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Native Mitochondrial Creatine Kinase Forms Octameric Structures

I. ISOLATION OF TWO INTERCONVERTIBLE MITOCHONDRIAL CREATINE KINASE FORMS, DIMERIC AND OCTAMERIC MITOCHONDRIAL CREATINE KINASE: CHARACTERIZATION, LOCALIZATION, AND STRUCTURE-FUNCTION RELATIONSHIPS*

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The mitochondrial isoform of creatine kinase (Mi-CK, EC 2.7.3.2) purified to homogeneity from chicken cardiac muscle by the mild and efficient technique described in this article was ≥99.5% pure and consisted of ≥95% of a distinct, octameric Mi-CK protein species, with a M_r of 364,000 ± 30,000 and an apparent subunit M_r of 42,000. The remaining 5% were dimeric Mi-CK with an apparent M_r of 86,000 ± 8,000. Octamerization was not due to covalent linkages or intermolecular disulfide bonding. Upon dilution into buffers of low ionic strength and alkaline pH, octameric Mi-CK slowly dissociated in a time-dependent manner (weeks-months) into dimeric Mi-CK. However, the time scale of dimerization was reduced to minutes by the addition to diluted Mi-CK octamers of a mixture of Mg^{2+}, ADP, creatine and nitrate known to induce a transition-state analogue complex (Milner-White, E. J., and Watts, D. C. (1971) *Biochem. J.* 122, 727–740). The conversion was fully reversible, and octamers were reformed by simple concentration of Mi-CK dimer solutions to ≥1 mg/ml at near neutral pH and physiological salt concentrations in the absence of adenine nucleotide. After separation of the two Mi-CK species by gel filtration, electron microscopic analysis revealed uniform square-shaped particles with a central negative-stain-filled cavity in the octamer fractions and "banana-shaped" structures in the dimer fractions. Mi-CK was localized inside the mitochondria by immunogold labeling with polyclonal antibodies. A dynamic model of the octamer-dimer equilibrium of Mi-CK and the preferential association of the octameric Mi-CK form with the inner mitochondrial membrane is discussed in the context of regulation of Mi-CK activity, mitochondrial respiration, and the CP shuttle.

Creatine kinase (CK)[1] transphosphorylates the phosphoryl group of phosphocreatine (CP) to ADP (for review see Ref. 1) to regenerate ATP, the primary source of energy in living systems. Besides the "cytosolic" brain-type (BB-CK), muscle-type (MM-CK) and heterodimer-type (MB-CK) isoforms, of which the latter usually is found in mammalian heart muscle (for reviews see Refs. 2 and 3), a fourth isoform of CK, mitochondrial CK or Mi-CK (4), is also present in significant amounts in skeletal muscle, brain, and heart (5–7) as well as in spermatozoa (8) and retina photoreceptor cells (9). The Mi-CK isoform is restricted to mitochondria and, as a prominent enzyme in tissues of high, sudden energy demand, seems to be well adapted to generate CP from ATP produced within the mitochondrial matrix by forming a functionally coupled microcompartment with the ATP/ADP-translocator (5, 10–17). The CP produced is then made available by a CP shuttle (10, 11, 18, 19, 21, 22) or by facilitated diffusion (23) to those fractions of CK that are localized at specific intracellular sites of high ATP turnover (for review see Ref. 21).

Mi-CK was found to be clearly different in amino acid composition (24), in DNA sequence (25), and in immunological properties (26) from B- and M-CK, but conflicting reports on the molecular structure, especially as far as oligomerization is concerned, had been published. Although higher M_r species than the 80–85-kDa dimeric forms usually reported for MM- and BB-CK have been found earlier for Mi-CK, the number of subunits of native Mi-CK is still a matter of debate. The following apparent molecular masses or oligomeric states have been reported, *e.g.* 250,000 (27), 340,000 (28), 180,000–200,000 (29), 256,000 hexamer, (30), all from bovine heart; 184,000 from rat brain (7); 350,000 from malignant human liver (31); and recently, 355,000 from bovine heart (32); hexamers from beef heart (33); octamers from pigeon skeletal muscle (34); 240,000 hexamer from rat cardiac muscle (35). Some of these oligomeric forms were explained as being products of intermolecular S–S bonding (30) or were simply dismissed as artifacts generated by unspecific aggregation (36). The contradictory data on the oligomeric structure of Mi-CK made a thorough biochemical and structural characterization of the enzyme seem necessary, especially since a defined oligomeric state could be expected to give important clues to possible structure-function relationships of the enzyme. A major prerequisite for such a characterization was the development of a fast and mild purification scheme. A phosphate extract of mitoplasts, which had been enriched for Mi-CK by ammonium sulfate fractionation was directly loaded onto a Blue Sepharose CL-6B affinity matrix where Mi-CK was separated from contaminating cytosolic BB-CK and then was specifically eluted by a pulse of ADP (38). Final enzyme purification was achieved by fast protein liquid chromatography (FPLC) on a Mono-S column.

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[1] The abbreviations used are: CK, creatine kinase; FPLC, fast protein liquid chromatography; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BME, 2-mercaptoethanol; Mi-CK and (Mi)-CK refer to the general term mitochondrial CK and to the individual subunit of Mi-CK, respectively; M- and B-CK refer to muscle- and brain-type CK isoforms, respectively; CP, phosphocreatine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

We present evidence that, unlike BB-CK and MM-CK, native Mi-CK forms highly ordered octameric Mi-CK molecules with a M_r of 360,000 ± 30,000 by association of four Mi-CK dimers each consisting of two identical (Mi)-CK subunits with an apparent M_r of 42,000. A reversible octamer-dimer equilibrium was observed in solution that is influenced by protein concentration, pH, ionic strength, and millimolar concentrations of adenine nucleotides. Mi-CK was identified in several tissues of sudden high energy demand by immunoblotting and was localized within the mitochondria by immunogold labeling. The octamer-dimer equilibrium as well as the rebinding of Mi-CK to the inner mitochondrial membrane may represent important regulatory parameters in controlling the energy flux in mitochondria.

Parts of this work have been presented in abstract form (39, 40).

EXPERIMENTAL PROCEDURES

Purification of Mi-CK

Extraction of Mi-CK from Mitoplasts—Muscle tissue (450 g) from fresh chicken hearts was put through a meat grinder and suspended in 3.2 liters of cold MSH buffer (220 mM mannitol, 70 mM sucrose, 10 mM Hepes, 0.2 mM EDTA, 1 mM 2-mercaptoethanol (BME), 1 mM NaN_3 titrated to pH 7.4 with KOH). The suspension was homogenized by Polytron mixer (Kinematica, Kriens, Switzerland). After centrifugation of the homogenate for 10 min at 600 × *g* and resuspension of the pellet in a total volume of 1 liter of MSH-buffer followed by an additional centrifugation at low speed (10 min at 600 × *g*) the two low-speed supernatants were combined and centrifuged for 30 min at 14,000 × *g*.

After washing the crude mitochondrial pellet with the above buffer and centrifugation for 30 min at 14,000 × *g*, the highly enriched mitochondria were swollen by resuspension in 600 ml of distilled water with a Sorvall-Omnimixer, followed by incubation for 15 min on ice. The mitoplasts were pelleted by centrifugation at 18,000 × *g* for 40 min, resuspended in distilled water at a 1:3 ratio (v/v), and centrifuged for 90 min at 27,500 × *g*. The resulting pellet was resuspended in distilled water at a 1:1 (v/v) ratio (by Sorvall) to give 525 ml of a suspension consisting mostly of mitoplasts and some swollen mitochondria. In order to extract Mi-CK, the mitoplast suspension was diluted 1:1 with phosphate-extraction buffer (50 mM sodium phosphate, 10 mM BME, pH 9.0) to give a final phosphate concentration of 25 mM and a pH of 8.3 (41) and incubated overnight at 4 °C under slow stirring. Similar extraction yields were achieved by extraction at pH 8.8 for 2 h at 4 °C.

Precipitation of Mi-CK by Ammonium Sulfate—After ultracentrifugation of the extract for 4 h at 41,000 × *g*, the 860-ml supernatant containing about 80% of the total mitochondrial CK activity was made 35% in ammonium sulfate and centrifuged for 15 min at 27,500 × *g*. The supernatant was made 55% in ammonium sulfate in order to precipitate Mi-CK. The precipitated protein was centrifuged at 27,500 × *g*, resuspended in 30 ml of Blue-Sepharose buffer (50 mM sodium phosphate, 1 mM $MgCl_2$, 1 mM NaN_3, 1 mM BME, 0.2 mM EGTA, pH = 6.0) and dialyzed against the same buffer.

Affinity Chromatography on Blue-Sepharose CL-6B—The dialyzed material was loaded at a flow rate of 1.5–2 ml/min onto a Blue-Sepharose CL-6B column (Pharmacia, Sweden, 2.3 × 27 cm) which previously had been equilibrated with 2 liters of Blue-Sepharose buffer (see above) at pH 6.0. Then the column was rinsed with 540 ml of Blue-Sepharose buffer and subsequently with 1200 ml of the same buffer changed to pH 8.0 in order to elute several contaminants, *e.g.* BB-CK (the amount of BB-CK depended on how well the mitochondria were washed) and other main contaminants migrating at 30, 50, and 70 kDa on SDS-gels (Figs. 1 and 3). Subsequently, Mi-CK was eluted by 540 ml of Blue-Sepharose buffer supplemented with 10 mM ADP, and the eluate was concentrated to a volume smaller than 5 ml (by AMICON), using a PM-10 membrane or by Centricon 10 microconcentrator (Amicon, Danvers, MA) and dialyzed against FPLC buffer (25 mM sodium phosphate, 0.2 mM EDTA, 1 mM BME, 50 mM NaCl, pH 7.0).

Cation Exchange Chromatography on FPLC—One to 5 aliquots of 500 μl each of the dialyzed material were loaded onto a Mono-S column (0.5 × 10 cm, Pharmacia, Sweden, at a flow rate of 0.5 ml/min) that had been equilibrated with FPLC buffer. After rinsing the Mono-S column with 5 ml of FPLC buffer, a linear salt gradient from 50 to 480 mM of NaCl was applied to elute pure Mi-CK between 190–240 mM NaCl.

Gel Permeation Chromatography—Gel filtration experiments were performed on a Sephacryl S-300 superfine column (2.2 × 80 cm) in 25 mM NaP_i, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM BME, at pH 8.0. Ferritin (M_r = 440,000), catalase (M_r = 232,000), aldolase (M_r = 158,000), MM-CK (M_r = 80,000), and α-chymotrypsinogen (M_r = 24,500) were used as M_r calibration standards, and the void volume was determined by Blue-Dextran 2000. CK activity and optical density at 280 nm of each fraction (30 drops) were measured as described below.

In order to improve speed and sensitivity of resolution of Mi-CK octamers and dimers and to be able to quantitate the reversible interconversion of the two oligomeric Mi-CK species on a faster time scale, gel filtration on Sephacryl S-300 has been replaced by FPLC gel permeation on Superose-12 calibrated by the same M_r standards in a buffer containing 50 mM sodium phosphate, 150 mM NaCl, 0.2 mM EDTA, 2 mM BME, 1 mM NaN_3 at pH 7.2.

CK Activity Measurements—CK activity was measured by the pH-stat method described earlier in detail (42) with the exception that the addition of bovine serum albumin to the assay mix was not necessary to stabilize Mi-CK.

Cellulose-Polyacetate Electrophoresis—Zymograms of CK isoenzymes were made on Gelman-Sepraphore strips in veronal buffer, pH 8.6 (4 °C), containing 1.5 mM BME. Five-μl samples were applied in the middle of the strips and electrophoresis was performed at constant voltage (250 V, 1–2 mA/strip) for 2 h at 4 °C. Enzyme activity was detected by a coupled enzyme overlay gel system (±CP as substrate and ± P′,-P^5-di(adenosine-5′-)pentaphosphate as myokinase inhibitor) (9, 43).

Negative Staining of Single Molecules—Purified Mi-CK was diluted to 10 μg/ml in 1 mM $MgCl_2$, 0.2 mM EGTA, 10–50 mM NaP_i at pH 8.0 with or without BME (1–100 mM). Drops of the diluted enzyme solution were put on parafilm, and glow-discharged carbon-filmed copper grids were floated on the diluted enzyme solutions for 1 min to adsorb Mi-CK molecules. The specimens were washed by transfer onto drops of the above buffer and finally onto a drop of distilled water for 20 s each. For negative staining specimens were floated on 1% acidic or neutralized uranyl acetate for 30–60 s. Some specimens were fixed with 0.5% glutaraldehyde at 0.1–1.0% before negative staining. Negative stain was blotted off by filter paper and the preparations allowed to dry at 20 °C. Electron micrographs were taken by a Jeol JEM 100C electron microscope equipped with an anticontamination device at an acceleration voltage of 100 kV.

Preparation of Antibodies—Highly purified chicken cardiac Mi-CK (200 μg) was emulsified in complete Freund's adjuvant and injected intracutaneously and subcutaneously at multiple sites on the back of rabbits or near the axillary and inguinal lymph node regions of a goat. A first boost was given after 28 days and a second one after an additional 14 days with 100 μg of protein each. After 14 days the animals were bled at weekly intervals and the sera stored frozen. Control sera were collected from the same animals prior to immunization. For affinity purification antisera (5–10 ml) were passed over columns with purified Mi-CK that had been immobilized on CNBr-activated Sepharose-4B matrix (3 mg of Mi-CK/ml bed volume) and specific anti-Mi-CK-IgG was eluted by 1 M propionic acid at pH 3.0.

Ultracryosectioning and Immunogold Labeling—Freshly excised chicken skeletal muscle was cut into small fibers and fixed for 2 h with 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M KCl, 1 mM EGTA, 5 mM EDTA at pH 7.0. The fixed tissue was infused overnight with 2.3 M sucrose, in PBS at 4 °C (44) and then frozen on specimen supports (bolts) by immersion into liquid nitrogen-cooled Freon 22. Ultrathin sections (900 Å) were cut at −95 °C with an Ultracut-E ultramicrotome (Reichert-Jung, Vienna, Austria) fitted with a Cryokit FC-4. The sections were picked up with a droplet of 2.3 M sucrose in PBS and mounted on collodion-coated copper grids. To wash away the sucrose and quench traces of glutaraldehyde, the grids were placed face down on a buffer surface containing 0.1 M glycine in PBS at pH 7.4. Immunolabeling was performed as described (43) using affinity-purified rabbit anti-chicken Mi-CK IgG, diluted to 1–5 μg/ml in PBG (PBS including 0.2% gelatine and 0.5% bovine serum albumin at pH 7.4), followed by goat anti-rabbit IgG conjugated with 5-nm colloidal gold particles (Janssen Pharmaceutica, Beerse, Belgium) (diluted 1:100 in PBG) for 2 h at 20 °C each. After extensive washing with PBG, followed by 0.1 M glycine in PBS, and finally with water, the sections were stained for 10 min first with neutral

and then for 2 min with acidic 1% uranyl acetate. Mechanical support for the sections on the collodion film was provided by allowing them to dry in a 1.3% aqueous solution of methyl cellulose. Sections were examined by a Jeol JEM 100C electron microscope.

Low Temperature Embedding and Immunolabeling—Low temperature embedding was performed according to Ref. 45. In this particular case chicken tissues were fixed with 3% paraformaldehyde, 0.1% glutaraldehyde in PBS for 2 h on ice. Dehydration and embedding in Lowicryl K4M was carried out in a low temperature embedding apparatus (LTE, Balzers, Liechtenstein) with a graded series of ethanol (30–100%) and progressively lowering the temperature to −35 °C. Infiltration with liquid resin K4M was performed at −35 °C, and polymerization was achieved by indirect UV-irradiation (360 nm) in a deep-freeze overnight followed by 2 days at 20 °C. Sections of 70-nm thickness were cut with an Ultracut E (Reichert, Vienna, Austria) and processed for immunolabeling using affinity purified rabbit anti-chicken Mi-CK antibodies followed by goat anti-rabbit IgG coupled to 5 nm colloidal gold (Janssen Pharmaceutica, Beerse, Belgium). Sections were stained with 2% aqueous uranyl acetate for 5 min and with lead citrate for 45 s.

Other Methods

PAGE in the presence of $NaDodSO_4$ was performed according to Ref. 46 and the protein bands stained by Coomassie Brillant Blue R-250 or by silver staining (Bio-Rad method derived from Ref. 47). Electrotransfer of separated protein bands (48) and subsequent immunoblotting with antibodies specific for Mi-CK were performed as described (49). After incubation for 4 h with 1:500 diluted affinity-purified rabbit anti-chicken Mi-CK antibodies followed by incubation for 2–4 h with 1:2000 diluted goat anti-rabbit IgG conjugated with horseradish peroxidase, washes were done with PBS containing 0.1% Tween-20 to reduce unspecific binding.

Protein concentrations were determined by the BCA-protein reagent method (Pierce) (50) or according to the Bio-Rad method (51) using a bovine serum albumin standard (2 mg/ml, Pierce).

RESULTS

Purification of Mi-CK

Besides significant amounts of Mi-CK, highly enriched and washed mitochondria from chicken cardiac muscle always contained some BB-CK (not shown), which is the predominant cytosolic form of CK in chicken heart (52). Whether this fraction of BB-CK is specifically associated with the mitochondrial outer membrane or just represents a contaminant is unclear at present. However, increasing evidence has been gathered over the past few years showing that M- or B-CK are ambiguitŏŭs enzymes (53) partially bound in an isoenzyme-specific way to subcellular structures (8, 9, 43, 54). BB-CK, differing in electrophoretic mobility on cellulose acetate strips (not shown), was separated from the mitochondrial isoform by an affinity chromatography step on Blue-Sepharose (38) (Fig. 1). BB-CK was almost fully removed by swelling the mitochondria and washing them several times in distilled water. Mitoplasts washed this way still retained ≥90% of the Mi-CK bound originally (0.6 IU·mg^{-1} of mitochondrial protein) which was rather selectively removed by phosphate extraction at alkaline pH (Ref. 5, Fig. 3, *lane c*, and Table I). Extraction at a final phosphate concentration of 25 mM at pH 8.3 overnight (41) or at pH 8.8 for 2 h, respectively, was most efficient for chicken cardiac mitochondria. Although Mi-CK activity has been extracted directly from whole muscle homogenates by a similar extraction procedure (24), the fact that Mi-CK is highly enriched in phosphate extracts of isolated mitochondria makes it worth the trouble of several centrifugations to obtain mitochondria first. After applying the phosphate extract, enriched for Mi-CK by a 35–55% ammonium sulfate fractionation, onto the Blue-Sepharose column at pH 6.0, a number of contaminants including BB-CK, were eluted by changing the pH of the Blue-Sepharose buffer from pH 6.0 to 8.0 (Fig. 1, *first peak* and *inset, lane b*). Then, Mi-CK was specifically eluted by a pulse of 10 mM ADP added to the pH 8.0 Blue-Sepharose buffer (Fig. 1, *second peak* and silver-stained gel of *inset lane c, arrow*). SDS-PAGE followed by Coomassie-Blue staining of this ADP-eluted material (Fig. 3, *lanes e* and *g*) and the elevated specific CK activity of 120 IU/mg at this step (Table I) indicate that this material obtained by a simple two-step procedure is pure enough for a number of biochemical purposes. Remaining impurities could be removed by cation

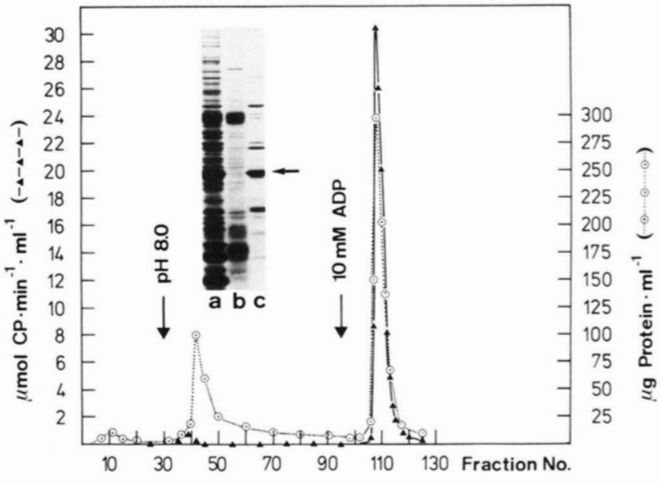

FIG. 1. **Affinity chromatography of mitochondrial extract on Blue-Sepharose, separation of CK isoforms, and specific elution of Mi-CK by ADP.** Phosphate extract of chicken cardiac mitoplasts enriched for Mi-CK loaded onto Blue-Sepharose column at pH 6.0 as shown after PAGE in SDS and silver staining for protein (*inset, lane a*). Material eluted in the first peak as the pH of the Blue-Sepharose buffer was changed to pH 8.0 (*inset, lane b*). Material eluted at a flow rate of 1.5–2 ml/min in the second, large peak (*fraction 110*) after a pulse of 10 mM ADP (*inset, lane c*). The majority of protein bands not found in the two eluted peaks remained bound to the column and eluted upon regeneration of the Blue-Sepharose resin (not shown). Note the presence of a strong protein band at 42 kDa (*lane c, arrow*) representing Mi-CK which is highly enriched in the ADP eluate. *Solid line* connecting *black triangles* represents CK enzymatic activity, *dotted line* connecting *open circles* represents protein concentration as determined by measurement of OD at 280 nm. The small amount of BB-CK still present after extensive washing of mitoplasts is eluted in the first peak at pH 8.0.

TABLE I

Purification of Mi-CK from chicken cardiac muscle

Summary of purification protocol indicating purification steps, amount of protein material, total CK activity, increase in specific CK activity and Mi-CK yield.

Purification steps	Protein	Total CK activity	Specific CK activity	Yield
	mg	*IU*[a]	*IU/mg*	%
Total mitoplasts	8600[b]	5350	0.6	100
Supernatant of phosphate extract	2750[b]	4300	1.6	80
35–55 Ammonium sulfate	124[c]	2340	19[d]	43
Sepharose-Blue	21.5[c]	2500	120	47
Mono-S-FPLC	15.2[c]	2000	130	38
	10.7[b]		180[b]	

[a] One international enzyme unit (IU) is equal to 1 μmol of CP transphosphorylated/minute at pH 7.0 and 25 °C.

[b] Protein concentration determined by Pierce BCA Protein-Assay (50).

[c] Protein concentration determined by Bio-Rad Protein-Micro-Assay (51).

[d] Measurement of the activity is not reliable because trace amounts of ammonium sulfate are still effective as inhibitor of Mi-CK-activity.

exchange chromatography on a FPLC Mono-S column yielding Mi-CK by salt gradient elution as a symmetrical peak (Fig. 2). Mi-CK judged to be more than 99.5% pure by densitometry of SDS-polyacrylamide gels (Fig. 2, *inset, lane c*; Fig. 3, *lanes f* and *h* stained by Coomassie Blue, and *lanes f'* and *h'* silver-stained) and by the Mono-S column peak profile (Fig. 2 *second peak*) was obtained. The breakthrough peak seen in the Mono-S elution profile monitored as absorption at 280 nm (Fig. 2) contained several contaminants. If purified Mi-CK was rechromatographed after prolonged storage at low protein concentration, an increase in material eluting in the breakthrough peak was observed that was identified by gel permeation on Superose 12 to contain some dimeric Mi-CK as well. A full account of this purification procedure carefully optimized for speed and ease is given in Fig. 3 where the protein composition and purity of Mi-CK is demonstrated by Coomassie-Blue- and silver-stained SDS-10% polyacrylamide gels at low and high loading of the protein samples. A single very minor contaminating polypeptide with an apparent M_r of approximately 80,000 seen on silver-stained gels (Fig. 3, *lane h'*), amounting to less than 0.5% of the purified main Mi-CK band, did cross-react with polyclonal as well as with monoclonal anti-Mi-CK antibodies generated in this laboratory (55). It is therefore likely to be an extremely stable Mi-CK dimer cross-linked by some sort of covalent bonding. Table I illustrates that starting with 450 g of cardiac tissue the purification schedule yielded 10–15 mg of purified pooled Mi-CK with a specific enzyme activity of 130–180 IU/mg at pH 7.0 and 25 °C depending on the protein assay used. This corresponds to a 200–300-fold enrichment of the enzyme from mitoplasts. The specific activity of chicken cardiac Mi-CK at 25 °C compares favorably with published data (24, 56) and is approximately five times higher at 42 °C, which is the physiological temperature of chickens.

Amino Acid Composition and N-terminal Sequence of Mi-CK—The amino acid composition of the purified chicken cardiac Mi-CK shows very high similarity to those of Mi-CK from other species including human cardiac Mi-CK (24). The first 20 amino acids of our 26-amino acid N-terminal sequence agreed well with the N-terminal 20 amino acids from chicken cardiac Mi-CK published recently (56). Furthermore our entire 26-N-terminal amino acid sequence is identical to the 5' end of the completed cDNA sequence of a Mi-CK cDNA clone derived from a chicken skeletal muscle cDNA library (25). The results concerning the protein data of chicken cardiac Mi-CK (55), together with the complete nucleotide sequence of Mi-CK obtained in this laboratory, have been described elsewhere (25). Amino acid composition, N-terminal amino acid sequence, and the cDNA sequence of Mi-CK are similar but different from those of M- and B-CK. Also if the immunological data from immunoblots and the immunolocalization of the enzyme, which is found exclusively in mitochondria, are considered it is evident beyond doubt that the protein purified here is indeed Mi-CK. The biochemical and immunological differences observed between Mi-CK and the cytosolic isoforms provide a basis for understanding the structural deviations displayed by Mi-CK, as compared with the common dimeric arrangement of MM-, MB-, or BB-CK isoforms.

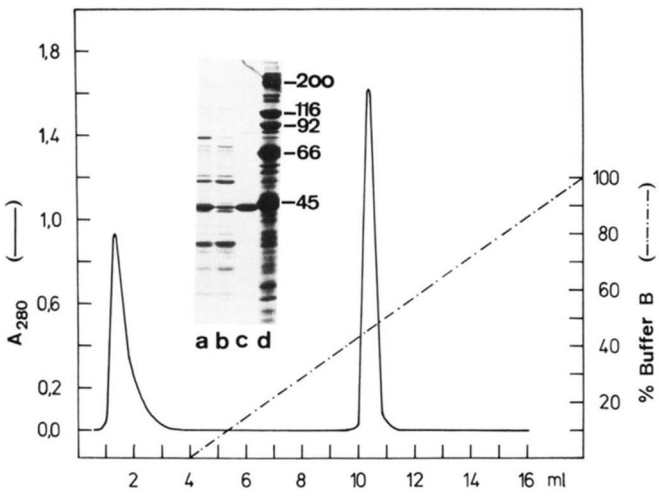

FIG. 2. **Purification of Mi-CK by FPLC Mono-S cation exchange chromatography.** Protein composition of ADP eluate from Blue-Sepharose affinity column that was loaded at a flow rate of 0.5 ml/min onto Mono-S resin at pH 7.0 is shown here after PAGE in SDS and silver staining (*inset, lane a*); of breakthrough peak (fractions 1–3 ml) (*inset, lane b*); and of material eluted by linear salt gradient (fractions at 10–11 ml) (*inset, lane c*). Mi-CK of greater than 99% purity with a subunit M_r of 42,000 was eluted as is indicated by silver staining of protein (*inset, lane c*). Bio-Rad high M_r standards visualized by silver staining for reference (*inset, lane d*, myosin heavy chain, 200,000; β-galactosidase, 116,000; phosphorylase *b*, 92,000; bovine serum albumin, 66,000; and ovalbumin of 45,000). *Solid line* in chromatogram represents protein concentration that was measured by OD at 280 nm; *dotted line* represents linear salt gradient from 50 to 480 mM NaCl.

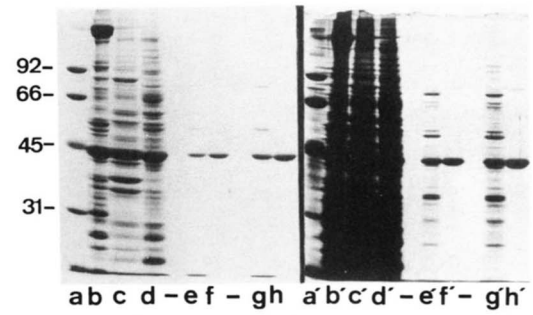

FIG. 3. **Documentation of purification procedure for Mi-CK by PAGE in SDS and staining for protein.** *Left-hand gel panel* (*a–h*) stained for protein by Coomassie Brillant Blue; and corresponding gel panel at *right* (*a'–h'*) stained by silver staining. Bio-Rad high M_r standards as indicated (*a* and *a'*, 2 μg of protein/band); total proteins of washed and swollen mitoplasts (*b* and *b'*, 60 μg of total protein); supernatant of alkaline phosphate extract of mitoplasts (*c* and *c'*, 60 μg of protein); same after fractionation by a 35–55% ammonium sulfate cut (*d* and *d'*, 60 μg of protein); material eluted from Blue-Sepharose matrix by 10 mM ADP (*e* and *e'*, 2 μg of protein); and (*g* and *g'*, same but 4 μg of protein loaded); purified Mi-CK eluted by salt gradient from Mono-S resin (*f* and *f'*, 2 μg of protein), and (*h* and *h'*, 4 μg of protein). The very faint band at a position of approximately 80 kDa only seen after silver staining is likely to be a covalently linked Mi-CK dimer species (see text). M_r standards (*a* and *a'*) as in Fig. 2 except for carbonic anhydrase with an apparent M_r of 31,000.

Mi-CK Forms Distinct, Monodisperse High M_r Octamers

Gel Permeation Chromatography of Mi-CK—When preparations of Mi-CK were subjected to gel permeation chromatography on Sephacryl S-300, two symmetrical protein peaks with CK activity were resolved (Fig. 4). The first Mi-CK peak with an apparent M_r of 360,000 ± 30,000 (Fig. 4, *I*) eluted well ahead of a second Mi-CK peak with apparent M_r of 78,000 ± 7,000 (Fig. 4, *II*), the latter comigrating with soluble MM-CK with a known M_r of 80,000 ± 5,000. This was an indication that native Mi-CK with high specific activity, purified to homogeneity according to our procedure, forms a defined high molecular weight species corresponding to an octameric structure that consists of four Mi-CK dimers composed of two identical (Mi)-CK subunits each of an apparent M_r of 42,000 as determined by SDS-PAGE. Subsequently, it

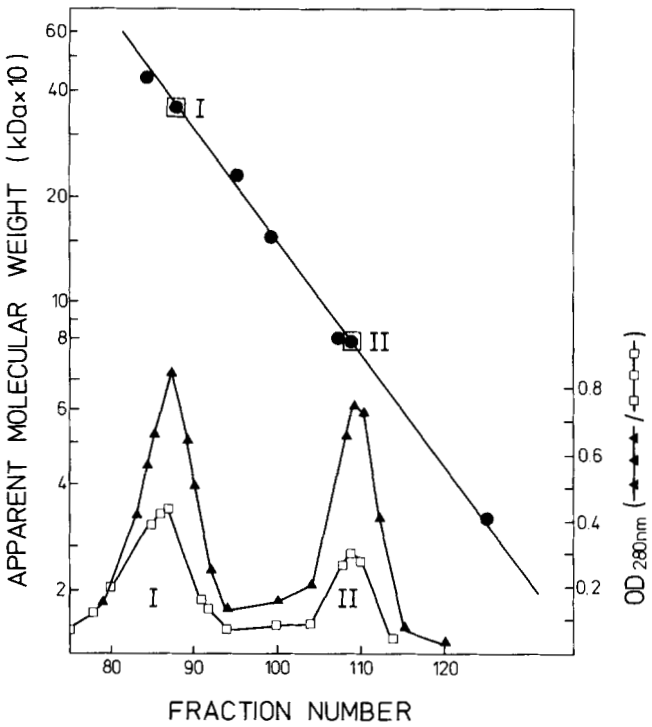

FIG. 4. **Separation of Mi-CK octamers and dimers by gel filtration on Sephacryl S-300 and apparent molecular weight of Mi-CK octamers and dimers.** Elution profiles on Sephacryl S-300 of a mixture of Mi-CK octamers and dimers (obtained by storage of octameric Mi-CK for 3 months at a concentration below 0.5 mg/ml) indicated by filled black triangles (▲). *Peak I* and *II* both contained CK activity and correspond to the octamer and dimer population of Mi-CK, respectively. Log of apparent M_r plotted against fraction number of peak fraction obtained by gel filtration on Sephacryl S-300 indicating M_r of 360,000 ± 30,000 for the octamer (, *peak I*) and 78,000 ± 8,000 for the dimer (, *peak II*) of Mi-CK. Position of M_r standards: ferritin (440,000), catalase (232,000), aldolase (158,000), chicken MM-CK (80,000), and α-chymotrypsinogen (24,500) as indicated by filled circles (●). An aliquot of the same Mi-CK mixture re-run after 45 min of incubation with 20 mM dithiothreitol showing similar octamer/dimer ratio (□).

was found that storage of purified Mi-CK, especially at low protein concentration (lower than 0.3 mg/ml) and alkaline pH (above pH 8.5), or repeated freezing (−20 °C) and thawing of the preparation, resulted in a quantitative shift in the ratio of peak I to II (seen in Fig. 4), indicating that octameric Mi-CK had a tendency to dissociate into dimers, the common structure of all creatine kinases and some of the other phosphagen kinases as well. In Fig. 4, a gel permeation run of a Mi-CK preparation which had been stored for approximately 3 months at 4 °C at a dilution of lower than 0.5 mg/ml is shown to demonstrate the existence of the two distinct oligomeric species of Mi-CK (Fig. 4, *filled triangles*). An aliquot of the same Mi-CK sample incubated with 20 mM dithiothreitol for 45 min at 20 °C, pH 8.0, prior to gel permeation is also shown (Fig. 4, *open squares*). In both cases two peaks of similar proportions were resolved, and similar elution profiles were obtained even in the presence of 0.1 M BME (not shown). Thus, it seems unlikely that artifactual aggregation of Mi-CK subunits via –S–S– cross-linkage or oxidation is the reason for the appearance of high M_r Mi-CK complexes as had been suggested earlier (29, 30, 36). If freshly purified Mi-CK or properly stored Mi-CK (in liquid N_2) was subjected to gel permeation, a high molecular weight peak consisting of ≥95% of octameric Mi-CK with an apparent M_r of 364,000 ± 30,000 was observed, as shown by FPLC Superose-12 chromatography (Fig. 5*a*, *upper tracing*). This confirms our idea that highly purified native Mi-CK, if treated gently and stored properly, consists of a homogeneous population of octameric molecules (Fig. 5*a*, see symmetry of peak). Most importantly, if a phosphate extract of freshly prepared mitoplasts was directly subjected to gel permeation chromatography on Superose-12, more than 90% of Mi-CK was indeed found in the octamer peak indicating that Mi-CK is eluted from mitoplasts as octamer, thus favoring the hypothesis that the octameric form of Mi-CK is bound to the inner mitochondrial membrane as had been postulated earlier (60). The octameric nature of Mi-CK was confirmed by analytical ultracentrifugation, electron microscopy, image reconstruction, and direct mass measurements as shown later (57, 58). These results indicate the existence of the octameric Mi-CK as a distinct molecular species unique to the mitochondrial isoform of CK which had not been characterized in detail before.

Stability of Octameric Mi-CK—Mi-CK purified from chicken cardiac muscle according to the method described herein was stable as octameric Mi-CK and enzymatically active for months if either stored frozen under liquid nitrogen or, after sterile filtration, even at 4 °C, provided that the protein concentration was high (≥1 mg/ml) and oxidation was minimized by addition of BME. Typically, after 3 months of storage the loss of specific activity leveled off at 80–110 IU/mg of protein whereas the octamer/dimer ratio was stable at 9:1 in samples stored directly in either Blue-Sepharose or FPLC column buffer containing 0.26 M NaCl both at pH 7.0 and 8.0 (see "Experimental Procedures"). Keeping Mi-CK octamer preparations at high dilution (≤0.3 mg/ml), alkaline pH (≥pH 8.5) or repeatedly freezing and thawing them led to a slow but progressive decay of the octamers into dimers (within days to weeks). Conditions close to physiological ionic strength (addition of 0.1–0.25 M NaCl) and pH values near neutrality did stabilize octameric Mi-CK but could not prevent the slow formation of dimers upon dilution of the protein.

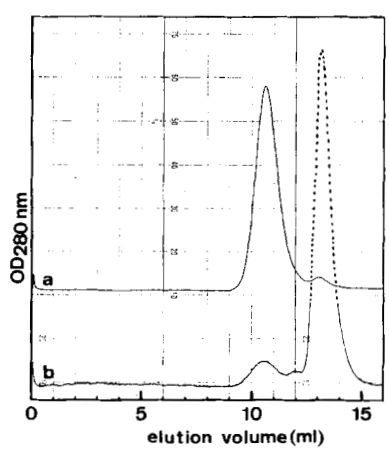

FIG. 5. **Octameric nature of Mi-CK and octamer-dimer conversion.** Actual absorption tracings (at A_{280}nm) of gel permeation runs on FPLC Superose-12 column with purified Mi-CK. *a*, Mi-CK pooled from FPLC-Mono-S peak (see Fig. 2) diluted to 0.1 mg/ml just before use is shown in the *upper solid-line tracing* (*a*) indicating an actual octamer-dimer ratio of Mi-CK of 19 to 1. Note the big symmetrical octamer peak eluting first and the small dimer peak eluting second. *b*, same Mi-CK material after addition of Mg^{2+}, ADP, creatine, and nitrate to induce an enzymatic transition-state analogue complex (59) and, at the same time, a quantitative conversion of Mi-CK octamers into dimers as shown by the lower superimposed tracing (*b*) of a consecutive FPLC run on Superose-12. Note the very small octamer peak remaining and formation of a huge dimer peak (*dotted line*). Sample application onto column at position 0 ml; octamer and dimer elution at volumes 10–11 and 13–14 ml, respectively.

Two Interconvertible Forms of Mi-CK: the Mi-CK Octamer and the Mi-CK Dimer—The most striking effector for reversible interconversion of octamers into dimers was protein concentration itself. As already mentioned above, Mi-CK octamers may be converted into dimers by dilution, but this process was slow and took months. It is remarkable, however, that Mi-CK dimers could be quantitatively octamerized on a much faster time scale, that is within minutes, by simply concentrating the enzyme to concentrations above 0.5–1 mg/ml. Thus, under a variety of buffer conditions octamers seem to be the favored Mi-CK structure, provided the protein concentration is high enough. On the other hand, addition of 4 mM ADP, 5 mM $MgCl_2$, 20 mM creatine, and 50 mM KNO_3 at pH 7.2, reagents known to form a transition-state analogue complex within the active site (59), to a diluted Mi-CK octamer solution (≤0.5 mg/ml) proved to be very effective on a minute time scale in completely converting octamers into dimers (Fig. 5*b*, *lower tracing*). This combination of reagents had been recently used to specifically elute chicken cardiac Mi-CK from ADP-hexane-agarose (56). Incidentally, these authors have found Mi-CK to be exclusively of dimeric nature with no evidence of higher M_r forms. From our results, however, this is fully compatible with the fact that these reagents led to almost 100% dimer formation. The nucleotides ADP and ATP alone with or without $MgCl_2$ had a significantly weaker dimer formation effect (not shown). A detailed quantitative analysis of several effectors that influence the dimer-octamer equilibrium is in progress.[2] Marcillat *et al.* (60) had shown a stabilizing effect of *p*-aminobenzamidine on Mi-CK octamers preventing the conversion of octamers into dimers. In accordance with this, we found that the same compound as well as benzamidine, to an even higher degree, both not only prevented dimer formation but actively shifted the dimer/octamer equilibrium toward octamer formation by a hitherto unknown mechanism. Gel permeation chromatography runs of aliquots of the same sample during octamer-dimer conversion experiment taken at different time points provided no indication for stable intermediate forms of Mi-CK such as hexamers or tetramers (not shown). The small peak or shoulder to the left hand side of the dimer peak seen after octamer-dimer conversion by the Mg-ATP, creatine, nitrate mixture (Fig. 5*b*) is unlikely representing tetrameric Mi-CK, for rechromatography of these pooled fractions revealed mostly dimers. This shoulder must have been derived from that fraction of Mi-CK octamers which was being dissociated into dimers while on the column during gel permeation and, therefore, giving rise to the shoulder seen in Fig. 5*b*. Thus, there is conclusive evidence that Mi-CK exists as two structurally distinct interconvertible forms differing in the extent of oligomerization, that is octameric and dimeric Mi-CK.

Electron Microscopy of the Two Mi-CK Forms—If the same Mi-CK sample used in Fig. 4 for gel permeation chromatography was negatively stained and visualized by electron microscopy, a mixture of two distinct molecular forms was resolved (Fig. 6*a*). First, structures that can best be described as square-shaped perforated particles of 10-nm side width displaying a 4-fold symmetry and a stain-filled central cavity of approximately 2.5–3.0 nm were most striking (Fig. 6*a*, *circled molecules*). Second, significantly smaller banana-shaped structures (Fig. 6*a*, *inside the square boxes*) were also apparent. After gel permeation of this material on Sephacryl S-300, the peak I fractions (Fig. 4) containing Mi-CK octamers with an apparent M_r of 360,000 consisted exclusively of square-shaped molecules (Fig. 6*b* and *inset b'* at higher magnification) whereas peak II (Fig. 4) containing dimeric Mi-CK material of apparent M_r of 80,000 consisted almost exclusively of the banana-shaped structures (Fig. 6*c*, with *inset c'*). Thus, the two distinct structures revealed by electron microscopy were attributed to two biochemically defined (by size) populations of molecules, one of which was the Mi-CK octamer, a highly ordered regular structure unique for Mi-CK. Addition of 20 mM dithiothreitol or 0.1 M BME to a sample of Mi-CK octamers did not result in disintegration of octamers (not shown). This was confirmed also by gel permeation (see Fig. 4).

Immunological Properties of Mi-CK—The polyclonal rabbit anti-chicken cardiac Mi-CK antibody generated by a low-dose immunization schedule proved to be monospecific for Mi-CK on immunoblots as shown in Fig. 7, *lane b*, where only one single protein in the position corresponding to the electrophoretic mobility of the Mi-CK subunit (at 42 kDa) was recognized by the antibody. In addition, immunoblot analysis of different chicken tissues revealed the presence of significant amounts of Mi-CK in striated muscle, cardiac (*c*), and skeletal muscle (*d*), as well as in brain (*e*), (including retina, not shown) and spermatozoa (*f*), but very little or none in gizzard (*g*) (as judged by loading 10 times more protein for blotting than shown here) and none in liver (not shown), indicating, in support of the CP shuttle hypothesis, that Mi-CK is an essential isoform present in tissues of sudden, high energy requirement. In accordance with earlier observations (26) anti-Mi-CK antibodies did not cross-react with native B- and M-type CK (not shown) and showed only weak, in most cases undetectable, cross-reactivity with M- or B-CK isoforms on immunoblots as shown earlier with extracts from spermatozoa (8), retina (7), and chicken cardiac muscle (38). Despite the extensive sequence homology of Mi-CK with both B- and M-CK (25) this confirms the unique tertiary and quaternary structure of Mi-CK compared to the latter two isoforms.

Ultrastructural Immunolocalization of Mi-CK—Immunogold labeling of prefixed frozen and ultrathin-sectioned muscle tissue led to specific staining of mitochondria with no staining at all of nonmitochondrial structures, *e.g.* the myofibrillar apparatus (Fig. 8), confirming an exclusive isoenzyme-specific localization of Mi-CK restricted to mitochondria. They are compatible with the postulated location of Mi-CK at the exterior face of the inner mitochondrial membrane as had been shown by biochemical means (5, 61, 62). The immunogold-labeling method on cryosectioned tissue (Fig. 8*a*) did not allow more precise localization, *e.g.* to determine the sidedness of the inner membrane localization. However, the first immunolabeling of Lowicryl-embedded mitochondria (in this case from retina photoreceptor cells) with anti-Mi-CK antibodies demonstrated at an ultrastructural level showed a clear delineation of gold clusters following the inner mitochondrial membranes and to some extent along the outer membrane (Fig. 8*b*). Most interestingly, clustered labeling was often observed at those peripheral regions where the inner mitochondrial membranes met the outer membrane (Fig. 8*b*, *arrowheads*). The corresponding controls using cryosectioned muscle (Fig. 8*c*) or Lowicryl-embedded tissues (not shown) showed no significant gold labeling.

DISCUSSION

The Octameric Structure As a General Feature of Mi-CK—At least two factors were important for the purification of Mi-CK to greater than 99.5% purity in more than 95% octameric form: (*a*) Mi-CK from chicken cardiac mitochondria turned out to be more stable than its mammalian counterparts and (*b*) the development of a fast, but mild purification method involving the adsorption of a mitochondrial phos-

[2] M. Wyss, J. Schlegel, and T. Walliman, unpublished results.

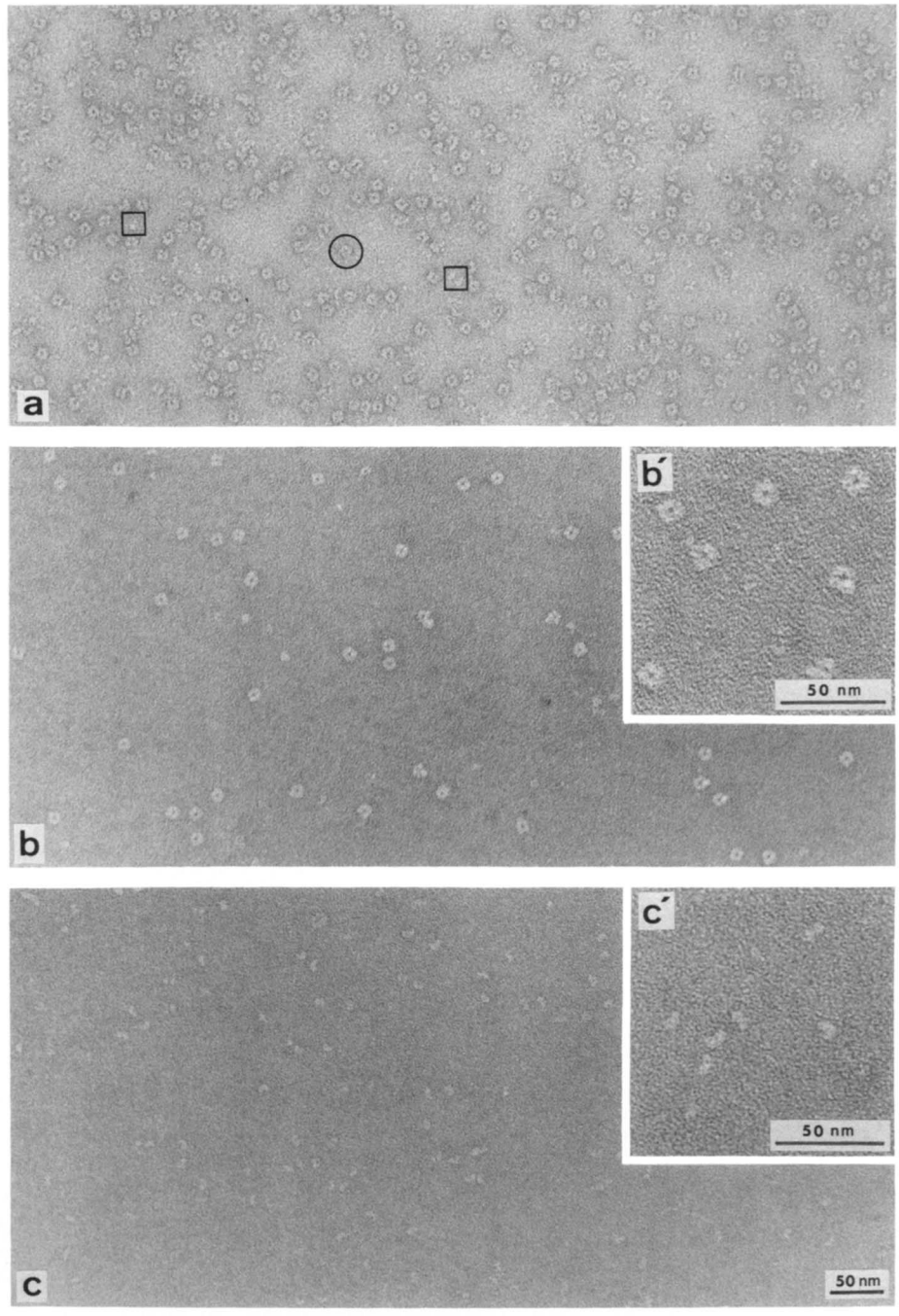

FIG. 6. **Electron micrographs of negatively stained Mi-CK molecules.** Negative stain of an aliquot of the Mi-CK preparation used for M_r determination (see Fig. 4), before separation by gel filtration on Sephacryl S-300, consisting of a mixture of Mi-CK octamers (this figure, *circles*) and Mi-CK dimers (this figure, *square-boxes*). Same after separation by gel filtration on Sephacryl S-300: sample from peak I (Fig. 4) containing exclusively octamers (*b* and inset *b'* at higher magnification); sample from peak II (see Fig. 4) containing exclusively dimers (*c* and *inset c'* at higher magnification). Note: square-shaped structures with central stain-filled cavity or channel of octamers (*a*, *b*, and *b'*) and banana-shaped structures of dimers (*a*, *c*, and *c'*). Magnification as indicated by *bars*.

phate extract onto Blue-Sepharose CL-6B and a specific desorption step by ADP yielding highly enriched Mi-CK in quantities of 10–20 mg within 36–48 h (38). The presence of Mi-CK octamers was not restricted to chicken cardiac muscle, for recently we have isolated and characterized Mi-CK from chicken brain as well and found it to form very similar octamers (55, 63). Surprisingly, brain Mi-CK turned out to be another tissue-specific mitochondrial isoform as judged from N-terminal amino acid sequence analysis and comparison with a full length cDNA clone of cardiac Mi-CK (25, 55). Thus, even though brain Mi-CK differed from cardiac Mi-CK by several protein-chemical criteria, *e.g.* in isoelectric point, tryptic peptide maps, immunological cross-reactivity with anti-cardiac Mi-CK antibodies, and in kinetic properties (55), the structure was very similar to that of cardiac Mi-CK. Octamers were also identified in Mi-CK preparations from brain by gel permeation, analytical ultracentrifugation, and direct mass measurement by scanning transmission electron microscopy.[2]

The two forms of native Mi-CK, octamers and dimers, were separated by FPLC gel permeation chromatography on Superose 12 in a matter of minutes and visualized separately by electron microscopy. Negatively stained material from the octamer and dimer peak revealed square-shaped structures with a central stain-filled cavity or channel, and elongated banana-shaped structures, respectively (Fig. 6). The fact that several laboratories have not found Mi-CK octamers at all or only "aggregates" of higher M_r most probably reflects species differences with respect to Mi-CK octamer stability or results from the rather harsh purification methods used earlier, like hydrophobic interaction chromatography (24). However, our findings are fully consistent with results published in an earlier abstract (28), which demonstrated the presence of octamers in preparations of bovine cardiac Mi-CK by analyt-

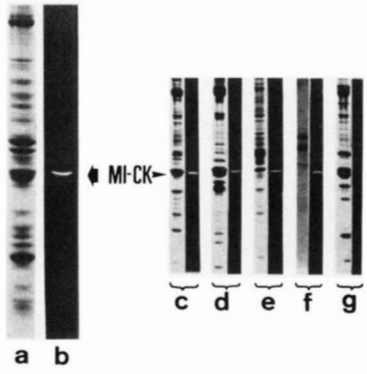

FIG. 7. **Specificity of rabbit polyclonal anti-chicken Mi-CK antibody on immunoblots: presence of Mi-CK in tissues of high, sudden energy requirement.** Total mitochondrial protein (50 μg) from cardiac mitochondria separated by PAGE in the presence of SDS and stained for protein by Coomassie Blue (*lane a*); same after electrophoretic transfer to nitrocellulose filter and indirect immunostaining with polyclonal rabbit anti-chicken Mi-CK antibody at 1:200 dilution, followed by fluorescein isothiocyanate-conjugated second antibody at 1:100 dilution and photographed under UV illumination (*lane b*). Note the specificity of the anti-Mi-CK antibody for a single protein band at position with M_r of 42,000. Similar immunoblots with extracts (1:4, w/v) of different tissues showing presence of immunoreactive Mi-CK in cardiac muscle (*c*), skeletal muscle (*d*), brain (including retina) (see Refs. 9, 43) (*e*), washed spermatozoa (*f*), but not in gizzard (*g*, where 10 × more protein was loaded for the parallel immunoblot) and liver (not shown).

ical ultracentrifugation. This indicates that Mi-CK octamers are also found in mammals. A sample of cardiac Mi-CK from rat (kindly provided by Dr. D. Cheneval, Zürich) which had been characterized by standard gel filtration techniques and negative staining to be a hexamer with a M_r of 240,000 (35) turned out to be also of octameric nature when tested under our conditions with FPLC and negative staining. Although rat cardiac Mi-CK had a somewhat lower apparent M_r than the chicken enzyme (340,000 *versus* 364,000 as determined by FPLC on Superose-12) the individual negatively stained Mi-CK molecules from rat were very similar to the ones shown for chicken cardiac Mi-CK (Fig. 6) and did clearly show 4-fold symmetry (not shown). The conclusion of these authors (35), albeit published very recently, that native Mi-CK of rat must be a hexamer is therefore no longer tenable. But their results can be explained by the fact that Mi-CK from rat tends to dissociate on a much faster time scale compared with chicken Mi-CK and, if not analyzed by FPLC, is giving the impression of an overall lower M_r of the octamer due to continuous dissociation into dimers during conventional gel filtration used by them.

Our results showing that Mi-CK, irrespective of animal species, can indeed exist as octameric molecules are supported by recent evidence from Marcillat *et al.* (60) and Lipskaya *et al.* (32) who consistently found a higher M_r species of Mi-CK in mitochondria of rat and pig cardiac muscle which was different from the commonly observed dimer. However, the fact that Mi-CK octamers, also from chicken heart, had a tendency to dissociate into dimers or were quantitatively converted within minutes into dimers by a mixture of Mg^{2+}, ADP, creatine, and nitrate (Fig. 5) is indicative of the existence of Mi-CK as two interconvertible forms, the octamer and dimer, with no long-lived intermediate species, *e.g.* tetramers and hexamers. This also indicates a higher cooperativity of dimer association to form octamers. Even though one can isolate Mi-CK in greater than 95% octameric form, protein concentration being the major factor favoring octamer formation *in vitro*, it should be stressed that we do by no means imply that the only form of native Mi-CK is the octamer, but would rather suggest that two interconvertible forms of Mi-CK also exist *in vivo*. This notion makes the molecule even more intriguing, for this very same octamer-dimer interconversion shown here *in vitro* by changing protein concentration or by addition of nucleotides may actually also take place *in vivo*. Although the local concentrations of adenine nucleotides in the mitochondrial intermembrane space are not known exactly and a detailed dose-effect study with adenine nucleotides on the octamer-dimer interconversion is still missing, it is conceivable that the reversible octamer-dimer interconversion and the preferential interaction of the octamer with mitoplasts (60)[3] may represent regulatory parameters affecting (i) the diffusion and lateral mobility of Mi-CK in the intermembrane space and on the inner mitochondrial membrane and (ii) the interaction of the enzyme with mitochondrial membranes or with ATP/ADP-translocators, respectively (Fig. 9, see below).

In summary, we believe that the highly ordered Mi-CK particles with 4-fold symmetry obtained from chicken cardiac muscle do not represent artifactual high M_r complexes formed by fortuitous aggregation, covalent bonding, or disulfide bridges, because only one type of octameric structure shown to be monodisperse by all criteria presented herein and in the following paper (58), namely by gel filtration, analytical untracentrifugation, conventional electron microscopy, and direct mass measurements with scanning transmission electron microscopy was consistently observed. Although the octamers were certainly much more susceptible to harsh treatment, *e.g.* to fluctuation in pH, to dilution or denaturing agents, they were found under a variety of buffer conditions as well as in the presence of a large excess of reducing agents. Most important of all, they were stabilized by physiological ionic strength. In addition, octamers could be readily converted into dimers and *vice versa* as described above without generating long-lived intermediate forms or aggregates of higher M_r than octamers. Finally, negative staining of Mi-CK crystals obtained recently in our laboratory clearly revealed that Mi-CK crystallizes as densely packed octamers in a regular tetragonal lattice (82).

Tissue-specific Distribution of Mi-CK—Mi-CK has been identified by immunological means in tissues of high, sudden energy demand such as skeletal and cardiac muscle, brain, retina photoreceptor cells, spermatozoa (Fig. 7) and also in kidney (not shown). In all these tissues Mi-CK is always expressed in conjunction with one of the cytosolic forms of CK, either MM- or BB-CK (8, 9, 21, 43). However, during differentiation the synthesis and accumulation of Mi-CK is delayed in most cases relative to the other isoforms, and expression begins just before hatching in birds[4] or postnatally in mammals (64). In addition, Mi-CK levels may be increased by chronic stimulation (65) or decreased (in muscle) as a function of disease (66). Surprisingly, chicken gizzard, a smooth muscle tissue known for its high content of BB-CK did not seem to contain Mi-CK nor did enriched mitochondrial preparations thereof show Mi-CK amenable to immunoblotting. Whether this lack of Mi-CK may indicate that smooth muscle, working slowly and continuously, does not need mitochondrial CK and that the high amounts of BB-CK were sufficient for ATP-regeneration or whether gizzard may contain a totally different Mi-CK isoform that would not cross-react with anti-cardiac Mi-CK antibodies is unclear at the moment. The latter possibility is unlikely, since the brain

[3] J. Schlegel, M. Wyss, and T. Walliman, unpublished results.

[4] P. Hossle, E. Wegmann, T. Wallimann, and J. C. Perriard, unpublished results.

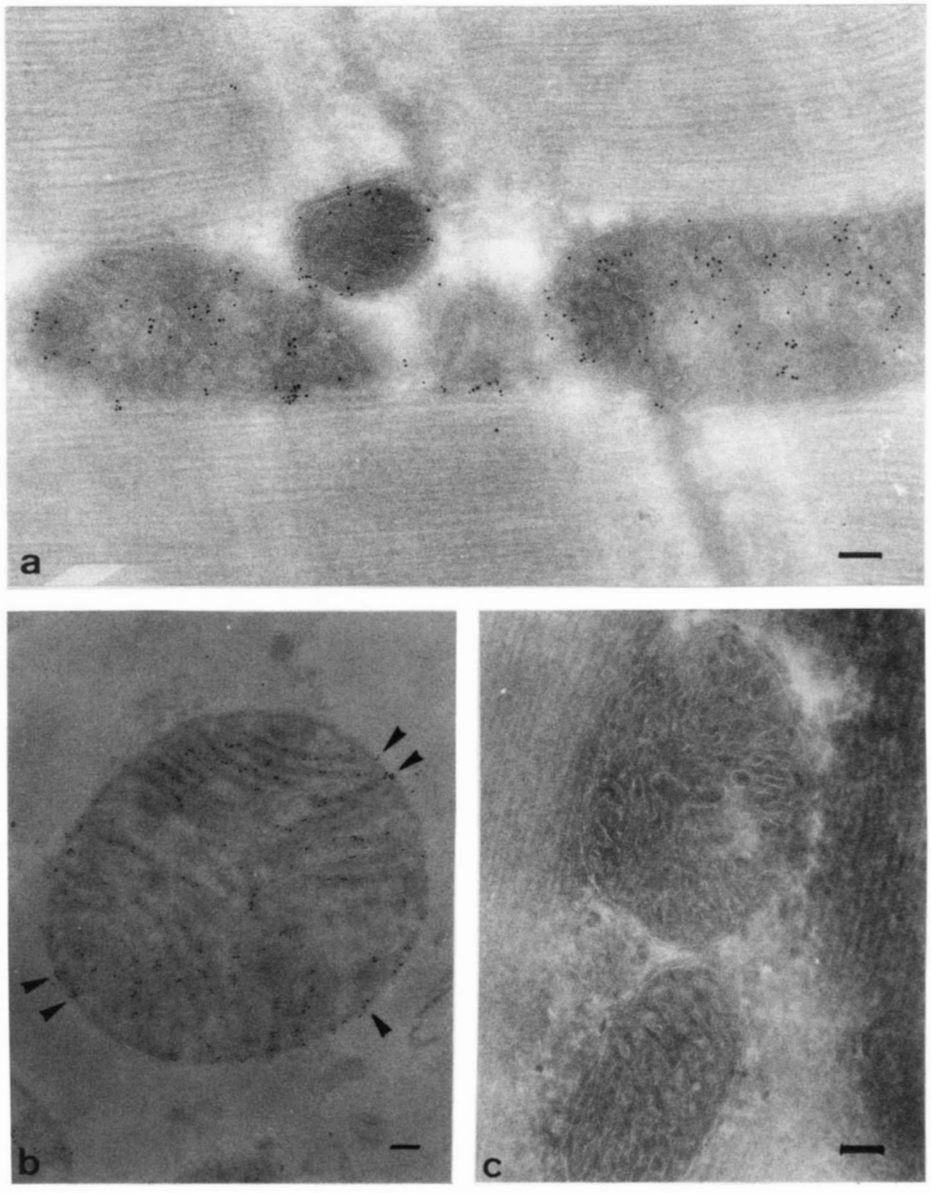

FIG. 8. **Immunolocalization of Mi-CK in ultrathin cryosections of chicken pectoralis muscle.** Ultrathin cryosections (700 Å) of frozen chicken pectoralis muscle (*a*) and plastic section of mitochondria of photoreceptor cells from chicken retina embedded in Lowicryl (K4M) (*b*), both stained by rabbit anti-chicken Mi-CK antibody at 1:100 dilution (*a* and *b*), or control antibody at the same dilution (*c*), and all followed by 1:100 diluted goat anti-rabbit IgG conjugated to 5-nm colloidal gold particles. *Bar* represents 0.1 μm. Note specific staining of the inside of mitochondria by anti-Mi-CK antibody in cryosections and more detailed localization in Lowicryl-sections along the inner mitochondrial membranes, and to some extent along the outer membrane, where Mi-CK is clustered peripherally at places where the inner membrane is in close vicinity with the outer membrane (*arrowheads*).

Mi-CK isoform shown to be distinct from the cardiac Mi-CK isoenzyme (25, 55) did clearly cross-react, albeit to a lesser extent, with the polyclonal antibodies used. However, the possibility exists that some of the bulk BB-CK present in gizzard may interact, like hexokinase, with the outside of gizzard mitochondria and thus connect mitochondrial ATP to CP production.

Amounts of Mi-CK and Stoichiometry with the ATP/ADP-Translocator—In chicken cardiac mitochondria a minimum of approximately 0.6 IU of Mi-CK activity/mg of mitochondrial protein were found. Assuming a specific activity of 130 IU/mg of Mi-CK as measured *in vitro* by pH-stat at 25 °C and pH 7.0, Mi-CK represents some 0.5% of the total mitochondrial protein. Considering the amount of Mi-CK octamer (0.02 nmol of Mi-CK octamers/mg of mitochondrial protein) and the volume of the intermembrane water space (2 μl/mg of mitochondrial protein (32, 34)), the concentration of Mi-CK, if it were in free state in the intermembrane space, can be estimated to be approximately 3.5 mg/ml. Hence, this theoretical value of protein concentration is clearly favoring octamer formation which, *in vitro*, is rapidly induced by concentrating Mi-CK dimer solutions to ≥1 mg/ml, but of course does not preclude dimerization induced by regulatory factors, *e.g.* by the simultaneous presence of adenine nucleotides and substrate (Fig. 9 and "Discussion" in the following paper (58)). The relatively low amount of Mi-CK found in chicken cardiac mitochondria is in agreement with earlier results (66, 67) but is lower by a factor of 4–5 compared to mitochondria from rat heart.

In contrast to the proposed 1:1 stoichiometry of Mi-CK to ATP/ADP-translocator (67), the molar ratios of translocator dimers to Mi-CK octamers was estimated to be approximately 40–60 in chicken and 10–20 in rat cardiac mitochondria. The ATP/ADP-translocator (ANT) is the most prominent protein species of mitochondria amounting to 8–12% of the total mitochondrial protein (68–70). These calculations clearly argue against the formation of stable complexes of 4 or 8 translocator dimers and 1 Mi-CK octamer at the inner mitochondrial membrane (58), but do not preclude a transient formation of such channeling units envisaged in Fig. 5 of the paper following (58). By contrast, the relatively high V_{max} and turnover rate of Mi-CK in intact mitochondria of at least 20,000–30,000 CP/min/Mi-CK octamer at 25 °C (calculated from Ref. 13) indicate that one single Mi-CK octamer could

serve as many as 40–50 ATP/ADP-translocators that are known to operate at relatively low speed (approximately 600 ATP/min/translocator dimer at 25 °C, calculated from Ref. 69). However, ATP/ADP-translocators have to supply ATP also to other mitochondrial kinases as well (hexokinase, nucleoside diphosphate kinase). Thus, the relatively low amount of Mi-CK in mitochondria may be sufficient to transphosphorylate in a very efficient way most of the ATP channeled through the inner mitochondrial membrane by ATP/ADP-translocators to finally form CP as the net energy-rich phosphate product of oxidative phosphorylation in tissues of high, sudden energy demand. The produced CP then is made available to sites of high ATP utilization where some of the cytosolic CK isoforms are bound specifically for regeneration and regulation of local ATP levels, thus completing the CP shuttle (for review see Refs. 20, 21, 37, 79).

Ultrastructural Localization of Mi-CK—Biochemical data suggest that Mi-CK remains associated, supposedly as octamers, with hypotonically swollen mitoplasts. Since Mi-CK can be released by phosphate at alkaline pH, it was thought to be exclusively located at the outer side of the inner mitochondrial membrane (4, 62) presumably by interaction with free (71) or ATP/ADP-translocator-bound cardiolipin (72) or with translocator protein itself. However, our direct ultrastructural localization data using immunogold labeling of cryosubstituted, low temperature-embedded intact mitochondria or whole tissue (Fig. 8) suggest that Mi-CK is not only localized along the inner mitochondrial membrane but also appears to be clustered at the periphery where inner and outer mitochondrial membrane are in close vicinity (Fig. 8*b*). The specificity of the antibodies used was tested by enzyme-linked immunosorbent assay (not shown) and immunoblotting (Fig. 7) and the antibodies stained only mitochondria in ultrathin frozen sections (Fig. 8*a*). This finding is supported by recent evidence that isolated membrane boundary contact sites are enriched in Mi-CK (73, 81) and that the outer mitochondrial membrane is necessary (15) to facilitate compartmented coupling of Mi-CK to the ATP/ADP-translocator (74, 75).

Function of Mi-CK—We propose 1) that the majority of Mi-CK in isolated inactive mitochondria or swollen mitoplasts is bound to the inner mitochondrial membrane as octamers and 2) that release of Mi-CK from the inner mitochondrial membrane does not necessarily involve the formation of dimers from octamers for the following reasons. (*a*) If a crude phosphate extract of mitochondria containing extracted Mi-CK is immediately applied to an FPLC Superose-12 sizing column, more than 80% of CK activity was found in the fractions corresponding to the octamer peak of purified enzyme. (*b*) Phosphate at concentrations higher than 10 mM (at pH 8.5–8.8) released Mi-CK from mitoplasts within minutes, but significant amounts of dimers were only found if octameric Mi-CK was stored for days in the very same buffer.

Thus, dimer formation is not a prerequisite for dissociation of Mi-CK from mitoplasts. However, if creatine and nitrate were added together with ADP, a mixture known to induce a transition-state analogue complex by mimicking a catalysis state intermediate (1, 59), the octamers at protein concentrations of ≤0.5 mg/ml were quantitatively dimerized within minutes. The conversion was no longer quantitative at high protein concentration (≥1 mg/ml).

Even though the specific activity of octamers and dimers (130–140 IU/mg), separated by gel permeation chromatography, were very similar in solution, it is likely that the kinetics of the octamer which rebinds preferentially over the dimer from a mixture of both, octamers and dimers, to the inner mitochondrial membrane[3] may change in favor for the octamer. A precedent of such a scheme has recently been demonstrated with phosphofructokinase that associates with F-actin in a reversible manner involving a dimer-to-tetramer transition (76). Concomitantly with the structural association of the tetramer, the K_m of the enzyme for fructose-6-diphosphate is reduced and its inhibition by high concentrations of ATP is relieved (76). Such considerations suggest that within mitochondria, which are actively engaged in oxidative phosphorylation and ATP transport, there must be an equilibrium of Mi-CK states as far as dimer/octamer as well as free- and membrane-bound states are concerned. In this equilibrium the inner mitochondrial membrane, the intermembrane space, and most likely also the outer membrane as well are involved (73, 77) (see also "Discussion" of the paper following (58)). The scheme presented in Fig. 9 opens up a great number of theoretical possibilities for regulation of either Mi-CK activity itself or energy channeling and oxidative phosphorylation in general. However, the exact pathways of events are not known yet. It is tempting, though, to speculate that Mi-CK octamers are transiently forming nucleotide transport and transphosphorylation complexes together with ATP/ADP-translocators by connecting the mitochondrial matrix space via translocations and outer membrane pores to the cytoplasm (see Fig. 5 of Ref. 58). Mi-CK of such complexes would utilize the local ATP and release CP to the cytoplasm. A dynamic interaction (Fig. 9, and Fig. 5 of Ref. 58) would be necessary because there are simply not enough Mi-CK octamers relative to ATP/ADP-translocator dimers to form stable stoichiometric 1:1 Mi-CK/translocator complexes. Alternatively, only distinct subpopulations of translocators, recently found by molecular cloning (80) may specifically interact with Mi-CK, whereas other translocators may be specialized to serve the various other mitochondrial kinases. This dynamic scheme (Fig. 9) involving newly formed boundary membrane contact sites where inner and outer mitochondrial membranes are close (77) is consistent with the fact that Mi-CK seems to be highly enriched at such isolated contact sites, (73, 81) and that by immunogold labeling of intact mitochondria, Mi-CK was localized not only on the inner mitochondrial membrane but was also found to be clustered where such peripheral contact sites would be expected (Fig. 8*b*). A quantitative evaluation of the parameters influencing and regulating mitochondrial respiration, *e.g.* the high energy phosphate potential (ATP/ADP·P_i), the ATP/ADP ratio, the availability of ADP at translocator sites, the availability of creatine to Mi-CK, the intramitochondrial pH value, and inorganic phos-

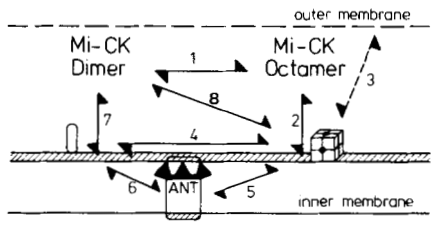

FIG. 9. **Schematic model of regulatory events for CK activity and mitochondrial respiration.** Possible regulatory mechanisms by equilibrium of free dimers and octamers in the intermembrane space (as measured in solution, see also Fig. 7) (*1*) by association of the octamer and dimer with the inner mitochondrial membrane (*2* and *7*) by possible involvement of outer membrane in the formation of contact-site-complexes to inner membrane (*3*), by equilibrium of octamer-dimer association while on the membrane (*4*), and by interaction of the respective Mi-CK species with adenosine nucleotide translocator molecules (*ANT*) and the cardiolipin collars (*filled triangles*) around ANT (*5* and *6*). Mi-CK octamer indicated by a cubic structure with 4-fold symmetry and central cavity or channel (for details see "Discussion" and data of the following article).

phate concentration (37, 78), exclusively or in combination, on the octamer-dimer equilibrium and on the rebinding of Mi-CK to mitoplasts will be necessary to dissect this complicated network which may be of physiological significance as a key regulatory system for energy channeling in tissues of sudden, high energy demand.

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