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Review

Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism

Markus Wyss [a], Jan Smeitink [b], Ron A. Wevers [c] and Theo Wallimann [a]

[a] *Institute for Cell Biology, ETH Hönggerberg, Zürich (Switzerland),* [b] *Wilhelmina Kinderziekenhuis, Utrecht (Netherlands) and* [c] *Laboratory of Neurology and Pediatrics, University Hospital Nijmegen, Nijmegen (Netherlands)*

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Correspondence to: J. Smeitink, Wilhelmina Kinderziekenhuis, Nieuwegracht 137, 3512 LK Utrecht, Netherlands.

Abbreviations: ANT, adenine nucleotide translocator; CS, contact sites between inner and outer mitochondrial membranes; CK, creatine kinase; Mi-, M- and B-CK, mitochondrial, muscle cytosolic and brain cytosolic isoforms of CK; Cr, creatine; PCr, phosphorylcreatine; GPA, β-guanidinopropionic acid; GPAP, phosphorylated GPA; GBA, β-guanidinobutyric acid; cCr, cyclocreatine; PcCr, phosphocyclocreatine; AK, arginine kinase; CSF, cerebrospinal fluid.

I. Introduction

The primary source of energy for many crucial processes in living cells is ATP. However, even though cellular pools of ATP are rather small, no significant decrease in [ATP] is detected during cell activation (e.g., muscle contraction, brain stimulation, phototransduction in retina or initiation of sperm motility,

for reviews, see Refs. 318,417). In all these tissues or cells with high and fluctuating energy requirements, ATP is continuously replenished from phosphorylcreatine (PCr) by the action of the creatine kinase (CK, EC 2.7.3.2) system. The CK isoenzymes catalyze the transphosphorylation reaction between PCr and ADP,

$$PCr^{x-} + MgADP^{(x-2)-} + (x)H^{+} \rightleftharpoons MgATP^{2-} + Cr$$

The indicated charges are approached above pH 6.5 where x approximates 1. When the pH is lowered below 6.5, the charges change considerably and x decreases.

Five CK isoenzymes are currently known in avian and mammalian tissues. Three of them are found within the cytoplasm and two are strictly mitochondrial. The cytosolic CK isoenzymes form only dimeric molecules, namely MM-CK, MB-CK and BB-CK, composed of two types of subunits, the M or muscle type subunit and the B or brain type subunit [95,115]. MM-CK is predominantly found in mature skeletal muscle and mammalian myocardium; BB-CK in mammalian brain and neural tissues, embryonic skeletal and cardiac muscle and avian myocardium; and MB-CK in adult mammalian heart as well as in striated muscles during the developmental transition from BB- to MM-CK [115,227,530]. Subcellular fractionation, e.g., isolation of myofibrils, sarcoplasmic reticulum, plasma membranes, etc., as well as direct in situ immunolocalization studies revealed a cellular and subcellular compartmentation of the cytoplasmic CK isoenzymes (for reviews, see Refs. 38,379,491,492). In muscle, for example, a small but significant fraction of MM-CK is specifically associated with the myofibrillar M-band where it directly rephosphorylates ADP generated by the actin-activated myosin ATPase [488].

The two mitochondrial CK (Mi-CK) isoenzymes, due to their tissue-specificity of expression on one hand and their relative isoelectric points on the other hand, were called either ubiquitous and sarcomeric Mi-CK [162,163,244,344] or Mi_a- and Mi_b-CK (only for chicken) [196,397,516], respectively. Both Mi-CK isoenzymes are located within the mitochondrial intermembrane space and form, in contrast to the cytosolic CK isoenzymes, octameric as well as dimeric molecules.

In recent years, the obvious importance of the CK isoenzymes for cellular energetics has attracted considerable attention and three main functions were assigned to the CK/PCr system [491,492]: (1) In many tissues, the concentrations of Cr and PCr are much higher than those of ADP and ATP, thus, enabling efficient buffering of the ADP and ATP concentrations within these cells. (2) The higher concentrations, together with the higher diffusion coefficients of Cr and PCr relative to ADP and ATP [524,525], ensure a significantly enhanced maximal rate of delivery of 'high-energy phosphates' to sites within the cells where energy is consumed and where ATP has to be locally regenerated (transport function of the CK system). (3) Since the CK system is only involved in one particular reaction pathway and since the CK isoenzymes are subcellularly compartmentalized, the CK system offers an attractive opportunity to specifically regulate local ATP/ADP ratios as well as cellular energy metabolism in general.

For reviews on the physiology of the CK isoenzyme system and on the biochemistry and evolution of the cytoplasmic CK isoenzymes, the reader is referred to Refs. 37,38,209,218,230,240,261,311,379,416,488 and 491–494. The purpose of this review is to summarize the important findings on Mi-CK since its discovery in 1964 and to convey our current ideas on the physiological significance and on the structure-function relationships of this enzyme important for the bioenergetics of cells with high and fluctuating energy turnover.

II. Biochemical studies of Mi-CK

II-A. Purification of Mi-CK isoenzymes

In order to obtain homogeneous starting material for biochemical and biophysical experiments, as well as for the production of polyclonal and monoclonal antibodies, Mi-CK has been purified from a variety of animal and human tissues, namely bovine heart [123,166,167,280], chicken heart [63,396], dog heart [363–365], human heart [49,50,156,231,457], pig heart [355], rabbit heart [132], rat heart [81,82,387], chicken brain [516], human liver [231], pigeon pectoralis muscle [281] and sea urchin spermatozoa [464]. Most of the purification procedures described take advantage of the intracellular localization of Mi-CK which is attached to the outer surface of the inner mitochondrial membrane [213,411]. In a first step, mitochondria are enriched by differential centrifugation. Then, Mi-CK is released from the mitochondrial inner membrane ('extraction') and finally, it is separated from contaminating proteins by ethanol- or ammonium-sulphate fractionation, chromatofocussing, affinity-, ion-exchange-, hydrophobic-interaction-, or gel-permeation chromatography. Some aspects of the purification procedures will now be discussed in more detail.

Simply rupturing the outer mitochondrial membrane, followed by extraction of the enzyme under iso-osmotic conditions, does not give satisfactory yields of Mi-CK. Instead, swelling of the mitochondria by incubation in a hypotonic medium or in an isotonic phosphate solution prior to extraction is a prerequisite for an optimal release of Mi-CK from the inner mitochondrial membrane [122,130]. Upon incubation with sodium phosphate, mitochondria swell because of a passive uptake of sodium as well as phosphate ions.

Swelling with potassium phosphate is only observed in respiring mitochondria, since the uptake of potassium ions is an active process. Accordingly, respiratory inhibitors block potassium-induced swelling of the mitochondria [122,130].

Release of Mi-CK from swollen mitochondria can be achieved by a variety of conditions. In most studies, 10–100 mM phosphate was used at pH values of 6.5–9.0 [31,63,81,82,101,123,130,132,156,166,167,210,213,231, 243,296,324,363–365,374,377,396,401,409,434,457,481, 482,487,498,502,504,516]. Extraction of Mi-CK by phosphate was shown to be strongly pH-dependent, being more efficient at pH values higher than 7.0 [481]. Besides phosphate ions, adenine nucleotides [265,296,385,482], negatively charged organic mercurials [81,82,130,131,324,482,498], and 100–250 mM KCl [265,280,481,482,498] were also used to release Mi-CK from the inner mitochondrial membrane. Organic mercurials like *p*-hydroxymercuribenzoate and mersalyl at concentrations as low as 10 μM seem to be the most potent releasing agents resulting in more than 80% solubilization of Mi-CK [482]. Since mersalyl blocks the uptake of phosphate ions into mitochondria, however, it should not be used in combination with phosphate. Organic mercurials have the additional disadvantage that they inhibit the enzymatic activity of Mi-CK, probably by reacting with the 'essential' sulphydryl group of the enzyme, but enzymatic activity can easily be recovered by incubation with excess amounts of reducing agents (2-mercaptoethanol or dithiothreitol). Because KCl extracts Mi-CK, the binding of the latter to mitochondrial membranes was believed to depend on ionic interactions and, therefore, to be sensitive to the ionic strength of the medium [281,498]. Instead, Saks et al. [385] suggested that the decisive factor is not the ionic strength of the medium by itself but rather the ion composition. Whereas in their experiments 125 mM KCl released more than 50% of Mi-CK, a 'physiological salt solution' containing only 10 mM chloride, but with higher ionic strength and osmolarity, released only 12%. Chloride (and other anions), therefore, seem to have a special effect on the release of Mi-CK, probably by binding to the active site of the enzyme [316,494].

Three types of affinity matrix have been used up to now for Mi-CK purification, namely Cibachrome-blue-based matrices [50,132,396,487,516], ADP-hexane-agarose [63,132] and ATP-hexane-agarose [167]. Whereas only 2–5 ml columns of the latter two matrices were used, Blue Sepharose CL-6B columns of up to 250 ml were routinely employed for the purification of the chicken Mi-CK isoenzymes [140,396,516], thus, allowing the application of large amounts of protein. Nevertheless, the most powerful affinity chromatography technique is probably the transition state-analogue affinity chromatography procedure developed by Brooks et al. [63]. In this method, the interaction of Mi-CK with the ADP-hexane-agarose matrix is selectively strengthened by the addition to the application buffer of magnesium ions, creatine and nitrate, thus, inducing a transition state-analogue complex of the enzyme. The nitrate ion mimics the phosphate group to be transferred during catalysis [316], which is thought to be planar in the transition state of the reaction.

A considerable problem in the purification of Mi-CK is its separation from the cytosolic CK isoenzymes, because in many tissues, Mi-CK makes up only a few percent of the total CK activity. A large part of the contaminating cytosolic CK can be eliminated by differential centrifugation. In this respect, brain is more delicate, because synaptosomes formed during homogenization of the tissue contain cytosolic CK [136,504] and co-sediment with mitochondria. In chicken and rat, however, swelling of the mitochondrial (and synaptosomal) suspension breaks up the synaptosomes and a first separation of the mitochondrial and cytosolic CK isoenzymes can be achieved by an additional centrifugation step [516,518]. Mi-CK can also be separated from the cytosolic CK isoenzymes by ethanol fractionation [166,503] or, because Mi-CK isoenzymes of most species have much higher isoelectric points (IEP > 8, see II-D) than the cytosolic CK isoenzymes (IEP ≤ 7) [185,483,526], by ion-exchange chromatography. In addition, mitochondrial and cytosolic CK isoenzymes can be separated by hydrophobic interaction chromatography [9,49,50,401,499], indicating that Mi-CK is more hydrophobic than cytosolic CK. Finally, both a complete separation of the isoenzymes as well as an additional purification of Mi-CK itself were achieved by affinity chromatography [167,396,487,516]. A second serious problem during purification emerges from the 'oligomeric heterogeneity' of Mi-CK. It is well established now that Mi-CK isoenzymes form octameric as well as dimeric molecules which are – depending on protein concentration, substrate concentrations, pH, etc. – readily interconvertible (see III-D). Mi-CK octamers and dimers behave quite differently in most of the purification methods mentioned above so that one oligomeric form has to be strongly favoured by appropriate experimental conditions in order to obtain maximal yields (Schlegel, J., Wyss, M. and Wallimann, T., unpublished data).

The purification procedures for two Mi-CK isoenzymes differ from the 'general scheme' described above. The purification of human heart Mi-CK by Blum et al. [50] includes three chromatography steps, but no prior enrichment of mitochondria by differential centrifugation. The low specific activity of their Mi-CK preparation may be explained by the rather harsh and lengthy procedure used. A purification scheme based on differential centrifugation may turn out to be superior if one takes into account the fact that an optimal release of

TABLE I

Molecular masses, sedimentation coefficients and Stokes radii of Mi-CK isoenzymes

References: a, 26–29,122,123,211,212,280,282,283,286; b, 63,396,397,403; c, 397,516; d, 23,347,348,363–365; e, 49,50,156,231,243; f, 242,296,483; g, 281,283; h, 132,296,353,439,482,483; i, 81,82,266,344,382,387,396; j, 463,464,517.

Tissue	Molecular masses (kDa)			Sedimentation coefficients (S)		Stokes radii (Å)		Refs.
	protomer	dimer	octamer	dimer	octamer	dimer	octamer	
bovine heart	41–44	80–89	317–355	5.3–5.4	11.8–12.4	37	59–65	a
chicken heart	42–43	78–87	321–378	5.0	12.8–13.5	38.3 *	62.4 *	b
chicken brain	42	85	306–352	4.9–5.3	11.6–12.0	36.3 *	60.3 *	c
dog heart	41–44	82–84	–	–	–	–	–	d
human heart	41–42	80–85	350	5.0	–	–	–	e
pig heart	–	75	342	–	(11.7)	–	–	f
pigeon pectoralis	44	87–91	331–380	5.4	12.0	37.4	59.3	g
rabbit heart	40–45	81	332–377	–	–	36–39 * *	56 * *	h
rat heart	42–43	82–87 *	340–345 *	–	–	37.7 *	61.1 *	i
sea urchin sperm	44–50	–	353	–	12.4	–	62.8	j

* Wyss, M., Schlegel, J. and Wallimann, T., unpublished data; * * Vial, C., unpublished data.

human sarcomeric Mi-CK from the mitochondrial membranes can only be achieved by addition of 0.05% Triton X-100 to the extraction buffer [434]. Because differential centrifugation of spermatozoa is almost impossible, this step was also omitted from the purification scheme of sea urchin sperm Mi-CK [464]. Furthermore, phosphate failed to release significant amounts of Mi-CK from the sperm membranes and instead, 5% of the non-ionic detergent Nonidet P-40 was used [463,464]. Additional purification of sperm Mi-CK was achieved by selective precipitation in a buffer of low ionic strength [464].

The specific activities of the purified Mi-CK isoenzymes shall not be listed here, since they strongly depend on the reaction direction and on the experimental conditions used. The most reasonable range in the direction of ATP synthesis at 30°C and pH near neutrality seems to be 100–200 μmol/min per mg protein [26,63,281,287].

II-B. Molecular size

Whereas it is generally accepted that cytosolic CK isoenzymes exclusively form dimeric molecules (MM-, MB- and BB-CK) [95,115,494], the naturally occurring oligomeric forms of Mi-CK were a matter of debate for quite a long time. Some research groups presented evidence that Mi-CK, like the cytosolic CK isoenzymes, is also exclusively a dimeric molecule with an M_r of 80500–84000 [23,49,50,63,156,364,365,439] and even claimed that the higher M_r aggregates reported by others represent 'induced artifacts' [156,365]. Nevertheless, an increasing body of evidence, starting with the pioneering studies of Farrell et al. [122], Saks et al. [374] and Jacobs [211], demonstrated that Mi-CK, at least in vitro, forms stable dimeric and octameric molecules. Dimeric and octameric Mi-CK are readily interconvertible (see III-D) and have molecular masses of 75–91 kDa and 306–380 kDa. Stokes radii of 36–39 Å and 59–65 Å and sedimentation coefficients of 4.9–5.4 S and 11.6–13.5 S, respectively (Table I).

The most thorough analysis concerning the molecular mass was performed on the Mi-CK isoenzymes from chicken, namely ubiquitous Mi_a-CK from brain and sarcomeric Mi_b-CK from heart. Gel-permeation chromatography and analytical ultracentrifugation of the purified isoenzymes revealed two oligomeric forms each with M_r values of 83000–86000 and 306000–364000, respectively, with no indication for the existence of an intermediate form [396,397,403,516]. Considering the protomeric M_r of approx. 43000 (Table I), the cDNA sequences (see II-F) and hybridization experiments [516], the lower M_r form in all likelihood corresponds to dimeric Mi-CK molecules. In contrast, the higher M_r value, which in addition was confirmed by direct mass measurements of single Mi-CK molecules by scanning transmission electron microscopy [403,516], is strongly indicative for an octameric molecule. This view is also supported by electron microscopical examination of Mi-CK molecules (see II-C), which suggests that four ellipsoid Mi-CK dimers are arranged in parallel to each other to form an octamer [407,408].

In several studies [26,27,82,100,122,156,167,278,464,504], the higher M_r value of Mi-CK was underestimated to be in the range 180000–250000. The reasons for these underestimations seem to be manifold: (i) already in 1972, Farrell et al. [122] published a Stokes radius of 65 Å for beef heart Mi-CK which is fully in line with the available data for octameric Mi-CK (Table I). However, probably due to inappropriate M_r standards, the molecular mass was calculated by the same authors to be only 250 kDa. (ii) Due to its recommended M_r fractionation range of 5000–250000,

Sephacryl S-200 clearly represents a bad choice for the estimation of Mi-CK oligomers with high M_r values [100,156,167]. In addition, dimeric Mi-CK revealed a M_r of only 65000 on Sephacryl S-200, but one of 80000 on Sephadex, indicating that the choice of the gel-filtration matrix is crucial for molecular mass determination of Mi-CK [156]. (iii) Using a FPLC Superose-12 column from Pharmacia, Tombes and Shapiro [464] determined a Stokes radius of only 45 Å for sperm Mi-CK of the sea urchin *Strongylocentrotus purpuratus*, whereas we [517] obtained a Stokes radius of 62.8 Å and a M_r of 353000 for the same protein from the sea urchin *Psammechinus miliaris* (Table I). Since Tombes and Shapiro for their ultracentrifugation experiments used a calculation method where the M_r is a function of the Stokes radius, whereas the sedimentation coefficient is not, they underestimated the M_r as 235000, but obtained a 'correct' sedimentation coefficient of 12.4 S. Correction for a Stokes radius of 62.8 Å gives a M_r of 328000 instead of 235000. (iv) The M_r of 240000 reported for rat heart Mi-CK [82] can be explained by partial dissociation of the Mi-CK octamers during the gel-filtration run. First, in the very same experiments, a M_r > 100000 was obtained for dimeric Mi-CK [81], second, faster gel-permeation chromatography on a FPLC Superose-12 column revealed a M_r of 340000 [396] and third, purified rat-heart Mi-CK octamers proved to readily dissociate [398]. (v) The observations on bovine heart Mi-CK made by Belousova and co-workers [26–29,123] are an exception, since these authors are the only ones who provided experimental evidence for two different high M_r forms, namely 'hexameric' (M_r 240000) as well as 'octameric' Mi-CK (M_r 340000). In their most recent article, however, 'hexameric' and 'octameric' Mi-CK both were shown to have a sedimentation coefficient of 12.3–12.4 S and to display a four-fold symmetry on electron micrographs. Since a hexamer would be highly inconsistent with a four-fold symmetry, one is now tempted to suggest that the two forms represent different conformations of octameric Mi-CK [28,29].

In vitro translation of Mi-CK mRNA [162,348] as well as determination of the cDNA sequences of the two human and rat Mi-CK isoenzymes [162,163,344] revealed that Mi-CK is synthesized as a precursor protein of M_r 47000–48000 containing an N-terminal mitochondrial target sequence which is proteolytically removed after the import into the mitochondria to yield mature Mi-CK subunits with a M_r of approx. 42000. A M_r of 42000 was also reported for Mi-CK protomers from rooster spermatozoa [489] and chicken retina [490,496]. Gel-filtration experiments performed by Yasui et al. [523] revealed two oligomeric forms of Mi-CK from human skeletal muscle, heart and stomach having M_r values of 80000 and 370000, but only one form of M_r 370000 for human brain Mi-CK. Similarly, two different oligomeric forms with M_r values of 80000 and 350000 were described for human liver Mi-CK [231]. However, since liver only contains minute amounts of CK [40,518], the assumption that this Mi-CK in fact originated from liver cells is unlikely. Instead, the extracted Mi-CK may be derived from blood vessels because vascular and intestinal smooth muscle cells have recently been shown to contain appreciable amounts of octameric Mi-CK [207]. As a last point, it seems worth mentioning that already in 1968, Keto and Doherty [242] enriched a CK form with a sedimentation coefficient of 11.73 S from a particulate fraction of pig heart. Even though the authors claimed that this form was not of mitochondrial origin, they were, in hindsight, probably the first to describe octameric Mi-CK.

II-C. Three-dimensional structure: Electron microscopy and protein crystallography

By electron microscopy, the octameric forms of Mi-CK isoenzymes from all species investigated so far reveal a very similar structure. Bovine heart [28,29, 122,286], rat heart [82], chicken heart [396,397,403–407,507], chicken brain [397,404] as well as sea urchin sperm Mi-CK [517] seem to be cube-like molecules with a side-length of 10 nm, displaying a four-fold symmetry and a central stain-filled indentation or cavity. The fact that not only the oligomeric state but also the overall three-dimensional structure of Mi-CK has been conserved throughout evolution from sea urchins to mammals points to an important physiological role of this structure.

At least two different projections have to be assumed for an octameric, cube-like molecule. However, negative staining (Fig. 1A,B) [396,397,403,404,507] and rotary shadowing (Fig. 1C,D) [403,407,507] of single molecules of chicken sarcomeric Mi_b-CK revealed only one single view of the molecule, indicating that the top and bottom faces of the octamer are identical and have a distinctly higher affinity for a variety of support films used for electron microscopy than the side faces [407]. Since the cross-like surface depression of Mi_b-CK visualized by rotary shadowing (Fig. 1C,D) is slightly twisted in clock-wise direction relative to the side faces of the octamer [407,507] and, since computer averaging of the structure of negatively stained and rotary shadowed Mi_b-CK octamers revealed a four-fold symmetry [507], a single view for all four side faces has to be assumed. This side view of the octamer has only recently been obtained under one set of particular conditions. Overnight incubation of chicken Mi_b-CK with neutral uranyl acetate, followed by dialysis against distilled water, resulted in the formation of linear unbranched Mi-CK filaments, in which Mi-CK octamers were stacked by their top and bottom faces on top of each

other (Fig. 1E) [407]. The side view of the filaments, therefore, also displays the side face of the Mi-CK octamers.

One possibility for the overall three-dimensional structure of Mi-CK octamers is that a central cavity is present inside the octamer (Fig. 1F) is connected with the exterior of the molecule by two smaller channels, protruding from the midst of the top and bottom faces right into the middle of the octamer. This possibility is favoured by the facts that negative staining, visualizing a projected view of the entire volume of the molecule, reveals a central accumulation of stain with a diameter of about 2.5 nm (Fig. 1A,B), and that rotary shadowing, reflecting the surface structure of Mi_b-CK, gives rise to a small orifice only (Fig. 1C,D). Alternatively, the side view displayed by the linear Mi-CK filaments (Fig. 1E), as well as the recent observation of a second projection of single Mi-CK octamers (Kaldis, P., Schnyder, T. and Wallimann, T., unpublished data), favour the notion that the banana-shaped dimers are arranged in such a way that they form funnel-like indentations in the middle of the top and bottom faces (Fig. 1F). Further insight into the structural organization within the octamer was gained from experiments on the formation of heterooctameric molecules of chicken ubiquitous Mi_a- and sarcomeric Mi_b-CK [516]. Mi_aMi_b-CK heterooctamers were formed out of a mixture of Mi_a- and Mi_b-CK homodimers and subsequently stored for 3 months at 4°C. Upon re-dissociation of the octamers, cellulose polyacetate electrophoresis only revealed Mi_a- and Mi_b-CK homodimers, but no Mi_aMi_b-CK heterodimers, thereby proving that within the octamer, discrete dimers are the stable building blocks, with no subunit exchange between them.

Though crystallization of cytosolic CK isoenzymes has already been reported by several groups [69,151,185,304] and though these crystals diffracted to up to 2.0 Å resolution, no three-dimensional structure has been solved up to now. One reason for this probably is the microheterogeneity of purified MM- and BB-CK preparations [185,356] which is primarily due to posttranslational modifications. This problem may be overcome by crystallizing chicken sarcomeric Mi_b-CK [404,405,408] for which no posttranslational modifications are currently known. Precipitation of Mi_b-CK with poly(ethylene glycol) 1000, either in the presence or absence of ATP, yielded two different types of tetragonal crystal with the space groups $P42_12$ and P422, containing one octamer and one dimer per asymmetric unit, respectively. The former crystal type diffracts to at least 3 Å resolution [405]. A change in the crystal form in the presence of ATP might be indicative for a conformational change induced by substrate binding, a phenomenon that has attracted much interest in the study of the cytosolic CK isoenzymes [39]. As far as Mi-CK is concerned, conformational changes induced by substrates were assumed to influence the dimer to octamer ratio [280,281,296], since in a variety of studies, formation of a 'transition state-analogue complex' of Mi-CK with MgADP, Cr and nitrate resulted in the complete dissociation of the octamers into dimers (see III-D). Conformational differences of octameric Mi-CK were also thought to be the basis for the apparent 'hexameric' and 'octameric' forms of bovine heart Mi-CK described by Belousova and co-workers [28,29,123] (see II-B). The finding that the modification of Mi-CK dimers, 'hexamers' and octamers with SH group reagents is biphasic, with the first half of the subunits being more readily modified than the second half, was taken as an argument for an asymmetric association of the subunits within the dimer [26,27,123], but might be explained as well by a conformational change of discrete dimers induced by modification of only one of their subunits. Surprisingly, however, only half of the active sites within the octamer bound MgADP in the presence of Cr and nitrate [124].

II-D. Isoelectric point

With the exception of sea urchin sperm Mi-CK [464], all Mi-CK isoenzymes currently investigated have higher isoelectric points than the cytosolic CK isoenzymes of the respective species. Whereas p*I* values of 8.2–9.7 were reported for most Mi-CK isoenzymes (Table II), p*I* values of 6.2 and 7.0 were obtained for sea urchin sperm [464] and frog heart Mi-CK [273]. The results on the human Mi-CK isoenzymes are somewhat contradictory. From the cDNA sequences of cardiac and placental Mi-CK [162,163], p*I* values of 7.42 and 7.39 can be calculated for the mature subunits. These values agree quite well with those determined for native human heart and brain Mi-CK (6.8–7.0) [50,502,504] but are distinctly lower than the value of 9.35 reported for human heart Mi-CK by Khuchua et al. [243]. Besides the differences between particular Mi-CK isoenzymes, distinct isoelectric points were also

Fig. 1. The three-dimensional structure of chicken sarcomeric Mi-CK octamers deduced from electron micrographs. (A), Negative staining of Mi-CK octamers; (B), contour representation of (A) after circular harmonic averaging [507]; (C), Mi-CK octamers rotary-shadowed with Ta/W at low temperature and ultra-high vacuum [407]; (D), contour representation of Mi-CK octamers rotary-shadowed with Pt/Ir/C after circular harmonic averaging [507]; (E), linear filaments of positively stained Mi-CK octamers. The inset on the right side displays an averaged stretch of the filaments. The side view of one octamer is outlined by dots [407]; (F), model representations of the structure of an octamer. Above: top or bottom view; below: the two possible side-views displayed as sections through the center of an octamer (see text).

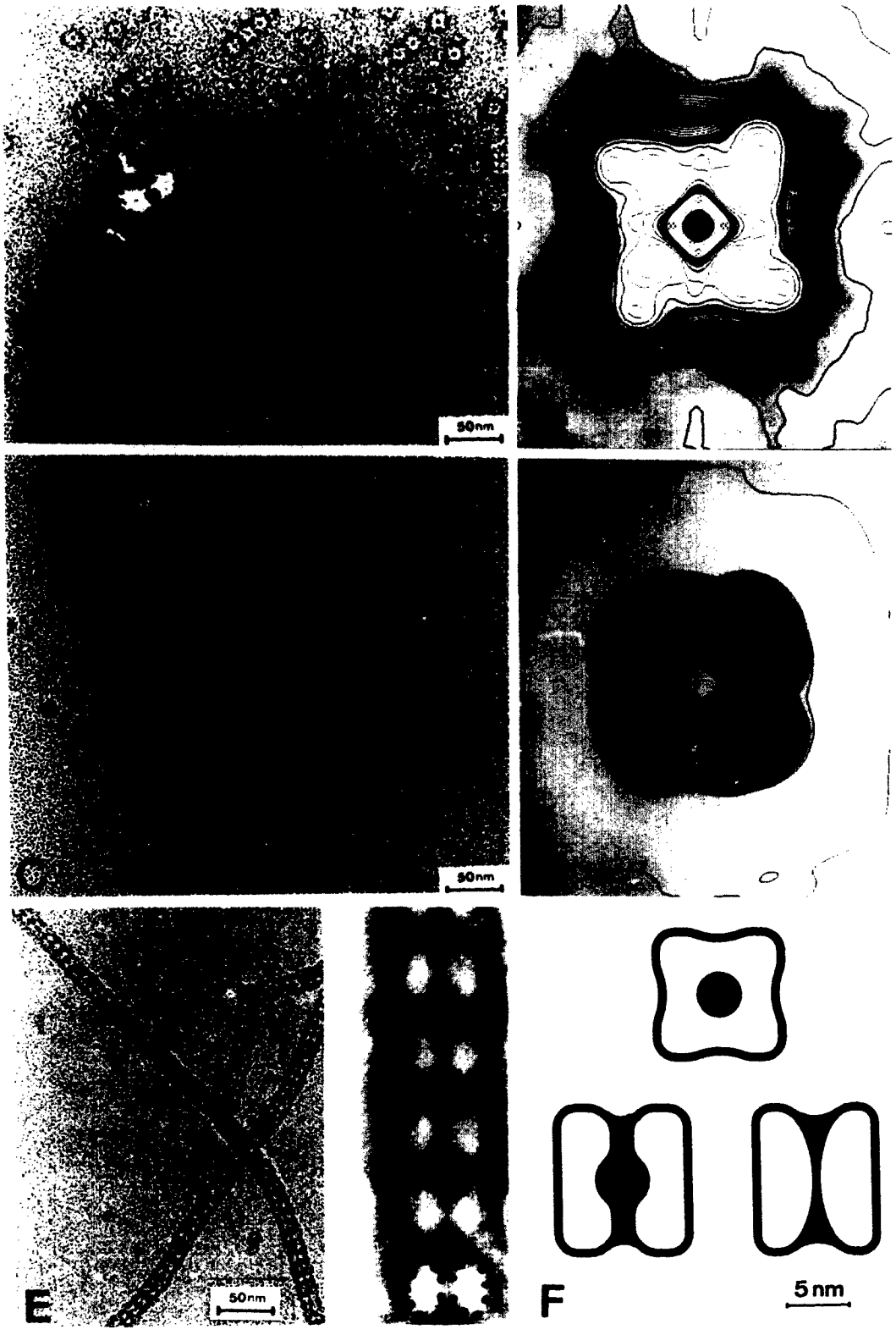
50nm
50nm
50nm
E
F
5nm

TABLE II

Isoelectric points of Mi-CK

Included in the column 'other forms' are values determined for Mi-CK which was either monomeric or for which the oligomeric form was not determined. Values marked with a * were calculated from the cDNA sequence. References: a, 26,27,208,211,282; b, 196,399,516; c, 399,508,516; d, 273; e, 50,163,243,502; f, 162,504; g, 354; h, 282; i, 354,483; j, 266,344,387; k, 344; l, 464.

Tissue	dimeric Mi-CK	octameric Mi-CK	other forms	Refs.
bovine heart	9.67	8.93	9.2–9.6	a
chicken heart	9.3	9.4–9.5	8.25 *	b
chicken brain	8.4–8.5	8.7–9.0	7.94 *	c
frog heart	-	-	7.0	d
human heart	6.94	-	6.8; 9.35; 7.42 *	e
human brain	7.01	-	7.49 *	f
pig heart	8.20	-	-	g
pigeon pectoralis	9.56	8.91	10.25	h
rabbit heart	8.24	8.83	-	i
rat heart	-	-	9.4; 7.88 *	j
rat brain			7.73 *	k
sea urchin sperm			6.2	l

observed for dimeric and octameric Mi-CK. Whereas a higher p*I* for the octamer than for the dimer was reported for Mi-CK from rabbit heart, chicken heart and chicken brain, an inverse relationship was found for the isoenzymes from bovine heart and pigeon pectoralis muscle (see Table II). However, these latter results have to be questioned, since in cellulose-acetate electrophoresis experiments, octameric bovine heart Mi-CK migrated further towards the cathode than dimeric [100,167] (see also III-D), thus, suggesting that the octamer is also more positively charged in bovine heart.

Mi-CK can be released from the inner mitochondrial membrane by high salt concentrations (see II-A) and is, therefore, thought to be bound via ionic interactions [82,369,395,498]. In fact, the basic p*I* of Mi-CK indicates that, within the mitochondrial intermembrane space, positively charged Mi-CK may bind to negatively charged membrane phospholipids. Accordingly, octameric Mi-CK from chicken heart, probably due to its higher p*I*, was found to interact more strongly with mitoplast and model membranes than dimeric Mi-CK [369,399]. Even though ionic interactions of Mi-CK with mitochondrial membranes are important, hydrophobic interactions can not be excluded, especially in the case of sea urchin sperm Mi-CK which can only be detached from the membranes by relatively high concentrations of detergents, but not by any other treatment known to release Mi-CK isoenzymes [464]. This may be due to its acidic p*I* of 6.2, indicating that ionic interaction with the mitochondrial membranes is reduced, but compensated for by increased hydrophobic interaction.

Because of the higher p*I*, Mi-CK isoenzymes can be nicely separated from the cytosolic CK isoenzymes under native conditions by electrophoresis on cellulose-polyacetate strips as well as on starch or agar gels, with Mi-CK migrating more cathodically than cytosolic CK [13,50,208,222,326,338,374,390,410,441,516,518]. The only exceptions known are human and frog heart Mi-CK. Human heart Mi-CK, because its p*I* is very similar to that of MM-CK, may be obscured by the latter isoenzyme [173]. Frog (*Rana esculenta*) heart Mi-CK, though having a p*I* which is distinctly higher than those of its cytosolic counterparts (7.0 vs. 5.5–5.8), migrated in between the cytosolic CK isoenzymes on cellulose polyacetate strips [273], as did the isoenzyme termed CK-V of *Xenopus* frogs which is expressed in lung and heart [511]. Since CK-I of *Xenopus* is supposed to be of mitochondrial origin as well, but is expressed in eye, brain and stomach, these results may indicate that frog, like chicken, rat and man, also contains two different Mi-CK isoenzymes. Interestingly, the fact that the mitochondrial isoenzymes have a higher isoelectric point than the cytosolic ones is not restricted to CK, but seems to be a rather general phenomenon [175]. Therefore, the hypothesis has been raised that the increased p*I* is either a prerequisite for an efficient import of the precursor proteins into the mitochondria or an adaptation to the metabolic conditions within these organelles.

II-E. Kinetic constants

Since the kinetic constants of the Mi-CK isoenzymes strongly depend on the calculation method used, on the species investigated, on the purity of the enzyme preparation, as well as on buffer composition, temperature and pH, it is beyond the scope of this review to mention all of the available data (for discussion, see Ref. 419). Instead, those studies are selectively picked out which compared different purified CK isoenzymes under exactly the same conditions, in which a variety of kinetic constants were determined for a single isoenzyme, or which allow interesting conclusions.

As already mentioned, the CK isoenzymes catalyze the transphosphorylation reaction between PCr and ADP (see I). Most studies on the reaction mechanism were performed on the cytosolic CK isoenzymes (for a review, see Ref. 240). For Mi-CK, it was only shown that at pH 7.4 the reaction mechanism is of the 'rapid-equilibrium random' type [287,375], that one cysteine [27,123] as well as two arginine residues per subunit [323,420] are 'essential' for catalytic activity and that probably Asp-335 is involved in substrate binding [223] (see also II-F).

The pH optima of the reaction catalyzed by Mi-CK from bovine heart [100,167], chicken heart [516], human heart [49,50], rabbit heart [439], guinea-pig heart

[338], rat heart [213], chicken brain [516], guinea-pig brain [456] and sea urchin spermatozoa [464] all range from 6.0–7.0 in the direction of ATP synthesis and from 7.5–9.0 in the direction of PCr synthesis, therefore, being in about the same range, or slightly lower than the respective values of the cytosolic CK isoenzymes [338,341,439,464,494] as well as of all other known phosphagen kinases (for a review, see Ref. 322). These findings are fully in line with pH studies on rabbit MM-CK which revealed that a single group with a pK_a near 7, probably a histidine residue, acts as an acid-base catalyst and must be unprotonated in the direction of PCr formation and protonated in the direction of ATP formation [90].

All purified Mi-CK isoenzymes investigated so far have a lower K_m value for MgADP than for MgATP and a lower K_m value for PCr compared to Cr [23,26, 50,63,100,167,231,287,397,401,410,448,464,523]. The same relationships have been described for the cytosolic CK isoenzymes (for a review, see Ref. 48). The K_m values of the Mi-CK isoenzymes were reported to be: 42 μM–1.7 mM for MgATP, 15–150 μM for MgADP, 3.4–62 mM for Cr and 0.23–4.1 mM for PCr. Human and chicken ubiquitous Mi-CK display a 2-fold and 4-fold lower K_m value for PCr than the respective sarcomeric Mi-CK isoenzymes [397,523], possibly reflecting a metabolic adaptation to the lower PCr concentrations in brain compared to cardiac and skeletal muscle. Furthermore, the fact that in intact mitochondria, Mi-CK has a lower K_m for ATP produced in the matrix compartment and presented by the adenine nucleotide translocator (ANT) than for exogeneously added ATP was taken as an argument that Mi-CK has privileged access to intramitochondrially synthesized ATP due to a direct interaction of Mi-CK with ANT [169,266,267,382,385,387]. However, the differences in K_m values could also be explained by restricted diffusion of adenine nucleotides across the outer mitochondrial membrane, signifying that especially at high velocities of the Mi-CK reaction, the substrate concentrations within the intermembrane space would not correspond to the extramitochondrial ones [146–148,257]. This controversy between 'direct interaction of Mi-CK with ANT' and 'restricted diffusion of adenine nucleotides' will be discussed in more detail in Section III-C. The indications that the ANT has a much lower K_m for ADP, but a distinctly higher K_m for ATP than Mi-CK were thought to explain why PCr is the high-energy phosphate compound leaving the mitochondria as the net product of oxidative phosphorylation [167]. However, on the basis that the K_m values of Mi-CK and ANT should not be compared directly, since MgADP and MgATP are the effective substrates of Mi-CK, whereas only the uncomplexed adenine nucleotides are transported by ANT, this interpretation has to be seriously questioned.

Different cooperativities of substrate binding between individual subunits might be a distinguishing feature of dimeric and octameric Mi-CK and thereby represent an effective means of regulating the enzymatic activity of the Mi-CK isoenzymes. However, the Hill coefficients of sarcomeric and ubiquitous Mi-CK octamers for the binding of PCr in the presence of excess MgADP are only 1.2 and 1.0, respectively [397], meaning that almost no communication between the subunits in the binding of this substrate occurs. Cooperativity in the binding of the adenine nucleotides is more likely to occur, since ATP and ADP are not only more tightly bound to Mi-CK, but are also more potent in inducing conformational changes of CK isoenzymes [238].

Arrhenius plots of the temperature-dependence of the enzymatic activity were used to calculate the activation energies of the CK reaction in the direction of ATP formation. For the human isoenzymes, significantly higher activation energies of 101–142 kJ/mol were found for ubiquitous and sarcomeric Mi-CK than for MM-, MB- and BB-CK (49–76 kJ/mol) [164,340,448,449,523], indicating that the substrates PCr and ADP are more strongly bound by the mitochondrial than by the cytoplasmic CK isoenzymes [231,401,448,503]. Whether this 2-fold difference in activation energies is of physiological importance, for example reflecting an adaptation to lower PCr or ADP concentrations in the intermembrane space compared to the cytosol, remains to be elucidated. Yet, a higher activation energy by all means causes a lowered maximal reaction velocity (V_{max}). Accordingly, lower V_{max} values in the direction of ATP formation were indeed reported for the chicken mitochondrial than for the cytosolic CK isoenzymes [518].

II-F. Nucleic- and amino-acid sequences

Compared to the cytosolic CK isoenzymes (for references, see Ref. 223), Mi-CK sequence data are rather sparse. Besides several partial protein sequences [50,82,137,196,223,464,516], six cDNA sequences [162,163,196,344,508] and two genomic sequences [162,244] were published up to now. Originally, determination of the amino-terminal sequences of chicken heart and brain Mi-CK proved that these tissues contain two different Mi-CK isoenzymes [196], as had been suggested earlier for the rat from agarose gel-electrophoresis experiments [391]. In the meantime, publication of two different Mi-CK cDNA sequences each for chicken [196,508], man [162,163] and rat [344] confirmed these findings and further demonstrated that they are due to two different nuclear genes rather than differential splicing of a single gene. Since on RNA blots, specific probes for the sarcomeric and ubiquitous human Mi-CK isoenzymes only detected minute

amounts of Mi-CK RNA in uterus and testis, tissues known to contain significant CK activities including Mi-CK. Payne et al. [344] assumed that a third Mi-CK isoenzyme is expressed in these tissues. This might indicate, together with the finding that in rainbow trout a CK isoenzyme termed TCK1 is expressed mainly in testis but only faintly in other tissues displaying CK activity [142], that testis has its own particular set of CK isoenzymes.

Since in a few instances contradictory results were published for Mi-CK sequences, the respective references shall be shortly mentioned to avoid confusion in case the original literature is studied. In the cDNA sequence for human ubiquitous Mi-CK presented in Fig. 3 of the article written by Haas et al. [162], the 11 amino-terminal residues were erroneously replaced by the 10 corresponding residues of human 'sarcomeric' Mi-CK, which the authors already knew at that time [162]. Furthermore, 4 of the 11 amino-terminal amino acids determined for human heart Mi-CK [50], 5 of the 120 amino acids specified for chicken ubiquitous Mi-CK [516] and 7 of the 40 amino-terminal amino acids determined for rat sarcomeric Mi-CK [82] by protein-chemical methods do not agree with the respective cDNA sequences [163,344,508]. Finally, Benfield et al. [30] published a partial sequence of a rat CK gene differing from M- and B-CK which probably corresponds, in spite of a variety of differences, to rat ubiquitous Mi-CK [344].

The gene structures of human ubiquitous [162] and sarcomeric Mi-CK [244] differ considerably from those of the cytosolic CK isoenzymes. The former genes span 5.5 and 37 kb, are located on chromosomes 15 [447] and 5 [244] and contain 9 and 11 exons, respectively. The lengths of the coding region exons as well as the locations of the exon junctions are absolutely identical between the two human Mi-CK isoenzymes, but differ considerably from those of the cytosolic CK isoenzymes, indicating that from an evolutionary point of view, a first duplication event of a common primordial CK gene resulted in ancestral mitochondrial and cytosolic CK genes. Only at a later stage, further gene duplications gave rise to the four different CK isoforms known for avian and mammalian species [244]. Very interestingly, exons 1 and 2 of the human sarcomeric Mi-CK are untranslated and followed by an 11- and 7-kb intron, respectively [244]. Since sarcomeric contractile proteins were found to share a similar 5′ organization, with an untranslated first exon followed by a relatively large intron [250], it has been hypothesized that this common feature is responsible for the coordinate transcriptional activation of sarcomeric Mi-CK and contractile proteins [244].

In contrast to M- and B-CKmRNA, Mi-CK mRNAs code, in addition to the native protein, for an amino-terminal mitochondrial target peptide which is proteolytically removed after import into the mitochondria [162,163,196,344,347]. This target peptide is 39 amino acids long, rich in serine, threonine and basic amino-acid residues and has a characteristic tripartite structure [174]. Its amino-terminus (residues 1–16) is positively charged and hydrophilic and likely represents a mitochondrial matrix-targeting signal. Residues 17–32 constitute a stretch of uncharged amino acids which might act as a stop-transfer signal, therefore, directing the protein into the intermembrane space. And finally, residues 33–39 are again positively charged and hydrophilic and are probably responsible for proper cleavage to yield the native enzyme.

Sequence comparisons show that within the native protein, within the mitochondrial targeting peptide, as well as within the 3′ and 5′ untranslated regions, the 'sarcomeric' Mi-CK isoenzymes on one hand and the ubiquitous Mi-CK isoenzymes on the other hand of chicken, man and rat are more closely related to each other than the sarcomeric and ubiquitous Mi-CK isoenzymes of one particular species, indicating that two different Mi-CK isoenzymes already appeared before divergent evolution of birds and mammals occurred. This is in line with the likely presence of two different Mi-CK isoenzymes in frog tissues (see II-D). By the Needleman-Wunsch method, the native sarcomeric Mi-CK isoenzymes display 89–96% amino-acid sequence identity and the ubiquitous isoenzymes 91–96%, whereas within the same species, the two Mi-CK isoenzymes have only an identity of 82–84%. The fact that in rat and man the mitochondrial targeting peptides are much more conserved between the sarcomeric (87%) and the ubiquitous Mi-CK isoenzymes (92%), respectively, as compared to both Mi-CK isoenzymes of the same species (37% in rat, 38% in man), was taken as an argument for tissue-specific mitochondrial import receptors [344]. Furthermore, the marked isoenzyme-specific rather than species-specific conservation of the 3′ untranslated regions indicates an important function of these stretches of DNA for Mi-CK gene expression.

The mitochondrial and cytosolic CK isoenzymes represent a class of highly conserved proteins. When the sequences of the known isoenzymes are compared, one finds six blocks of very high homology separated by seven regions which are less conserved [196]. Among the latter are the amino- and the carboxy-terminus. The highly conserved regions are likely to be involved in essential functions of the enzyme like catalytic activity and dimer formation. For example, affinity labelling of chicken sarcomeric Mi_b-CK with the ATP analogue γ-[4-(*N*-2-chloroethyl-*N*-methylamino)]benzylamide ATP (ClRATP) led to the selective modification of Asp-335, a residue which is conserved in all Mi-CK and the great majority of the cytosolic CK sequences [223]. Since in ClRATP the reactive label is attached to the γ-phosphate group of ATP and on the basis of struc-

tural comparisons with adenylate kinase, Asp-335 was suggested to be involved in the binding of Mg^{2+} ions coordinated to the phosphate groups of ATP. The findings (i) of a partial loss of enzymatic activity of chicken sarcomeric Mi_b-CK when Asp-335 is changed to a Thr-, Asn-, Ala- or Lys-residue by site-directed mutagenesis (Furter, R., unpublished data) and (ii) of a considerable loss of enzymatic activity upon selective cleavage by proteinase K of M-CK between Ala-328 and Ala-329 [271,321] as well as of chicken sarcomeric Mi-CK between Ala-324 and Val-325 (Wyss, M., Schlegel, J. and Wallimann, T., unpublished data) further substantiate that besides the region around the 'essential' Cys-278, stretches around Asp-335 are also important for substrate binding and catalysis.

In contrast, the regions less conserved between the CK isoenzymes might be responsible for isoenzyme-specific properties like octamer formation, binding to cellular membranes, or binding to the myofibrillar M-band. In experiments with purified Mi-CK peptides, for example, Cheneval and Carafoli [82] found only one peptide, corresponding to the 25 amino-terminal residues, which bound to cardiolipin-containing liposomes. Accordingly, the positively charged residues Arg-19, Lys-20 and His-21 have been implicated to mediate the binding of Mi-CK to the negatively charged phospholipids of the mitochondrial membranes [82]. As a matter of fact, these three amino-acid residues are absolutely conserved among all amino-termini known for the 'sarcomeric' and ubiquitous Mi-CK isoenzymes from chicken [196,508,516], man [162,163] and rat [82,344], as well as for mouse brain Mi-CK [344], but not among the cytosolic CK isoenzymes.

III. Functional studies of Mi-Ck

III-A. Species and tissue distribution

Highest enzymatic activities and mRNA levels of Mi-CK are present in tissues with high and fluctuating energy demands like heart [13,50,82,156,167,210,213, 338,344,363,377,396,410,411,441], skeletal muscle [31, 34,173,196,210,281,293,326,344,377,410], brain [53,210, 213,268,344,397,456,504,512,516], retina [41,268,490, 496] and spermatozoa [463–465,489]. Smaller amounts of Mi-CK and Mi-CK mRNA are found in smooth-muscle-containing tissues like uterus [163,344], placenta [344], intestine [41,63,162,206,207,213,239,344], vas deferens as well as aorta [207]. In contrast, no Mi-CK activity was detected in chicken gizzard in spite of its high cytosolic BB-CK activity [207,396]. It has been hypothesized that this difference in Mi-CK content between distinct smooth muscle types is due to the different physiological functions of these muscles [207,492]; whereas most smooth muscles respond to stimulation with very long tonic contractions, carbachol and electric-field stimulation in chicken gizzard elicited only phasic contractions without a tonic component. Maximal force was developed within 10–15 s and almost complete relaxation was accomplished in less than a minute [126]. Therefore, the CK/PCr system in chicken gizzard has to 'buffer' [ATP] which can easily be accomplished by the extraordinarily large amounts of BB-CK present, with no need for an additional mitochondrial isoenzyme. In contrast, 'transport' of PCr and Cr between Mi-CK and cytosolic CK isoenzymes may be a prerequisite for the proper functioning of tonic smooth muscles (see also IV-B).

In contrast to earlier studies [210,213], significant amounts of Mi-CK activity and mRNA were also observed in rat kidney [1,41,137,344], where Mi-CK, together with BB-CK, was found in cortex and outer medulla, probably supporting sodium transport in the distal nephron [137]. Liver was reported to contain either no or only minute amounts of Mi-CK activity and mRNA [41,163,210,213,344,518]. Nevertheless, Kanemitsu et al. [231] succeeded in purifying Mi-CK from human liver and provided some, albeit weak evidence indicating that this isoenzyme differs from human heart Mi-CK. Finally, Mi-CK might become increasingly important as a valuable diagnostic tool since it was also found in certain tumor cells as well as in serum (see III-G). These data show that Mi-CK expression is regulated in a tissue- and cell-type-specific fashion. In all instances, however, Mi-CK is coexpressed with at least one of the cytosolic CK isoenzymes MM-, MB- or BB-CK. As discussed in II-F., it is now clear that at least two different Mi-CK isoenzymes exist and are products of distinct genes. Northern blot analysis revealed that mRNA for the isoenzyme termed sarcomeric Mi-CK is almost exclusively expressed in heart and skeletal muscle [163,196,244,344]. In contrast, mRNA for ubiquitous Mi-CK was mainly found in kidney, placenta, intestine and brain. It seems that sarcomeric Mi-CK is the counterpart of M-CK and ubiquitous Mi-CK that of B-CK.

The relative proportions of Mi-CK vary considerably between different tissues and species. Mi-CK amounts to 0.5–15% of total CK activity in brain [48,245,335, 391,423] and to 10–15% in kidney [137]. Since in fast-twitch skeletal muscle the percentage of Mi-CK is rather low [48,173,294,326,410,434,520], while it is considerably higher in slow-twitch skeletal muscle [210,520], the proportion of Mi-CK can be increased within a particular muscle by endurance training [9] or chronic stimulation [400]. The highest proportions of Mi-CK are found in the heart, where Mi-CK was reported to constitute < 1–59% of total CK activity [4,13,40,43,47,48,103,133,202,204,210,245,273,327,335, 338,350,374,377,378,390,391,410,439,441,459,460,480]. Both the upper and lower limit clearly represent over-

and underestimations due, for example, to incomplete release of Mi-CK from the mitochondrial membranes. The real range may be approx. 10–35%, with the actual value depending on the species and the direction of the CF reaction. Very likely, the proportion of Mi-CK correlates very well with the relative importance of the 'transport function' of the CK/PCr system expected for a given tissue (for discussion, see IV-B). In absolute terms, highest total CK activities are present in skeletal muscle, 2–4-times less in heart and about 10-times less in brain [391]. However, in terms of IU/mg mitochondrial protein, the specific activity of Mi-CK was found to be equal for all Mi-CK-containing tissues of a given species, with a ratio of Mi-CK to ANT molecules of exactly one [265,266]. If these findings were true, changes in the absolute amount of Mi-CK would directly reflect changes in the percentage of whole cellular protein made up by mitochondria. However, a Mi-CK to ANT ratio of 1:1 is unlikely, since other researchers determined the concentration of Mi-CK to be much lower than that of ANT. ANT was reported to make up about 10% of total mitochondrial protein (for a review, see Ref. 248), whereas the relative proportion of Mi-CK is < 1% [355,396,482], maximally 2.5% [265,384].

As far as the evolutionary aspect is concerned, Mi-CK was found in echinoderms [359,463–465], fish [50], amphibians [273,511], birds [34,210,281,396,397,489, 490,516] and mammals (see for example Refs. 50,167,210,213,268,296,363,374). Since the CK/PCr system seems to be most advantageous for energy metabolism of spermatozoa of the primitive type (see IV-B) and, since Cr was detected in sperm of invertebrate species [493], these highly specialized cells appear to be most promising for a search for Mi-CK in phyla 'below' the echinoderms. Mitochondrial arginine kinase (Mi-AK) has been described for crustacean and crab muscles [78,105,106,113] as well as for *Drosophila* [325], whereas most of the molluscs, insects and annelids lack Mi-AK (for a review, see Ref. 113).

III-B. Intracellular localization of Mi-CK: binding to mitochondrial inner and outer membranes

In most studies, Mi-CK was found to be enriched in the mitochondrial fraction [103,210,326,338,410,441] and immunohistochemistry with antibodies directed against Mi-CK also revealed an accumulation of this isoenzyme exclusively within mitochondria [396,489, 490,496]. Nevertheless, CK of cathodic mobility resembling Mi-CK was found in the nuclear fractions of rat, human and pig heart and skeletal muscle [117,228].

Since some mitochondrial subfractionation experiments were performed in phosphate- [246,350,509] or KCl-containing solutions [278,498], these conditions being known to release Mi-CK from the mitochondrial membranes (see II-A), it is not surprising that Mi-CK was suggested to be soluble in the mitochondrial intermembrane space. However, in a number of subsequent studies it was proven, instead, that Mi-CK is bound to the outer surface of the inner mitochondrial membrane. Rotenone, a strong inhibitor of the respiratory chain, inhibited the solubilization of Mi-CK from mitochondrial membranes by phosphate [122]. Subfractionation studies in hypotonic Tris-phosphate, hypotonic Tris-HCl or isotonic sucrose solutions as well as with digitonin revealed that most of the Mi-CK remains associated with the inner membrane/matrix fraction [3,64,104,208,213,409,411]. Furthermore, the fact that Mi-CK activity could not be inhibited by atractyloside, a competitive inhibitor of adenine nucleotide transport across the inner membrane [213,480] and that Mi-CK can be released by phosphate only from mitoplasts, but not from intact mitochondria [130] both demonstrate that Mi-CK is located inside the outer membrane, but on the 'cytoplasmic' side of the ANT. Finally, electron microscopical cytochemistry and immunohistochemistry revealed an accumulation of Mi-CK protein along the inner mitochondrial membrane [13,380,396,421, 496,497].

Recent evidence suggests that Mi-CK is not distributed at random over the inner membrane, but may be accumulated in contact sites (CS) between the inner and outer mitochondrial membrane. CS are thought to play an important role in the mitochondrial production of high-energy phosphates, because they were found to increase in extent and number during oxidative phosphorylation [40,58,68,249]. In subfractionation experiments of rat brain and kidney mitochondrial membranes, Mi-CK was predominantly found, in association with hexokinase I, in a fraction enriched for CS [1,59,256,257]. It is likely that Mi-CK in that fraction binds simultaneously to both membranes and is functionally coupled to the ANT in the inner and to porin in the outer membrane [61] (see III-C). These conclusions are corroborated by the following facts: (i) 40–50% of the total Mi-CK in rat brain mitochondria was not readily accessible to external substrates and could only be released from the membranes by rather high concentrations of digitonin [1,33,257]. (ii) After selective disruption of the outer membrane by digitonin, or in isolated CS fractions, König's polyanion, known to inhibit the transport of adenine nucleotides through porin [32], inhibited Mi-CK activity by 50% [1,257]. (iii) In inhibition experiments on intact or digitonin-treated mitochondria, 50% of the Mi-CK was not accessible to externally added PCr and iodoacetate, the latter substance being known efficiently to inhibit purified Mi-CK. A reasonable explanation for these findings is that about half of the total Mi-CK is buried between the two mitochondrial membranes in the CS, where porin is thought to be in its cation-selective state, thus,

creating a microcompartment that is fairly inaccessible to externally added anions [257].

The localization of Mi-CK within the CS of intact mitochondria in situ was further corroborated by electron microscopical immunohistochemistry showing an accumulation of gold particles at places where inner and outer membranes approach each other [396,496], and by electron microscopical cytochemistry displaying a CK-dependent trapping of stain within CS of rat heart, brain, retina photoreceptor cells, skeletal muscle and kidney [40,41]. Dinitrophenol and amytal, known to reduce the number of CS [58], also diminished the proportion of Mi-CK-derived stain within CS [40,41].

The ability of Mi-CK to simultaneously interact with two different membranes was directly investigated in an in vitro system generated (i) by a monolayer of outer membrane lipids or spread outer membranes, and (ii) by addition to the subphase of radioactively-labelled large unilamellar vesicles (LUVs) of inner membrane lipids or inner membranes, respectively. Mi-CK proved to be highly efficient in inducing close contacts between the monolayer and the vesicles, the octameric form being more potent than the dimeric one [333,370]. In contrast to Mi-CK, polylysines not only mediated contacts between LUVs and monolayer, but in addition caused membrane fusion between them, suggesting that the rather large side-length of the Mi-CK octamer is an essential prerequisite to avoid fusion between two opposing membranes. These results, however, do not imply that Mi-CK is an essential prerequisite for the formation of membrane contacts, since CS were also found in rat liver mitochondria known to be devoid of Mi-CK [68,249]. Instead, they clearly indicate that the Mi-CK octamer is a structure ideally suited for a CS localization and possibly also for a stabilization of such contacts. Electron micrographs of Mi-CK are fully in line with this interpretation, displaying two identical top and bottom faces for the octameric molecule and, therefore, suggesting that both faces have the same potential to interact with membranes (see II-C).

Once it was established that Mi-CK interacts with mitochondrial membranes, the biochemical nature of the 'receptor' for Mi-CK binding was investigated. Because most Mi-CK isoenzymes can be released from the mitochondrial membranes by a variety of different salts in a ionic strength-dependent manner (see also II-A), a mainly electrostatic interaction of positively charged Mi-CK with negatively charged phospholipids of the membranes was assumed [64,278]. Cardiolipin seems to be the most likely candidate, since (i) experiments on artificial liposomes composed of defined mixtures of phospholipids demonstrated that Mi-CK preferentially binds to cardiolipin [324,369], (ii) the binding of Mi-CK to cardiolipin-containing liposomes, aqueous dispersions of cardiolipin, as well as to heart and liver mitoplasts was inhibited by adriamycin [64,83,324,331], a clinically useful anticancer drug which was suggested to specifically interact with cardiolipin [80], (iii) treatment of rat heart mitochondria with phospholipase A_2, but not with phospholipase C led to the release of Mi-CK, therefore, also indicating that the interaction is critically dependent on cardiolipin [395], (iv) both inner and outer mitochondrial membranes, but not other cellular membranes, contain cardiolipin [197] and (v) Mi-CK (see above) as well as cardiolipin [10] are highly accumulated in CS. In experiments with cyanogen bromide-digested rat heart Mi-CK, only one peptide representing the 25 amino-terminal residues bound to cardiolipin-containing liposomes, whereby this interaction was also inhibited by adriamycin [82]. In addition, chemical modification of arginine and lysine residues of Mi-CK drastically reduced the binding of the enzyme to the liposomes. Therefore, it was suggested that the positively charged residues Arg-19, Lys-20 and His-21 are responsible for the specific binding of Mi-CK to cardiolipin [82,344]. In fact, these three residues are absolutely conserved among all known Mi-CK sequences (see II-F). However, it should be kept in mind that the interaction of Mi-CK with cardiolipin might not be as specific as originally thought. First, Mi-CK proved to bind also to other negatively charged phospholipids like phosphatidylserine or phosphatidylinositol [81,369] as well as to monolayers of spread microsomal membranes [369] known to be virtually devoid of cardiolipin [94]. Furthermore, two different classes of Mi-CK binding sites were found on mitoplasts, one with high and the other with low affinity [285,395]. The number of high-affinity binding sites approximately equalled the amount of Mi-CK in heart mitochondria [395], but in total, 2–14-times this amount could be rebound to heart and liver mitoplasts [64,131,168,324,395]. Therefore, cardiolipin may constitute the low-affinity binding sites. The isoelectric point of octameric and dimeric Mi-CK may be a last argument for a ionic interaction of this isoenzyme with the mitochondrial membranes [354]. Mi-CK octamers have a higher isoelectric point than the corresponding dimers (see II-D) and, as a matter of fact, octameric Mi-CK was found to be the exclusive or predominant oligomeric form being bound to the mitochondrial membranes (see III-D).

Whereas Cheneval et al. [83] obtained no evidence for penetration of Mi-CK into a membrane bilayer, monolayer experiments with spread mitochondrial membranes or membrane lipids clearly showed an increase in surface pressure upon binding of chicken sarcomeric Mi_b-CK, suggesting that Mi-CK penetrates into the hydrophobic domain, at least under the experimental conditions used [369,370]. This phenomenon can be ascribed to the hydrophobic nature of Mi-CK (see II-A) and is also in agreement with experiments on the extraction of Mi-CK from chicken heart mito-

plasts which were indicative for a small nonpolar effect [64]. Furthermore, detergents had to be used for an optimal release of Mi-CK from rat heart [395], human skeletal muscle [434] and sea-urchin-sperm mitochondria [463,464,517].

As an alternative to cardiolipin, ANT may be suggested to act as a 'receptor' protein for Mi-CK [376,380], since a variety of studies revealed a functional coupling between Mi-CK and ANT of the inner membrane (see III-C). However, incubation of Mi-CK with ANT-containing liposomes [324] as well as cross-linking experiments [406] failed so far to demonstrate such a relationship. In addition, electrostatic repulsion of the two very basic proteins was taken as an argument against a direct interaction between Mi-CK and ANT [278]. However, a specific interaction of certain domains of both proteins can not be ruled out by the mere fact that both proteins have an overall positive charge.

In conclusion, Mi-CK is primarily attached to the outer surface of the inner mitochondrial membrane, but, within CS, additionally interacts with the outer membrane as well. Binding of Mi-CK molecules exclusively to the outer membrane was never observed in situ. The interaction of Mi-CK octamers with the mitochondrial membranes seems to be mostly due to an electrostatic interaction of positively charged Mi-CK with negatively charged phospholipids like cardiolipin, but hydrophobic interactions, as well as the existence of a specific receptor (protein), can not yet be ruled out. The question of whether the release of Mi-CK from the membranes into the intermembrane space by phosphate ions or other effector molecules is of physiological importance remains to be elucidated. Rojo et al. [370] recently showed that Mi-CK octamers and, to a much lesser extent, Mi-CK dimers are able to mediate contact formation between two mitochondrial membranes. This data, together with the finding of functional coupling between Mi-CK and ANT of the inner, as well as porin of the outer mitochondrial membrane, provoked alternative ideas about the functional involvement of Mi-CK in CS, as will be discussed in the next Section.

III-C. Functional coupling

Besides the still existing controversy in assigning an exclusive 'buffer' or 'transport function' to the CK/PCr system (see IV-B), the question of how PCr production is coupled to mitochondrial oxidative phosphorylation is also heavily disputed. Therefore, this topic will be discussed here in more detail. Already in 1911, Thunberg [461] realized that addition of Cr to a respiring muscle homogenate results in increased oxygen uptake. In isolated respiring mitochondria from cardiac and skeletal muscle, as well as brain, Cr together with catalytic amounts of ADP or ATP also stimulates oxygen consumption [34,122,153,213,319,377,381,439,480, 521]. This is due to Mi-CK continuously regenerating ADP from ATP, produced by oxidative phosphorylation, and Cr. The ADP, in turn, further stimulates oxygen consumption. The stimulatory effect is dependent on the concentration of Cr, with Cr-stimulated respiration approaching the state-3 rate [34,213]. Under the conditions described, PCr is the net product of oxidative phosphorylation [54,213,319,374,377]. Most importantly, the Mi-CK content of heart mitochondria is sufficient to transphosphorylate all of the ATP produced by oxidative phosphorylation to PCr at all rates of respiration [213,374,381,480].

A large number of subsequent studies demonstrated that on one hand, Mi-CK has 'privileged access' to ATP, produced in the mitochondrial matrix by oxidative phosphorylation, over externally added (extramitochondrial) ATP and that on the other hand, the ANT preferentially transports ADP liberated by Mi-CK over externally added ADP. In other words, Mi-CK and oxidative phosphorylation seem to be functionally coupled in some way or another. The major evidence supporting this view shall now be summarized:

(a) For PCr production, the K_m value for ATP of intact mitochondria [119,120,216,380,381] or mitoplasts [384] is significantly lower when ATP is produced within the matrix by oxidative phosphorylation, than when ATP is added externally. Due to this decreased K_m value for ATP, PCr synthesis by intact, respiring mitochondria, compared to rotenone- plus oligomycin-inhibited mitochondria with externally added ATP, is less susceptible to product inhibition by PCr [216,381]. In other words, at identical ATP and Cr concentrations in the surrounding medium, the rate of PCr production is much higher when oxidative phosphorylation is activated compared to when it is inhibited [375,376,381] and, therefore, is also much higher than calculated from the substrate concentrations in the medium and the kinetic constants of Mi-CK [375].

(b) Similarly, ADP produced in the intermembrane space by Mi-CK proved to be much more potent (lower K_m value) in stimulating oxidative phosphorylation than ADP which was externally added or produced by hexokinase [65,218,220,320]. Accordingly, much less atractyloside/mg mitochondrial protein was needed to suppress respiratory stimulation by externally added ADP than for stimulation by ADP produced by Mi-CK [320,384]. Furthermore, the uptake of ^{3}H-labelled ADP or ATP into respiring mitochondria was found to be high in the absence, but much lower in the presence of Cr or PCr, respectively, thus suggesting as well that ADP or ATP produced by Mi-CK has preferential access to the ANT over externally added ADP or ATP [20,266,385].

(c) Externally added phospho*enol*pyruvate (PEP) plus pyruvate kinase (PK) fully suppressed respiratory stimulation when ADP was produced in the extramitochondrial compartment by hexokinase, but only partially diminished the stimulatory effect of ADP produced in the intermembrane compartment by Mi-CK [145,147,148,257,384,386].

(d) After short incubations of respiring rabbit heart mitochondria with [γ-^{32}P]ATP and Cr, the specific radioactivity of PCr was only 36% that of ATP [522]. However, if mitochondrial respiration was uncoupled by carbonylcyanide *m*-chlorophenyl hydrazone or inhibited by atractyloside, the specific radioactivities of PCr and ATP were the same. These findings indicate that intra- and extramitochondrial ATP do not readily equilibrate and that Mi-CK has preferential access to intramitochondrial ATP. The same conclusions were drawn from similar labelling experiments with [γ-^{32}P]ATP or [^{33}P]phosphate [118–120].

(e) Under identical experimental conditions, intact rat heart mitochondria produced 2.3-times the amount of PCr as rat liver mitochondria containing no Mi-CK themselves, but to which the same quantity of Mi-CK had been added externally [380].

(f) Whereas all experiments described so far deal with kinetic effects, a thermodynamic approach was chosen by DeFuria et al. [98] and Saks et al. [384,386] to shed more light on Mi-CK function. Normally, the mass-action ratio of the CK substrates, $\Gamma = [MgADP^-][PCr^{2-}][H^+]/[MgATP^{2-}][Cr]$, relative to the equilibrium constant K, determines in which direction the reaction will proceed. An approximation to the equilibrium state of the reaction is reflected by a diminuition of the difference between Γ and K. Whereas partially purified Mi-CK or Mi-CK in inhibited, non-respiring mitochondria obeyed this thermodynamic law, PCr production in respiring mitochondria or mitoplasts still proceeded even when $\Gamma > K$.

Although some experiments described above have been criticized and conflicting reports have been presented [4,5,54,55,279], it is now generally accepted that a functional coupling between Mi-CK and oxidative phosphorylation indeed exists. Two alternative explanations have mainly been proposed as basis for this coupling. On one hand, Mi-CK may be bound to the inner membrane in close proximity to or directly associated with the ANT, thus creating a microenvironment enabling efficient metabolic channelling of substrates between the catalytic sites of both enzymes [20,145,218,220,266,267,320,375,376,380–382,384–387]. On the other hand, the outer mitochondrial membrane might act as a diffusion barrier for adenine nucleotides, leading to concentration gradients over the outer membrane [38,65,98,118,119,147,148,218,220]. In this latter case, substrate concentrations in the surrounding medium would not reflect those experienced in the intermembrane space.

Whereas most of the arguments mentioned above (a–f) are compatible with both ideas, several experiments favour a direct interaction between Mi-CK and ANT. Phosphate and high concentrations of chloride ions are known to release Mi-CK from mitoplasts (see II-A) and can, therefore, also be assumed to detach Mi-CK from the membranes in intact mitochondria. Incubation of intact mitochondria in phosphate- or chloride-containing media significantly raised the K_m values of Mi-CK for ADP and ATP [474] and drastically reduced the functional coupling between Mi-CK and oxidative phosphorylation [169,170,267,387], suggesting that the binding of Mi-CK to the membranes and possibly to ANT is mainly responsible for the kinetic coupling. The observation of functional coupling not only in intact mitochondria, but also in mitoplasts, supports this view [384,386]. Furthermore, in rat heart mitoplasts, rabbit anti-rat Mi-CK antibodies affected neither Mi-CK nor ANT activity, whereas chicken anti-rat Mi-CK antibodies inhibited Mi-CK and ANT activity, as well as the rate of oxidative phosphorylation, approximately to the same extent [266,382,385]. Removal of Mi-CK from the mitoplast membranes by 150 mM KCl plus 20 mM ADP completely abolished these inhibitory effects, thus suggesting an intimate functional and structural interaction of Mi-CK and ANT, with the catalytic sites of both enzymes lying side by side.

In contrast, the ANT inhibitors atractyloside and carboxyatractyloside had no effect on the kinetic properties of Mi-CK in non-respiring mitochondria, arguing that the active sites of Mi-CK and ANT do not simply overlap [146,474]. Instead, the outer mitochondrial membrane as a diffusion barrier for adenine nucleotides was suggested to be responsible for the functional coupling on the basis of the following experiments:

(a) Labelling studies with [^{33}P]phosphate demonstrated that selective damage or removal of the outer mitochondrial membrane by digitonin abolishes the functional coupling between Mi-CK and oxidative phosphorylation [119]. Furthermore, approximately equal K_m values of Mi-CK for ATP were found for respiring, digitonin-treated mitochondria, for atractyloside-inhibited, digitonin-treated mitochondria, as well as for respiring intact mitochondria, whereas the corresponding value for atractyloside-inhibited, intact mitochondria was significantly higher. (b) Under conditions where 70% of Mi-CK was membrane-bound, the K_m value of Mi-CK for ATP in respiring, as well as in oligomycin-inhibited mitoplasts was indistinguishable from the corresponding value of the soluble enzyme, while it was significantly lower in respiring and much

higher in oligomycin-treated intact mitochondria [65]. (c) As already mentioned above, externally added pyruvate kinase (PK) plus phospho*enol*pyruvate (PEP) fully suppressed the respiratory stimulation of ADP produced by hexokinase [145,147,148,386]. However, if ADP was liberated either by membrane-bound Mi-CK or by the intermembrane space protein adenylate kinase, PK/PEP in both cases inhibited the respiratory stimulation by ADP only partially and approximately to the same extent, indicating that diffusion limitation by the outer membrane is more likely to be responsible for the functional coupling of Mi-CK and ANT than interaction of Mi-CK with the inner membrane [148].

Finally, membrane energization, alkalinization near the mitochondrial membranes, as well as unstirred layer effects were also taken into consideration to explain the functional coupling, but most of these possibilities have been dismissed as sole explanation for the observed phenomena, although in combination with each other they may play an important role [147,169,218,220,320,381,474]. To conclude, the experiments performed so far do not allow a definite answer to be given on the true basis for the functional coupling between Mi-CK and oxidative phosphorylation. In fact, depending on the metabolic state, one, two or all of the mechanisms mentioned above may be operational at the same time.

However, many recent experiments argue for a third explanation of the functional coupling, that of microcompartmentation and metabolite channelling within the CS. Four new aspects have opened the door to this fascinating, alternative explanation (Fig. 2). First, porin of the outer mitochondrial membrane was recognized as a voltage-dependent channel for polar metabolites and is, therefore, also known as voltage-dependent anion-selective channel (VDAC) [87]. Below a membrane potential of 30 mV, porin is anion-selective, whereas above 30 mV it adopts a different state which is characterized by a lower conductance and cation-selectivity [125,135,291,371]. In this latter state, ADP and ATP are excluded from transport by porin [32,33,59,61]. It was assumed that within the CS between the mitochondrial inner and outer membranes, the cation-selective state of porin is induced by 'capacitive coupling' to the membrane potential of the inner membrane. Beyond the CS, where the distance between inner and outer membrane would be too large to allow capacitive coupling, porin is thought to be in its anion-selective state [33,61]. Second, Mi-CK was found to be enriched in mitochondrial CS where its activity is 'latent' [1,59,257] (see also III-B). In other words, Mi-CK within the CS is not readily accessible to externally added substrates. Furthermore, König's polyanion, known to block porin, reversibly inhibited the activity of Mi-CK within the CS by cessation of the adenine nucleotide diffusion through the outer membrane [257]. And finally, Mi-CK within the CS was neither accessible to product inhibition by the negatively charged substrate PCr nor to irreversible inhibition by negatively charged iodoacetate [257]. Third, structural analysis of Mi-CK revealed an intriguing octameric structure with two identical top and bottom faces and either central indentations in these two faces or a channel through the molecule (see II-C). And fourth, Mi-CK was found to be able to interact simultaneously with two different membranes probably due to its identical top and bottom faces [370].

In the two alternative models depicted in Fig. 2, all of these findings were taken into account. Within the CS, Mi-CK is buried between the two mitochondrial membranes where it is functionally (and structurally?) coupled to ANT of the inner and porin of the outer mitochondrial membrane. It may even be hypothesized that octameric Mi-CK, tetrameric ANT [484], oligomeric (tetrameric?) porin [259,276] and possibly also tetrameric hexokinase [519] form a highly-ordered multi-enzyme complex allowing efficient substrate channelling. In the CS, Mi-CK has preferential access to ATP produced by oxidative phosphorylation within the mitochondrial matrix. According to the first model [61,492] (Fig. 2A), Cr is 'presented' to Mi-CK by porin in its cation-selective state. Mi-CK activity liberates ADP, which is preferentially and directly re-taken up into the matrix, as well as PCr, which as the net product of oxidative phosphorylation leaves the mitochondria beyond the CS through porin in its anion-selective state. This model has the disadvantage that newly synthesized PCr would equilibrate with the substrate pools in the remaining intermembrane space before its export out of the mitochondrion. Accordingly, it seems unlikely that the cytosolic phosphorylation potential in this case could be sensed by the mitochondria, either within or beyond the CS.

This problem is circumvented by the second model (Fig. 2B). ANT, Mi-CK and porin form a contiguous channel spanning both mitochondrial membranes. PCr is produced within the channel and is directly exported to the cytosol where it rapidly equilibrates with the cytosolic PCr/Cr and ATP/ADP pools through the action of the cytosolic CK isoenzymes which are thought to be in a near equilibrium state [205,262,298,310]. PCr production even at a high cytosolic phosphorylation potential may be accomplished by 'active' expulsion of PCr from the channel possibly due to electrostatic repulsion. On the other hand, the cytosolic phosphorylation potential could be sensed by the mitochondria in regions beyond the CS. Clearly, elucidation of the structural and functional involvement of octameric Mi-CK in the CS will be an exciting task for future studies and may establish the existence of a multi-enzyme complex suited for metabolic channelling [442,443].

Independent of both models, Mi-CK is not re-

stricted to the CS, as was shown by in situ localization studies [396,496,497] and biochemical experiments. In rat brain mitochondria, only 50% of the total Mi-CK activity was 'latent' and not susceptible to inhibition by PCr or iodoacetate [1,257]. Furthermore, since CS are dynamic structures changing in number and extent depending on the metabolic state of the mitochondria (see III-B), the proportion of Mi-CK within the CS is also supposed to correlate with the respiratory rate. Accordingly, cipher 1 in Fig. 2 indicates the dynamic equilibrium between the two Mi-CK localizations 'in' and 'beyond' the CS. Whereas it is highly likely that exclusively octameric Mi-CK is present in the CS [257,370], reversible changes in the dimer to octamer ratio (ciphers 2 and 4), as well as dissociation and reassociation of Mi-CK from and to the inner membrane (ciphers 3 and 5) may be important regulatory parameters beyond the CS [399] (see also III-D). Usually, dissociation of Mi-CK octamers into dimers is accompanied by the release of Mi-CK from the membranes and vice versa [296,399]. In turn, release of Mi-CK from the inner membrane may abolish the functional coupling between Mi-CK and ANT, thus, potentially diminishing the overall Mi-CK reaction rate. Finally, Mi-CK dimers, when bound to the inner membrane, reassociate to octamers, a process which is probably facilitated by lateral diffusion of the dimers on the membrane [399].

The involvement of Mi-CK in a specialized structure like the CS is highly indicative for a metabolic advantage of this location. The facts that Mi-CK within the CS is 'buried', that it is protected from inhibition by PCr and iodoacetate and that its activity is 'latent' strongly suggest that CS represent a special microcompartment with substrate concentrations differing from those in the remaining intermembrane space, as well as in the extramitochondrial compartment(s), an assumption that has been corroborated by experiments on hexokinase and glycerol kinase bound to mitochondrial CS (Refs. 36,58,155,161; for reviews, see Refs. 1,60,61). In liver mitochondria, for example, hexokinase and adenylate kinase usually share the same adenine nucleotide pool. However, 5 μM Ca^{2+}, known to increase the number of CS, resulted in metabolic separation of the two enzymes [58]. Interestingly, in intact respiring rabbit heart mitochondria containing both hexokinase, bound within the CS at the outer surface of the outer

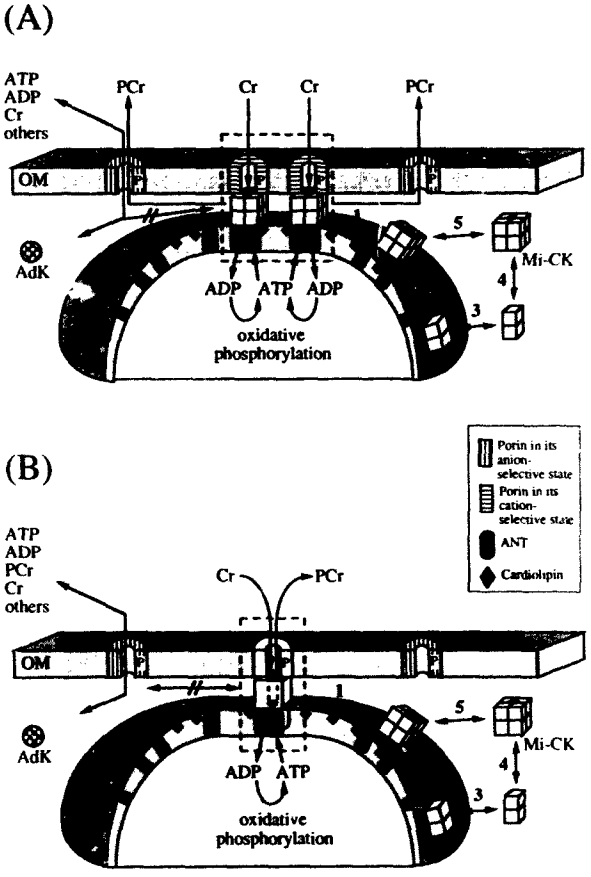

Fig. 2. Structure and function of Mi-CK within the mitochondrial intermembrane space with special reference to the contact sites. Within CS (surrounded by a dashed line), exclusively octameric Mi-CK interacts with both the inner (IM) and the outer mitochondrial membrane (OM) and is thought to be functionally coupled to ANT of the inner and porin (P) of the outer membrane. Below the contacts, Mi-CK may be dimeric or octameric and it may be either bound to the inner membrane or free in the intermembrane space. In contrast to Mi-CK, adenylate kinase (AdK) is not firmly bound to the membranes. In both models, it is schematically indicated that two different microcompartments for CK substrates exist within and beyond the CS. Due to this micro-compartmentation, PCr production by Mi-CK may still proceed even at a high cytosolic ATP/ADP ratio. Note that by the schematic representation it is not intended to suggest that Mi-CK is only active within the CS. Furthermore, it should not be inferred that below the CS, the two mitochondrial membranes are far from each other. In fact, electron microscopic analysis of in situ mitochondria revealed that the mitochondrial inner and outer membrane form a 5 layered structure with a width of only 12 nm, whereas a 7-layered structure would be expected for two separated membranes [295]. (A) Within CS, porin is in its cation-selective state and, therefore, only permeable to Cr. Porin beyond the CS is in its anion-selective state and, thus, allows diffusion of ATP, ADP and PCr through the outer membrane. (B) Tetrameric ANT, octameric Mi-CK and (oligomeric) porin form a highly organized multi-enzyme complex and thereby create a microcompartment allowing efficient substrate channelling between the three enzymes. PCr is produced within the central channel of the octameric Mi-CK molecule and is directly pulled out of the mitochondria by electrostatic repulsion through the superimposed porin molecule. For simplicity, the subunit 'boundaries' were omitted in the Mi-CK octamer localized within the CS. For further details, see the text.

membrane to porin, as well as Mi-CK, the formation of glucose 6-phosphate was depressed by creatine at a low ATP/ADP ratio. However, glucose had no effect on PCr production, indicating that Mi-CK has preferential access over mitochondrial hexokinase to ATP produced in the matrix [55]. Accordingly, even within the CS, rapid equilibration of substrate concentrations does not occur.

The assumption of a separate microcompartment for adenine nucleotides within the CS (see also IV-C) has dramatic consequences for total cellular energy metabolism. First, it may explain why the cytosolic ATP/ADP ratio is considerably lower in liver than in muscle [179,247,440]. In liver lacking Mi-CK, the inner membrane potential seems to be the primary determinant of the cytosolic ATP/ADP ratio. In muscle, however, the functional coupling of Mi-CK and ANT within the CS, the particular substrate concentrations experienced in this microcompartment and the vectorial export of PCr out of the mitochondria before equilibration with the substrate concentrations beyond the CS may allow the build-up of a higher ATP/ADP ratio and therefore also of a higher phosphorylation potential in the cytosol. Second, the localization of Mi-CK, hexokinase, glycerol kinase and nucleoside diphosphate kinase within the CS [1,2,59] seems to be especially relevant, since all of them, due to their involvement in the regulation of the energy metabolism and/or in the export of high-energy phosphates out of the mitochondria, influence the cytosolic phosphorylation potential. In contrast, adenylate kinase, thought to protect the cell from an accumulation of ADP [492], is not found within the CS and is not or only partially bound to mitochondrial membranes. And third, microcompartmentation within the CS may account for the finding that mitochondrial PCr production still proceeds even if, on the basis of total (intra- and extramitochondrial) substrate concentrations, the opposite CK reaction direction is favoured [98,384,386].

To conclude, it is now largely accepted that functional compartmentation of Mi-CK within the mitochondrial intermembrane space is an essential prerequisite for efficient mitochondrial PCr production. However, it is not yet completely clear whether this compartmentation is due only to an interaction of Mi-CK with ANT, to diffusion limitations by the outer mitochondrial membrane, to both of these effects, or to micro-compartmentation and metabolic channelling within the CS. A variety of recent studies favour the latter hypothesis. Consequently, in any description or mathematical modelling of mitochondrial PCr production in particular and of total cellular energy metabolism in general, the particular substrate concentrations experienced in the CS, together with the fact that the substrate concentrations within the CS are not in equilibrium with those of the cytosol, have to be taken into account. The same holds true for the interpretation of in vivo ^{31}P-NMR measurements.

III-D. Dynamic regulation of the octamer to dimer ratio

Before anything was known about the molecular size of Mi-CK, two distinct cathodically migrating bands with Mi-CK activity were often observed using cellulose-acetate, agarose- and acrylamide gel-electrophoresis at pH 8.0–8.8 [166,222,268,374,502]. These two bands were readily interconvertible, therefore indicating that they were not due to different Mi-CK isoenzymes [166]. Instead, they could be assigned to two distinct oligomeric forms of the same isoenzyme [100,167,504]. The band moving faster to the cathode corresponds to octameric Mi-CK with a M_r of approx. 350000, and the more slowly migrating band represents dimeric Mi-CK with a M_r of approx. 85 000 [231,296,516]. The existence of two different, readily interconvertible oligomeric forms was subsequently corroborated by several techniques (see II-B). In only two experiments, acrylamide gel-electrophoresis [100] and isoelectric focussing of bovine heart Mi-CK [282], additional Mi-CK bands were observed which were interpreted as tetrameric, hexameric and/or aggregated Mi-CK. However, in the light of the uncertainties inherent in the methods employed and the overwhelming body of evidence demonstrating no intermediate forms between dimers and octamers, these findings have to be seriously questioned.

Once it was realized that the two distinct oligomeric forms of Mi-CK might be of physiological importance, several groups searched for factors influencing the dimer to octamer ratio in vitro. This ratio is not influenced by the temperature (5–20°C) and the nature of the monovalent anions (chloride, nitrate or acetate) or cations (Na^+ or K^+) in the buffer used at ionic strengths between 0.02 and 0.25 M [280,281]. In contrast, it is strongly dependent on the Mi-CK concentration itself. The proportion of octamers rises as the Mi-CK concentration is increased [26,100,123,166,167, 287,396,397,516]. The dimer to octamer ratio also depends on the pH value of the medium, with dissociation into dimers being more pronounced at alkaline pH values [280–282,296,396,483]. Furthermore, partial or complete dissociation of octamers into dimers can be achieved by exceedingly high concentrations of 2-mercaptoethanol [100,166,167,448,502,504], freezing and thawing [281,396], low ionic strength conditions [281, 282], 1 M KCl [296], *p*-hydroxymercuribenzoate (Wyss, M. and Wallimann, T., unpublished data) and 1–8 M urea [26,123,156,231,296,403]. The mode of action of 2-mercaptoethanol is unclear, since several weak and strong oxidizing agents did not reverse its effect [502]. Upon incubation of Mi-CK octamers with urea, dimers are first formed and only on a much slower time-scale,

or at higher urea concentrations, monomers also appear [26,403], indicating that the intersubunit interactions are much stronger within a dimer than between adjacent dimers within an octamer (see also II-C).

Single CK substrates and 'unproductive' substrate combinations on one hand were reported not to influence the dimer to octamer ratio [280,281,296,396,483]. On the other hand, 4 mM MgADP or MgATP led to partial dissociation of octameric chicken ubiquitous Mi_a-CK into dimers [398], suggesting that the stability of octamers differs between Mi-CK isoenzymes. In contrast, equilibrium substrate combinations (MgADP + MgATP + Cr + PCr) and formation of a transition-state-analogue complex of Mi-CK (Mi-CK + MgADP + Cr + nitrate) in all instances resulted in fast dissociation of the octamers [280–282,287,296,396,399,483, 516]. However, even in the presence of MgADP, Cr and nitrate, the dimer to octamer ratio still depended on the Mi-CK concentration, but at any given concentration, the proportion of octamers was lower than in the absence of substrates [516]. At last, Lipskaya and co-workers [280,281] concluded from a limited set of data that at physiological concentrations of ATP and ADP, the dimer to octamer ratio of bovine heart and pigeon breast muscle Mi-CK correlates with the Cr/PCr ratio. This finding may be explained by the fact that with changes in the Cr/PCr ratio, the probability that Mi-CK simultaneously binds two complementary substrates (ADP + PCr or ATP + Cr) also varies. Simultaneous binding of two complementary substrates in its turn is expected to have the same effect as formation of a transition-state-analogue complex of Mi-CK, namely to dissociate octamers into dimers.

As far as the kinetic aspect is concerned, the new equilibrium state between dimers and octamers after formation of a transition-state-analogue complex of Mi-CK is already reached after 15 min [296,396,483], whereas it is approached very slowly in the absence of substrates [281,396]. Such a time-scale (minutes) is clearly sufficient to envisage a physiological role for the dimer to octamer interconversions of Mi-CK (see III-C, and Fig. 2). Compared to all these findings, virtually no factors stabilizing octamers are known. *p*-Aminobenzamidine was reported to inhibit octamer dissociation induced by formation of a transition state-analogue complex of Mi-CK [296]. Furthermore, *p*-aminobenzamidine, as well as benzamidine, result in partial reassociation of dimers into octamers [396]. Finally, inorganic phosphate leads to an increased proportion of octamers as well, but only in the presence of CK substrates [280].

Now that in vitro some factors influencing the dimer to octamer ratio are known, the questions of whether changes in the oligomeric state of Mi-CK also occur in vivo and if they have any physiological bearing have yet to be answered. Radiation inactivation [353,483] and cross-linking experiments [284] revealed that the Mi-CK octamer very likely is the only oligomeric form bound to intact bovine and rabbit heart mitochondria. This was further corroborated with antibodies against purified pig- and rabbit-heart Mi-CK which allowed the discrimination between dimeric and octameric Mi-CK [355,483]. In extraction experiments, the vast majority of Mi-CK was released from the mitochondrial membranes in its octameric form, independent of the releasing agent used [284,296,399,483]. Dissociation into dimers, if it happened, only occurred in solution and on a slower time-scale than the release of Mi-CK from the membranes. Whereas exclusively octameric Mi-CK could be rebound to pig- and rabbit-heart mitoplasts [296,483], both dimers and octamers rebound to chicken-heart mitoplasts [399]. The rebinding of both oligomeric forms was strongly pH-dependent and sharply decreased between pH 7.5 and 8.1 for dimeric Mi-CK, but only above pH 8.1 for octameric Mi-CK. Therefore, Mi-CK octamers rebound preferentially over dimers to mitoplasts at intermediate pH values around 8.0. Since octamers have a higher isoelectric point than dimers (see II-D), and since the interaction of Mi-CK with membranes seems to be mostly ionic in nature (see III-B), octameric rather than dimeric Mi-CK is indeed expected to bind more strongly to negatively charged groups of the membranes [354,399]. Finally, Mi-CK dimers, once bound to the mitochondrial membranes, partially reassociate into octamers [399], thus emphasizing that octameric Mi-CK may be favoured even more so under in vivo conditions.

The above findings were further extended by differential digitonin extraction of Mi-CK from rat brain mitochondria [257]. Mostly octameric Mi-CK was present in intact mitochondria, and dimeric Mi-CK could be released from the membranes more easily than octameric. Most importantly, exclusively octameric Mi-CK seems to be enriched in CS between mitochondrial inner and outer membranes where it is thought to play an essential role in mitochondrial PCr production (see III-C). Octamers, in contrast to dimers, appear to be ideally suited for a CS localization. Due to their identical top and bottom faces, they can simultaneously interact with two opposing membranes, as was directly shown in in vitro experiments where octameric proved to be much more potent than dimeric Mi-CK in inducing close contacts between a spread membrane monolayer and large unilamellar membrane vesicles [370]. To conclude, the octameric form of Mi-CK seems to be an indispensable prerequisite for the efficient functional coupling between Mi-CK and oxidative phosphorylation (see also III-C).

The existence and physiological importance of dimeric Mi-CK in vivo may be questioned by the fact that the Mi-CK concentration in the intermembrane

space was estimated to be in the range of 3.5–17.2 mg/ml [280,396]. At these same concentrations in vitro, Mi-CK is predominantly octameric, even under conditions most strongly favouring dissociation into dimers, namely formation of a transition-state-analogue complex [516]. Thus, one is inevitably led to the question of whether dimeric Mi-CK represents only a test-tube 'artifact' or not. The possibility of its involvement in the regulation of mitochondrial energy metabolism should, however, not yet be dismissed, since (i) interaction of Mi-CK octamers and dimers with mitochondrial membranes, as well as the octamer to dimer ratio are sensitive to physiologically important parameters like pH and the concentration of Mi-CK substrates; (ii) a small, but significant fraction of Mi-CK is always released in its dimeric form from intact mitochondria; and (iii) Mi-CK isoenzymes from different species and tissues form octamers varying in stability. E.g., pig heart and chicken ubiquitous Mi-CK dissociate much more readily into dimers than rabbit-heart and chicken sarcomeric Mi-CK, respectively [296,397,483,516].

Alternatively, different conformations of Mi-CK rather than two oligomeric forms may be envisaged as a decisive factor for the regulation of mitochondrial energy metabolism. Changes in the octamer to dimer ratio may simply reflect changes in conformation, with octamers being more stable in one conformation rather than the other(s) [281,296,398]. This view is supported by a variety of experiments (fluorescence- and EPR spectroscopy, inhibition and crystallization experiments, etc), suggesting that CK isoenzymes undergo conformational changes upon substrate binding [39, 238,405,506]. Furthermore, conformational changes might also explain why the effects of some agents on the dimer to octamer ratio and on the release of Mi-CK from the mitochondrial membranes parallel each other. In fact, MgADP, formation of a transition-state-analogue complex, high chloride concentrations, as well as *p*-hydroxymercuribenzoate, all efficiently release Mi-CK from the inner mitochondrial membrane and, in addition, dissociate octamers into dimers (Refs. 281,296 and Wyss, M., Schlegel, J. and Wallimann, T., unpublished data) (see also II-A). However, this parallelism may not be that strict, since in the case of rabbit-heart Mi-CK, *p*-aminobenzamidine only inhibited the dissociation of Mi-CK octamers into dimers, but not its release from the mitochondrial membranes [296].

In dimeric bovine heart Mi-CK, the 'essential' sulphydryl groups of both subunits react readily and with the same rate with alkylating agents [123]. Additionally, both subunits of rabbit MM-CK bind substrates [124]. Conversely, only half of the subunits of octameric bovine heart Mi-CK bind substrates and are readily accessible to sulphydryl-group reagents [123,124]. Clearly, also in this case, conformational changes may be the underlying basis for the observed phenomena.

Finally, bovine heart Mi-CK octamers were found to have a more than 2-fold higher specific enzymatic activity than dimers [123], whereas others observed no significant difference in this parameter for dimeric and octameric chicken- and bovine-heart Mi-CK [100,396]. To conclude, the question of whether changes in the dimer to octamer ratio or simply in the conformation of Mi-CK are essential for the regulation of mitochondrial oxidative phosphorylation and PCr production deserves full attention in the future. Recombinant techniques are likely to be a powerful tool for the direct comparison of properties between native (octameric) Mi-CK and dimeric cytosolic CK, to which by genetic engineering a mitochondrial target peptide is attached, thus allowing its import into the intermembrane space.

III-E. Developmental changes

Studies on the developmental changes of the CK isoenzyme system were conducted mainly with heart- and skeletal muscle, as well as with brain and retina of a variety of species, including man [18,107,108,115,165, 199,200,292,335,435,455,496,500,530]. In all cases, these studies revealed a complex pattern of isoenzymes appearing and disappearing during developmental maturation [345]. Total CK activity considerably increases during the last stages of fetal development of cardiac and skeletal muscle of mouse, rat, rabbit, sheep and man [199,200,202,435]. Whereas total CK activity continues to rise postnatally in the mammalian heart [18, 107,108,199,335,473], the specific CK activity was either found to increase [107,108,473] or to remain constant [192]. During postnatal brain-development, total CK activity increases markedly at a time when a greater coordination of complex nervous activity is becoming apparent. Furthermore, the similar developmental patterns of CK and hexokinase suggest that CK is involved in the overall coordination of energy metabolism and neurotransmission in the fully active adult brain [53,335].

The CK isoenzyme distribution is both tissue-dependent and developmental-stage-specific. In mammalian brain, as well as in heart and brain of birds, BB-CK is the major CK isoenzyme at all stages of development. In contrast, in skeletal muscle of birds and mammals as well as in mammalian myocardium, a developmental transition from B-CK to M-CK mRNA and, therefore, also from BB-CK over MB-CK to MM-CK protein dimers takes place prenatally [115,292,466]. In mouse heart, the transition from BB-CK to MM-CK happens during the last trimester of fetal development, whereas after birth, comparatively small changes in the proportion of the cytosolic CK isoenzymes are observed [165,199]. In human skeletal muscle, the BB- to MM-CK

transition takes place around week 8 of fetal development, in contrast to human heart where MM-CK predominates from the earliest stage examined onward, i.e., $4\frac{1}{2}$ weeks of embryonic development [500]. Similarly, the developmental transition from B-CK to M-CK expression occurs earlier in heart compared to skeletal muscle of the rat [466]. Finally, stage-dependent regional differences in the expression of cytosolic CK isoenzymes were observed in the prenatal development of the rat heart [176]. For example, MM-CK was first observed in the outflow tract and the trabeculae of the right ventricle at embryonic days 12–14 and only at later developmental stages in other parts of the heart.

As far as Mi-CK is concerned, developmental studies stress its importance for energy metabolism. In all tissues and species examined, the Mi-CK isoenzymes are accumulated at later stages than M- and B-CK, indicating that cytosolic and Mi-CK isoenzymes are subject to different regulatory programs [195,202,345]. In contrast, M- and Mi-CK mRNA were coordinately induced during differentiation of mouse muscle cells in culture [163].

In myocardial tissue of altricious animals like mouse, rat, rabbit and chicken, very low amounts of Mi-CK and Mi-CK mRNA are found before birth [165,192, 195,199,335,418]. Cardiac Mi-CK activity rises sharply between 6 and 25 days of neonatal life in the mouse [165,199], up to about 3–9 weeks of age in the rat [107,108,199,202,418] and between about 1 and 20 days in the rabbit [192,510], thereby reaching adult values. However, during these periods of time, no changes in a variety of mitochondrial parameters (e.g., mitochondrial ATPase activity) were observed [107,108]. In contrast to these altricious animals, Mi-CK is already present in fetal heart and/or skeletal muscle of precocious animals such as sheep and guinea pig, but astonishingly also in man [199,200,435,479], and accumulation of Mi-CK to the adult concentrations occurs mostly before birth. In quadriceps muscle of preterm-born infants, for example, Mi-CK activity, as well as protein content, increased significantly with gestational age [435]. Adult concentrations of Mi-CK were reached soon after birth in both sheep and man.

Most interestingly, Mi-CK in cardiac muscle appears at a time when, during postnatal development, incorporation of MM-CK into the myofibrillar M-band of the mammalian heart muscles begins [71,72,192]. During the same period of time, a general maturation of the heart muscle towards its full contractile potential takes place [18,192,194]. The coordinate postnatal appearance of Mi-CK on the PCr production side and of M-line-bound MM-CK on the PCr consumption side of the PCr 'circuit' emphasizes the need for a functional coupling of the two systems for optimal muscle function [492]. In addition, the developmental accumulation of 'total Cr' (Cr + PCr) in the mouse, rat and possibly also sheep heart occurs in two consecutive steps [199]. First, total Cr concentration increases prenatally, almost in parallel with the accumulation of MM-CK, thereby reaching a plateau which is maintained for a certain period of time. Then, a further several-fold increase in total Cr pool size parallels the accumulation of Mi-CK, indicating that the two processes are linked in some way. This assumption is fully in line with experiments on cell cultures derived from neonatal rat hearts showing that addition of 20 mM Cr to the culture medium stimulates the synthesis of Mi-CK [418,473]. Besides, the relative proportion of Mi-CK increases with age in culture and with age of animal from which the culture is derived.

In chicken-leg muscle, but not in chicken heart, amounts of Mi-CK mRNA similar to those in adult heart were already found at embryonic day 19 [195]. In rat brain, Mi-CK is undetectable at birth and increases also postnatally to the adult proportion of 15% of total CK activity [335]. In chicken retina, BB-CK content was high in late stages of embryonic development, decreased slightly around hatching and remained high during adulthood [496]. Mi-CK content, however, was low during development in ovo, rose just before hatching, at a time when visual functions have to become operational in autophagous birds to enable them to find food, and remained high throughout the following developmental periods. Mi-CK was accumulated predominantly within the ellipsoid portion of the inner photoreceptor cell segments [490].

^{31}P-NMR measurements were performed to test the functional consequences of the development of the CK system. In neonatal rat brain lacking Mi-CK, ischemia led to an almost parallel decrease in the concentrations of ATP and PCr [335]. In the adult brain, however, first PCr and, after a delay, ATP concentration also decreased, indicating that Mi-CK might be essential for efficient rephosphorylation of ADP. This conclusion is corroborated by the finding that the capacity of the rodent brain to modulate the rates of glycolysis and tissue respiration in response to sudden changes in energy demand increases in the narrow time-window between days 12 and 15 of postnatal development when also the CK-catalysed reaction rate increases [193]. To monitor the developmental changes of fluxes through the CK reaction, ^{31}P-NMR saturation transfer experiments were performed with hearts of 3–18-day-old neonatal rabbits at different levels of cardiac performance [300,346]. Parallel biochemical experiments demonstrated that during this developmental period total CK activity and adenine nucleotide pool size in the heart remained constant, the proportions of MM-CK and Mi-CK, as well as the Cr pool size increased (see also Ref. 199) and the proportions of MB-CK and BB-CK decreased [346]. The ^{31}P-NMR measurements revealed that the CK reaction flux in the direction of

ATP production was positively correlated with the relative proportion of Mi-CK, with the Cr pool size and with cardiac performance [300,346]. Furthermore, the fluxes in both directions of the CK reaction were identical under all conditions tested [300].

In conclusion, the expression of the four known CK subunit isoforms is differentially regulated during development on the transcriptional and possibly also on the translational level. The reasons for these developmental changes and the functions of the different isoenzymes at the various stages of development are not fully understood. However, the transition in muscle from B- to M-CK has to do with the isoenzyme-specific subcellular localization of CK isoenzymes. In sharp contrast to MB- or BB-CK, homodimeric MM-CK is capable of binding to the myofibrillar M-band where it fulfils its specialized function as an intramyofibrillar ATP regenerator and as a structural component of the M-band [488]. The level of expression of M- as well as as Mi-CK strongly depends on the physiological requirements and is, therefore, developmental-stage-specific. In addition, maturation of the CK system differs quite dramatically between altricious and precocious animals.

Since Mi-CK appears only after birth in a variety of small animals, it seems not to be essential for life per se or for basic muscle function at a low workload. Strikingly, however, the flux through the CK reaction, the mechanical performance of the heart, the fraction of M-line-bound M-CK, as well as the proportion of Mi-CK increase concomitantly, in spite of a constant total CK activity [192,346]. These latter results strongly suggest that Mi-CK is crucial for energy supply at high workloads where ADP diffusion may become a limiting factor [525].

III-F. Adaptive changes

Two main primary functions were ascribed to the CK/PCr system (see IV-B). First, it is involved in buffering of ATP and especially ADP during abrupt changes in workload, with this function certainly being most crucial for fast-twitch skeletal muscles. Second, the CK/PCr system is involved in the transport of high-energy phosphates from sites of ATP production (mitochondria, glycolysis) to sites of ATP consumption. This function is more pronounced in 'endurance' tissues like myocardium and slow-twitch skeletal muscles and, in contrast to the 'buffer' function, is thought to be facilitated by the presence of Mi-CK.

In accordance with these ideas, training for long-distance running and even a marathon race itself increased the proportion of Mi-CK relative to total CK activity in human gastrocnemius muscle, with the increase being larger in female than in male runners [9]. Total CK activity, however, remained unchanged. In addition, activity and relative proportion of Mi-CK were found to increase almost linearly with the duration of chronic stimulation of fast-twitch rabbit muscle (type II, white fibers) [400], whereas they decreased in human quadriceps femoris muscle during 6 weeks of leg immobilization after knee surgery [224]. In contrast to these results, endurance training was reported not to influence total activity and relative proportion of Mi-CK in rat gastrocnemius muscle [342].

In most instances, the changes in Mi-CK activity were either directly paralleled or even surpassed by changes in the mitochondrial protein content or in the activities of mitochondrial marker enzymes like citrate synthase [224,400]. Incidentally, chronic stimulation of fast-twitch rabbit muscle was found to increase the activities of citric acid cycle enzymes, as well as of total mitochondrial volume [361]. Furthermore, several mitochondrial enzymes were shown to increase after exercise [358]. Finally, Mi-CK, as well as MB-CK activity were found to be positively correlated with the oxidative capacity of a muscle [457,459]. All these findings strongly indicate that the changes in Mi-CK activity induced by training or chronic stimulation are not specific events, but are due to general changes in mitochondrial content, thus, reflecting a transition from a more glycolytic to a more oxidative, fatigue-resistant energy metabolism.

In vivo and in vitro, ischemia affects a variety of functional as well as structural properties of heart and skeletal muscles, some being reversible and others not (see for instance Ref. 388). In rabbit heart, for example, total ischemia resulted in a progressive loss of Mi-CK activity, which was closely paralleled by a decline in left ventricular pressure [43,215,462]. Within 60 min of ischemia, the ratio of Mi-CK to mitochondrial malate dehydrogenase decreased by more than 70%, indicating that the loss is not due to a general decrease in mitochondrial content, but to a specific release or selective inactivation of Mi-CK. In addition, the functional coupling between Mi-CK and ANT was depressed [462] and Mi-CK was detected in blood after ischemia (see III-G). Inorganic phosphate was suggested to cause a selective release of Mi-CK from the inner mitochondrial membrane [462]. During prolonged, severe ischemia, intracellular [P_i] may approach 50 mM [260], a concentration that is in the range used for in vitro solubilization of Mi-CK from the mitochondrial inner membrane [169]. However, since the loss of Mi-CK appeared to be irreversible and, since Mi-CK could not be detected in the post-ischemic supernatant, Bittl et al. [43] concluded that Mi-CK is not just released from the mitochondrial inner membrane, but is irreversibly inactivated during ischemia. Again in contrast to these results, Saks et al. [388] in ischemic rat hearts found only a transient, reversible decrease in Cr-stimulated respiration and,

thus, in Mi-CK activity. In addition, even though ventricular performance and metabolite contents of isolated perfused rat hearts were permanently depressed, a variety of respiratory parameters proved to be highly tolerant to ischemia, thus suggesting that, at least under the experimental conditions used, mitochondrial injury is not a major component of ischemic damage [388]. Clearly, more experiments are needed to unravel the apparent discrepancies.

Compared to heart muscle, more pronounced mitochondrial changes were noticed in ischemic skeletal muscles [172]. For example, ischemia resulted in the appearance of giant mitochondria containing paracrystalline inclusions. These intramitochondrial inclusions had the appearance either of accumulations of finely granular material distending the intracristae space, or of plate-like structures sandwiched between the outer and the inner mitochondrial membranes or between two leaflets of the inner membrane. The inclusions were suggested to derive from aggregation of enzymes present in the intermembrane space of muscle mitochondria, such as Mi-CK (see below).

To evaluate whether or not Cr is essential for normal muscle function and structure, animal models have been developed to test the effect of Cr depletion. An almost complete Cr depletion can be achieved by feeding animals with Cr analogues like cyclocreatine (cCr: 1-carboxymethyl-2-iminoimidazolidine) [513], β-guanidinobutyric acid (GBA) [269] and β-guanidinopropionic acid (GPA) [127], which act as competitive inhibitors of Cr uptake into the cell and are in general poor substrates for the CK isoenzymes [6,129]. Instead of PCr, large amounts of phosphorylated cCr (PcCr) and GPA (GPAP) are accumulated inside the cells. In contrast, GBA is not phosphorylated by the CK isoenzymes [531]. As a result of Cr depletion, important biochemical, functional, as well as morphological alterations occur, as will be discussed now in more detail.

The effects of cCr on brain, heart and skeletal muscle metabolism were mainly investigated in relation to ischemia [7,366,469,470,513]. Most importantly and also very surprisingly, ATP levels during total ischemia were found to be sustained substantially longer in several tissues of cCr-fed animals as compared to controls [366,469,470], even though the V_{max} value of CK with PcCr as substrate is about 160-fold lower than that with PCr [6]. Upon cCr-feeding, delayed ATP depletion was observed for mouse brain [513], as well as for chicken breast-muscle [470], chicken heart [469] and rat heart [366], these muscles displaying a relatively homogeneous fiber population. On the other hand, mixed-fiber leg-muscles of cCr-fed mice [7] or chicken [470] for unknown reasons did not exhibit a directly measurable ATP-sustaining activity during ischemia. The capacity of dietary cCr to sustain [ATP] may be attributed to the unique thermodynamic properties of the accumulated PcCr. Because its free energy of hydrolysis is roughly 2 kcal/mol lower than that of PCr [6], PcCr may continue to buffer the adenine nucleotide concentrations and to transport high-energy phosphates throughout the muscle fibers even at cytosolic pH values and phosphorylation potentials well below the range where the CK/PCr system can function effectively. In contrast to cCr, homocyclocreatine and GPA feeding did not delay ATP depletion [366,469].

GPA-fed animals also seem a suitable model to study the consequences of Cr depletion, although the possibility that GPA or GPAP are toxic for muscle has to be considered [349]. GPA feeding results in a variety of adaptive changes similar to those observed in transitions from a more glycolytic to a more oxidative energy metabolism, as occurs for example during endurance training (see above). In rat skeletal muscle, GPA feeding decreased the concentrations of total Cr, PCr and ATP by up to 75, 90 and 50%, respectively [127,154, 313,422,427,428], whereas [P_i] remained unchanged [428]. The glycogen content, as well as the activities of aerobic enzymes, such as citrate synthase, 2-oxoglutarate dehydrogenase and 2-hydroxyacyl-CoA dehydrogenase were found to be increased in all fast-twitch (plantaris and gastrocnemius) muscle regions except the superficial gastrocnemius, but not in the slow-twitch soleus muscle [427]. In contrast, the activities of CK, phosphofructokinase and glycogen phosphorylase decreased in all skeletal muscle regions, except the deep gastrocnemius [427]. As far as contractile characteristics are concerned, plantaris muscles of GPA-fed animals exhibited no abnormalities, except for a slight decrease in initial strength [349]. Surprisingly, the endurance of soleus muscle was prolonged. In addition, the isometric twitch characteristics in this latter muscle were altered and the maximum velocity of shortening was decreased [346].

^{31}P-NMR experiments revealed that in resting gastrocnemius muscle of GPA-fed rats, the flux through the CK reaction is reduced in the direction of ATP synthesis [424] and that there is no measurable phosphate-exchange between GPAP and ATP [313]. In addition, the rate of GPAP hydrolysis in stimulated GPAP-loaded muscle was much less than that of PCr in control muscles [313]. Intracellular pH decreased more rapidly during stimulation and recovered more rapidly afterwards in GPAP-loaded muscles compared with controls. However, despite buffering by PCr hydrolysis, the pH ultimately decreased more in control muscles. This finding is very likely due to the 2-fold greater lactate accumulation in stimulated control gastrocnemius muscles as compared to GPAP-loaded muscles. These results were taken as an argument that in skeletal muscle, PCr is not essential for steady-state energy production, but that phosphate release by PCr

hydrolysis is essential for maximum activation of glycogenolysis and/or glycolysis [313]. However, recent evidence suggests that GPAP in working heart muscle may be used quite efficiently as a CK substrate [88].

Heart muscle of GPA- and GBA-fed rats exhibited contractile failure, as evidenced by cardiac hypertrophy [305] or by a rise in the left ventricular diastolic pressure (LVDP) [235,236,531]. The latter, probably by impairing left-ventricular filling, may be responsible for the diminished cardiac performance observed in GPA- and GBA-fed animals [235,531]. The extent of contractile failure was found to depend on the functional load and on the degree of Cr depletion. However, ^{31}P-NMR saturation transfer experiments revealed that a 80–90% depletion of PCr results in only a 2–4-fold reduced flux through the CK reaction [235,531], thus, rendering final conclusions about the involvement of the CK/PCr system in contractile failure difficult. Finally, the expression of myosin isoenzymes in the left ventricle of the heart was recently shown to change from the fast form V_1 to the slower forms V_2 and V_3 during GPA feeding for several weeks [305], thus, reflecting changes in contractile properties similar to those observed in skeletal muscles (see above). Taken together, the available data stronly suggest that, at least in the heart, the CK/PCr system is essential for proper muscle function.

In addition to the biochemical and functional alterations discussed so far, Cr depletion in muscle also caused several morphological changes, thus suggesting that Cr metabolism is important for sustaining normal muscular structure. GPA feeding caused an increase in the relative proportion of type-I muscle fibers (red, slow-twitch), in soleus muscle, for example, from 81% in control rats to 100% in GPA-fed rats [427]. In addition, GPA-feeding in general decreased type-II fibres (white, fast-twitch) in size and weight, while type-I fibres were unaffected [349,422,427]. Accordingly, the largest change in relative muscle size was observed for the gastrocnemius muscle displaying the greatest proportion of type-IIb fibres (62%) [427]. Selected fibres of the pectoralis and gastrocnemius muscles of chicken fed with GBA exhibited loss of thick and thin filaments, disruption of the Z-band, dilated mitochondria, as well as dilated and displaced sarcoplasmic reticulum [269]. These ultrastructural changes are attributable to an abnormality of Cr metabolism, since GBA by itself seems not to be toxic. Accordingly, when chicken were given extra dietary Cr in addition to GBA, muscle [PCr] was found to be normal and no significant ultrastructural alterations occurred.

As a result of Cr depletion, abnormal mitochondria were observed in slow-twitch skeletal muscles of rats [154,339]. These mitochondria often were enlarged and contained crystal-like inclusions like those frequently observed in human mitochondrial myopathies [121,429,444], ischemia [172,180], as well as in muscles exposed to mitochondrial poisons [307,373] (see also III-G). When adult rat cardiomyocytes were cultured in a medium devoid of Cr or in a medium supplemented with GPA, two populations of mitochondria could be distinguished [116]. Giant, cylindrically-shaped mitochondria were randomly distributed over the cell and contained inclusions highly enriched in Mi-CK, as shown by immuno-gold labelling. In contrast, small, 'normal'-sized mitochondria without inclusions were localized between the myofibrils and contained much lower amounts of Mi-CK. Addition of Cr to the culture medium caused the disappearance of the giant mitochondria, as well as of the crystal-like inclusions, accompanied by an increase in the intracellular concentration of total Cr. It is not readily understood why only part of the mitochondria are affected by Cr depletion and form inclusions. One possibility is that subsarcolemmal mitochondria are more susceptible to metabolic alterations and react to a Cr deficit first by fusion to form giant mitochondria and second by a compensatory accumulation of Mi-CK, the latter resulting in the formation of Mi-CK-containing crystal-like inclusions [116]. Taken together, these results corroborate the suggestion that changes in Cr metabolism in mitochondrial myopathies play an important role in the formation of abnormal mitochondria, as well as of mitochondrial inclusions. However, in a preliminary study among six patients, no correlation was found between the occurrence of abnormal mitochondria and total [Cr] [436].

Phosphate depletion in rats produced by dietary phosphorus restriction resulted in a decreased concentration of inorganic phosphate in skeletal muscle, in an elevated phosphorylation potential and in reduced oxygen uptake [57]. Furthermore, the specific activities of Mi-CK and of myofibrillar MM-CK were reduced. Accordingly, addition of Cr to state-4-respiring mitochondria did not increase the rate of oxygen-consumption. The mechanism by which phosphate depletion may induce the observed alterations is unknown. Since Mi-CK is of prime importance in regulation of cellular energy production and transport, and since these steps are impaired in skeletal muscle during phosphate depletion, the reduction in the activity of Mi-CK may be the key biochemical disturbance in the myopathy of phosphate depletion [57].

III-G. Mi-CK in pathology

III-G.1. Neuromuscular diseases

In recent years, a variety of studies focussed on the possible involvement of Mi-CK in the pathology of several neuromuscular disorders, such as muscular dystrophies and mitochondrial myopathies [31,293]. Mus-

cular dystrophy is a heterogeneous group of disorders of which the Duchenne muscular dystrophy is most frequently observed in humans. However, no extensive studies on Mi-CK were performed in human muscular dystrophy. In skeletal muscle of dystrophic chicken, compared to normal age-matched controls, Mi-CK activity progressively decreased during the course of the disease, with the pectoralis muscle being more affected than the gastrocnemius [31,293]. Furthermore, Cr-stimulated mitochondrial respiration in dystrophic chicken breast muscle was found to be decreased [31]. Consequently, a decrease in Mi-CK activity may ultimately cause the functional loss in breast muscle fibers by decreasing the efficiency of trapping available mitochondrial ATP as PCr. An inborn error of metabolism comprising Mi-CK deficiency would clearly be very helpful in testing this hypothesis.

A mitochondrial myopathy can be defined as a muscle disease characterized by structurally or numerically abnormal mitochondria and/or abnormally functioning mitochondria. In about 30% of the patients with an in vitro observed disturbed pyruvate-oxidation rate, no single-enzyme deficiency of the mitochondrial respiratory chain is found in the skeletal muscle mitochondria. As an alternative, Mi-CK activity may be affected in these patients. However, quantitative measurement of Mi-CK activity by a newly developed method [434] revealed no deficiency of Mi-CK in skeletal muscle specimens of 11 patients with disturbances in the pyruvate oxidation rate in whom no defect in the pyruvate dehydrogenase complex or in complexes of the respiratory chain could be established [437]. In two patients with an established single-enzyme deficiency of the mitochondrial respiratory chain, the specific activity of Mi-CK was clearly enhanced. A possible explanation is that this increase in the specific activity of Mi-CK reflects some kind of adaptation. A similar compensatory increase was also observed for cytochrome *c* oxidase and citrate synthase activities in patients with single enzyme deficiencies of the respiratory chain [372].

Electron microscopical inspection of muscle samples revealed that abnormal mitochondria and intramitochondrial inclusions are a typical feature of neuromuscular diseases in general and of mitochondrial myopathies in particular [121]. These crystal-like mitochondrial inclusions were frequently found in the 'ragged red' muscle fibers of patients suffering from ocular myopathies with clinical manifestation of progressive involvement of the external ocular muscles (chronic progressive external ophthalmoplegia; CPEO). In patients with mitochondrial myopathies, two distinct types of crystals are observed, which can be distinguished by shape, size, pattern, unit-cell dimension, specific localization in the intermembrane space and their occurrence in different muscle-fiber types (Fig. 3). So-called type-I crystals (Fig. 3A) are usually present in the intracristae space, between two folds of the mitochondrial inner membrane, whereas type-II crystals (Fig. 3B) are preferentially located in the intermembrane space between outer and inner mitochondrial membranes. Type-I crystals occur only in type-I muscle fibers (slow twitch type with high oxidative capacity) and type-II crystals in type-II muscle fibers (weak or intermediate staining for mitochondrial enzymes) [121,444]. Only recently, it was shown that these crystals are labelled by antibodies directed against Mi-CK (Fig. 3C, see also III-F) [445,446]. The Mi-CK immunolabelling of these crystals was uniform and irrespective of the orientation of the crystals to the plane of sectioning. However, type-II crystals were always more heavily labelled than type-I crystals [445,446]. In a preliminary study among six CPEO patients, no relationship was found so far between the concentration of total Cr, free Cr or PCr and the occurrence of mitochondrial crystals in the muscle [436]. This holds true especially for one CPEO patient with an extremely low free Cr content in muscle, in whom despite thorough electron microscopical inspection, no crystals were observed. In two patients with CPEO in which Mi-CK-containing crystals were found in the quadriceps muscle, Mi-CK activity was significantly enhanced despite a normal pyruvate oxidation rate [436].

Recently, it was realized that long-term zidovudine therapy, used for the treatment of patients with the acquired immuno-deficiency syndrome (AIDS), can cause a toxic mitochondrial myopathy with depletion of muscle mitochondrial DNA [11,93]. Besides inflammatory changes, crystal-like mitochondrial inclusions were also observed in muscle biopsies of zidovudine-treated patients. It would be worthwhile to further study these inclusions in relation to the CK/PCr system. In addition, further studies are necessary to elucidate the mechanism of crystal formation and to clarify if crystal formation is causative to or only a consequence of mitochondrial myopathies.

III-G.2. Cardiomyopathies

A cardiomyopathy may be defined as a dysfunction of the myocardium caused by a primary disorder within the myocardium or by secondary disorders, like for example hypertension. In experiments on rat hearts, where arterial hypertension was induced by suprarenal aortic banding, total CK activity in the left ventricle rose within 4 days by about 70% [133]. In this model of a short-term cardiomyopathy, the expression of M-, B- and Mi-CK was concomitantly increased and, therefore, no significant change in the relative proportion of the different CK isoenzymes occurred. It seems that the increased energy requirements in acute pressure overload are met by a generalized induction of expression and synthesis of all CK isoenzymes.

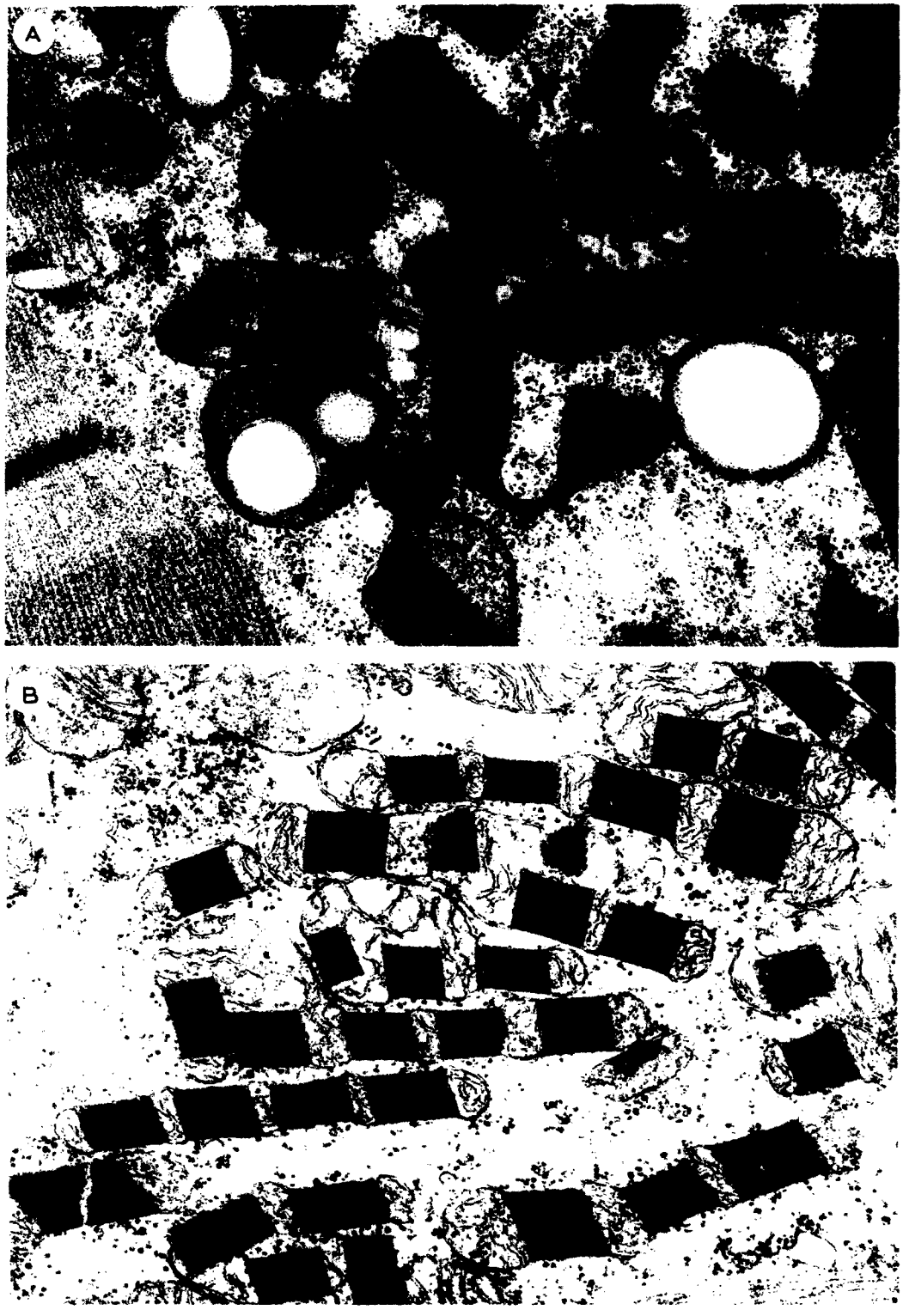
A
B

Fig. 3. Accumulation of Mi-CK in intramitochondrial inclusions. Mitochondrial crystals in human skeletal muscle biopsies (m. quadriceps). (A) Transversely sectioned Type-I crystals in mitochondria of 'ragged red' fibers from a patient suffering from chronic progressive external ophthalmoplegia (CPEO). Note the presence of the crystals in the intracristae spaces. (B) Type-II crystals in muscle fibers of a patient suffering from unclassified mitochondrial myopathy. (C) Strong anti-Mi-CK immunogold labelling of longitudinally sectioned Type-I crystals from a patient with CPEO. Magnification: (A) 36500 × (B) 51000 × (C) 39000 ×. For further details, see the text.

In contrast, long-term cardiomyopathies (left-ventricular hypertrophy due to aortic stenosis, volume overload, or hypertension; coronary artery disease; hereditary and diabetic cardiomyopathies) are characterized by an unchanged or decreased total CK activity, by a decreased in vivo flux through the CK reaction, by an increase in the relative proportions of MB- and BB-CK and by a decrease in the total Cr content [202–205,243,390]. With the single exception of Bio 14.6 Syrian hamsters [12], cardiac Mi-CK activity was considerably decreased in all sorts of long-term cardiomyopathies in both animals and humans [12,45,202, 205,243,390,393]. Accordingly, Cr-stimulated mitochondrial respiration was also found to be decreased [237,390,476,477].

Most interestingly, total CK and Mi-CK activity, as well as the flux through the CK reaction were normal in spontaneously hypertensive rats during the first 12 months of life, this period being characterized by a stable compensated hypertrophy of the myocardium [45,202,205]. However, between the 12th and 18th month of life, a transition from compensated hypertrophy to failure occurred, whereby in parallel with the functional capacity of the heart muscle, also total CK and especially Mi-CK activity drastically decreased. Finally, in various hereditary and experimental cardiomyopathies (induced by auto-immunization or by treatment of rats with adriamycin, norepinephrine, GPA, or streptozotocin), [ATP + PCr] was decreased [237]. With the exception of GPA-treated animals, cardiac output at standard load conditions was also substantially lowered, probably due to mild bradycardia, elevated left ventricular (LV) diastolic pressure and stiffness that limited cardiac contractile adaptation to volume or resistance overloads. The LV diastolic stiffness at maximal functional load was inversely correlated with the high-energy phosphate content. Its increase in cardiomyopathic hearts may be explained by the increased myofibrillar sensitivity to Ca^{2+} and by the loss of functional coupling of Mi-CK to oxidative phosphorylation. Since in another study, the myofibrillar MM-CK activity of the cardiomyopathic heart was found to be normal [477], these results clearly indicate that loss of Mi-CK activity may be of prime importance for the development of cardiac failure.

III-G.3. Tumor tissues

Mi-CK was detected in several types of human [101,190,233,340,368,467] and animal tumors [275], in the human carcinoma cell line HeLa [473], as well as in murine Ehrlich ascites tumor cells [25]. In these tissues or cells, Mi-CK is frequently coexpressed with BB-CK, indicating a de-differentiation of transformed cells, which is reflected in the appearance of an embryonic isoenzyme pattern. In Ehrlich ascites tumor cells, however, CK activity was found to be exclusively associated

with mitochondria. The authors, therefore, suggested that the 'transport' function of the CK/PCr system (see IV-B) is not crucial for tumor cells [25]. To our knowledge, there are no reports on the Mi-CK content of brain tumors.

No differences in the molecular mass, the electrophoretic mobility, or the kinetic characteristics were observed between Mi-CK in tumors of the digestive tract and Mi-CK in adjacent normal tissue [340]. Accordingly, Mi-CK of malignant and normal liver displayed the same M_r of 320000–350000, similar heat stability and a similar behaviour in 2 M urea, whereas the electrophoretic mobility, for unknown reasons, differed clearly [233]. Interestingly, tumors of the digestive tract were shown to contain significantly higher amounts of Mi-CK than surrounding normal tissue [340]. This may reflect that a higher expression of Mi-CK is required to meet the increased energy demands of the tumor cells. However, the facts that (i) stomach adenocarcinomas displayed lower Mi-CK activity than the surrounding normal tissue [190] and that (ii), tumors depend more on anaerobic rather than aerobic energy metabolism, make this latter interpretation unlikely.

III-C.4. Body fluids

Normally, no Mi-CK is detectable in normal human serum [234,288,352] or cerebrospinal fluid (CSF). This holds true for most species, although sheep may be an exception [24]. Under certain pathological conditions, however, Mi-CK is released into the blood or CSF in man. This may cause diagnostic confusion, as Mi-CK interferes in many of the methods that are routinely used in clinical chemistry for the determination of MB-CK as an indicator of myocardial damage. Serum Mi-CK is generally thought to originate from the mitochondrial compartment. Therefore, the finding of Mi-CK in a patient's serum forms an index of mitochondrial damage. It should be kept in mind, however, that the cell nucleus of heart and skeletal muscle has been suggested to contain Mi-CK forms as well [228]. Clinically, the presence of Mi-CK in serum should not be ignored, for it may be of help in finding the proper diagnosis.

Serum CK isoforms with a M_r of >80000 are called macro CKs [527]. According to the nomenclature of Stein and co-workers [52,448], macro CK type 1 represents an autoantibody complex of BB-CK. In contrast, macro CK type 2 is generally assumed to be Mi-CK [449] because of similarities in electrophoretic mobility [52,232,449], molecular mass [232,449], activation energy [448,449], enzyme kinetics [232,448] and antibody studies [232]. The M_r of the principal form of macro CK type 2 was estimated as 287000–350000, which is similar to the M_r of Mi-CK isolated from tissues [231,449]. In addition, smaller quantities of a 80 kDa form [231,449] and surprisingly also of a >750 kDa form of CK were found in serum [449], whereby for the latter, nothing is known about the number and stoichiometry of its constituents. To avoid confusing terminology in the following section, macro CK type 2 is referred to as Mi-CK whenever possible.

The occurrence in serum of a CK isoenzyme of mitochondrial origin was first described for one out of two patients examined with Reye's syndrome [367]. The occurrence of Mi-CK in serum was later confirmed [22,222] and considered an ominous sign, since 10 out of 14 positive cases died shortly after Mi-CK was detected in the serum [222, see also Refs. 234,352]. In a prospective study among 2954 consecutive patients in a hospital for internal diseases, the prevalence of Mi-CK in serum was found to be 3.7% among hospitalized patients and 1.1% among outpatients [450]. In this study, Mi-CK was found predominantly in severely ill patients of all ages, mainly with malignancies (41%) and liver diseases (25%). In a study of 5000 random patient sera, malignancies were found in 25 of the 26 adult patients that were positive for Mi-CK in serum [514,515]. Of course, the prevalence figure depends both on the sensitivity of the test procedure for Mi-CK and on the patient group that is screened.

The occurrence of Mi-CK in serum has been studied extensively in neoplastic diseases [183,317]. Serum of patients with malignancies may contain Mi-CK, sometimes in combination with mitochondrial aspartate aminotransferase, thus indicating mitochondrial damage in the tumor. Serum Mi-CK has been found in patients with primary tumors in liver [73,158,232–234, 515], pancreas [515], lung [158,234,258,274,303,452,515], breast [73,158,234,258,288,352,450,453,515], gastro-intestinal tract [158,190,234,258,289,303,308,317,340,352, 368,452,515], prostate [158,234,452,453,515], gallbladder [225,234,452], ovaries and uterine cervix [234]. However, Mi-CK was not found in serum of 120 leukemia or lymphoma patients [73]. In addition, Mi-CK has never been described in patients with renal tumors. In some cases, the tumor and its metastases were shown to contain the same macromolecular Mi-CK as the patient's serum [232,450], thus suggesting that tumor tissue itself can release Mi-CK and sometimes also BB-CK into the serum. Histological typing of tumors probably releasing Mi-CK into the blood has been performed by Kanemitsu et al. [234]. However, a patient can have a tumor expressing Mi-CK without displaying measurable quantities of Mi-CK in the serum. This was evidenced by two patients with liver metastases, in which Mi-CK was released in measurable quantities into the serum only after embolization of the hepatic artery [432]. Although hypoxia may play a role, the exact mechanism for the pathological release of Mi-CK from tumors is unknown. Xenografting tumor lines into athymic mice may be a rewarding

model for studying Mi-CK release from tumors into the blood [101].

Several authors have suggested the use of Mi-CK in serum as a tumor marker [234,275,288,303,308,368, 450,468], or more specifically as a marker for gastrointestinal cancer [258,308,368], metastatic prostatic carcinoma [453] and adenocarcinoma [340,352]. Serum Mi-CK activity roughly seemed to reflect the tumor burden [232,275,450,452,453], so that in individual cases, serum Mi-CK may be used to monitor the initial response to therapy [303]. However, the appearance of Mi-CK in serum alone is not a specific signal for neoplastic disease (see below). Accordingly, the diagnostic sensitivity of serum Mi-CK activity for neoplastic disease in general seems rather poor [73]. The presence of Mi-CK in serum was shown to be related to the clinical stage of neoplastic disease for some tumors [308], while it was not so for others [73].

Apart from a few patients with various chronic and acute diseases [367,450], Mi-CK was frequently found in serum of patients with liver diseases, more in particular liver cirrhosis [73,450]. In these patients, serum Mi-CK apparently originated directly from liver cells, which in this special case were found to contain Mi-CK [232]. Normal liver cells, in contrast, do not contain measurable amounts of Mi-CK (see III-A).

Myocardial damage was early recognized as an additional potential cause of Mi-CK efflux into the blood [22,222]. Mi-CK was found in sera of children with myocarditis [362,515], congestive heart failure and cardiomyopathy after aortic valve surgery [515]. Mi-CK appeared to be present in serum of patients who have experienced periods of poor tissue perfusion [22], myocardial ischemia [158] and cardiorespiratory arrest [92]. These findings are in line with the observation that in rats during hypoxia, Mi-CK activity in the blood increased in parallel with a decrease in the heart [178]. Furthermore, Mi-CK was found in serum of individual cases after acute myocardial infarction (AMI) [158,222,289,448,458]. Peak values for serum Mi-CK were observed 24 h after AMI [458]. The activity of Mi-CK approximated 20% of that of MB-CK, the traditional and established indicator of myocardial damage [458]. In larger series, however, serum Mi-CK could not be detected at all after AMI or was only found in sporadic cases [73,122 352,362,367,441,450, 503]. Therefore, release of Mi-CK into the blood circulation after AMI seems to be the exception rather than the rule. Besides, it remains principally unclear why Mi-CK is released into the blood in some patients with AMI and not in others. Interestingly, the drug theophylline was suggested to induce Mi-CK release into the serum in vivo [99]. As this drug is often given to patients suffering from cardiac diseases, it can not be decided yet whether in fact theophylline or simply myocardial damage is the actual cause resulting in Mi-CK release into the serum.

In CSF, Mi-CK was found under several pathological conditions [75,76,503,505]: hypoxic-ischemic brain damage [76]; after surgery in relation with various central nervous system tumors [503]; apoplexia caused by a hypophysis tumor [505]; and meningitis [503]. Due to its presence in human brain [75,277,504], Mi-CK in CSF seems to derive directly from the central nervous system. Rather surprisingly, Mi-CK in CSF is not necessarily accompanied by BB-CK [503]. As of yet, the diagnostic potential of determining Mi-CK in CSF remains an open question and should therefore be a topic of further research.

As far as clinical chemistry is concerned, electrophoresis was often used for the detection and quantitation of Mi-CK. However, interpretation of the serum CK zymograms is complicated for several reasons: (1) The human Mi-CK isoenzymes either migrate cathodally to or comigrate with MM-CK [52,289,502]. Incubation of Mi-CK in normal human serum results in modification of the most cathodal human heart Mi-CK band [231,502], with the modified Mi-CK comigrating with MM-CK [502]. In contrast, human liver Mi-CK is not influenced by serum incubation [231]. Blocking of all M-CK activity in a zymogram with inhibiting antibodies generally is very informative to discriminate between MM-CK and Mi-CK [52] (see below). (2) Various authors have observed one to three Mi-CK bands in serum upon electrophoresis [52,183,231,289, 329,432,449,453,502] or even more in isoelectric focussing experiments [433,449]. This multiplicity is poorly understood. (3) Co-migration of adenylate kinase isoforms [241] and in single cases of a macro CK type 1 complex [52,528] with Mi-CK may further complicate the zymograms.

There are several reports in the literature where the authors relied exclusively on the electrophoretic mobility to classify a cathodally migrating form as Mi-CK. However, in any publication on Mi-CK, it should adequately be shown that there is no interference with alternative enzymatic activity at stake. Since the mere presence of the inhibitors AMP and diadenosine pentaphosphate does not always guarantee full inhibition of adenylate kinase [241], a control zymogram of the electrophoresed samples without PCr in the reaction mixture is required to exclude the presence of adenylate kinase. Proper discrimination between macro CK type 1 and Mi-CK, on the other hand, can be achieved by published methods [52,448].

Mi-CK in serum was often found just by chance, because (i), it interferes with most methods that are commonly used for the detection of MB-CK and (ii), most patients with Mi-CK in serum (81–88%) have a normal total serum CK activity [73,234,450]. As the

Mi-CK activity in serum often is below 10 IU/l [450], the sensitivity of the assay used is a crucial point. Improvement of sensitivity may be achieved by using bioluminescence in combination with immuno-inhibition of MM-CK [303,505]. Unfortunately, anti-Mi-CK antibodies for a direct determination of this isoenzyme in serum have been raised on a limited scale and have been available for research purposes only.

Since Mi-CK is not inhibited by antibodies against M-CK, a clinical chemist may be alerted for the possible presence of Mi-CK in serum by the often abnormally high ratio (residual CK activity after immuno-inhibition of M-CK)/(total CK activity) [450]. Accordingly, many researchers have used immuno-inhibition of M-CK, thus measuring all the non-M-CK activity [303,450,505,514], as a reliable first step in establishing the presence in serum or CSF of abnormal CK isoenzymes in general and of Mi-CK in particular. A good methodological alternative is the commercially available reagent kit that combines immuno-inhibition with precipitation of the immune-complexes by a second antibody [514]. This set-up allows direct discrimination between MB-CK on one hand and macro CK forms on the other. In all techniques mentioned, however, further tests are necessary to definitely confirm the presence of Mi-CK. Among these, determination of the apparent activation energy [448,449], the molecular mass [431,448,449], the isoelectric focussing pattern [433,449] and the electrophoretic behaviour [52] would seem to be most convincing.

IV. Integration of Mi-CK in cellular energy metabolism

IV-A. Advantages of the CK/PCr system

Some of the aspects mentioned below have been discussed in the recent review of Wallimann et al. [492], but are reinforced here in order to be able to fully understand the new arguments and to get a comprehensive picture of the physiological importance of Mi-CK. First, the potential advantages of the CK/PCr system shall be elucidated. Clearly, the most evident advantage of this system is that PCr and Cr allow a much higher flux of 'high-energy phosphates' from sites of ATP production to sites of ATP utilization than ADP and ATP, since (i) within tissues with high and fluctuating energy demands, Cr and PCr are accumulated to much higher concentrations than the adenine nucleotides and, since (ii) Cr and PCr are smaller-sized and less negatively charged than ADP and ATP. Accordingly, in model solutions, as well as in frog muscle, the diffusion coefficients of PCr and Cr were found to be 1.3–2.3-times higher than those of ATP and ADP [219,328,524,525].

Diffusion of ATP is unlikely to be hindered by binding to subcellular structures, since in the cytosol, its diffusion was restricted to the same extent as that of other small molecules. The diffusion coefficients of ATP and PCr were both about 60% lower in frog muscle than in model solutions [525] which is in perfect agreement with the observations that the diffusion coefficients of several molecules with M_r 170–24000 are 2–5-fold lower in the cytoplasm of mammalian cells than in water [297] and that living cells have a fluid phase viscosity 3–4-times greater than water [290]. In contrast to ATP, the diffusion of ADP in the cytosol seems to be severely hindered [389,390]. As much as 97% of the ADP may be tightly bound and non-diffusable in skeletal muscle and heart (see Refs. 74,219), a finding that may also explain the apparent discrepancy between biochemically measured [ADP] (approx. 100–500 μM) and effective in vivo [ADP] of 1–50 μM calculated from ^{31}P-NMR spectra [42,141,143,312].

Assuming appropriate diffusion coefficients, substrate concentrations and concentration gradients of 5%, Jacobus [219] calculated the maximal flux rates of the respective substrates to be (in μmol/min/mg cardiac tissue): 35 for MgATP, 0.112 for MgADP, 57 for P_i, 103 for Cr and 123 for PCr. Evidently, MgADP is the most diffusion-restricted of all substrates, a fact that is also reflected by ^{31}P-pulsed-gradient NMR experiments yielding mean-square lengths of diffusion of 1.8 μm for ADP, 22 μm for ATP, 57 μm for PCr and 37 μm for Cr [525]. However, the values obtained for ADP are still in the same range as the maximum measured rate of ATP utilization in the heart (0.135 μmol/min per mg tissue) [219] and the diameter of a single myofibril of approx. 1 μm [525].

Since the free energy of PCr hydrolysis (ΔG_{obs} = −45 kJ/mol) [270] is consistently higher than that of ATP hydrolysis (ΔG_{obs} = −30.5 kJ/mol) [157], the CK/PCr system efficiently 'buffers' [ADP] and [ATP] and, therefore, also the ATP/ADP ratio, as well as the phosphorylation potential in the cytosol [230,454]. This is especially important for tissues with abrupt changes in energy demand like cardiac and skeletal muscle, as well as brain. During work or anoxia, first [PCr] decreases at relatively constant levels of ATP and ADP and only when a large part of the PCr is depleted, [ATP] decreases as well [141,159,226,330,381,417]. Since ATP and ADP are key regulators of many fundamental metabolic pathways, whereas Cr and PCr are likely not to be involved in allosteric regulation of intermediary metabolism [128], the CK/PCr system, by damping fluctuations of [ATP] and [ADP] upon abrupt changes of energy demand, allows a better fine-tuning of whole cellular metabolism and, therefore, protects the cell from energy dissipation (see also IV-B).

By keeping [ADP] low, the CK/PCr system further protects the cells from a net loss of adenine nucleotides [144,209,226,492]. Accumulation of ADP activates adenylate kinase (myokinase) which catalyzes the

transfer of a phosphate group between two molecules of ADP to give ATP and AMP. Especially in white and red fast-twitch muscles, AMP is degraded into inosine monophosphate (IMP) and ammonia by AMP deaminase [309] which is bound to the myofibrils at both ends of the A-band [91]. Cytosolic or sarcolemma-bound 5'-nucleotidase dephosphorylates both AMP and IMP into adenosine and inosine, respectively. These latter substances ultimately leave the cell, since the sarcolemma is permeable to the latter two compounds (see Ref. 226).

As can be directly seen from the chemical equation of the CK reaction,

$$PCr^{2-} + MgADP^{-} + H^{+} \rightleftarrows MgATP^{2-} + Cr,$$

the CK/PCr system also avoids acidification of the cytosol during periods of high workload [110]. As long as PCr is present in significant amounts, [ATP] remains almost constant and, thus, $PCr^{2-} \rightarrow Cr + P_i^{1-}$ is the net reaction supporting work. Since P_i at a pH around 7.0 has a mean charge between -1 and -2, PCr hydrolysis may at least in part be responsible for the tissue alkalinization observed during the first stages of muscular work [89,191,264,310]. Only when almost all PCr is exhausted, lactate production by glycolysis, as well as net ATP hydrolysis lead to a considerable acidification of the cytosol. Acidification has three main consequences: (i) it decreases the maximal force of a muscle, either by itself or in combination with diprotonated inorganic phosphate, $H_2PO_4^-$, which itself is favoured over HPO_4^{2-} at low pH values [336]; (ii) it reduces the glycolytic flux by inhibiting phosphofructokinase (see Ref. 77), thereby also avoiding further acidification, exhaustion of high-energy phosphates and, thus, irreversible damage of the cell; and (iii) it shifts the CK equilibrium towards ATP synthesis, as can also be seen directly from the chemical equation. Thus, the CK/PCr system ensures an almost constant ATP/ADP ratio over quite a wide range of energy demands and pH values which is essential for the proper functioning of all cellular ATPases.

In tissues with high and fluctuating energy demands, at least two potential sites of regulation are introduced by the CK/PCr system. Whereas the Cr + PCr pool size is likely to be only important for long-term regulation and adaptation, the cytosolic and Mi-CK isoenzymes are attractive candidates for short-term regulation of the overall flux through the CK reaction. Since the cytosolic CK activity can cope easily with the maximal rates of ATP production or ATP consumption and, thus, the cytosolic CK system is likely to be in a near-equilibrium state [205,262,298,310], regulation of cytosolic CK activity was suggested to have no influence on energy metabolism.

In contrast to the cytosolic CK isoenzymes, Mi-CK is thought to be involved in metabolic channelling of high-energy phosphates from the mitochondrial matrix to the cytosol and is, therefore, likely to be displaced from thermodynamic equilibrium [262]. Accordingly, regulation of Mi-CK activity would directly influence the export of PCr out of the mitochondria. The potential implications of regulation of CK isoenzymes can only fully be appreciated if one considers that it allows very specific and efficient regulation of whole cellular energy metabolism. Though there have been no convincing reports up to now proving regulation of CK activity in vivo, the recent findings that phosphorylation of BB-CK reduces the K_m for PCr by about a factor 2 [84,356], that BB-CK is a possible substrate of protein kinase C [85] and that a variety of CK isoenzymes are subject to autophosphorylation [184] suggest that CK regulation may be of physiological relevance.

As a last point, PCr shall be compared with other phosphagens. In all vertebrate and some invertebrate species, PCr is the sole phosphagen. In contrast, a variety of different phosphagens like phosphoarginine (PAr), phospholombricine (PL), phosphotaurocyamine (PTc), phosphohypotaurocyamine (PHTc) and phosphoglycocyamine (PGc) were found in lower phyla, either alone or in combination with each other or with PCr [112,322,493]. Interestingly, exclusively PCr is found in spermatozoa of a large number of 'lower' species having other phosphagens in other tissues. Determination of the apparent equilibrium constants of the phosphagen kinase reactions by biochemical and ^{31}P-NMR methods revealed that at pH 7.25, the apparent equilibrium constant of CK ($K'_{CK} = [Cr][ATP]/[PCr][MgADP]$) is 3–8-times higher than the respective K' values for arginine kinase (AK), glycocyamine kinase (GK), taurocyamine kinase (TK) and lombricine kinase (LK) [112]. In other words, the free energies of hydrolysis of PAr, PGc, PTc and PL are 2.9–5.2 kJ/mol lower than that of PCr. This property can be explained by the methyl group attached to the guanidine moiety of PCr which eliminates almost all resonance states and, thus, decreases the thermodynamic stability of PCr [112,114]. Due to the higher ΔG_{obs} value of PCr hydrolysis, the ATP/ADP ratio can be buffered at a higher value which seems especially relevant in the light of experiments on vertebrate skeletal muscle, showing that the reciprocal of the relaxation rate constant is directly proportional to the cytosolic phosphorylation potential and, thus, also to the ATP/ADP ratio [97,112]. In addition, maintaining a high affinity (free energy change) for ATP hydrolysis has been shown to be essential for a variety of cellular ATPases [229] (see also IV-B). On the other hand, when the cellular pH is lowered, phosphagen kinase reactions with a lower K' value will show a smaller degree of net hydrolysis of the respective phosphagen [112]. Consequently, a pool of the highly labile phosphagen PCr

would be rapidly dissipated under conditions of intracellular acidosis which especially in molluscs is a commonly observed phenomenon. In contrast, AK/PAr may act as an effective buffer system under these circumstances.

These latter reflections shed some light on the functions and evolutionary relationships of the different phosphagens. It has been suggested that PAr is the most primitive of the phosphagens, representing an evolutionary precursor. PCr, present mostly in vertebrates, was thought to represent a functional improvement over PAr, because PAr/Ar interfere with amino-acid metabolism, whereas Cr represents an end-product of a distinct metabolic pathway not interfering with amino-acid metabolism [495]. No satisfactory explanations for the occurrence of a variety of different phosphagens in lower phyla have been presented so far. However, the differential distribution in the animal kingdom of PCr on one hand and PAr, PL, PTc, etc. on the other hand may also be explained as follows. PCr is predominant found in vertebrates, which almost perfectly maintain intracellular homeostasis. In contrast, intracellular homeostasis is less pronounced in lower phyla, causing larger fluctuations of pH, temperature, substrate concentrations, etc. upon changes in the actual environment. Changes in intracellular conditions might lead to hydrolysis of the highly labile PCr pool and, thus, to energy dissipation. Consequently, under conditions of poor intracellular homeostasis, more stable phosphagens might be better suited than PCr.

In conclusion, the CK/PCr system has the following major advantages over a system based exclusively on ATP/ADP diffusion: (i) it allows a larger flux of high-energy phosphates between sites of ATP production (mitochondria and glycolysis) and ATP utilization (all sorts of ATPases), (ii) it allows the maintenance of a higher ATP/ADP ratio throughout the cell, (iii) it avoids a net loss of adenine nucleotides, (iv) it keeps the pH almost constant during the first stages of cellular work and (v) provides two additional potential sites for the very specific regulation of energy metabolism. Most of the models of CK function discussed in the next Section are based primarily on only one or just a few of these advantages. Consideration of all advantages, of various models and of changes in metabolic demand specific for different developmental stages and metabolic adaptations will lead to a more thorough understanding of CK function in whole cellular energy metabolism.

IV-B. Models of CK function

Since the discovery of PCr in 1927 [111], the ideas and models about the involvement of the CK/PCr system in energy metabolism have changed several times. The fact that it is not possible to explain all physiological findings with one single of these models may explain why confusion about the 'real' function of the CK/PCr system still exists in the literature and why in most textbooks, energy metabolism is simplified by omitting the CK/PCr system and by assuming exclusive diffusion of ATP and ADP between sites of ATP production and ATP consumption (Fig. 4A). This model may be correct for cells and tissues lacking CK like liver, but it is clearly incomplete for tissues with high and fluctuating energy demands like heart and skeletal muscle, brain, spermatozoa, retina, kidney, etc. (see Ref. 492). For a historical overview on the development of alternative models, the reader is referred to Refs. 35,37 and 214. In this Section, the various models will be discussed only for ATP production by oxidative phosphorylation within mitochondria. However, no differences in the qualitative aspects of the models and in conclusions result from a replacement of mitochondrial oxidative phosphorylation by glycogenolysis or glycolysis, because functional coupling, like that of Mi-CK to oxidative phosphorylation (see III-C), has also been observed between cytosolic CK isoenzymes and glycolysis (for reviews, see Refs. 381,492).

Since during work, [PCr] decreases whereas [ATP] remains relatively constant, it was long believed that PCr is the direct source of energy for muscular contraction, with ATP being responsible for the regeneration of PCr. Thirty years ago, however, rather specific inhibition of CK in frog skeletal muscle by 1-fluoro-2,4-dinitrobenzene caused a contraction-dependent decrease in [ATP] at constant [PCr] and, therefore, proved that ATP hydrolysis directly supports muscular contraction [70]. Because CK inhibition lowered the number of normal contractions of a muscle fiber from > 30 to approx. 3, it was hypothesized that the CK/PCr system represents a back-up system for very efficient 'buffering' of [ATP] and especially [ADP] [70,302,485] (Fig. 4B). However, a large body of evidence challenged the validity of the 'buffer model' [149,159,160, 414] so that instead an (exclusive) 'transport' function was proposed for the CK system [35,37,38,213,301,328, 379,415,416,463,486,488]. According to this model, which was also termed 'PCr shuttle hypothesis', Mi-CK bound to the outer face of the inner mitochondrial membrane catalyzes the transfer of the γ-phosphate group of ATP, synthesized by mitochondrial oxidative phosphorylation, to Cr. PCr then diffuses out of the mitochondria to sites within the cell where energy is consumed and PCr continuously regenerates ATP. Diffusion of Cr back to the mitochondria closes the cycle (Fig. 4C). In other words, the CK/PCr/Cr system is shunted in between sites of ATP production and ATP consumption.

Many experiments were interpreted as favouring one of the two models and dismissing the other. However, within a cell, both 'buffer' and 'transport' func-

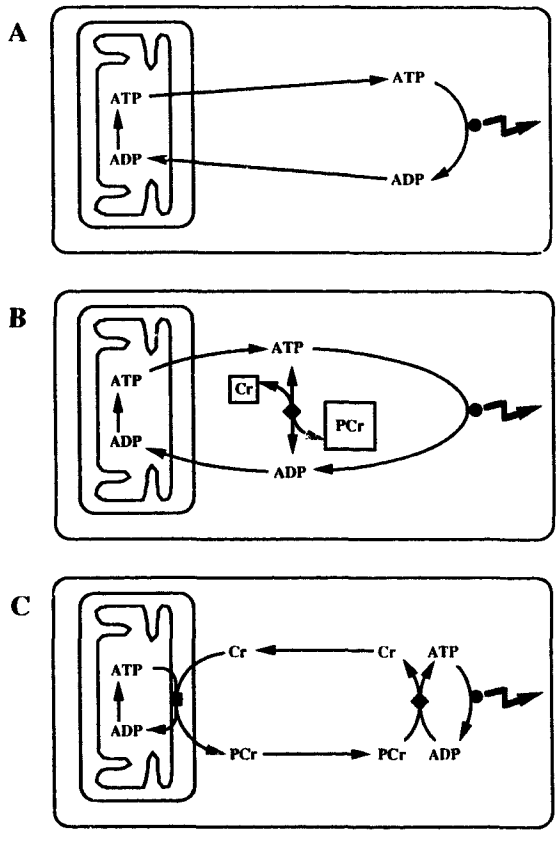

Fig. 4. Models of CK function. (A) Classical 'textbook' model of energy transport between sites of ATP production (mitochondria, glycolysis) and ATP consumption (all sorts of ATPases). This model may roughly reflect the situation in tissues devoid of CK, PCr and Cr like liver, but is clearly inappropriate to describe the situation in CK-containing tissues like skeletal or cardiac muscle, brain, retina and spermatozoa. (B) 'Buffer' (storage) function of the CK/PCr system. A large pool of PCr is available for immediate regeneration of ATP hydrolyzed during short periods of intense work. Due to the high cytosolic CK activities, the CK reaction remains in a near-equilibrium state and, thus, keeps [ADP] and [ATP] almost constant. In other words, the CK/PCr system efficiently 'buffers' the concentrations of ATP and especially of ADP. (C) 'Transport' function of the CK/PCr system. The CK/PCr system fulfils the function of a 'transport device' shunted in between sites of ATP production and ATP consumption. Note that for the 'buffer' function no Mi-CK isoenzyme is needed, whereas for the 'transport' function, Mi-CK may be an essential prerequisite, especially if there were diffusion limitations for adenine nucleotides across the outer mitochondrial membrane [32,33,147,148]. In addition, it should be kept in mind that the two models (B) and (C) represent extremes, with the actual situation in a cell or tissue being somewhere in between. Accordingly, the physiological requirements of a tissue determine the relative importance of the 'buffer' and 'transport' function of the CK/PCr system and, therefore, also the relative proportion of Mi-CK. For further details, see the text. (●), sites of ATP hydrolysis, e.g., myosin ATPase or ion pumps; (■), Mi-CK; (◆), cytosolic CK.

tions of the CK/PCr system may be operational at the same time, with the relative contributions of these two aspects depending on the metabolic demands of a tissue. For example, the buffer function is likely to be more pronounced in fast-twitch muscles where bursts of ATP breakdown have to be buffered immediately and very efficiently to allow short periods of maximal work. These muscles fatigue rapidly because regeneration of high-energy phosphates is achieved on a much slower time-scale mostly by glycolysis. In slow-twitch muscles and especially in heart, however, the transport function seems more important, since in these muscles, high rates of ATP consumption and, therefore, also of ATP production and transport have to be ensured for longer periods of time. For each workload, a steady-state is attained where ATP production and consumption are efficiently regulated and balanced [17].

The 'buffer' function of the CK/PCr system is supported by the following facts: (i) Non-excitable cells and organs like liver with a relatively high, but continuous flux of high-energy phosphates contain only small amounts of CK and PCr or even none at all [41,518]. This might indicate that the CK/PCr system is not essential for maintaining high flux rates of high-energy phosphates, but rather for buffering of sudden changes in energy demand (see below). (ii) For a transport function, comparable amounts of Mi-CK and cytosolic CK activities are expected within a cell. However, the proportion of Mi-CK was found to be only 0–2% of total CK activity in some skeletal muscles and brain [8,9,48,245,294,434]. In addition, no mitochondrial isoenzymes of arginine kinase have so far been found in a variety of arthropod flight and squid mantle muscles [113,402,451], suggesting that in all these tissues, the CK/PCr and AK/PAr systems are not so important for energy 'transport'. However, though insect flight muscles are capable of extremely high aerobic energy fluxes without the participation of a mitochondrial arginine kinase isoenzyme, these results do not exclude a transport function of phosphagen kinase systems, since first, rows of densely packed mitochondria are lined up in close apposition to individual myofibrils of insect flight muscles in such a way that diffusion distances from mitochondria to myofibrils are minimized [438] and second, a mitochondrial isoenzyme is less important for a transport function of an AK/PAr than for a CK/PCr system. Theoretical considerations have shown that due to the lower equilibrium constant of AK compared to CK, the proportion of high-energy phosphate flux carried by PAr at the same ATP/ADP ratio is higher than that carried by PCr [311]. (iii) PCr content, total CK and soluble MM-CK activity were found to correlate with the glycolytic potential of a muscle (Ref. 332, for a review, see Ref. 492). Consequently, they were highest in fast-twitch muscles with high glycolytic, but low oxidative potentials where ATP breakdown due to muscular work occurs on a much faster time-scale than regeneration by glycolysis [264], indicating that the transport

function of the CK/PCr system is nearly irrelevant for fast-twitch glycolytic muscles. (iv) Biochemical and ^{31}P-NMR experiments demonstrated that in brain, heart and skeletal muscle, the cytosolic CK activity, as well as the overall flux through the CK reaction are much higher than the maximal rates of ATP synthesis and ATP consumption, suggesting that the CK system in the cytosol is in a near-equilibrium state [42,88,205, 262,299,310,346,381,423,475]. Under near-equilibrium conditions, ATP and ADP are efficiently buffered, as is corroborated by physiological experiments revealing that during muscular and nervous work, [ATP] remains almost constant, whereas [PCr] decreases (Ref. 381; for a review, see Ref. 417). (v) No functional coupling was detected between Mi-AK and mitochondrial oxidative phosphorylation in the heart of the horseshoe crab *Limulus polyphemus*, meaning that this isoenzyme is in free equilibrium with the cytosolic substrate concentrations [106]. Thus, the function of this Mi-AK isoenzyme is likely to ensure near-equilibrium conditions of the phosphagen kinase reaction in the intermembrane space rather than to participate in a 'shuttle mechanism' for high-energy phosphates.

A 'transport' function for the CK/PCr system is favoured by the following arguments: (i) The presence of Mi-CK, as well as of cytosolic CK isoenzymes within the same cells suggests that they have different functions. Most attractively, Mi-CK is responsible for PCr synthesis and significant fractions of cytosolic CK for ATP regeneration (Fig. 4C). (ii) The proportion of Mi-CK increases with the oxidative potential and, therefore, also with the expected relevance of the transport function for the proper functioning of a tissue (see also III-E, and III-F). It is higher in slow-twitch than in fast-twitch muscles and highest in heart (up to 50%, see III-A). In brain, depending mostly on glycolysis, the relative proportion of Mi-CK is low [48,391,423]. Furthermore, during development, the proportion of Mi-CK and muscle performance rose in parallel (see III-E). Finally, chronic stimulation [400] and endurance training [9] increased the relative proportion of Mi-CK, while Mi-CK activity decreased in the immobilized human leg after surgery [224]. (iii) In the neonatal rabbit heart, the flux through the CK reaction directly correlated with the relative proportion of Mi-CK [346]. Furthermore, model calculations suggested that upon heart stimulation, the flux through Mi-CK increases several-fold [251,529]. (iv) Whereas the overall flux through the CK reaction is probably independent of the metabolic state in skeletal muscle [529], it increases with workload in the heart [42,44,46,47,262,300,346, 386,529], suggesting that the CK/PCr system in working heart is no longer in a near-equilibrium state. This conclusion was also corroborated for several tissues suffering or recovering from hypoxia or ischemia [19,21,141,159,160,206,334], indicating that merely buffering the concentrations of adenine nucleotides is not the sole function of the CK/PCr system. (v) Jacobus [218] calculated that the total ATP pool would only suffice for 10 s of cardiac work at normal rates of energy utilization. Even with PCr present, the 'resting' heart turns over the total high-energy phosphate pool 2–4 times per min. Similarly, ATP plus PAr would only suffice for 1.5 s of flight of *Locusta migratoria L.* [402]. Assuming concentration gradients of 5%, maximal flux rates (in μmol/min per mg heart) of 35 for MgATP, 57 for P_i, 123 for PCr and 103 for Cr were calculated. However, the flux of ADP (0.112 μmol/min per mg), primarily due to its very low concentration, is in the same range as the maximum measured rate of ATP utilization (0.135 μmol/min per mg), indicating that especially at higher workloads, ATP-ADP flux alone might be insufficient to maintain appropriate ATP/ADP ratios throughout the cell [219]. (vi) In an elegant series of experiments it was shown that in sea urchin spermatozoa, very specific inhibition of the mitochondrial and flagellar CK isoenzymes by 1-fluoro-2,4-dinitrobenzene (FDNB) attenuated flagellar movement in the two distal thirds of the sperm tail, indicating that ATP and ADP diffusion alone are only sufficient to ensure dynein ATPase activity in the proximal third of the tail. Indeed, when ATP was added externally to permeabilized, FDNB-treated sperms, they were able to swim again normally [463], indicating that the CK/PCr system is essential for energy supply in these highly polar cells. Evolutionary studies support this conclusion, since high CK activities were found in sperms of the primitive type, whereas 10–100-fold lower CK activities were observed in sperms with a modified morphology [465]. 'Primitive' sperms have long flagellae, depend on aerobic energy metabolism, are typical for external fertilization and the mitochondria are localized exclusively within the head. 'Modified' sperms are typical for internal fertilization, depend also on glycolytic energy flux, and the mitochondria are localized in head and tail. That the CK/PCr system is essential for highly polarized cells is also supported by the finding of high CK activities in chicken and frog photoreceptor cells [465,496], as well as in ciliated cells from rabbit oviduct epithelium [465]. (vii) The finding of intracellular compartmentation of adenine nucleotides and Cr (see IV-C) has the direct consequence that communication between the various compartments (mitochondria, myofibrils, etc) and, thus, transport of CK substrates must happen. (viii) If only the buffer function were important for the CK/PCr system, a homogeneous distribution of CK isoenzymes would be expected in the cytosol. However, different proportions of cytosolic CK isoenzymes were found to be tightly bound to the myofibrillar M-band, the plasma membrane and the sarcoplasmic reticulum membrane where CK is thought to locally regenerate ATP, as well

as to be functionally coupled to myosin ATPase, Na^+/K^+-ATPase and Ca^{2+}-ATPase, respectively (Refs. 51,120,301,357,383,386,455, for reviews, see Refs. 38, 379,488,492). Co-localization of CK with ATP-requiring enzymes together with the likely potential that CK itself is a regulated enzyme might be an effective means of high-energy phosphate channelling. (ix) Theoretical considerations have shown that even under near-equilibrium conditions, most of the high-energy phosphate will be transported in the form of PCr at physiological ATP/ADP ratios [311]. Accordingly, Meyer et al. [311] hypothesize that the binding of CK near sites of ATP production or utilization 'serves to raise the local enzyme activity where the flux is greatest, thus, ensuring overall near-equilibrium with less total enzyme activity than would be necessary with uniformly distributed enzyme'.

Clearly, there were also some arguments raised against a transport function of the CK/PCr or AK/PAr systems. First, the AK activities in flight muscles of four insect species, as well as in squid mantle muscle, were found to be considerably smaller than the maximal ATP turnover rate [332,451]. However, comparison with mammalian tissues is difficult due to profound morphological differences (see above). And second, feeding of rats with the creatine analogue β-guanidinopropionic acid (GPA) for 6–10 weeks resulted in the heart in a nine-fold decrease in [PCr], as well as in a 4-fold decrease in the flux through the CK reaction, while GPA and GPAP were accumulated and $[P_i]$, [ATP], intracellular pH, oxygen consumption and cardiac performance remained more or less unchanged [424,426]. Since in GPA-fed rats, the measured rates of ATP turnover were 1.5–3-times greater than the flux through the CK reaction, it was concluded that PCr cannot be an obligatory intermediate of energy transduction in the heart [426]. However, it has to be stressed that during the rather long feeding periods, compensatory metabolic adaptations take place [427] and that GPAP can serve as a CK substrate to buffer ATP quite efficiently during transitions between work states [88]. Furthermore, the left ventricular developed pressure was significantly lower in rats fed GPA or β-guanidinobutyric acid compared to controls [531].

Many studies in the past aimed to prove that the CK/PCr system in some way is essential for energy metabolism. However, the GPA experiments suggest that vital mammalian tissues (except spermatozoa and retina?) still function reasonably well at greatly reduced concentrations of Cr and PCr (see III-F), this fact simply reflecting the overcapacity of the CK/PCr system under normal conditions. Nevertheless, the CK/PCr system in all likelihood has both a transport and buffer function and thereby increases the thermodynamic efficiency of energy metabolism, as will be discussed below. A second misleading aim was the attempt to demonstrate that a 'sufficient' flux of high-energy phosphates can not be attained by ADP and ATP alone. Evidently, with sufficiently steep concentration gradients, adenine nucleotide turnover and ADP-ATP flux might be balanced as well [219]. Therefore, the function of the CK/PCr system may not simply be to guarantee a sufficient flux of high-energy phosphates from sites of ATP production to sites of ATP consumption, but rather to ensure a sufficient flux of high-energy phosphates to maintain appropriate ATP/ADP ratios throughout the cell in order to ensure a proper functioning of all ATPases in tissues with rapidly changing energy demands [96,97,143,157,229, 230]. High ATP/ADP ratios point to high phosphorylation potentials and, thus, to high affinities (free energy changes) for ATP hydrolysis. In resting skeletal muscle, as well as in heart at a basal metabolic rate, the affinity for ATP hydrolysis was found to be in the range of 55–64 kJ/mol (Refs. 143,229, for a review, see Ref. 230), while in other tissues, it amounts to only 45–51 kJ/mol [230,440]. The facts that a variety of cellular ATPases require energies of 41–44 kJ/mol [96,97,229], that the affinity for ATP hydrolysis seems to be very efficiently buffered between 45 and 50 kJ/mol [229] and that the mechanical performance of the heart drastically decreases below 48 kJ/mol [229] strongly corroborate that the ATP/ADP ratio is of prime importance for energy metabolism. Most important in this respect is the finding that the Ca^{2+}-ATPase of the sarcoplasmic reticulum depends on a very high affinity for ATP hydrolysis to be able to reduce the cytoplasmic $[Ca^{2+}]$ to 100 nM and, thus, to ensure muscle relaxation [229]. Cessation of proper Ca^{2+} sequestration may even be the biochemical basis for muscular fatigue [97,157]. Kammermeier [230] further hypothesized that the affinity for ATP hydrolysis provides an explanation why CK is present in heart and skeletal muscle, as well as in brain, but not in liver. In tissues with affinities of 55–64 kJ/mol, [ADP] would have to be kept very low. Under these conditions, the diffusion gradients of ADP and ATP required to maintain the desired high-energy phosphate flux would cause an affinity gradient of 2–3 kJ/mol per μm. In tissues with affinities below 51 kJ/mol, [ADP] would be higher and the affinity gradients much smaller. Therefore, the CK/PCr system may have the additional function of flattening affinity gradients for ATP hydrolysis and, thus, avoiding energy dissipation [311]. However, this interpretation raises some additional questions: Why do brain, heart and skeletal muscle depend on a higher affinity for ATP hydrolysis? What are the processes or chemical reactions requiring affinities of 55–64 kJ/mol? And how is the affinity for ATP hydrolysis regulated within a cell? Answering these questions may provide some deeper insight into the 'real' functions of the CK/PCr system.

Doubts about the real functions of the CK/PCr system might also be raised by the finding of cytosolic, as well as Mi-CK isoenzymes in smooth muscle tissues [207] displaying much lower maximal performances than striated muscles. However, in analogy to striated muscles, 'fast' and 'slow' smooth muscles may be discriminated. Most visceral and vascular smooth muscles are characterized by long-lasting tonic contractions, contain cytosolic and Mi-CK isoenzymes and consequently can be compared with slow-twitch skeletal muscles. In contrast, chicken gizzard displays only phasic contractions without a tonic component, where maximal force is developed after 10–15 s of stimulation [126]. Since chicken gizzard, in addition, has no Mi-CK, but considerable amounts of BB-CK [207], it may be compared with fast-twitch skeletal muscles. The obvious difference between slow- and fast-twitch skeletal muscles and 'slow' and 'fast' smooth muscles is the time-scale of metabolic changes. An attractive explanation to investigate is that diffusion of CK substrates is much more hindered in smooth than in striated muscles, but that the CK/PCr system in both muscle types has the same functions.

The last function of the CK/PCr system to be discussed is to 'accelerate' and 'smooth' transitions between different work states. If ADP liberated by muscular contraction or by all sorts of cellular ATPases were the signal for ATP production by mitochondrial oxidative phosphorylation (see IV-D) and if the system under these conditions were non-linear, then oscillations around the new steady-state level would be expected following changes in workload. These oscillations would be paralleled by fluctuations in [ADP], [ATP] and in the ATP/ADP ratio. Since ATP and ADP are key regulators of many of the fundamental metabolic pathways, the net result might be a destabilization of whole cellular metabolism. Because near-equilibrium conditions only allow for a linear system, where oscillations do not occur, the CK/PCr system may dampen the oscillations mentioned above and consequently stabilize whole cellular metabolism. It has to be stressed that the oscillations discussed here are completely different from those described in several practical and theoretical studies [21,134,139]. The former are due to an approach to a new steady-state level, whereas the latter result from regularly fluctuating energy demands characteristic of cardiac muscle, or from alternating perfusion with O_2 and N_2.

By accelerating 'communication' between sites of ATP production and ATP consumption, the CK/PCr system may, in addition, reduce the transient times for reaching a new steady-state. Transient times can be shortened in two different ways, either by elevating enzyme activities in such a way as to guarantee near-equilibrium conditions [109,182] or by metabolite channelling [343]. Both of these mechanisms are probably operational in the CK/PCr system, thus indicating as well that reducing transient times is important for energy metabolism in tissues with rapidly changing energy demands. The cytosolic CK isoenzymes are likely to be in a near-equilibrium state (see above) and it seems that Mi-CK is involved in metabolic channelling of high-energy phosphates out of the mitochondria (see III-C). All of these latter interpretations are strongly favoured by the fact that the CK/PCr system is predominantly found in tissues with high and fluctuating energy demands like brain, cardiac and skeletal muscle, but not in tissues with high but more or less constant energy demand like liver.

In conclusion, the CK/PCr system has two main primary functions: (1) it buffers the concentrations of ADP and ATP and (2) accelerates the transport of high-energy phosphates between sites of ATP production and ATP consumption, thereby accelerating also communication and feedback regulation between the two complementary parts of the CK isoenzyme system. The remaining functions of the CK/PCr system are direct consequences of the 'buffer' and 'transport' functions. Besides buffering H^+ and preventing loss of adenine nucleotides (see IV-A), (3) the CK/PCr system, due to keeping [ADP] low, maintains a high affinity for ATP hydrolysis which seems to be crucial for various ATPases. (4) Furthermore, transient times between different workloads are shortened by the CK/PCr system due to metabolite channelling by Mi-CK and near-equilibrium conditions of the cytosolic CK isoenzymes. (5) Finally, due to the CK reaction being in a near-equilibrium state in the cytosol, oscillations in the concentrations of high-energy phosphates may be avoided upon abrupt changes in workload.

IV-C. Subcellular compartmentation of CK substrates

The function of the CK/PCr system to accelerate the 'communication' between sites of ATP production and ATP consumption, as it was proposed in the preceding Section, would be even more crucial if discrete subcellular pools of CK substrates (ADP, ATP, PCr, Cr, H^+) occurred. In fact, much evidence for microcompartmentation of CK substrates has been accumulated over the last thirty years (for reviews, see Refs. 38,492). Incubation of striated muscle of the frog with tritium-labelled adenine or Cr, followed by fixation of the tissue (in the absence or presence of a lanthanum salt to 'precipitate' the high-energy phosphates) and autoradiography of thin sections, revealed an accumulation of adenine nucleotides and PCr at discrete locations within the myofibrils [186–189]. Depending on the conditions of fixation, adenine nucleotides were found in a narrow disk either in the I-band or the A-band, but in both instances close to the A-I boundary [187,188]. PCr was concentrated in a

narrow disk within the I-band, approximately halfway between the A-I boundary and the Z-line [189]. For this latter region, the local [PCr] was calculated to be 166 mmol/kg muscle. MM-CK in chicken pectoralis or rat muscles was also found to be loosely bound to the I-band, together with glycolytic enzymes, in addition to its localization within the M-band of the sarcomeres [491,497]. Even though serious reservations have to be made about the method employed by Hill [186–189], the co-localization of MM-CK and its substrates nevertheless points to an important role of microcompartmentation of the CK/PCr system within the I-band of the myofibrils.

After incubation or perfusion of rabbit or rat heart with ^{14}C-labelled Cr, the specific radioactivity of PCr (SA_{PCr}), surprisingly, was significantly higher than the SA_{Cr} (SA_{PCr}/SA_{Cr} 1.24–1.87) [272,392]. Subsequent washout of excess Cr or anoxia even increased the SA_{PCr}/SA_{Cr} ratio to 5–11, which is a clear indication for microcompartmentation. Savabi [392] from her data concluded that 55% of the total Cr (Cr + PCr) in spontaneously beating rat-heart atria is PCr, 9% constitute the Cr-pool 1 which is readily accessible to phosphorylation by (Mi-) CK and 36% constitute the Cr-pool 2. Cr in this latter pool is rather inaccessible to phosphorylation and may be bound to subcellular structures. Incubation of the atria with [^{14}C]Cr causes a selective uptake of radioactive Cr into pool 1. PCr hydrolysis due to anoxia also leads to an accumulation of Cr in pool 1, suggesting that only a small proportion of the total Cr is metabolically 'active'. If these findings were true, the validity of the conclusions from the GPA experiments would have to be seriously questioned. For example, only about 80% of PCr and 60% of total Cr were depleted by feeding animals with GPA for 8 weeks [531]. If GPA-feeding selectively diminished only the Cr-pool 2, pool 1 and, therefore, also energy metabolism in general might remain almost unaffected.

Besides sequestration of ATP and ADP in membrane-enclosed dense granules of blood platelets [471] and accumulation of ATP in the nucleus of frog oocytes [315], as much as approx. 30% of the intracellular ATP was found to be 'trapped' in cardiac and liver mitochondria (Ref. 144, for reviews, see Refs. 177,247,430). Furthermore, subcellular fractionation of rat heart and liver in non-aqueous media revealed the mitochondrial matrix [ATP]/[ADP] ratio to be much lower than the cytosolic [ATP]/[free ADP] ratio [177,430]. Together with the findings of separate mitochondrial and cytosolic CK isoenzymes and of diffusion limitations of adenine nucleotides across the outer mitochondrial membrane [147,148], these results clearly point to distinct adenine nucleotide pools within the mitochondria and in the cytosol.

The terms 'functional coupling' and 'metabolite channelling' automatically imply microcompartmentation. Since much evidence has been accumulated for functional coupling between Mi-CK, ANT and porin, as well as between cytosolic CK isoenzymes and myosin-ATPase of the myofibrils, Ca^{2+}-ATPase of the sarcoplasmic reticulum and Na^+/K^+-ATPase of the sarcolemma and, since the different CK isoenzymes are in part bound to these subcellular structures in an isoenzyme-specific manner (see III-C. and IV-B), it seems very likely that not only between mitochondrial matrix, intermembrane space and cytosol, but also in the cytosol itself, different microcompartments for CK substrates exist, at least at high workloads. The latter suggestion is strengthened by experiments on hypoxic or ischemic heart and smooth muscle, indicating that in spite of the high cytosolic CK activities, the CK substrates are not in a near-equilibrium state [21,159,160, 206]. The most likely explanation is microcompartmentation of adenine nucleotides at the myofibrils [160,301,379,478,488] where ADP in resting muscle was supposed to be tightly bound to actin [144,501].

^{31}P-NMR data were also interpreted in favour of subcellular compartmentation of CK substrates. In conventional saturation transfer experiments under steady-state conditions, the apparent high-energy phosphate flux in the forward direction of the CK reaction (PCr synthesis) was in most cases considerably higher than that in the reverse direction (ATP synthesis) [42, 44,48,141,252,299,310,337,423]. Two alternative explanations were given for this difference: (1) microcompartmentation of CK substrates [251,252,337,529]. Two models accounting for microcompartmentation were developed. According to the model of Koretsky et al. [251], differences in flux rates between the forward and reverse direction of the CK reaction are observed in conventional saturation transfer, but not in 2-D or inversion transfer NMR experiments, with these differences being a direct measure of microcompartmentation. In fact, the forward and reverse fluxes through the CK reaction were found to be equal in 2-D-NMR experiments [15]. On the other hand, the model of Zahler et al. [529] is based on the assumption that some pools of cellular adenine nucleotides are NMR-invisible, and consequently, not saturable in conventional saturation transfer experiments. This model correctly predicts that the flux PCr → ATP increases with workload in the heart [42,44,46,47,262,300,346,386], but not in skeletal muscle [67,141,360,425]. Furthermore, this model was shown to be consistent with a NMR-invisible ATP pool at the mitochondria, but not with localization of NMR-invisible ATP exclusively within the myofibrils. The notion that mitochondrial adenine nucleotides, probably due to the extremely high viscosity of the mitochondrial matrix [394], are restricted in rotational diffusion and are, therefore, NMR-invisible, is in support of this second model [529]. (2) Alternatively, the participation of ATP in various side-reac-

tions has been proposed as basis for the unequal forward and reverse fluxes through the CK reaction [141,299]. This interpretation is favoured by the finding that under steady-state conditions, the difference between both fluxes vanished in multiple saturation transfer experiments, when for the determination of the ATP → PCr flux, the P_i-, as well as PCr resonances were concomitantly saturated [472]. Nevertheless, more work is needed to define clearly which of the two interpretations or which of the two models of microcompartmentation is correct. Finally, ^{31}P-NMR experiments on intact cells and tissues, as well as anoxic muscle revealed different pH environments for P_i (Ref. 412; for a review, see Ref. 201) which may be explained by pH gradients of up to 0.5–1.0 pH units between the cytoplasmic and mitochondrial compartments.

In conclusion, telling arguments for distinct mitochondrial and cytosolic CK substrate pools have been accumulated over the years. In contrast, only indirect evidence is currently available for microcompartmentation of CK substrates in the cytosol itself. Even though it is difficult to achieve, unequivocal corroboration of this latter type of microcompartmentation will be an important task for future research, since it critically determines the functions of the CK/PCr system and likely also a variety of cellular processes.

IV-D. Regulation of mitochondrial oxidative phosphorylation

Since the CK/PCr system in some tissues seems to be an essential part of the energy metabolism, its possible involvement in the regulation of mitochondrial oxidative phosphorylation shall briefly be discussed. Up to now, mitochondrial respiration was proposed to correlate with (and, therefore, to be regulated by) [ADP], the [ATP]/[ADP] ratio, the phosphorylation potential, the 'adenylate reaction pressure', the [NADH]/[NAD^+] ratio, [Ca^{2+}] or O_2 supply (Refs. 152,217,263, for reviews see Refs. 17,56,102,150,171, 177,181,218,253,294,306]. Whereas experiments on isolated mitochondria, as well as on liver, skeletal muscle and newborn sheep hearts revealed a good correlation between the respiration rate and the concentration of ATP hydrolysis products [17,181,351], these findings were challenged by experiments on in vivo or perfused adult heart, brain and kidney, which indicated a poor correlation between these parameters [16,17,74,138, 181]. Instead, especially for the heart, the [NADH]/[NAD^+] ratio and [Ca^{2+}] were favoured as primary regulators of oxidative phosphorylation, since Ca^{2+} at physiological concentrations was found to stimulate efficiently a variety of mitochondrial dehydrogenases [17,181].

At this point, a serious problem emerges. If reduction of transient times is an important function of the CK/PCr system (see IV-B), then the ATP hydrolysis products must be primary determinants of the respiration rate in adult heart and brain as well. How can the apparent discrepancy be explained? Let us consider the possible consequences of introducing the CK/PCr system in a cell. If, for example, [ADP] (or [ATP]/[ADP] or the phosphorylation potential) determined the mitochondrial respiration rate [217,218] and if the diffusion of ADP were restricted, ADP concentration gradients would exist between sites of ATP consumption and ATP production, with the steepness of the gradient increasing with workload. The larger the diffusion restrictions for ADP, the higher the apparent K_m of oxidative phosphorylation for ADP will be. Introduction of the CK/PCr system would drastically reduce the concentration gradient of ADP and, therefore, decrease the apparent K_m of oxidative phosphorylation for ADP. This latter effect would be even more pronounced in the case of diffusion restrictions for adenine nucleotides across the outer mitochondrial membrane [32,33,147,148]. In other words, when [ADP] is plotted against the respiration rate or the rate-pressure product, a much flatter line will be obtained in the presence of the CK/PCr system than in its absence, with this flatter dependency probably being barely detectable by ^{31}P-NMR techniques [74].

These considerations may explain (1) why a clear correlation between the respiration rate and the concentration of ATP hydrolysis products was found in liver and isolated mitochondria, but not in adult heart exhibiting a fully developed CK system; (2) why the regulation of oxidative phosphorylation by ATP hydrolysis products is apparently lost during postnatal development of the sheep heart [351], at about the same time when the CK system develops to its full maturity [199] and (3) why training or chronic stimulation, known to increase the proportion of Mi-CK (see III-F), also resulted in an apparent loss of respiratory control by the phosphorylation potential [86].

These reflections suggest that the ATP hydrolysis products ADP, P_i and H^+ are more important for respiratory control in the heart than currently believed and that [Cr], [PCr] or the [Cr]/[PCr] ratio represent some sort of intermediate feedback signal for oxidative phosphorylation [42,221,314,376,379,414,415]. Since Mi-CK is likely to be displaced from equilibrium, a prerequisite for metabolic control, it may even be envisaged that oxidative phosphorylation is rate-limited by the Mi-CK reaction [294]. Nevertheless, the ATP hydrolysis products are clearly not the sole determinants of the respiratory rate [17,177,181]. They may be regarded as primary regulators of oxidative phosphorylation, with the [NADH]/[NAD^+] ratio or [Ca^{2+}] playing a more modulatory role, or vice versa. For further investigation of respiratory control in the heart, it will be essential to determine the diffusion limita-

tions for ADP, be it in the cytosol or across the outer mitochondrial membrane.

V. Perspectives

The aim of this review was to summarize the current knowledge about the biochemistry, physiology and pathology of mitochondrial creatine kinase and to present working hypotheses for future research. In addition to the 'buffer' and 'transport' function of the CK/PCr system, a third main function is proposed here, namely to reduce the transient times of the system to reach a new steady-state upon abrupt changes in workload (see IV-B). Transient times can be reduced in two different ways: (1) by increasing the enzymatic activities in such a way as to guarantee near-equilibrium conditions. The cytosolic CK activity in heart and skeletal muscle was shown to be several-fold higher than the maximal rates of ATP production or ATP consumption so that the cytosolic CK system is in a near-equilibrium state. (2) By metabolic channelling of substrates. Many recent findings support the notion that octameric Mi-CK within mitochondrial contact sites (CS) is involved in metabolic channelling of high-energy phosphates across both mitochondrial membranes (see III-C). First, kinetic and thermodynamic experiments revealed microcompartmentation of CK substrates within the mitochondrial intermembrane space which was either explained by enzyme-enzyme proximity of Mi-CK and ANT or by diffusion limitations for adenine nucleotides across the outer mitochondrial membrane. Second, it was realized that CS may play an important role in the export of high-energy phosphates out of the mitochondria. The extent of CS is variable and increases with mitochondrial stimulation. Third, Mi-CK was found to be enriched in mitochondrial CS. And finally, the highly symmetrical 3-D structure of the Mi-CK octamer, with two identical top and bottom faces, seems to be ideally suited for a CS localization, since the top and bottom faces are likely to have the same potency to interact with membranes (see II-C). Together with the apparent channel through the octameric molecule, it may be proposed that octameric Mi-CK within CS simultaneously binds to both the inner and outer mitochondrial membrane and is functionally (and physically?) coupled to ANT of the inner and porin of the outer membrane (see Fig. 2B). This highly ordered multi-enzyme complex could be an effective means of displacing the CK reaction in the intermembrane space far from equilibrium and, thus, of allowing PCr synthesis even at high cytosolic ATP/ADP ratios. In addition, functional coupling was also demonstrated for cytosolic CK with myosin ATPase of the myofibrils, Ca^{2+}-ATPase of the sarcoplasmic reticulum and Na^+/K^+-ATPase of the plasma membrane.

A further function of the CK/PCr system may be to dampen oscillations of [ATP] and [ADP] upon abrupt changes in workload (see IV-B). Inherent to the latter two ideas is the assumption that Cr acts as some sort of 'signal transducer' for the feedback regulation of mitochondrial oxidative phosphorylation by ATP hydrolysis products (see IV-D). Whereas the 'transport' and 'transient-time reduction' functions of the CK/PCr system are thought to predominate in tissues with a high proportion of Mi-CK like heart and slow-twitch skeletal muscles, the 'buffer' function is clearly more important in fast-twitch skeletal muscles with low amounts of Mi-CK. To dampen oscillations of [ATP] and [ADP] may be a crucial function of the CK/PCr system in all CK-containing tissues.

In order to scrutinize these ideas and to get a deeper insight into the 'real' functions of the CK/PCr system, it will be indispensable to define clearly (i) the diffusion limitations for ADP, ATP, PCr and Cr in the cytosol of heart, brain, skeletal and smooth muscle, retina and spermatozoa in comparison to liver; (ii) the permeability properties of the outer mitochondrial membrane for all CK substrates and P_i; (iii) the functional and/or physical coupling of Mi-CK to ANT and porin, as well as the stoichiometries of the three proteins within and beyond the CS; (iv) the three-dimensional structure of the Mi-CK octamer at atomic resolution in order to see if a channel through the molecule really exists and if the active sites of the subunits are directed towards this channel, and (v) whether changes in the dimer to octamer ratio of Mi-CK as well as dissociation and reassociation of dimeric and octameric Mi-CK from and to mitochondrial membranes (see III-D) also occur under in vivo conditions and how they influence mitochondrial PCr synthesis or feedback regulation of oxidative phosphorylation. These experiments may then serve as basis for mathematical modelling of the CK/PCr system which is an essential prerequisite for asking further questions.

From the point of view of comparative biochemistry and physiology, it will be intriguing to investigate at which stage of evolution two distinct Mi-CK isoenzymes in different tissues of the same species appeared and how widespread the octameric structure of the Mi-CK isoenzymes is. In this respect, the human Mi-CK isoenzymes are of particular interest, since up to now, no detailed characterization of the higher M_r forms of the purified isoenzymes has been published. In addition, ubiquitous and sarcomeric Mi-CK may serve as valuable tools to clarify whether heart and brain have different mitochondrial import machineries for precursor proteins.

A variety of recent studies suggest that the significance of the CK system in human pathology is currently underestimated. Culturing adult rat cardiomyocytes in a medium devoid of Cr or supplemented with

GPA led to the formation of large cylindrical mitochondria with crystal-like inclusions enriched in Mi-CK (see III-F). Similar abnormal mitochondria containing crystal-like inclusions enriched in Mi-CK were also observed in human mitochondrial myopathies (see III-G. and Fig. 4). In this case, however, formation of mitochondrial inclusions did not seem to correlate with [Cr] in the tissue. Therefore, it will be an important task for future studies to determine the conditions causing the formation of abnormal mitochondria in myopathic tissues. Furthermore, animals fed with GPA or GBA may serve as models for the investigation of mitochondrial myopathies. Since the CK/PCr system appears to be very important for spermatozoa, the relevance of defects within this system for male infertility will also be a promising subject for further research [198]. Finally, studies of patients with decreased levels of Mi-CK or with decreased tissue [PCr] may provide new views on the 'real' functions of the CK/PCr system.

In addition to all these challenges, the combination of established biochemical, biophysical and physiological methods with new, investigative techniques like ^{31}P-NMR, overexpression of cloned CK genes in *Escherichia coli* or yeast [14,62,79,140,254], site-directed mutagenesis, or generation of transgenic animals [66,255] will open the doors to new playing-fields for CK research.

Note added in proof (Received 18 August 1992)

Very recent work by LeFurgey et al. (J. Microsc. 165 (1992) 191–223), applying quantitative X-ray microanalysis to freeze-dried sarcomeric muscle, showed that phosphate compounds, presumably represented mostly by PCr and ATP, are highly compartmentalized in muscle, with a preferential occupancy of the I-band (sparing the Z-disc) as well as the H-zone (weaker signal as compared to the I-band). Incidentally, these are the very same intracellular locations where MM-CK was localized in situ in frozen muscle [497].

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