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Functional Studies with the Octameric and Dimeric Form of Mitochondrial Creatine Kinase

DIFFERENTIAL pH-DEPENDENT ASSOCIATION OF THE TWO OLIGOMERIC FORMS WITH THE INNER MITOCHONDRIAL MEMBRANE*

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Jörg Schlegel‡, Markus Wyss, Hans M. Eppenberger, and Theo Wallimann§

From the Institute of Cell Biology, Swiss Federal Institute of Technology, ETH-Hönggerberg, Zürich, CH-8093, Switzerland

Phosphate extraction of mitochondrial creatine kinase (Mi-CK, EC 2.7.3.2) from freshly isolated intact mitochondria of chicken cardiac muscle, after short swelling in hypotonic medium, yielded more than 90% of octameric and only small amounts of dimeric Mi-CK as judged by fast protein liquid chromatography-gel permeation analysis of the supernatants immediately after extraction of the enzyme. In extraction buffer, octameric Mi-CK displayed a tendency to dissociate, albeit at a slow rate with a half-life of approximately 3–5 days, into stable dimers.

Experiments with purified Mi-CK octamers or dimers, or defined mixtures thereof, incubated under identical conditions with Mi-CK-depleted mitoplasts revealed that *both* oligomeric forms of Mi-CK can rebind to mitoplasts. However, the association of Mi-CK was strongly pH-dependent and, in addition, octameric and dimeric Mi-CK showed different pH dependences of rebinding. Therefore, it was possible under certain pH conditions to rebind either both oligomeric forms or selectively the octamers only. Furthermore, evidence is presented that Mi-CK dimers partially form octamers upon rebinding to the inner membrane.

The differential association of the two oligomeric Mi-CK forms with the inner mitochondrial membrane together with the dynamic equilibrium between octameric and dimeric Mi-CK (Schlegel, J., Zurbriggen, B., Wegmann, G., Wyss, M., Eppenberger, H. M., and Wallimann, T. (1988) *J. Biol. Chem.*, 263, 16942–16953) suggest that both oligomeric forms are physiologically relevant. A change in the octamer to dimer ratio may influence the association behavior of Mi-CK in general and thus modulate mitochondrial energy flux as discussed in the phosphoryl creatine circuit model (Wallimann, T., Schnyder, T., Schlegel, J., Wyss, M., Wegmann, G., Rossi, A.-M., Hemmer, W., Eppenberger, H. M., and Quest, A. F. G. (1989) *Prog. Clin. Biol. Res.* 315, 159–176.

Creatine kinase (CK,[1] EC 2.7.3.2, creatine phosphotransferase) catalyzes the following reaction.

$$MgADP^- + PCr^{2-} + H^+ \overset{CK}{\rightleftharpoons} MgATP^{2-} + Cr$$

Three cytoplasmic isoenzymes, composed of two different subunits, have been described (1, 2): MM-CK (M standing for the muscle-type CK subunit); BB-CK (B standing for the brain-type CK subunit); and the heterodimeric form MB-CK found transiently during differentiation of skeletal muscle and permanently in adult mammalian heart (3). These isoforms of CK are expressed tissue specifically and localized subcellularly in an isoenzyme-specific manner (for review see Refs. 4 and 5). CK activity was also found in the mitochondrial fraction where mitochondrial CK or Mi-CK (6) is localized on the outer surface of the inner mitochondrial membrane (7). Recently, two Mi-CK isoenzymes were described which are expressed tissue specifically as well (8–10). The more basic Mi_b-CK and the more acidic Mi_a-CK isoforms are accumulated in mitochondria of cardiac muscle and brain, respectively (9). The different cytosolic and mitochondrial CK isoenzymes are thought to be involved in energy buffering and transport of "energy-rich" phosphoryl compounds as described in the phosphocreatine shuttle model (11–14) as well as in the regulation of local subcellular ATP levels as discussed within the PCr circuit model (15).

Two interconvertible oligomeric forms, an octameric and a dimeric form of Mi-CK, have been isolated and characterized both from cardiac muscle (Mi_b-CK) and brain (Mi_a-CK (9, 10, 16)). Since no obvious difference in specific enzyme activity was observed between the two oligomeric forms, the possible functional significance of these two molecular structures with respect to their interaction with the inner mitochondrial membrane was investigated. A number of authors have studied the solubilization and reassociation of Mi-CK from and with mitoplasts, respectively, long before anything about the existence of the octameric Mi-CK species was known (11, 17–23). From these results it became clear that the extraction as well as the rebinding of Mi-CK was strongly dependent on the source of mitochondria and on the buffer conditions used. Under certain *in vitro* conditions a multiple of the quantity

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‡Present address: Laboratory of Biochemistry, ETH Zentrum, Zürich CH-8092, Switzerland.

§To whom correspondence should be sent.

[1] The abbreviations used are: CK, creatine kinase; Mi-CK, mitochondrial creatine kinase; Mi_a- and Mi_b-CK, more acidic (a) and more basic (b) subunit isoforms found in brain (Mi_a-CK) and cardiac muscle (Mi_b-CK), respectively; Cr, creatine; PCr, phosphoryl creatine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, [ethylenebis(oxgethylenenitrilo)]tetraacetic acid; BSA, bovine serum albumin; BME, β-mercaptoethanol; IEF, isoelectric focusing; IEP, isoelectric point; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; ANT, adenine nucleotide translocator.

of Mi-CK released from a given amount of mitochondria could be rebound to extracted homologous mitoplasts or even to liver mitoplasts that do not show any Mi-CK activity *in vivo* (18, 23). These and further results (22) indicated that mitochondria from a variety of tissues show low as well as high affinity binding sites for Mi-CK.

Marcillat *et al.* (24) presented evidence that only the high M_r form of Mi-CK with a molecular weight of 350,000 did rebind to extracted mitoplasts but not the lower M_r or dimeric form. In two independent experiments, these authors tried to rebind either dimeric or octameric Mi-CK from crude extracts to mitoplasts, but the reassociation conditions chosen were different for the two forms. Rebinding experiments with octamers were done at pH 7.4, whereas pH 8.8 and *p*-chloromercuribenzoate were used for rebinding of dimers. As we know now, pH 7.4 is favoring rebinding of Mi-CK in general, that is octamers as well as dimers. In contrast, alkaline pH (25) and sulfhydryl reagents (19) are conditions known to release chicken or rat Mi-CK from mitoplasts. Thus, conclusive results about the rebinding behavior of the two oligomeric forms of Mi-CK are still missing.

We have shown recently that the octamer/dimer equilibrium of Mi-CK can be influenced *in vitro* and that at low protein concentrations the addition of substrates and cofactors inducing a transition state analogue complex leads to a complete conversion of octamers into dimers (10). In addition, it is now possible to generate mixtures of octameric and dimeric Mi-CK with relatively stable ratios of the two forms.

In order to investigate the association behavior of Mi-CK octamers and dimers with the inner mitochondrial membrane, we performed a series of experiments with highly purified Mi-CK octamers, dimers, or mixtures thereof with a concomitant quantitative analysis of the octamer to dimer ratio before and after the experiment using FPLC-gel permeation chromatography. With these new tools at hand, one was able to study in detail the reassociation behavior of Mi-CK octamers and dimers to mitoplasts under controlled conditions.

Strong evidence for an association of both oligomeric Mi-CK forms with mitoplasts was found, but at the same time clear functional differences in the association behavior between Mi-CK octamers and dimers were discovered. This may be relevant for regulation of energy transfer from mitochondria to cytosolic compartments where part of the cytosolic CK is specifically associated with subcellular structures (26) as discussed within the framework of the PCr circuit model (15).

Parts of this work have been presented as abstract (27).

MATERIALS AND METHODS

All chemicals used were from Boehringer Mannheim, Merck, Serva, Sigma, or Fluka and were at least of analytical grade quality.

Isolation of Mitochondria—Fresh chicken hearts were rapidly placed into cold isolation medium containing 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 0.2 mM EDTA (sodium salt), and 0.05% BSA (pH 7.4, adjusted with KOH), washed, minced through a Moulinex meat grinder, and homogenized with a Polytron mixer (Kinematica, Kriens, Switzerland) at position 5 for 15 s. Subsequently, the mitochondria were enriched by differential centrifugation with a first centrifugation step for 10 min at 530 × *g*, followed by filtration of the supernatant through a 100-μm nylon filter and two consecutive centrifugation steps for 10 and 8 min each at 10,000 and 8,500 × *g*, respectively. For the resuspension of the pellets from these two centrifugation steps, isolation medium containing 0.1 mM instead of 0.2 mM EDTA was used.

For all extraction and rebinding experiments only freshly prepared mitochondria were used which were centrifuged again for 7 min at 8500 × *g* directly before the experiment. The supernatant and the upper light brown layer of the pellet, consisting mainly of myofibrillar debris and damaged mitochondria, were removed, and the brown pellet consisting of highly enriched intact mitochondria was resuspended in isolation medium containing 0.2 mM EGTA instead of EDTA but no BSA.

Typical respiratory control ratios (quotient from respiration in state 3 to respiration in state 4) and P/O quotients (moles of ADP/mol oxygen) of isolated cardiac mitochondria, determined by a Clark-type oxygen electrode at 25 °C, were 6–7 and 2.5–3.5, respectively, indicating that the mitochondria were intact and well coupled.

Fast Extraction of Mi-CK from Mitochondria by Alkaline Phosphate Buffer—Freshly prepared intact mitochondria were centrifuged for 5 min in a Heraeus Christ Biofuge A at 13,000 rpm, resuspended, and swollen in cold distilled water for 2 min and extracted for 3 min by 1:1 dilution in a 2-fold concentrated extraction buffer at pH 8.6–8.8 (final concentrations: 25 mM sodium phosphate, 5 mM BME). Subsequently, the extracted mitochondria were pelleted in a Beckman Airfuge™ for 13 min at 30 p.s.i. (30 ° rotor, 178,000 × *g*). This high speed centrifugation was shown not to sediment solubilized octameric or dimeric Mi-CK. The supernatant obtained by this very fast extraction procedure contained about 80–85% of the total Mi-CK activity present in mitochondria and small amounts of other proteins that were also extracted by this treatment. However, the extraction was rather specific for Mi-CK which represented a prominent major protein band after analysis by SDS-PAGE as shown earlier (10, 24).

Preparation of Larger Amounts of Mitoplasts—Freshly prepared intact mitochondria were centrifuged for 15 min at 10,000 × *g*, resuspended, and swollen by incubation for 15 min in cold distilled water on ice. The resulting mitoplasts were washed once by centrifugation for 15 min at 20,000 × *g* and resuspension in cold distilled water.

Experiments on the Rebinding of Mi-CK to Extracted Mitoplasts—For all rebinding experiments phosphate-extracted mitoplasts were used which retained only 10–20% of the Mi-CK bound originally. In order to rebind Mi-CK exclusively to the physiological binding sites ("high affinity binding sites") of mitoplasts and not to overload them by an excess of Mi-CK binding to "low affinity binding sites" (see Ref. 22), only 60% of the total amount of Mi-CK extracted from a given aliquot of mitoplasts was readded to the same aliquot of Mi-CK-depleted mitoplasts. In detail, for the rebinding experiments, mitoplasts isolated as described above were extracted once by incubation on ice for 15 min with 25 mM sodium phosphate, 5 mM BME at pH 8.8 (final concentrations) and subsequent centrifugation for 20 min at 30,000 × *g*. Then, the extracted mitoplasts were washed twice in *rebinding buffer* containing 10 mM sodium phosphate and 5 mM BME at pH 7.0. These extracted CK-depleted mitoplasts were incubated with purified active Mi-CK for 2 min on ice in rebinding buffer at different pH values ranging from 7.0 to 8.8 followed by centrifugation in a Beckman Airfuge™ for 13 min at 30 p.s.i. (30 ° rotor, 178,000 × *g*). From each supernatant, 250 μl were loaded immediately on a Superose 12 column for gel filtration analysis by which Mi-CK octamers and dimers were nicely resolved (10). Subsequently, supernatants and pellets of the Airfuge centrifugation runs and the fractions from the gel filtration were analyzed for CK activity. For certain experiments mitoplast pellets were re-extracted as above (25 mM phosphate, 5 mM BME, pH 8.8) 30–60 min after rebinding of Mi-CK, and the ratio of octamers and dimers released was determined again by gel permeation on Superose 12.

For those experiments where purified and separated octameric or dimeric Mi-CK was rebound separately, octameric Mi-CK was taken from a Mi-CK sample stored at high protein concentration (≥3 mg/ml) in liquid nitrogen, which consisted of ≥90% octameric Mi-CK, whereas dimeric Mi-CK was generated by incubation of octameric Mi-CK for 4 h at low protein concentration (0.4 mg/ml) with a mixture of 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, 50 mM KNO_3, pH 7.2, inducing a transition state-analogue complex (see Ref. 10). Directly before use, the buffer of the sample was changed within 10 min by a FPLC fast desalting column HR 10/10 (Pharmacia LKB Biotechnology Inc.) which was equilibrated with the rebinding buffer at the pH value used for the experiment. Desalted dimeric Mi-CK freed of MgADP, creatine, and nitrate always displayed the same specific activity as octameric Mi-CK.

Creatine Kinase Assay—Creatine kinase (EC 2.7.3.2) activity was determined in the direction of ATP synthesis in a pH-stat (26) at 25 °C or, if higher sensitivity was needed, at 42 °C.

Assay of Protein Concentration—Protein was determined by the Bio-Rad protein assay, based on the method of Bradford (28), using BSA as a standard.

FPLC-Gel Permeation Chromatography—Gel filtration experiments were performed on a FPLC-Superose 12 HR 10/30 column

(Pharmacia) in a buffer containing 50 mM sodium phosphate, 150 mM sodium chloride, 2 mM BME, 0.2 mM EDTA-sodium salt, 2 mM sodium azide at pH 7.2. The column was calibrated using ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), chicken BB-CK (80 kDa) and α-chymotrypsinogen (24.5 kDa) as standards. Activity of creatine kinase in each fraction was determined as described above. Each Superose run was completed in less than 30 min.

Gel Electrophoresis and Isoelectric Focusing—PAGE in the presence of SDS was performed according to Laemmli (29). The isoelectric points (IEP) of Mi-CK from brain and heart were determined by isoelectric focusing on IEF 3–9 gels with the PhastSystem™ of Pharmacia and with the IEF calibration kit "broad" (Pharmacia, Sweden) as reference.

RESULTS

Extraction of Creatine Kinase from Freshly Prepared Intact Heart Mitochondria—The analysis of a fresh phosphate extract at pH 8.6 of intact swollen mitochondria on a FPLC-gel filtration column (Superose 12) with subsequent measurement of the CK activity of the different fractions (Fig. 1*A*) clearly showed that Mi-CK analyzed immediately after extraction consists of 90% octameric and only 10% dimeric Mi-CK (Table I). The extraction conditions (phosphate buffer at high pH) did not favor octamer formation, since after 2 days 23% (Table I and Fig. 1*B*) and after 10 days 50% of the Mi-CK was dissociated into dimeric Mi-CK (Table I and Fig. 1*C*). At pH 8.8 this dissociation process was even faster than

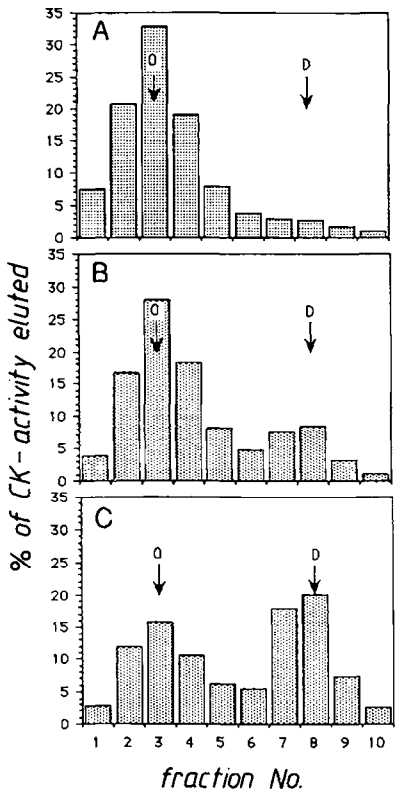

FIG. 1. **Extraction of Mi-CK by phosphate from freshly purified mitochondria of chicken cardiac muscle followed by analysis of the Mi-CK oligomers by gel permeation chromatography.** The enzymatic activity of the fractions obtained by gel permeation chromatography on a FPLC-Superose 12 HR 10/30 column of a phosphate extract of freshly purified mitochondria is given in percent of the total amount of Mi-CK activity eluted from the column. *O* and *D* refer to the fractions with the highest activity in the octamer and dimer peak of Mi-CK, respectively. *A*, gel permeation analysis immediately after phosphate extraction; *B*, 2 days after extaction; and *C*, 10 days after extraction. Samples were always kept at 4 °C in phosphate extraction medium. Under these conditions no decrease in total enzyme activity was noticed (see Table I).

TABLE I

Enzymatic activity of Mi-CK in the phosphate extract of freshly isolated swollen mitochondria

Intact mitochondria were extracted by the fast extraction method and subsequently centrifuged in a Beckman Airfuge as described under "Materials and Methods." The supernatants were analyzed on a FPLC gel filtration column immediately ($t = 0$), 2 days, and 10 days after centrifugation. The activity of the different fractions was determined as described (see also Fig. 1).

Time after centrifugation of P_i extract	Activity of dimeric and octameric Mi-CK at 42 °C in the supernatant (in IU/ml and in % of total activity) of phosphate (=P_i) extracts		
	Dimeric Mi-CK	Octameric Mi-CK	Total activity
0	7.5 IU/ml (10%)	67.8 IU/ml (90%)	75.3 IU/ml
2 days	17.5 IU/ml (25%)	59.3 IU/ml (77%)	76.8 IU/ml
10 days	39.3 IU/ml (50%)	38.8 IU/ml (50%)	78.1 IU/ml

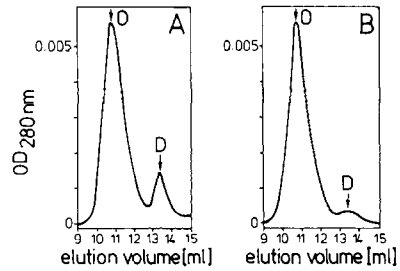

FIG. 2. **Elution profiles (absorbance at 280 nm) of Mi_b-CK on Superose 12.** *A*, analysis of the sample of Mi_b-CK used for the rebinding experiments consisting of a 15/85% mixture of dimeric and octameric Mi_b-CK; *B*, the same sample as in *A* but shown after concentration consisting of a significantly higher percentage (≥95%) of octameric Mi-CK than the sample before concentration shown in *A*, indicating that dimeric Mi-CK in *A* still is not denatured and capable of forming octamers. *O* and *D* refer to octamer and dimer elution positions, respectively.

at pH 8.6 with a half-life of approximately 2 days (not shown). It is therefore unlikely that under these conditions the high percentage of octamers *versus* dimers found immediately after extraction was due to immediate formation of octamers from dimers during the very fast extraction step. This octamer-dimer dissociation process was also not due to denaturation of the enzyme or degradation of the protein, since the enzymatic activity of Mi-CK remained constant during this time (Table I). This also indicates that the octamer-dimer dissociation has no effect on the specific activity of the enzyme. On SDS-polyacrylamide gels only one major band, corresponding to the intact polypeptide of Mi-CK with an apparent M_r of 43,000, and a very faint band at M_r 84,000 corresponding to the Mi-CK dimer (10) were seen which reacted on an immunoblot with specific anti-Mi-CK antibodies (not shown; see also Ref. 10).

Selective Rebinding to P_i-extracted Chicken Heart Mitoplasts of Octameric Mi-CK Out of a Mixture Containing Both Dimeric and Octameric Mi-CK—Purified and enzymatically active Mi_b-CK consisting of a mixture of both oligomeric forms was bound under a variety of conditions to freshly prepared extracted mitoplasts. The sample of Mi-CK chosen for the rebinding experiment consisted of 85% octameric and 15% dimeric Mi-CK as determined by gel filtration analysis on a FPLC-Superose 12 column (Fig. 2*A*). Using this fast gel permeation technique, which was completed in less than 30 min, it was found that almost 100% of the Mi-CK from this sample bound to the mitoplasts at pH 7.0 (Table II), whereas at pH 8.1 and 8.8 only 87 and 19%, respectively, of the total Mi-CK was rebound (Table II).

After rebinding of a mixture of dimeric and octameric Mi-

TABLE II

Binding of purified heart Mi-CK to extracted heart mitoplasts

Binding of purified heart Mi-CK (Mi_b-CK, 0.4 mg of protein/ml) consisting of 85% octameric and 15% dimeric Mi-CK to extracted mitoplasts.

pH[a]	Percent of enzymatic activity detected in the pellet after centrifugation (=bound Mi-CK)	Percent of enzymatic activity detected in the supernatant after centrifugation (=soluble Mi-CK)
7.0	99	1
8.1	87	13
8.8	19	81

[a] Rebinding conditions: 10 mM sodium phosphate, 5 mM BME.

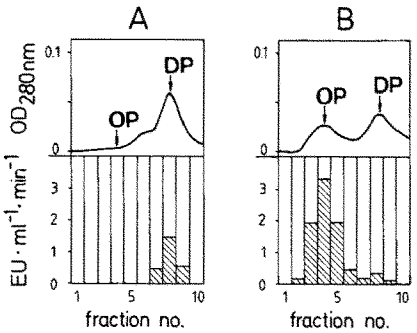

FIG. 3. **Rebinding of a mixture of octameric (85%) and dimeric (15%) Mi-CK to extracted mitoplasts under identical conditions.** *A*, Superose 12 elution profiles (absorbance at 280 nm and enzymatic activity in IU/ml) of the supernatant after selective rebinding in phosphate buffer at pH 8.1 of octameric Mi_b-CK out of a mixture of dimeric *and* octameric Mi_b-CK. Note that all octameric Mi-CK is removed from the supernatant due to selective rebinding to the mitoplasts, whereas under the same conditions most of the dimeric Mi-CK remains in the supernatant. *B*, Superose 12 elution profiles of the supernatant after re-extraction of the mitoplasts incubated with a mixture of dimeric and octameric Mi_b-CK. Note that after selective rebinding of octameric Mi-CK also mostly octameric Mi-CK is re-extracted from mitoplasts as seen in the *lower panel* of *B* showing CK activity of the different fractions. The absorbance shoulder at 280 nm at the dimer position (*DP*) seen in the *upper panel* of *B* is due to other proteins extracted from mitoplasts. *OP* and *DP* refer to the elution positions of octameric and dimeric Mi-CK, respectively.

CK to mitoplasts at pH 8.1, gel permeation analysis of the supernatant showed (Fig. 3*A*) that all the octameric Mi-CK (85% of the total Mi-CK present in the sample) had bound to the mitoplasts whereas the dimeric Mi-CK (15%) remained in the supernatant. This agrees well with the fact that 13% of the total Mi-CK activity measured did not bind to mitoplasts at pH 8.1 (Table II).

If mitoplasts, to which the 85/15% octamer/dimer mixture of Mi-CK was bound, were re-extracted and the extract thereof analyzed by gel permeation chromatography, mostly octameric Mi-CK was re-extracted as seen by the CK activity profile in Fig. 3*B*. Since by this second extraction of the mitoplasts a number of other proteins are also released in small quantities by alkaline phosphate buffer, the absorbance profile at 280 nm (Fig. 3*B*, *upper part*) does not reflect Mi-CK protein only. The absorbance shoulder (OD_{280nm}) at the Mi-CK dimer position is due to extracted proteins other than Mi-CK which eluted at a similar position but did not show any CK activity as shown on the *lower panel* of Fig. 3*B*. The finding that these fractions contain only very little Mi-CK but a number of other proteins was verified by SDS-PAGE and immunoblot analysis (not shown). With these rebinding and re-extraction experiments it was clearly proven that at pH 8.1 Mi-CK, which had bound to the mitoplasts, was of octameric nature (Fig. 3*B*), whereas the dimeric Mi-CK did not bind under these conditions (Fig. 3*A*).

The reason why dimeric Mi-CK did not bind to mitoplasts at pH 8.1 was not due to inactivation, denaturation, or degradation of the dimeric Mi-CK fraction for (i) the very same Mi-CK sample (shown in Fig. 2*A*) which was used for rebinding was readily converted into ≥95% octameric Mi-CK by simply concentrating the Mi-CK sample (Fig. 2*B*); (ii) the dimeric fraction present in the mixture (Fig. 2*A*) did bind to mitoplasts at pH 7.0 (Table II), was enzymatically active (Table II and Fig. 3*A*), and had the same specific activity as octameric Mi-CK; and (iii) sodium dodecyl sulfate-polyacrylamide gels with samples of Mi-CK shown in Fig. 2*A* displayed one single band only at the M_r position of 43,000 with no indication of proteolytic degradation (not shown).

The specificity of the rebinding of octamers only from a mixture containing both octamers and dimers, observed at alkaline pH (pH 8.1, Fig. 3), did not depend on the relative proportion of the two forms in the mixture added to extracted mitoplasts (see below).

Quantitation of the Rebinding of Separated Dimeric and Octameric Mi-CK in Dependence of the pH Value—In order to study the rebinding of the two oligomeric Mi-CK forms, dimers or octamers, separately but *under identical experimental conditions*, samples which consisted of ≥90% octameric Mi-CK or of 78% dimeric Mi-CK were used. Samples containing this very high percentage of octamers or dimers were obtained by concentrating the mixture shown in Fig. 2*A* or by the addition of MgADP, creatine, and nitrate, respectively, as described under "Materials and Methods." However, in order to rebind the two different Mi-CK samples under exactly the same conditions the buffer of the samples was changed within 10 min to rebinding buffer by desalting on a FPLC fast desalting column HR 10/10 (Pharmacia) directly before rebinding to the mitoplasts (see "Materials and Methods"). As shown in a parallel experiment by gel filtration analysis on FPLC, the ratio of dimeric to octameric Mi-CK in these desalted samples was not altered during the time course of the rebinding experiment. Rebinding itself was performed at the same protein concentrations in the very same rebinding buffer containing 10 mM sodium phosphate and 5 mM BME. Rebinding was measured at different pH values.

The rebinding experiments summarized in Fig. 4 were completed after 3–5 min of incubation of extracted mitoplasts with Mi-CK. Control experiments showed that, at all pH values used, no change at all in the octamer-dimer ratios occurred within this time range in the supernatants or with isolated Mi-CK in solution. The rebinding experiments showed that octameric Mi-CK (□——□) was quantitatively rebound to extracted chicken heart mitoplasts in rebinding buffer between pH 7.0 and 8.1. However, under identical conditions, as far as protein concentration, buffer composition, and pH were concerned, dimeric Mi-CK (o····o) did rebind quantitatively only at pH 7.0 with the percentage of rebound dimers decreasing drastically with increasing pH, *e.g.* at pH 7.5 and 8.1 only 80 and 22%, respectively, of the sample containing 78% dimeric Mi-CK were rebound (Fig. 4). The 22% of Mi-CK activity which did rebind at pH 8.1 correspond exactly to the fraction of octameric Mi-CK in the sample, indicating that at pH 8.1 dimeric Mi-CK did not rebind at all to extracted mitoplasts, whereas octameric Mi-CK, under the same conditions, bound to nearly 100% (Fig. 4). These results correspond well to the data shown in Fig. 3.

If mitoplasts, to which the mixture of 78% dimeric and 22% octameric Mi-CK was rebound at pH 7.0, were re-extracted

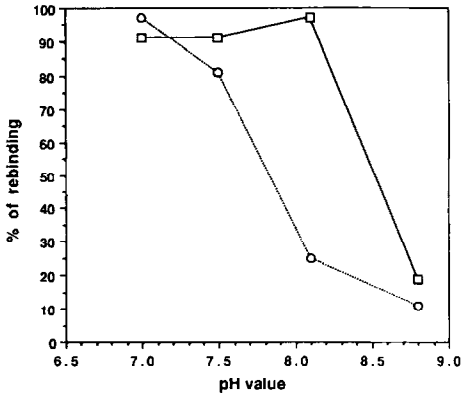

FIG. 4. **pH dependence of the rebinding of dimeric and octameric Mi_b-CK to extracted mitoplasts.** The percentage of rebinding of purified separated Mi-CK octamers (□——□) or dimers only (○···○) to phosphate-extracted washed mitoplasts using either a preparation containing ≥90% octameric or 78% dimeric Mi-CK, respectively, is indicated as a function of pH and was measured in percent of CK activity recovered in the mitoplast pellet after incubation of the mitoplasts with the two Mi-CK forms (see also Table II). At the same time, CK activity of the supernatants and total CK activity were measured. In addition, supernatants and extracted pellets of some experiments were quantitated in terms of the octamer/dimer ratio by gel permeation chromatography as described for the experiments shown in Fig. 3. No change in the octamer/dimer ratios was observed by incubation of Mi-CK at the pH values indicated over the time course of the rebinding experiments.

at pH 8.8 (as above) after 30–60 min and the extract analyzed by FPLC-gel permeation chromatography, the supernatant of the extract contained about 50% dimeric and 50% octameric Mi-CK (not shown here). This finding indicates that some of the dimeric Mi-CK did form octamers while bound to the mitoplasts. This provides evidence that under these experimental conditions some octamerization of dimeric Mi-CK can occur on the inner mitochondrial membrane, probably by frequent collision of dimers on the membrane.

DISCUSSION

Extraction of Freshly Purified Mitochondria from Chicken Heart—Phosphate extraction of freshly prepared swollen chicken heart mitochondria yielded 90% octameric Mi-CK in the supernatant (Fig. 1*A* and Table I). Since the extraction was done under conditions favoring dimer formation (see Fig. 1, *B* and *C*, and Table I), it is very unlikely that Mi-CK was bound in dimeric form to the mitochondria before extraction and subsequently would have formed octamers during extraction. The same holds true for rabbit heart mitochondria (24, 30), whereas from mitochondria of rat brain[2] and pig heart (30) a significant proportion of dimeric Mi-CK is also extracted. Furthermore, our rebinding and re-extraction experiments with mostly dimeric Mi-CK at pH 7.0 showed that the contact of dimeric Mi-CK with the inner mitochondrial membrane induces the formation of some octameric Mi-CK probably while attached to the inner membrane, because after rebinding of 78% dimeric Mi-CK to the mitoplasts, approximately 50% dimeric Mi-CK was also re-extracted. This new result is a further indication that the extraction process *per se* is not the reason for the fact that in a phosphate extract of freshly prepared mitochondria mostly octameric Mi-CK was found. From all these experiments it can be concluded that in intact heart mitochondria Mi-CK is present mostly in octameric form.

We have determined the M_r of isolated octameric chicken cardiac Mi-CK *in vitro* by different methods to be between 320,000 and 360,000 (10, 16). This is in good agreement with the recent findings of Quemeneur *et al.* (31) and of Lipskaya *et al.* (32) who provided evidence by radiation inactivation measurements of Mi-CK and cross-linking experiments with glutaraldehyde, respectively, that in isolated mitochondria Mi-CK is an oligomer with a M_r of about 330,000–380,000.

Rebinding of Mi-CK to Extracted Chicken Heart Mitoplasts—At pH 7.0, both oligomeric forms of Mi-CK were rebound to extracted chicken heart mitoplasts, whereas at slightly alkaline pH, octameric Mi-CK had a clearly higher affinity to the inner mitochondrial membrane than dimeric Mi-CK (Fig. 4). Due to this fact, it was possible to selectively rebind octameric Mi-CK out of a mixture of both dimeric and octameric Mi-CK back to extracted mitoplasts (Fig. 3). It was shown that both forms of Mi-CK, dimeric and octameric Mi-CK, were enzymatically active showing identical specific enzyme activities, that they were not degraded, and that both forms did rebind at pH 7.0 to extracted mitoplasts. Therefore, it can be concluded that the observed difference in binding behavior between octameric and dimeric Mi-CK was not a consequence of an experimental artifact due to degradation or aggregation of Mi-CK. Thus, dimeric and octameric Mi-CK differ not only in their size but depending on the pH also in their ability to bind to the inner mitochondrial membrane. In contrast, Marcillat *et al.* (24) claimed that only octameric Mi-CK could rebind to mitoplasts and Lipskaya and Trofimova (32) argued that the octamer is the only form bound to mitochondrial membranes during cross-linking.

The data available on the IEP of dimeric and octameric Mi-CK are somewhat conflicting and may reflect species differences. Whereas by IEF the IEPs of native rabbit heart Mi-CK were shown to be 8.83 and 8.24 for the octamer and dimer, respectively (30), a higher IEP (9.67) was reported for pigeon breast muscle Mi-CK dimers than for octamers (8.93) (33). In accordance with the first report (30) we found IEPs of 8.7–9.0 and 8.4–8.5 for chicken brain octamers and dimers and of 9.4–9.5 and 9.3 for chicken heart octamers and dimers of Mi-CK, respectively, as determined by IEF on the PhastSystem™. Native octameric Mi-CK from rabbit and chicken seems to have a somewhat higher IEP compared with native dimeric Mi-CK. Since the interaction of Mi-CK with the inner mitochondrial membrane is at least in part of ionic character for it is easily extractable by phosphate at alkaline pH (10), the observed difference in IEPs may to some extent be the reason for the very specific rebinding of octamers over dimers at slightly alkaline pH. At pH 7.0 and below, that is at pH conditions observed in muscle under a heavy work load, the specificity of rebinding is lost so that both oligomeric forms can rebind to the inner mitochondrial membrane.

From the facts mentioned above it may be assumed that dimeric and octameric Mi-CK are two distinct forms of Mi-CK, both with their own physiological role. It seems very likely that the importance of these two forms *in vivo* is related to the regulation of the energy transfer from mitochondria to the cytosol, depending on the energy requirement of the cell. However, since both oligomeric forms of Mi-CK have the same specific activity *in vitro*, the regulation of the energy transfer *in vivo* is unlikely to take place simply via the formation of different oligomeric states but perhaps may occur in combination with other relevant physiological parameters, *e.g.* by interaction of Mi-CK with mitochondrial membranes.

An interaction of Mi-CK with mitochondrial substructures and proteins of the inner and outer mitochondrial membranes is very likely, because Adams *et al.* (34) showed that Mi-CK from rat brain and kidney is accumulated within the contact

[2] V. Adams, personal communication.

sites between inner and outer mitochondrial membranes. This was also confirmed by direct immunoelectron microscopy where we found an Immuno-gold labeling with anti-Mi-CK antibodies along the cristae membranes as well as an additional accumulation of Immuno-gold labeling at those sites where inner and outer mitochondrial membranes are close, presumably at the mitochondrial contact sites (10). The localization of CK at the contact sites has recently been demonstrated also by histochemical methods (35). In addition, it was shown by morphometric measurements that the extent of both contact sites and CK activity present at these sites increased upon stimulation of cardiac muscle tissue (35, 36). This is in good agreement with recent results showing that the proportion of dimeric to octameric Mi-CK within mitochondria seems indeed to be regulated by the respiratory condition of the mitochondria and as such also by the energy requirement of the cell.[3]

Model of Regulation of Mi-CK Activity within Mitochondria—The facts that (i) Mi-CK octamers as well as dimers bind to mitoplasts, but the latter with lower affinity and in a strongly pH-dependent manner, that (ii) dimers, once bound to the inner membrane, seem to be able to form octamers, *e.g.* by frequent collision while attached to the membrane (see results of the experiment involving re-extraction of rebound Mi-CK dimers), and that (iii) octameric Mi-CK seems to accumulate within the contact sites where inner and outer mitochondrial membranes are in close vicinity (34, 35) all indicate that upon activation of mitochondrial respiration which is accompanied by an increase in the number of contact sites (35, 36) Mi-CK octamers are accumulated there (32). Furthermore, these results indicate that due to changes in the immediate environment beyond the contact sites, dimers floating freely in the intermembrane space get bound, octamerize on the membrane, and accumulate at the contact sites. These features are depicted in a model shown in Fig. 5. Thus, in this model, Mi-CK is accumulated in respiring mitochondria at the contact sites (10, 34, 35). There, as some recent evidence points out, Mi-CK is thought to interact with the outer mitochondrial membrane pore protein (34, 37), the inner membrane adenine nucleotide translocator (ANT) (11, 23, 34), and probably also with different lipids of both membranes, *e.g.* cardiolipin (▲), a prominent phospholipid of the inner mitochondrial membrane (38, 51). Mi-CK would receive ATP from ANTs, transphosphorylate freely diffusible uncharged creatine that can enter through the pore at the contact sites (39), and release the produced PCr into the cytosol. ADP produced by the creatine kinase reaction would be passed from Mi-CK back to the nearest ANT that would transport it through the inner membrane in exchange for ATP produced by oxidative phosphorylation.

In the model presented in Fig. 5, the functional coupling of Mi-CK and ANT and/or mitochondrial respiration, which is described in many publications (11, 13, 40–48), could be based on protein-protein interactions where Mi-CK at the contact sites is in close proximity or even in direct contact with the pore protein of the outer and the ANT of the inner membrane. Such a tightly regulated microcompartment would function as an efficient energy channeling unit (10, 15, 16). The protein-protein interactions within a common multienzyme microcompartment with close proximity or direct contact of the partners within the contact sites make possible an efficient functional coupling between Mi-CK and ANT (11, 16, 23, 45) on one hand and Mi-CK and the pore protein on the other hand (37, 49, 50). This would also explain why the functional interaction of these components is abolished by disturbing

[3] V. Adams and D. Brdiczka, personal communication.

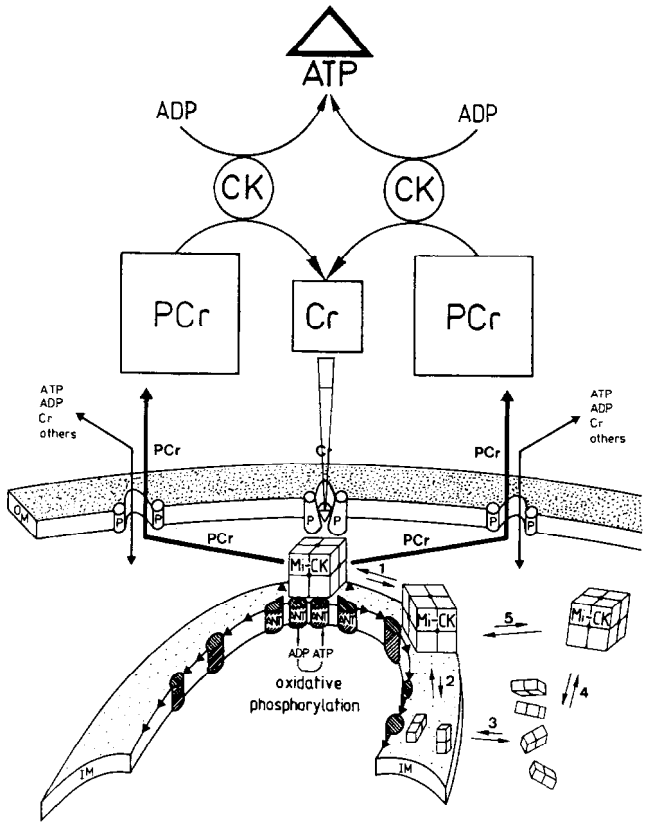

FIG. 5. **Model for the possible regulation of Mi-CK activity and transport of "high energy" phosphoryl compounds through the mitochondrial membranes.** The model depicting the producing side of the PCr circuit (15) is described under "discussion." *IM* and *OM* refer to the inner and outer mitochondrial membrane, respectively, and *P* and *ANT* to the pore protein and to the ADP/ATP translocator. The filled triangles (▲) represent free and translocator-bound cardiolipin (51). The location where the inner and outer mitochondrial membranes are in close vicinity and where Mi-CK octamers are thought to interact with ANT and *P* is depicting a mitochondrial contact site. The equilibrium constants of the various octamer-dimer and membrane association-dissociation reactions of Mi-CK are indicated semiquantitatively by the lengths of the *arrows* in the respective directions.

the integrity of the outer mitochondrial membrane, a phenomenon observed by several investigators (*e.g.* Refs. 47 and 48).

The model not only shows a state fixed in time of PCr-producing mitochondria but also indicates the possibilities of regulation by influencing different equilibrium reactions involved, for example *breaking up the contact sites* (equilibrium 1, Fig. 5), which are known to be regulated via an unknown pathway depending on the respiratory state of mitochondria (35, 36), could lead to interference of the functional interaction of Mi-CK with ANT and with pore protein, which would then lead to a reduced PCr-production of the mitochondria. The contrary events would take place upon stimulation of mitochondria accompanied by formation of contacts (36) and accumulation of octameric Mi-CK at these sites (16, 34). Furthermore, *influencing the dimer/octamer equilibrium of Mi-CK* (equilibrium 2, Fig. 5) could lead to accumulation of Mi-CK at the contact sites or to the "removal" of Mi-CK from the contact sites or even (see "Results") to the extraction of Mi-CK from the inner mitochondrial membrane (equilibriums 3 and 5, Fig. 5).

Thus, by influencing one of the five equilibrium constants described in Fig. 5 which in concert can generate a complex network of regulation based on the mutual influence of at least five different equilibrium reactions, the regulation of

Mi-CK activity and energy transport *in vivo* could occur by these parameters. The above equilibrium reactions could be influenced by one or more of as yet unknown regulatory substances, by post-translational modification of Mi-CK itself, or simply by changing the concentration of the CK substrates and of the pH value in the mitochondrial intermembrane space. The latter two factors have been shown *in vitro* to strongly affect the octamer/dimer equilibrium (10) as well as the association of the two oligomeric forms of Mi-CK with the inner mitochondrial membrane (see "Results"). Since lowering of pH, as observed in ischemia or metabolically stressed muscle, favors the association of Mi-CK dimers with the inner mitochondrial membrane, the pH value within the intermembrane compartment of mitochondria which is supposed to change locally as a function of the respiratory state may be an important regulatory factor influencing the proportion of free to bound Mi-CK. Both forms of Mi-CK, dimeric and octameric Mi-CK, each emerge to play an important physiological role within the mitochondrial energy metabolism whereby the octameric form of Mi-CK is likely to be the "active form" at the contact sites in respiring mitochondria. Furthermore, the involvement of contact sites forming multienzyme microcompartments for efficient transfer of energy is also emerging.

This complex regulatory network allows mitochondria to adjust the rate of PCr production to the energy requirements of the cell as illustrated by the PCr circuit model (15).

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