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The Subcellular Compartmentation of Creatine Kinase Isozymes as a Precondition for a Proposed *Phosphoryl-Creatine Circuit*

Theo Wallimann and Hans M. Eppenberger

Institute for Cell Biology, Swiss Federal Institute of Technology, CH- 8093 Zurich, Switzerland

I. INTRODUCTION

In higher eukaryotes three types of tissue-specific dimeric creatine kinase (CK) isozymes can be found: ubiquitous BB-CK, expressed at high levels in brain and smooth muscle, muscle-specific MM-CK present in striated muscle and, during muscle differentiation, a transitorily expressed heterodimer, MB-CK, which can persist in adult heart muscle [see Eppenberger et al, 1983]. A fourth CK isozyme, mitochondrial or Mi-CK, is specifically associated with the inner mitochondrial membrane [Jacobs et al, 1964; Scholte et al, 1973] and seems to be functionally coupled to the ATP/ADP- translocator (AN) [see Jacobus, 1985]. In differentiated tissues and cells which express CK, e.g., brain, muscle, photoreceptor cells, and spermatozoa, Mi-CK is usually co-expressed with either one of the cytosolic CK isozymes. Whereas Mi-CK is strictly

compartmented in mitochondria, BB-, MB-, and MM-CK have been considered in the past as "soluble" enzymes distributed over the entire cytoplasm. However, immunolocalization studies with isolated organelles have provided ample evidence that significant amounts of cytosolic CK isoforms are also associated with specific subcellular sites. For example, in muscle a relatively small, but physiologically significant amount (at least 3-7%) of the total MM-CK is specifically associated with the myofibrillar M-band [Turner et al, 1973; Wallimann et al, 1977a] where it forms a particular structural element, the so-called "m-bridges" which represent M-lines M4 and M4' [Strehler et al, 1983]. The M-line-bound CK was shown to be sufficient for intramyofibrillar regeneration of ATP hydrolyzed by the actin-activated Mg^{2+}-ATPase during *in vitro* contraction of myofibrils [Wallimann et al, 1984]. The binding of CK to the M-band region is isozyme-specific; that is, only the homodimers of M-CK, but neither BB- nor MB-CK, are capable of integrating themselves as enzymatically active structural components into the complex M-band architecture [Wallimann et al, 1983]. In chicken cardiac cells, as in other avian species, M-CK is not expressed and consequently myofibrils are devoid of an electron-dense M-band [Wallimann et al, 1977b]. However, a skeletal muscle-like M-band could be generated by microinjection of *in vitro* synthesized M-CK mRNA [Schäfer and Perriard, 1988], confirming that this isoprotein sorting is M-CK- specific. In addition, using a chimeric M-CK/B-CK mRNA, it was shown that the C-terminal half of M-CK was involved in the interaction of the enzyme with the m-band [Schäfer and Perriard, 1988].

CK likewise is found in association with sarcolemmal membranes (SL) where it seems to be functionally coupled to the Na^+/K^+-ATPase [Sharov et al, 1977]. It has also been shown that the CK associated with the sarcoplasmic reticulum (SR) [Levitzky et al, 1977] is bound specifically and tightly to purified SR vesicles. This MM-CK is indeed capable of supporting a significant fraction of CA^{2+}-pumping via P-Creatine (PCr) and ADP [Rossi et al, 1988; submitted]. These results further support the notion that a fraction of the "cytosolic" CK-isozyme is also compartmented, thus clearly favoring earlier models on CK function [Wallimann et al, 1975; 1977; 1985; Saks et al, 1978; Bessman and Geiger, 1981] which suggests that communication and transfer of "energy-rich" compounds between ATP- generating (mitochondria and glycolysis) and ATP-utilizing sites (myofibrillar actin-activated MG^{2+}-ATPase,

Na^+/K^+-ATPase and Ca^{2+}- ATPase, etc.) are facilitated via the PCr/CK system [Meyer et al, 1984; Wallimann and Eppenberger, 1985]. There are several problems concerning the subcellular localization and compartmentation of CK- isozymes that we will address in this review, but a major objective will be to propose – as a consequence of our data – the so-called "*phosphoryl-creatine circuit*" model.

II. COMPARTMENTATION OF CK ISOZYMES IN MUSCLE

A. M-Type Creatine Kinase

Indirect double immunofluorescence staining of chicken pectoralis muscle cryosections with anti-Mi-CK and anti-M-CK antibodies revealed that on the one hand Mi-CK is restricted to mitochondria. The fluorescence was seen clearly in areas along the myofibrils where mitochondria are clustered. On the other hand, staining of relaxed muscle tissue sections by anti-M-CK revealed a regular striation pattern with strong fluorescence not only within the M- band, where the enzyme has been localized earlier in isolated and washed myofibrils [Wallimann et al, 1977a], but also at the I-band, often sparing the Z-line [Wallimann et al, in press]. Most of the sarcoplasmic CK is unevenly distributed along the myofibrils and concentrated at the I-band where it only loosely interacts with the thin filament lattice region. Surprisingly, CK is almost completely absent from the actin-myosin overlap regions. Our interpretation of these results is that the actin-myosin overlap zone is not readily accessible to sarcoplasmic proteins which seem to be excluded from this region possibly by molecular sieving and electrostatic repulsion. Indeed, Donnan potential measurements have shown that the electric charges are quite different in the A-band as compared with the I-band region [Bartels and Elliott, 1985]. Permeabilization of muscle fibers by glycerol or saponin followed by washing at physiological ionic strength prior to fixation and cryosectioning resulted in a complete loss of I-band fluorescence while the strong M-band signal remained, thus confirming the earlier described strong binding of MM-CK to the M-band.

The soluble CK is co-localized together with glycolytic enzymes at the I-band where it is thought to be coupled to glycolysis [see Wallimann and Eppenberger, 1985] by forming loosely interacting, but functionally coupled, multienzyme complexes in this sarcomeric region as shown recently [Maughan and Lord, 1988]. Considering that there is no CK in the actin-myosin overlap

zone where the actual ATP-hydrolyzing events of muscle contraction take place, the localization of the enzyme between two of these zones, at the M- band, makes physiological sense, for cytoplasmic streaming due to cross-bridge cycling and piston-like interdigitation of the two myofilament lattices during contraction would tend to push the products of ATP-hydrolysis from both sides towards the M-band as had been postulated by Wallimann and Eppenberger [1985]. Thus, the M- line-bound CK shown to be an efficient intramyofibrillar *in situ* ATP-regenerator [Wallimann et al, 1984], seems to be ideally positioned in this respect. Direct experimental observations revealed a membrane-bound fraction of the M-CK isozyme within the sarcoplasmic reticulum giving evidence for still another case of compartmented CK. Based on the availability of highly purified SR vesicles and on a reliable test for Ca^{++} uptake, Rossi et al [1988; submitted], in our laboratory, were able to demonstrate that a functional coupling between MM-CK and the Ca-pump existed. *In vitro*, the amount of CK still bound to the SR at the end of the purification was sufficient to support about 25% of Ca pumping of SR vesicles in the presence of PCr and ADP, as compared to maximal pumping in the presence of an excess of ATP. The remaining CK was not removed either by high or low salt treatment of the SR vesicles.

B. Mitochondrial Mi-CK Forms Highly Ordered Octameres

From recent publications from our laboratory [Schlegel et al, 1988a,b; Schnyder et al, 1988] describing a new procedure for the isolation of Mi-CK and the subsequent biochemical and biophysical characterization of the enzyme, it has become clear that Mi-CK can form highly ordered octameric structures with a M_r 328-364'000 (Table 1) exposing a central cavity or channel. It has been shown that octameric Mi-CK is *in vitro* in dynamic equilibrium with dimeric Mi-CK which is affected by protein concentration, ionic strength, pH, and nucleotides, indicating that these parameters may also be regulatory factors *in vivo*. Under certain conditions Mi-CK octamers rebind preferentially and with higher affinity than Mi-CK dimers to the inner mitochondrial membrane, where Mi-CK is not evenly distributed over the membrane, but seems to be concentrated at contact sites (CS) between inner and outer mitochondrial membranes [Kottke et al, 1988; Adams et al, submitted] This was confirmed by immunoeletronmicroscopy showing Mi-CK clustered at the contact sites

[Schlegel et al, 1988a]. There it is thought to form functionally coupled multienzyme energy channeling complexes together with ATP/ADP translocators and voltage-gated ion-selective pores of the outer membrane as suggested in a recent model [Wallimann et al, in press]. The existence of octameric Mi-CK within mitochondria has recently been confirmed by radiation inactivation experiments [Quemeneur et al, 1988].

TABLE I. Biophysical Characterization of Purified Mi-CK [for details see Schnyder et al, 1988]

Measured by	M_r of Mi-CK octamer	M_r of Mi-CK dimer
a) Gel permeation chromatography	364'000 ± 30'000	86'000 ± 8'000
b) Analytical ultracentrifugation	328'000 ± 25'000	83'000 ± 8'000
c) Mass measurements by STEM[a]	340'000 ± 55'000	89'000 ± 27'000

[a]STEM = scanning transmission electron microscope.

III. THE PHOSPHORYL-CREATINE CIRCUIT MODEL

Based on our results and the work of many authors in the field we have proposed the model described in Figure 1 depicting the energy flux in muscle and its regulation, respectively, emphasizing the central role of Mi-CK in the energy channeling process and the importance of the subcellular compartmentation of cytosolic CK's as *in situ* regenerators and regulators of local ATP-levels [Wallimann et al, in press]. By implementing three main functions for the creatine kinase system, namely (i) energy buffering, (ii) energy transport and channeling, and (iii) regulation of local intracellular ATP-levels, and by extending the concept of the model from a simple "CP-shuttle" [Bessman and Geiger, 1981] to a more complex, regulated PCr-circuit, a realistic picture of the highly sophisticated network may be suggested. Depending on energy need, an excitable cell may have to put emphasis on either one, two, or all three aspects at any one time. Such a PCr-circuit model is supported by immunolocalization studies on CK isozyme, by recent structural work on Mi-CK

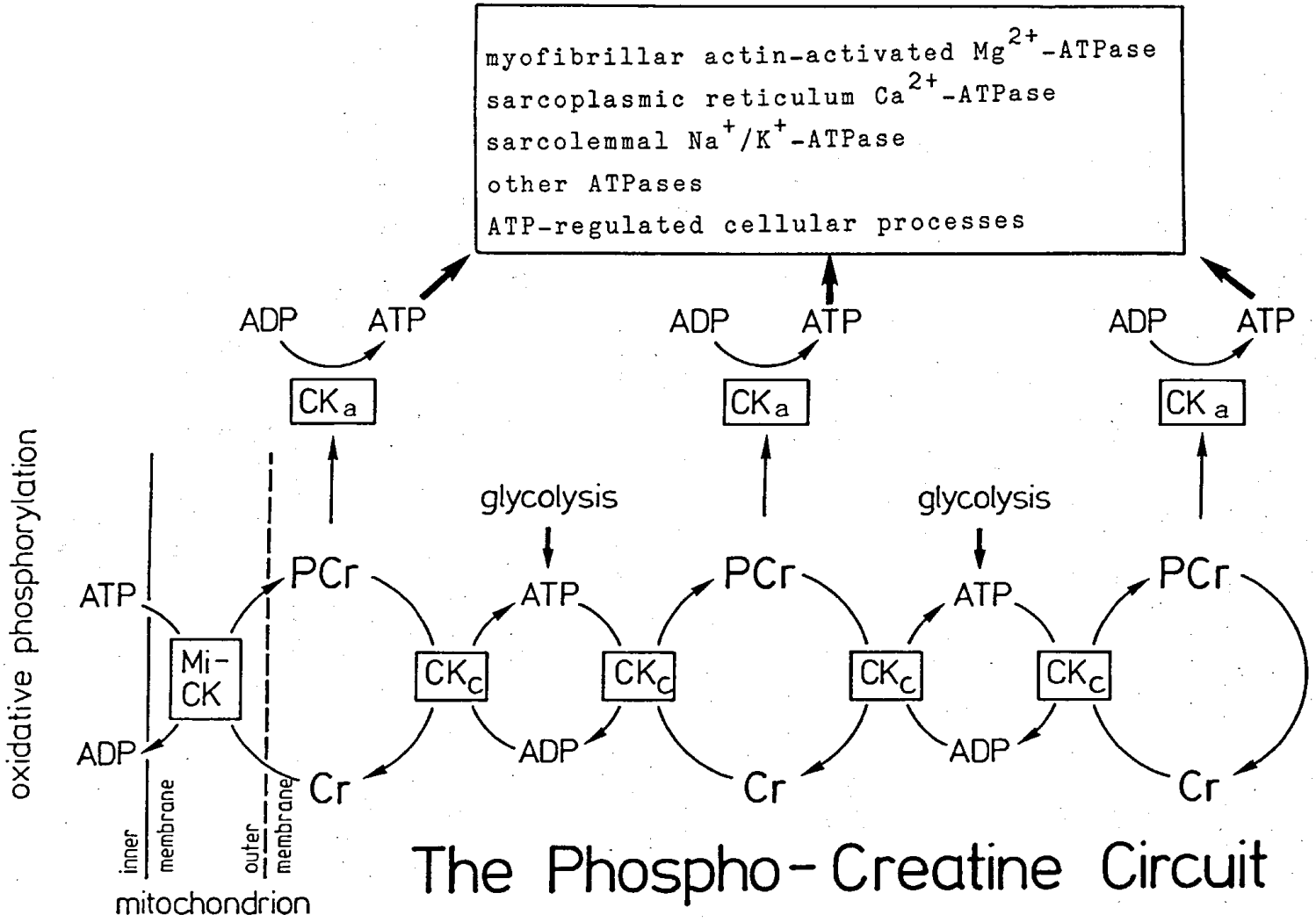
myofibrillar actin-activated Mg^{2+}-ATPase
sarcoplasmic reticulum Ca^{2+}-ATPase
sarcolemmal Na^{+}/K^{+}-ATPase
other ATPases
ATP-regulated cellular processes
ADP
ATP
CK_a
glycolysis
PCr
Cr
ATP
ADP
Mi-CK
CK_c
oxidative phosphorylation
inner membrane
outer membrane
mitochondrion
The Phospho-Creatine Circuit

Figure 1. A modified version of the CP-shuttle model by Wallimann and Eppenberger (1985) incorporates the facts of subcellular compartmentation of mitochondrial CK (Mi-CK) and cytosolic CK (CK_c) as well as the subcellular association of some fractions of CK (CK_a) at strategically important sites. Therefore, functionally and/or structurally coupled microcompartments are formed involving CK at the producing side of the PCr-circuit, e.g. Mi-CK coupled to ATP-ADP-translocators at the contact sites between inner and outer mitochondrial membranes, and at the consuming side of the PCr-circuit, e.g., CK_a at the sarcoplasmic reticulum coupled to the Ca^{2+}-ATPase, CK_a at the myofibrillar M-line coupled to the acto-myosin ATPase, CK_a at the sarcolemma coupled to the Na^+/K^+-ATPase and possibly to ATP-gated ion-channels. The model suggests that PCr produced by oxidative phosphorylation via mitochondrial matrix-generated ATP and via Mi-CK enters the cytosolic PCr/Cr-ATP/ADP equilibrium governed by the large amount of cytosolic CK_c which in muscle is localized together with lycolytic enzymes at the I-band where it is thought to be functionally coupled to the glycolysis, the second producing side of the PCr-circuit. This model visualizes the aspects of facilitated diffusion [Meyer et al, 1984], but in addition incorporates the aspects of the compartmented subcellular localization of CK isoforms. The PCr-circuit serves i) as an energy buffer, ii) as an energy transport and channeling system, and iii) as a regulator of local ATP levels.

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as well as by many physiological and ^{31}P-NMR studies with intact muscle (see also legend of Fig. 1).

We would like to stress the point that the PCr-circuit is not an absolute prerequisite for muscle function *per se*, for we have provided evidence ourselves, that (i) certain muscle types, although predominantly slow tonic ones which are lacking M-line-bound CK and at the same time are missing an electron-dense M-band structure [Wallimann et al, 1977; 1985], or that (ii) hearts of chicken [Wallimann et al, 1977b], of neonatal mammals [Perry et al, 1988] or of redifferentiating adult heart cells in culture [Eppenberger et al, 1988], which are lacking M-line-bound CK or both M-line CK plus mitochondrial CK, respectively, seem yet to function perfectly well within their appropriate physiological range. In addition, non- excitable cells and organs with a still relatively high, but continuous energy flux, e.g., liver, which contains only small amounts of CK or none at all, function without a PCr-circuit. However, it is our contention that the addition of the PCr-circuit system on top of the basic energy ground-state of a cell is likely to have a profound physiological impact for cellular energetics and performance that will be most advantageous at high work loads.

IV. SUMMARY AND CONCLUSIONS

The idea of a PCr-circuit is supported by the fact that in fully differentiated and highly specialized cells with high sudden energy turnover, e.g., skeletal and cardiac muscle [Wallimann and Eppenberger, 1985], brain and retina photoreceptor cells [Wallimann et al, 1986a], spermatozoa [Tombes and Shapiro, 1985; Wallimann et al, 1986b] and Torpedo electrocytes [Wallimann et al, 1985] mitochondrial CK is generally found in conjunction with cytosolic CK's with a significant fraction of the latter being associated subcellularly in a compartmented fashion at intracellular sites of high energy turnover. It is also becoming apparent that some of the cytosolic CK is specifically associated with membranes possibly via membrane anchors, e.g., with the SR-membrane where CK was shown to be functional by supporting a significant portion of the maximal Ca^{2+}-pumping rate [Rossi et al, 1988; submitted]. Similar membrane associations of CK have been shown with the post-synaptic acetylcholin-receptor-rich membrane, the invaginated, and non-innervated face membrane of electrocytes, rich in Na^+/K^+ATPase as well as with synaptic vesicles [Wallimann et al, 1985], with the sperm-tail plasma membrane [Wallimann et al, 1986a], and recently also with rod outer segment plasma membranes of bovine photoreceptor cells [Quest et al, 1987; Hemmer et al, 1989]. Thus, for all the above cells the PCr-circuit seems to represent an efficient, flexible, and highly responsive accessory, crucial not only as an energy back-up system, but also as a regulator of energy flux (channeling) and as a fine- tuning device of local ATP-levels. The strength of such a regulated channeling circuit operating at relatively low adenine nucleotide levels compared to the high total PCr and Cr pools, which are metabolically inert, is its high sensitivity towards ADP [Wallimann et al, 1984] that is preventing in excitable cells the accumulation of ADP and AMP unless severe stress, such as hypoxia or ischaemia is imposed. Additional details concerning the PCr-circuit model in muscle and our current ideas about the structure-function relationships of mitochondrial have been described elsewhere [Wallimann and Eppenberger, 1985; Schlegel et al, 1988; Schnyder et al, 1988].

While it is accepted that in living muscle the CK reaction velocity could be several-fold faster than the rate of ATP-hydrolysis by the acto-myosin ATPase and that of ATP-synthesis by oxidative phosphorylation, a positive correlation between oxygen consumption of perfused hearts and a concomitant increase of

the CK reaction velocity has only been recently verified by ^{31}P-NMR-techniques [Kupriyanov et al, 1984] or by direct biochemical measurements [Mahler, 1985] and model calculations [Connett, 1988]. This correlation factor seems to depend strongly on the relative amount of Mi-CK [Perry et al, 1988] which in mammalian cardiac muscle is gradually expressed in increasing amounts only during postnatal development. Thus, the flux of energy through the CK- and PCr-system increases with progressive differentiation of cardiac muscle where the accumulation of Mi-CK becomes physiologically relevant especially at higher work loads [Perry et al, 1988]. During chronic stimulation of skeletal muscle [Schmitt and Pette, 1986] or training for long distance running [Apple and Rogers, 1986] the conversion from fast- twitch to slow-twitch fibers is accompanied by a significant decrease in total CK activity, but at the same time by a several-fold elevation of Mi-CK at different times during this metabolic adaptation correlate with the oxidative potential of the corresponding muscles. In our opinion this is a very good example or even an experimental proof for the two main functions of the PCr- circuit that is, for the energy buffering function more prominent in glycolytic, fast-twitch white-muscles containing high levels of M- CK, and for the energy transport or channeling function more pronounced in oxidative slow-twitch red-muscles containing relatively high levels of Mi-CK. During the observed fiber-type conversion the gradual increase in the relative amounts of Mi-CK is characteristic for shifting emphasis from the energy-buffering towards the energy-channeling function, with Mi-CK assuming an increasingly more important role as an intra-extra-mitochondrial energy transfer system.

Thus, once again, the myfibrillar and mitochondrial compartmentation of CK and their *in situ* functions as intramyofibrillar ATP- regenerators and mitochondrial energy channeling transporters, respectively, may be critical parameters for optimal muscle function, especially in fast muscles and muscles under high work load.

To conclude, the best living example so far for the physiological significance of the PCr-circuit are spermatozoa where also two compartmented CK isoforms were identified, Mi-CK being localized in the midpiece and B-CK in the tail portion [Wallimann et al, 1986]. Shapiro and his coworkers were able to show that in sea urchin spermatozoa oxidative phosphorylation and flagellar motility were linked together via the CK reaction [Tombes and Shapiro, 1985] and that

inactivation of the CK-system with increasing, but very low concentrations of dinitro-fluorobenzene (DNFB), resulted in a progressive loss of sperm tail oscillation starting from the distal end of the sperm tails [Tombes et al, 1987]. Therefore, the PCr- circuit hypothesis remains a very attractive idea to be tested by further experimentation.

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