kinase System to Oxidative Damage

7.4.1

Molecular Damage of Creatine Kinase by Oxidative and Nitrosative Stress

Oxidative and nitrosative stress have been implicated in loss of cellular function in a number of pathological states. These include ischemia–reperfusion injury in heart or brain, acute and chronic heart failure (in particular, the heart failure induced by anthracyclins, a prominent class of anticancer drugs) [253–257] as well as inflammation [258], neurodegenerative and neuromuscular diseases [259, 260], and aging [261]. Elevated levels of reactive oxygen and nitrogen species (ROS and RNS, respectively), together with decreased anti-oxidative defense mechanisms, are at the origin of harmful modifications of various biomolecules, including proteins, lipids, and nucleic acids.

All CK isoenzymes are particularly susceptible targets of ROS and RNS (as superoxide anions, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite [PN]) as well as of anthracyclines, which are known to generate ROS and RNS especially in the presence of iron (see [8, 257, 262]). Oxidative modifications affecting CK functions are thought to play a critical role during pathologies involving oxidative stress. Oxidative inhibition of the CK system is a hallmark of cardiac ischemia–reperfusion injury [263]. In permeabilized rat heart fibers, cytosolic MM-CK has been shown to be the main target of ROS in the myofibrillar compartment. In a study with hydrogen peroxide or using a xanthine oxidase– xanthine system that generated superoxide anions, neither myosin ATPase nor

other myofibrillar regulatory proteins were affected [264]. Specific oxidation of CK, correlating with loss of muscular performance, was observed in skeletal muscles of patients with chronic obstructive pulmonary disease [265]. PN-mediated nitration of myofibrillar creatine kinase has been shown to impair myocardial contractility in different models of experimental heart failure as well as in heart failure induced by anthracyclines [266]. BB-CK was identified, together with glutamine synthase and ubiquitin carboxy-terminal hydrolase L-1, as one of the major proteins specifically oxidized in Alzheimer's disease brain [267] as well as in aged brain [261, 268]. Oxidative damage to the CK system was shown to contribute to impaired energy metabolism in amyotrophic lateral sclerosis (ALS) [269]. More recently, an important reduction in MtCK function was related to oxidative stress caused by endotoxin challenge in sepsis [270].

ROS and RNS induce not only inactivation, which is often irreversible and occurs with all CK isoenzymes, but also specific damage to the mitochondrial CK isoform, namely, interference with its oligomeric state and membranebinding capacity. In animal models of acute and chronic cardiac ischemia, impairment of heart function and mitochondrial energy metabolism was associated with a significant decrease in sMtCK octamer:dimer ratio and enzymatic activity, which were related to PN-induced damage [173]. MtCK has been recognized as a prime mitochondrial target of PN. Its inactivation in isolated heart mitochondria (after direct addition of PN) or in heart of anthracycline-treated mice (where PN is generated via redox cycling of the drugs) is observed before dysfunction of enzymes in oxidative phosphorylation becomes apparent [271, 272]. Finally, MtCK is a prime target of ROS and RNS not only because of high sensitivity but also due to its mitochondrial localization. Most of the reactive species originate directly or indirectly from the mitochondrial respiratory chain.

7.4.2

Molecular Basis of Creatine Kinase Damage

It is known that proteins that are highly sensitive to oxidative modifications have sulfur-containing amino acid residues, with the thiol (-SH) moiety on the side chain of the cysteine being particularly sensitive to redox reactions [273]. In CK isoforms, the active-site cysteine (Cys-283 for cytosolic CK and Cys-278 for MtCK) was identified as the prime residue responsible for oxidative inactivation [8, 274]. This cysteine is not only highly reactive and important for full enzymatic activity but also is easily accessible to modifying agents. Further modifications found at the dimer–dimer interface of MtCK involve oxidation of Met267 and nitration of Trp268 and/or Trp264, which is the most critical single residue for octamer formation. Finally, the C-terminal cysteine Cys358 was also found to be oxidized, which in turn can contribute to a reduced membrane affinity of the C-terminal phospholipid-binding motif [274].

The impact of oxidative modifications on protein function and the reversibility of these modifications depend on the character and concentration of the oxidizing molecule. During conditions of moderate oxidative stress, CK inactivation observed with ROS or anthracyclines is partially reversible by reducing agents (e.g., β -mercaptoethanol, dithiothreitol [DTT]) [172, 275]. However, at higher ROS concentrations or if caused by the very potent oxidant PN, CK damage is largely irreversible and less specific, involving other amino acid residues in addition to the active-site cysteine [172, 271, 274]. Recent studies on MtCK using site-directed mutagenesis and mass spectrometry showed unambiguously that PN induces reversible nitrosylation and irreversible oxidation of the active-site Cys278, the latter explaining the entire loss of enzymatic activity of the oxidized enzyme.

CK thiols have been shown to undergo reversible oxidative modifications, such as S-thiolation, S-nitrosylation [124], and formation of inter- or intramolecular disulfide bonds [172]. These modifications may have a potential regulatory significance in situations where pro-oxidizing conditions arise in a cell [273]. S-thiolation of CK, consisting of formation of mixed disulfides between CK thiols and the low-molecular-mass thiol glutathione, has been observed, and the active-site cysteine (Cys283) was identified as the site of thiolation [276]. CK S-thiolation, resulting in enzyme inactivation, can be reversed by reduced glutathione or DTT. It is not clear whether S-thiolation of CK would play a protective rather than a deleterious role *in vivo* [262]. Currently, protein Sglutathiolation is considered an adaptive response that protects critical regulatory molecules from permanent loss of function as a result of oxidative or nitrosative



Fig. 7.7 The catalytic site cysteine residue of CK is exquisitely sensitive to oxidation. Cysteine residues (space-fill representation, black balls) in chicken sarcomeric muscle sMtCK (left) [162] and human ubiquitous uMtCK (right) [161] monomers. Locations of the active site (*) of MtCK and the highly reactive cysteine (Cys278) within the active-site pocket of MtCK are indicated. The active-site cysteine in all CK isoenzymes is exquisitely sensitive to oxidative damage, and its oxidation leads to almost complete

inactivation of CK catalytic activity. The larger number of cysteines and their concentration around the active site in sMtCK (black balls, left) may contribute to the higher sensitivity of sMtCK to oxidative and nitrosative damage compared with uMtCK. Note that this may partly explain the specific cardiotoxic side effects of anthracyclines, a potent class of anticancer drugs, which inactivate sMtCK significantly more strongly than uMtCK (for details, see text).

insult [277]. In this case, the maintenance of appropriate intracellular levels of reduced glutathione, GSH, would be critical for regeneration of active CK from S-glutathionylated protein. Inactivation of purified MtCK by the anthracycline drug doxorubicin was accompanied by formation of cross-linked aggregates due to reversible intermolecular disulfide bridge formation between MtCK monomers [172]. The active-site cysteine does not seem to be essential for this process, because a C278G mutant CK protein shows doxorubicin-induced cross-link products quite similar to those of wild-type protein.

An interesting outcome of our studies with purified MtCK treated with PN or anthracyclines is the significantly higher sensitivity to oxidative damage found with sarcomeric sMtCK in comparison to ubiquitous uMtCK [172, 274], indicating that the deleterious effects of these compounds may be more pronounced in heart and skeletal muscles, both of which express sMtCK. This finding could contribute to the cardiac-specific side effects of anthracyclines. The different susceptibility of the two MtCK isoenzymes could be related to some differences in their molecular structures. Both isoenzymes share a number of conserved cysteine residues, including active-site Cys278 and Cys358, but sMtCK shows a distinct distribution, with more cysteines near the active site and one additional cysteine in total compared with uMtCK (Fig. 7.7).

7.4.3

Functional Consequences of Oxidative Damage in Creatine Kinase

Inevitably, ROS- and RNS-induced CK damage is at the origin of numerous deleterious processes that promote cellular dysfunction. Compromised MtCK functions, including inactivation, dimerization, and inhibition of its binding to cardiolipin, not only impair the energy channeling and signaling between mitochondria and cytosol [6–8] but also affect mitochondrial respiration. This molecular damage also contributes to destabilization of the mitochondrial contact sites consisting of ANT, MtCK, and VDAC [169, 185]. MtCK defects sensitize cells to mitochondrial permeability transition [182] and abolish MtCK function in prevention of radical generation, which was recently demonstrated [192]. In the cytosol, where MM-CK is functionally coupled to the Ca^{2+} pump of the sarcoplasmic reticulum, inhibition of this isoenzyme results in Ca^{2+} imbalance, which in turn interferes with muscle contraction and relaxation [4, 80] and could lead to apoptotic and/or necrotic cell death via chronic Ca^{2+} overload.

7.5

Enhancement of Brain Functions and Neuroprotection by Creatine Supplementation

7.5.1

Creatine Metabolism and Brain Energetics

The importance of the CK system for normal function *in vivo* of skeletal muscle [6, 80, 136] as well as heart muscle [79, 85, 278–280] has been established most convincingly by transgenic approaches. In addition, the ergogenic effects of oral

Cr supplementation on muscle performance and muscle power in humans, especially for repetitive, high-intensity tasks, are obvious [281–283], and the first positive effects of Cr supplementation on the skeletomuscular health status of elderly patients [284] as well as for those with muscle, neuromuscular, and heart diseases have been reported [190, 285–293].

As a recent development in the field, however, a plethora of new data provide evidence of the importance of the CK system and of Cr metabolism for normal function of the brain. Further, oral Cr supplementation shows remarkable positive effects on brain function and neuroprotective properties for acute and chronic neurological diseases (for review, see [262, 294, 295]).

As for skeletal and cardiac muscle, the specific functional properties of neuronal tissue require very high cellular energy resources. The brain may spend up to 20% of the body's energy resources. A very high turnover of ATP is necessary to maintain electrical membrane potentials, as well as signaling activities of the central nervous system (CNS) and peripheral neuronal system. Rapid changes in ATP demands occur during physiological function of neurons, while cellular energy reserves are small. Due to widely distributed cellular processes and sites of high-energy consumption that are localized at remote locations from the neuronal cell body, i.e., synapses, an effective coupling of ATP-generating and ATPconsuming processes is needed to maintain a sufficiently high energy transfer [296].

The CK–PCr circuit has been described as playing a key role in the energy metabolism of the brain and neurons [36, 46, 295, 297, 298]. Hence, this system is thought to play a pivotal role in neuronal ATP metabolism [298, 299]; consequently, Cr depletion in brain is associated with disruption of neuronal function, changes in morphology, and clinical pathology [300].

7.5.2

Disturbance of the CK System or Creatine Metabolism in the Brain

Interestingly, ablation of the cytosolic brain-type BB-CK or the ubiquitous mitochondrial uMtCK genes, individually or combined, leads to a significant neurological and brain-related phenotype in such transgenic mice. For example, mice with a gene knockout of cytosolic BB-CK show diminished open-field habituation, learn slower in the water maze, present with a delayed development of pentylenetetrazole-induced epileptic seizures, and anatomically show a loss in hippocampal mossy fiber connections [301]. Double-knockout transgenic mice, lacking both BB-CK and uMtCK, present with undetectable PCr and Cr levels in the brain [302] and display a strong phenotype that includes reduced body weight, severely impaired spatial learning, lower nest-building activity, and diminished acoustic startle reflex, as well as having lower brain weight and reduced hippocampal size [303].

On the other hand, if the CK substrate creatine (Cr) is depleted in the brain by feeding animals with the Cr analogue beta-guanidino propionic acid (β GPA) – a competitive inhibitor of the creatine transporter (CRT) – strong muscle and neurological phenotypes are also observed (for review, see [300]). Most importantly,

in humans a new creatine-deficiency syndrome that affects either endogenous Cr synthesis or transport has been discovered recently (for review, see [304]). Patients with this syndrome present with an almost complete lack of Cr in the brain and show a severe neurological phenotype (for details, see below). Thus, either ablating the CK isoenzymes or drastically reducing the substrate Cr in the brain leads to similar and rather severe phenotypes. This represents strong evidence in favor of the physiological significance of the CK–PCr system for normal brain function. Because Cr obviously is crucial for normal brain function, better knowledge about Cr metabolism in the human body and specifically in the brain is needed (for review, see [262]).

7.5.3

Body and Brain Creatine Metabolism

In omnivores, a certain fraction of Cr (1.5-2.0 g of Cr per day, representing approximately 50% of daily Cr requirement in humans) is endogenously synthesized by a two-step synthesis involving the enzymes arginine:glycine amidinotransferase (AGAT) (Fig. 7.8, 1a) and S-adenosyl-1-methionine: N-guanidinoacetate methyltransferase (GAMT) (Fig. 7.8, 2a). Whereas AGAT, which produces guanidino acetate (GAA) as an intermediate, is expressed preferentially in kidney and pancreas (Fig. 7.8, 1a), GAMT is found to some extent in the pancreas but mostly in the liver, the main organ of the final step of endogenous Cr synthesis (Fig. 7.8, 2a). Cr, which leaves the liver by some unknown mechanism, is then transported through the bloodstream and is actively taken up, via a specific creatine transporter (CRT) (Fig. 7.8, 3), by cells with high energy requirements, such as muscle (Fig. 7.8, 3) or brain cells, where Cr is charged up by CK to high-energy PCr (Fig. 7.8, 4a,b) (for review, see Ref. [262]). To get into the brain, Cr has to first pass the blood-brain barrier, where a CRT is localized in endothelial cells of microcapillaries (Fig. 7.8, 5) but not in astrocytes lining these microcapillaries [305, 306]. From there, Cr is taken up by those brain cells that express the CRT, mainly represented by neurons and oligodendrocytes but not astrocytes, which seem to lack the CRT [305-307]. A similar situation has been found at the blood-retina barrier, where CRT is expressed at the luminal and abluminal membranes of retinal capillary endothelial cells, explaining the transcellular transport of Cr through the endothelium [308]. On the other hand, alimentary Cr, which is present in fresh fish and meat, is taken up by an intestinal CRT (Fig. 7.8, 8) [309] and transported into the bloodstream, where it mixes with endogenously synthesized Cr.

In embryonic brain (and to a limited extent also in adult brain), AGAT and GAMT can be detected in all of the brain cells mentioned above (Fig. 7.8, **1b,2b**), such that there seems to be a limited potential for endogenous Cr synthesis in the adult brain as well [305]. As a consequence, due to the presence of CRT in neurons and oligodendrocytes but not in astrocytes, trafficking of Cr between astrocytes and neurons or oligodendrocytes has been suggested (i.e., Cr synthesized by astrocytes could be released and taken up by neurons; Fig. 7.8, **6**) [305, 310].



Fig. 7.8 Simplified scheme of whole-body creatine metabolism with emphasis on the brain. A certain fraction of Cr is endogenously synthesized by a two-step synthesis involving AGAT (1a,b) and GAMT (2a,b). AGAT, which produces guanidino acetate (GAA) as an intermediate, is expressed preferentially in the kidney and pancreas (1a). GAMT is found to some extent in the pancreas but mostly in liver (2a), the main organ of the final step of endogenous Cr synthesis [262]. Cr leaving the liver is transported through the bloodstream and is actively taken up via a specific creatine transporter (CRT) by cells with high energy requirements, such as muscle (3) or brain cells (4), where Cr is charged up by CK to

high-energy PCr (4a,b). To get into the brain, Cr needs to pass the blood-brain barrier by the CRT, which is localized in the endothelial cells of microcapillaries (5). From there, Cr is taken up by those brain cells that express the CRT (4), represented mainly by neurons and oligodendrocytes but not by astrocytes, which seem to lack the CRT [305-307]. On the other hand, alimentary Cr, present in fresh fish and meat, is taken up by an intestinal CRT (8) [309] and transported into the bloodstream, where it mixes with endogenously synthesized Cr. Trafficking of Cr between astrocytes and neurons or oligodendrocytes (6) and exocytotic release of Cr from neurons (7) [312] are also shown.

A similar situation has been found in retina, where CRT is not expressed in Mueller cells but in retinal photoreceptor cell inner segments, suggesting that, as in brain, neurons are independent of these glial cells in accumulating Cr because they can take it up via CRT [311]. A new finding shows that upon electrical stimulation of superfused brain slices, endogenous Cr is specifically released (Fig. 7.8, 7). This exocytotic release can be prevented by omitting Ca²⁺ from the medium

or by adding the Na⁺ channel blocker tetrodotoxin (TTX) [312]. These *in situ* data indicate that Cr not only is synthesized and taken up by central neurons but also is released in an action potential–dependent manner, providing strong evidence for its role as a neuromodulator in the brain (Fig. 7.8, 7). Several important questions concerning the details of Cr metabolism, such as the exact mechanisms and regulation of transcellular Cr transport, interorgan Cr exchange, release of synthesized Cr from the liver, uptake of Cr into the brain, and intracellular trafficking and exocytotic release of Cr inside the brain, remain to be answered.

7.5.4

Effects of Creatine on Memory, Mental Performance, and Complex Tasks

High expression of CK isoenzymes has been found in hippocampal pyramidal cells, which are involved in learning and memory [35]. This finding indicates that the CK-PCr system plays an important role in the energy metabolism of these cells and that Cr supplementation may lead to improved functions of these systems. Indeed, positive effects of orally administered Cr on mental performance have been reported in healthy volunteers in a controlled double-blind study [313]. Using infrared spectroscopy, the authors found correspondingly increased blood oxygenation in the Cr-treated group. Moreover, a double-blind study investigating Cr supplementation for six weeks on healthy vegetarians, who have a reduced nutritional Cr supply, revealed significantly better results in intelligence test and working memory performance in the Cr-treated subjects compared with control individuals [314]. In a recent test, Cr supplementation $(4 \times 5 \text{ g Cr per day for 7})$ days), prior to 18-36 hours of sleep deprivation, was shown to significantly improve the performance of complex central executive tasks [315, 316]. Thus, it can be concluded that Cr supplementation enhances brain function under normal and stress conditions. This may be relevant for promoting Cr supplementation as a brain performance-enhancing nutritional supplement for humans.

7.5.5

Creatine Supplementation and Neurodegenerative Diseases

Neurodegenerative disorders are characterized by a progressive loss of cells from one or multiple regions of the nervous system. Despite intensive research, the etiology of neuronal cell death in most neurodegenerative diseases still remains enigmatic. However, there are a number of similarities in the fundamental biochemical processes involved in the pathogenesis and progression of these otherwise different pathological states. The concepts of energy depletion, oxidative stress by ROS and RNS, excitotoxicity, and mitochondrial dysfunction have been implicated in neurodegenerative disorders [317, 318]. Although these processes may be directly or indirectly involved in the pathogenesis of a given disease, they converge in final common pathways of either necrosis or apoptosis. Substantial evidence indicates that energy dysfunction plays either a primary or secondary role in cell death in neurodegenerative disorders, and even in normal aging. Mitochondria are critical organelles in the regulation of cellular energy status. Mitochondrial dysfunction results in ATP depletion, which may contribute to neuronal cell death. Moreover, these organelles are also involved in excitotoxicity, generation of free radicals, calcium buffering, and apoptotic pathways. Mitochondrial mutations, particularly at complexes I and III, can lead to generation of ROS, and accumulation of mitochondrial DNA mutations in aging and Alzheimer's disease has been shown to be linked to oxidative stress [319, 320]. These processes provide potential targets for the therapy of neurodegenerative diseases.

Parkinson's Disease Parkinson's disease (PD) is a common neurodegenerative disorder affecting more than 1% of all individuals over the age of 50 years [321]. Clinical symptoms include resting tremor, bradykinesia, rigidity, and postural imbalance [322, 323]. PD is characterized by a progressive deterioration of dopaminergic neurons in the substantia nigra, leading to a profound loss of dopaminergic input into the striatum. An impaired function of the mitochondrial electron transport system, in particular complex I, is thought to be involved in the pathogenesis of PD [324-326], suggesting a mitochondrial basis for the disease. Recent research has focused on neuroprotective strategies for PD [327]. Using an experimental in vitro paradigm of PD, we observed neuroprotective properties of Cr against toxic insults induced by 6-hydroxydopamine (6-OHDA) [328] or 1-methyl-4-phenyl pyridinium (MPP+) [329] exposure in ventral mesencephalic rat cultures. Beneficial effects of Cr have also been demonstrated in an animal model of PD [330]. In clinical pilot studies, Cr supplementation resulted in improved patient mood and, in smaller doses, increased the effects of dopaminergic therapy [331] and reduced the progression of the disease [332]. These results make Cr a qualifier for clinical phase III trials with PD patients.

Huntington's Disease and Other Trinucleotide Repeat Disorders Huntington's disease (HD) is an autosomal, dominantly inherited, neurodegenerative disorder that clinically presents with progressive choreoathetotic movements in combination with severe cognitive and emotional dysfunction, finally leading to death [333]. The main pathologic finding is a loss of striatal GABA-ergic projection neurons. A defect in energy metabolism has been proposed as one of the potential pathogenetic mechanisms leading to neuronal death [334]. Studies on cerebral metabolism using ¹⁸F-fluorodeoxyglucose positron emission tomography (PET) showed typical patterns of diminished cerebral metabolic rates in the basal ganglia as well as in the frontal and parietal regions of HD patients, correlating with the severity of the disease [335]. Recently, evidence of impaired energy metabolism in HD due to reduced mitochondrial complex II and complex III activity has been reported [336, 337], resulting in increased cerebral lactate levels and a reduced PCr:Pi ratio in muscle. Corresponding mitochondrial defects have been described in the brains of patients suffering from HD, particularly in the basal ganglia [338]. Further evidence of mitochondrial respiratory chain dysfunction has been provided by studies of transgenic mouse models of HD [339]. Increas-

ing cellular PCr levels and thereby improving the impaired energy metabolism by exogenous Cr supplementation may therefore offer a feasible approach for reducing neuronal deterioration in the context of this severe disorder. Using an experimental in vitro model of HD, we detected that Cr supplementation provided significant neuroprotection against glucose and serum deprivation and against 3nitropropionic acid (3-NP)-induced toxicity in striatal cultures [340]. Cr directly injected intraperitoneally at 12 mg kg⁻¹ body weight was shown to protect experimental animals against convulsive behavior and lactate production elicited by intrastriatal injection of methylmalonate [341]. In addition, Cr administration increased survival, delayed motor symptoms, and significantly reduced brain lesion size in a transgenic animal model of HD [342, 343] and in 3-NP-exposed rats [344]. In recent clinical trials, it was reported that Cr is well tolerated and safe in HD patients [345]. Brain glutamate levels were significantly reduced after a Cr-enhanced diet [346], and serum 8-hydroxy-2'-deoxyguanosine (8OH2'dG) levels, an indicator of oxidative injury to DNA that is markedly elevated in HD, were reduced by Cr treatment [347], indicating some efficacy of Cr treatment for this devastating neurodegenerative disease. Finally, a Cr-supplemented diet significantly extends Purkinje cell survival in a spinocerebellar ataxia type 1 transgenic mouse model but does not prevent the ataxic phenotype [348].

Alzheimer's Disease Alzheimer's disease (AD) is a neurodegenerative disease of the brain leading to progressive dementia. Mutations in the amyloid precursor protein (APP) result in abnormal processing of APP and accumulation of betaamyloid peptide, the main constituent of amyloid plaques in the AD brain [349]. Brain-specific creatine kinase (BB-CK) is significantly inactivated by oxidation in AD patients, which may result in further compromising of the energetic state of neurons and exacerbate the disease process [350]. In addition, recently discovered Cr deposits in the brain of transgenic AD mice, as well as in the hippocampus of AD patients, indicate a direct link between cellular energy levels, mitochondrial function, Cr metabolism, and AD [351]. Neuroprotective effects of Cr have been observed in models of AD with cultured neurons undergoing neurotoxic insults by glutamate excitotoxicity or by exposure to beta-amyloid protein [36]. Hence, it is worth speculating whether Cr supplementation at an early time point of the disease may prevent or delay the course of AD-related neurodegeneration [297]. In fact, a direct connection between AD and MtCK was discovered by showing that MtCK forms a complex with APP family proteins that affects the correct import of MtCK into mitochondria and thus negatively interferes with cellular energetics [352].

Amyotrophic Lateral Sclerosis (ALS) Amyotrophic lateral sclerosis (ALS) is characterized by progressive loss of motor neurons in the brain and spinal cord [353]. Mitochondrial and energetic defects are implicated in the pathogenesis of motor neuron degeneration in ALS [354]. A marked reduction in cerebral cortex ATP levels was detected in a mouse model of ALS well before symptom onset [355]. Accordingly, reduced CK activity has been reported in transgenic ALS mice [269]. Consequently, it was found that Cr supplementation showed protec-

tive properties in mouse models of ALS [356–358]. This neuroprotection could be based on antioxidant effects exerted by Cr, given that Cr has the potential to act as a direct antioxidant against aqueous radical and ROS or RNS species [359], or it could be due to the action of MtCK and Cr in coupling mitochondrial respiration tightly to ATP synthesis, by efficient ADP cycling, and thus suppressing ROS formation in mitochondria [192].

Cr reverted the cholinergic deficit present in some forebrain areas at an intermediate stage of the disease [360]. In a follow-up study, additive neuroprotective effects of oral Cr supplementation together with a cyclooxygenase-2 inhibitor were demonstrated with the same ALS mice [361]. We were able to demonstrate CK immunoreactivity in choline acetyltransferase–expressing neurons in the developing [362] and adult human spinal cord (Andres et al., unpublished), supporting the hypothesis that Cr treatment might be beneficial in ALS or other motor neuron diseases. Despite the promising findings in experimental animal models, first clinical studies have failed to show a relevant benefit of Cr treatment in ALS patients [363, 364]. However, these trials have also posed unanswered questions about the optimal dosage of Cr. It must also be considered that Cr offers potential benefits in terms of facilitating residual muscle contractility in ALS patients, which is a factor that has not been investigated yet [365]. A large placebo-controlled, multi-center trial is currently underway to further investigate the efficacy of Cr supplementation in ALS.

Charcot–Marie–Tooth Disease Charcot–Marie–Tooth disease (CMT) is a common hereditary disorder characterized by slowly progressive sensomotor neuropathy that can lead to lifelong disability in patients. CMT represents a heterogeneous group of genetically distinct disorders with similar clinical presentations and a large number of responsible gene mutations [366]. In a recent study, it was shown that Cr supplementation alters muscle myosin heavy chain (MHC) composition in CMT patients undergoing resistance training and that MHC changes associated with Cr supplementation can improve muscle function [367].

7.5.6

Creatine Supplementation and Acute Neurological Disorders

Cerebral Ischemia Cerebral ischemia rapidly leads to neuronal cell death due to compromised energy metabolism [368], often resulting in disabling neurological sequelae. Neuroprotective effects of Cr supplementation have been reported in an experimental model of ischemia [369, 370]. In a recent study, it was found that Cr treatment of mice suffering from transient focal cerebral ischemia resulted in a reduction in stroke volume in the absence of significant changes in brain Cr, PCr, and ATP levels [371]. The authors presented some evidence that a positive effect of Cr on vasodilatory response in the brain might be responsible for the observed effects.

Traumatic Brain and Spinal Cord Injury Traumatic brain and spinal cord injury is known to initiate a series of cellular and molecular events in the injured tissue, leading to further damage in the surrounding area. This secondary damage

is partly due to ischemia and compromised cellular bioenergetics. Cr-mediated neuroprotection has been demonstrated in experimental brain injury [372, 373]. In a prospective randomized study investigating the effects of Cr in children and adolescents suffering from traumatic brain injury, administration of Cr for six months resulted in significantly better clinical recovery in the categories of cognitive, personality/behavior, self-care, and communication aspects [374]. Cr supplementation also has been shown to have protective effects after spinal cord injury [375, 376]. However, exogenous Cr is taken up slowly into CNS tissue; therefore, it seems unlikely that patients suffering from acute brain or spinal cord injury would immediately benefit from Cr supplementation, unless Cr could be brought directly to the sites of injury, e.g., by perifusion of the affected region or intracerebroventricular administration, which leads to a fast and marked increase of Cr levels in the brain [377].

Injury of the Peripheral Nerve Injury of the peripheral nerve causes denervation of the associated muscle fibers and may be treated by microsurgical nerve repair. Using an experimental paradigm of sciatic nerve transection in the rat, it has been shown that systemic Cr supplementation promotes reinnervation and functional recovery on both surgically repaired and unrepaired nerve injuries [378].

7.5.7

Inborn Errors of Metabolism

Mitochondrial Encephalomyopathies Mitochondrial encephalomyopathies are a multi-systemic group of disorders that are characterized by a wide range of biochemical and genetic mitochondrial defects and variable modes of inheritance. Among this group of disorders, the "mitochondrial myopathy, encephalopathy, lactic acidosis with stroke-like episodes (MELAS) syndrome" is one of the most frequently occurring, maternally inherited mitochondrial disorders. Clinical trials with Cr supplementation on MELAS patients reported an increase in muscle performance [379, 380]. Interestingly, Cr supplementation led to a reversal of the paracrystalline intramitochondrial inclusions, which were shown to consist mainly of crystallized MtCK [133], as seen in a muscle biopsy from one such patient [190].

Another mitochondrial encephalomyopathy is Leigh syndrome (LS), which results in characteristic focal necrotizing lesions in one or more areas of the central nervous system, including the brainstem, thalamus, basal ganglia, cerebellum, and spinal cord. Clinical symptoms depend on which areas of the central nervous system are involved. The most common underlying cause is a defect in oxidative phosphorylation [381]. In a recent study using oral Cr supplementation, improvement in behavioral and physiological functions in a child with LS [382] was seen. Another study investigating the possible benefit of Cr treatment on an inhomogeneous population of patients with different forms of encephalomyopathies revealed protective effects in all patients, suggesting that targeting the final common pathway of mitochondrial dysfunction favorably influences the course of these diseases [383].

Creatine-deficiency Syndrome with Defects in Creatine Synthesis and Transport

Brain Cr deficiency is involved in the pathogenesis of some severe inheritable neurological phenotypes [384-386]. These so-called Cr-deficiency syndromes form a group of inborn errors in either one of the two enzymes involved in endogenous Cr synthesis, i.e., AGAT or GAMT [387], or, alternatively, in the Cr transporter (CRT), leading to CRT deficiency [388] (Fig. 7.8). Patients with Crdeficiency syndrome clinically present with graded forms of developmental and speech delay, epileptic seizures, autism, and brain atrophy, leading to varying degrees of mental retardation and suggesting major involvement of cerebral gray matter. Due to the neurotoxic effects of guanidino acetic acetate (GAA) [389], which accumulates in the gray matter, patients with GAMT deficiency additionally show a dystonic-hyperkinetic movement disorder [390]. Cr-deficient patients are characterized by cerebral Cr deficiency, which especially in CRT-deficient patients is completely lacking in the brain, as can be demonstrated by noninvasive ¹H-NMR spectroscopy [391]. Measurement of the neurotoxic intermediate metabolite GAA in body fluids can be used to discriminate the extent to which GAMT (high concentration), AGAT (low concentration), and CRT (normal concentration) are affected. CRT-deficient patients present with a significantly elevated Cr:creatinine (Cr:Crn) ratio in the urine. GAMT and AGAT deficiency can be treated by oral Cr supplementation [304], while patients with CRT deficiency do not respond to this type of treatment [392, 393]. Once more, these observations emphasize the importance of the CK-PCr system for the brain and the absolute requirement of Cr for normal brain function.

7.5.8

Psychiatric Disorders

Posttraumatic stress disorder (PTSD) is an anxiety disorder that can develop in persons who have experienced highly traumatic situations. PTSD is linked to structural and neurochemical changes particularly in the limbic system, postulated as a substrate for stress-induced alterations in affective behavior [394]. Decreased Cr levels have been measured in the brains of patients suffering from anxiety disorders [395]. Consequently, Cr supplementation has beneficial effects in treatment-resistant PTSD patients, resulting in relief of symptoms as well as improved sleep and depression parameters [396]. Furthermore, in a patient suffering from PTSD, comorbid depression, and fibromyalgia, Cr treatment led to improvement of symptoms [397].

7.5.9 Neurorestorative Strategies

In order to repair the brain by replacing the neurons lost in acute or chronic pathological processes, transplantation of neuronal precursors or stem cells has attracted great attention. In cell replacement therapy for PD, it has been observed that some transplanted cells show long-term survival and structural and functional integration in the host brain [398, 399]. The grafted tissue is assumed to

release dopamine in a regulated fashion and to reverse many of the behavioral deficits seen in animal models of PD [400, 401] as well as in humans [402]. In HD, there is large body of experimental data showing the effectiveness of striatal transplants in experimental models [403, 404], while preliminary studies report that transient recovery can be promoted in patients as well [405, 406]. In stroke, it has been shown that transplanted neuronal stem cells survive, migrate, and differentiate into appropriate neuronal phenotypes [407, 408]. Transplanted motor neurons can survive in spinal cord and reinnervate the denervated target muscle [409]. However, at present, there are some specific obstacles that prevent widespread clinical application of cell replacement techniques. The main problems of neuronal transplantation include the limited availability of donor tissue and the poor survival of transplanted cells, as well as the suboptimal innervation of the targeted structures in host brain. In PD, less than 20% of the implanted cells survive the transplantation procedure [410, 411]. Studies in rats revealed that most implanted embryonic neurons died within one week after transplantation [412], mostly by apoptotic cell death [413]. Strategies for improving cell replacement therapies, therefore, include treatment of the cells with neuroprotective factors.

In this context, we were able to show significant neuroprotective effects of Cr on both dopaminergic mesencephalic and GABA-ergic striatal neuronal precursors [328, 329, 340]. Furthermore, Cr was identified as a potent differentiation factor for striatal precursor neurons, inducing differentiation towards the GABAergic phenotype. Interestingly, inhibition of mitogen-activated protein kinase and phosphatidylinositol-3-kinase significantly attenuated the effect on induction of the GABA-ergic phenotype [340]. These data suggest that Cr may play an important role in cell fate decision during development of neuronal cells, a finding that is also supported by a report describing the expression of CK in the developing zebra fish embryo. Expression of CK was shown to be highly dynamic, often being transiently expressed in specific cells for a short time period only, indicating a well-timed, cell type-specific function of CK during brain development [414]. Moreover, in a study describing pattern expression and localization of CK isoforms in fetal rat brain, CK was shown to be present throughout the central and peripheral nervous systems [35, 415, 416]. Research on neuronal precursors and stem cells continues with great hope for the treatment of acute and chronic neurological disorders. In this context, Cr holds a future potential to influence the survival and differentiation of these cells and is hoped to contribute to a better outcome to obtain functional transplants.

7.5.10

Future Prospects of Creatine Supplementation as Adjuvant Therapeutic Strategy

Cr supplementation has been shown to increase intracellular levels of PCr and thereby to stabilize ATP levels, not only in human muscle [281] but also in human brain [417]. Thus, Cr obviously can pass the blood–brain barrier via uptake by endothelial CRT in the brain capillaries [307]. As a result, the potential benefits of Cr supplementation also are likely to be realized in human patients with

neurological disorders in which cellular energy metabolism is impaired [294]. Indeed, Cr supplementation has been shown to attenuate neuronal cell loss in various experimental models of neurodegenerative diseases. So far, significant protective effects have been shown in animal models of PD [330], HD [342– 344], ALS [356–358], stroke [369, 370], cerebral hypoxia [418], and traumatic injury to the brain [372, 373] and spinal cord [375, 376].

Promising results in first clinical trials with human patients by application of oral Cr supplementation have been reported, particularly in chronic pathological conditions of the CNS such as PD [331, 332], HD [345–347], CMT [367], and inborn errors of metabolism [382, 383, 392]. In a recent study, remarkably beneficial effects were discovered on functional recovery after traumatic brain injury [374]. In addition, positive effects of Cr on mental performance and memory have been reported in healthy volunteers [314]. In conclusion, these results confirm the neuroprotective properties of Cr, particularly on damage due to hypoxia [418] or free ROS or RNS [357]. At present, it seems that there are various mechanisms by which neurons respond to an increase in their Cr levels [36, 419].

So far, however, the general experience with clinical Cr supplementation studies involving human patients with neurological diseases is that, at least for chronic diseases, the beneficial effects seen with Cr are less pronounced or partially disappointing compared with the neuroprotective effects observed in the corresponding animal models. On the other hand, these findings may be a result of Cr dosage, schedule, and time of supplementation, because in these animal studies, the Cr doses were approximately 10 times higher than those used in the clinical trials. In addition, the observation that chronic Cr supplementation failed to protect adult mice from stroke hints that adaptive mechanisms may counteract the beneficial effects of Cr supplementation; thus, further considerations are required if Cr should be used as a long-term nutritional supplement for patients [371]. Nevertheless, Cr remains a promising neuroprotective agent for further studies involving chronic neurological diseases, but its potential for such diseases may only be revealed by large multi-center studies with higher Cr doses over an adequate observation time [331]. This conclusion is supported by the recent futility clinical trial of Cr in early PD patients, where a clear positive effect of Cr on patient mood was discovered [332] that should be assessed in future clinical trials of patients with depression.

7.5.11

Non-energy-related Effects of Creatine

Besides its role in cellular energy metabolism [262] by enhancing cellular energy status [420], Cr is believed to have additional functions in the CNS. For example, there is evidence of the direct antiapoptotic effect of elevated cellular Cr levels. In conjunction with the action of MtCK inside mitochondria, Cr prevents or delays mitochondrial permeability transition pore opening [182, 184], an early event in apoptosis. Moreover, Cr supplementation was demonstrated to have antioxidant properties via a mechanism involving the direct scavenging of reactive oxygen

species [421] or, alternatively, reducing the production of mitochondrially generated reactive ROS. The latter is facilitated by the stimulatory effects of Cr on mitochondrial respiration [51], which allows for efficient recycling of ADP inside mitochondria by MtCK, leading to tight coupling of mitochondrial respiration with ATP synthesis and suppression of ROS formation because futile idling of the respiratory chain is avoided [192]. Important with respect to this finding, strong protective effects of Cr against oxidant and UV stress have been directly shown in keratinocytes and on human skin [422]. Furthermore, Cr was reported to normalize mutagenesis of mitochondrial DNA and its functional consequences caused by UV irradiation of skin cells [423], all pointing to effects of Cr on suppression of the generation of ROS and RNS and their congeners that lead to cell damage and inactivation of CK (see Section 7.4).

Further evidence that Cr-mediated neuroprotection can occur independently of changes in the bioenergetic status of brain tissue was reported in another recent study, suggesting an effect of Cr on cerebral vasculature that leads to improved circulation in the brain [371]. A very recent report showed that Cr could be released in an action potential-dependent, excitotic manner (Fig. 7.8) [7], suggesting a novel role for Cr as a neuromodulator [312]. This finding is supported by a study showing attenuation of the acute stress response in chickens by administration of Cr, which has been found to act through GABA-A receptors [424]. Finally, a recent study demonstrated that Cr is able to protect cultured cells from hyperosmotic shock by means of a significant increase in Cr uptake into cells, indicating that Cr can act as a compensatory osmolyte [425]. In contrast, Cr and taurine are released from cortical astrocytes and cortical brain slices after exposure to hypoosmotic perfusion, suggesting a link between brain energy reserve and brain osmoregulation, with Cr as one of the main brain cell osmolytes [426, 427]. An osmotic effect of Cr accompanied by a decrease in myoplasmic ionic strength was postulated to explain some of the beneficial effects of Cr on muscle that are seen before intramuscular PCr levels rise [428]. Thus, the subject of the actions of Cr on biological systems is not yet closed, and future discoveries about this endogenous body substance that has been known for more than 150 years now are likely to follow.

7.5.12

Creatine as a Safe Nutritional Supplement and Functional Food

Because Cr is endogenously synthesized and is a constituent of the regular diet, a potential benefit of Cr administration is assumed to be achieved without any major side effects, even at high dosage. Indeed, preliminary findings on athletes using high-dosage Cr supplementation for improving muscle strength revealed no kidney, liver, or other health problems [429, 430]. For oral dietary supplementation, a loading phase of 5–7 days with four doses of 5 g of Cr per day followed by a maintenance dose of 2–5 g per day for 3–6 months or even longer (up to two years) has shown an excellent safety profile [282, 431]. Even in premature newborns, Cr administration is well tolerated at high dosage [432], which is important for the treatment of inborn metabolic diseases. So far, with the limited research available, the data suggest that chronic Cr administration may be safe. Future studies will be required, however, to further address the potential of Cr supplementation for the treatment of neurological diseases and for improving neurorestorative strategies.

Recent data support the view that humans, born as omnivores, need a certain amount of nutritional Cr, either from fresh fish and meat or from creatine supplementation [294]. Total Cr levels (PCr plus Cr) are significantly lower in serum as well as in muscles of vegetarians [433], and thus Cr is to be highly recommended to vegetarian athletes [434]. Because Cr supplementation has been shown to improve muscle strength and rehabilitation after immobilization and muscle disuse atrophy [435], as well as to enhance mineralization of bone [436] and bone density [284], it should be considered a most valuable supplement within the context of a preventive strategy for seniors to retain muscle strength and bone health, thus extending the time to lead an independent life before entering disability [294]. Because Cr has been shown to improve mental performance [313], memory, learning, and intelligence [314], the beneficial effects of such prevention go beyond skeletomuscular health and positively affect the brain as well.

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Abbreviations

AD: Alzheimer's disease; AdK: adenylate kinase; AGAT: L-arginine:glycine amidinotransferase; ALS: amyotrophic lateral sclerosis; AMPK: AMP-activated protein kinase; ANT: adenine nucleotide transporter or mitochondrial ATP/ADP exchange carrier or antiporter; BB-CK: cytosolic brain-type creatine kinase; CMT: Charcot-Marie-Tooth disease; CK: creatine kinase; CL: cardiolipin; CNS: central nervous system; Cr: creatine; Crn: creatinine; CRT: creatine transporter; DPPE: dipalmitoyl PE; DSC: differential scanning calorimetry; GAMT: S-adenosylmethionine-guanidinoacetate N-methyltransferase; GAA: guanidino acetate; GABA: y-amino butyric acid; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HD: Huntington's disease; K⁺_{ATP}: sarcolemmal ATP-gated K⁺-channel; MCL: monolysocardiolipin; IM: inner mitochondrial membrane; MM-CK: cytosolic muscle-type creatine kinase; MS: multiple sclerosis; NDPK-D: mitochondrial nucleoside diphosphate kinase; OM: outer mitochondrial membrane; PFK-1: phosphofructokinase-1; PD: Parkinson's disease; PE: phosphatidylethanolamine; PEP: phosphoenolpyruvate; PCr: phosphocreatine; PN: peroxynitrite; POPE: 1-palmitoyl-2-oleyl PE; RNS: reactive nitrogen species; ROS: reactive oxygen species; sMtCK: sarcomeric mitochondrial creatine kinase; SR: sarcoplasmic reticulum; uMtCK: ubiquitous mitochondrial creatine kinase; VDAC: voltagedependent anion channel or mitochondrial porin.

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