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Mitochondrial Creatine Kinase from Chicken Brain

PURIFICATION, BIOPHYSICAL CHARACTERIZATION, AND GENERATION OF HETERODIMERIC AND HETEROOCTAMERIC MOLECULES WITH SUBUNITS OF OTHER CREATINE KINASE ISOENZYMES*

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In a recent study it has been shown that mitochondrial creatine kinase from chicken brain (Mi_a-CK) and heart (Mi_b-CK) are two distinct isoenzymes differing in ten out of the thirty N-terminal amino acids (Hossle, J. P., Schlegel, J., Wegmann, G., Wyss, M., Böhlen, P., Eppenberger, H. M., Wallimann, T., and Perriard, J.-C. (1988) *Biochem. Biophys. Res. Commun.* 151, 408–416). The present article describes the purification and biophysical characterization of the mitochondrial creatine kinase isoenzyme from chicken brain (Mi_a-CK). Gel permeation chromatography, direct mass measurements of individual molecules by scanning transmission electron microscopy, and analytical ultracentrifugation confirmed the existence of two different oligomeric forms, dimeric and octameric Mi_a-CK, with molecular masses of 85 kDa and 306–352 kDa and with sedimentation constants of 4.9–5.3 and 11.6–12.0 S, respectively.

In addition, it was tested if Mi_a- and Mi_b-CK can form heterodimeric and heterooctameric molecules with subunits of other CK isoenzymes. By denaturation in urea or guanidine hydrochloride and subsequent renaturation, Mi_aMi_b-CK and surprisingly also Mi_aM-CK heterodimers could be generated. In contrast, no heterodimers were obtained between Mi_b- and M- or B-CK. Furthermore, reoctamerization of a mixture of Mi_a- and Mi_b-CK homodimers led to the formation of Mi_aMi_b-CK heterooctamers. In these heterooctamers, the Mi_a- and Mi_b-CK homodimers remained the fundamental building blocks. No subunit exchange between adjacent dimers within the heterooctamer could be observed even after storage for 3 months at 4 °C. The relevance of these data on the structural organization of the Mi-CK octamer and on the physiological aspects of tissue-specific isoenzyme expression are discussed.

Creatine kinase (CK,[1] EC 2.7.3.2) is a key enzyme in the energy metabolism of tissues with high energy demand like cardiac and skeletal muscle, brain, retina, and spermatozoa (1–7). It catalyzes the reversible transfer of the γ-phosphoryl group of ATP to creatine (Cr) to yield phosphorylcreatine (PCr) and ADP.

In chicken tissues, four different subunit isoforms of CK are expressed (8–10): M-CK (M standing for muscle), B-CK (B standing for brain), Mi_a- and Mi_b-CK (Mi standing for mitochondria). Whereas on a subcellular level the former two are localized in the cytosol, the latter two are found within the mitochondria. According to the PCr circuit model (11), mitochondrial creatine kinase on the outer surface of the inner mitochondrial membrane metabolizes ATP, generated by oxidative phosphorylation in the mitochondrial matrix, and Cr to yield PCr and ADP. PCr is thought to be the actual "high-energy compound" diffusing, within the cells of the tissues mentioned above, to places where energy-consuming reactions take place. At these sites, the cytosolic CK isoenzymes regenerate ATP, and the Cr thereby produced diffuses back to the mitochondria.

Already in the 1960s it was proven that the cytosolic CK isoenzymes are dimeric molecules with a molecular mass of 80–85 kDa (12–18). In agar and starch gel electrophoresis experiments, a urea-denatured and subsequently renatured mixture of the muscle-type (MM-CK) and brain-type (BB-CK) isoenzymes revealed an additional band with intermediate mobility (8, 18). This MB-CK heterodimer was found also naturally in adult heart (8, 14) and during the developmental transition from BB-CK to MM-CK in striated muscle (14, 19, 20). A number of investigators tried to find a clear-cut correlation between the serum content of MB-CK and several heart diseases (21–25).

In contrast to these dimeric CK isoenzymes, the naturally occurring oligomeric form(s) of mitochondrial creatine kinase from bovine (26–30), chicken (31–33), dog (34), human (35, 36), pig (37), rabbit (37), and rat heart (38) as well as from pigeon breast muscle (39) has been a matter of debate for many years. Whereas some groups claimed that Mi-CK is always a dimeric molecule (80–90 kDa) like all the cytosolic CK isoenzymes (32, 34–36), others found oligomeric forms with higher molecular masses (240–360 kDa, Refs. 26, 33, and 37–39). Recently, extensive studies on bovine, chicken, pig, and rabbit heart Mi-CK revealed that the higher M_r form of Mi-CK is composed of eight subunits (10, 33, 37, 40–42), and that probably both dimeric and octameric Mi-CK are physiologically important (43). A change in the dimer to octamer ratio might be an effective means of regulating the energy metabolism of a cell (29, 39, 43–45).

The present study was undertaken for two principal reasons: (i) Since chicken brain and chicken heart are known to

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[1] The abbreviations used are: CK, creatine kinase; M- and B-CK, the subunit isoforms of muscle-type and brain-type creatine kinase; Mi_a-CK, mitochondrial creatine kinase isoform isolated from chicken brain; Mi_b-CK, mitochondrial creatine kinase isoform isolated from chicken heart; Cr, creatine; PCr, phosphorylcreatine; STEM, scanning transmission electron microscopy; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; BME, β-mercaptoethanol.

contain two distinct Mi-CK isoenzymes (Mi_a-CK in brain and Mi_b-CK in heart) differing in 10 out of the 30 N-terminal amino acids (9), and since up to now only the sizes of the Mi_b-CK oligomers were properly investigated, the molecular masses of dimeric and octameric Mi_a-CK were determined by three different techniques. (ii) For a variety of oligomeric proteins, different subunit isoforms are known to yield, upon random combination, several distinct isoenzyme species. In the case of lactate dehydrogenase, for example, two different subunit isoforms, the M- (muscle) and H- (heart) type, combine to give five different tetrameric lactate dehydrogenase isoenzymes: M_4, M_3H, M_2H_2, MH_3, and H_4 (for reviews see Refs. 46 and 47). Metabolic adaptation was shown to be accompanied by changes in the relative proportion of these five isoenzymes. Therefore, we determined in this study the extent of heterodimer and heterooctamer formation of Mi_a- and Mi_b-CK with each other as well as with chicken M- and B-CK.

M- and B-CK are known to form MB-CK heterodimers. In addition, several investigators have tried to generate heterodimeric molecules between cytoplasmic and mitochondrial CK isoenzymes from bovine (28), dog (34), and human tissues (35, 36, 48, 49), but failed with one exception. Wevers *et al.* (48) reported the formation of a heterodimer between human heart Mi-CK and brain B-CK. However, it cannot be ruled out that the intermediate band apparent in their preparation was due to very small amounts of contaminating MB-CK, since these authors used crude mitochondrial phosphate extracts for their heterodimer formation experiments. Using purified human CK isoenzymes, Grace *et al.* (35) and Blum *et al.* (36) were not able to reproduce the formation of a MiB-CK heterodimer.

EXPERIMENTAL PROCEDURES

The disodium salts of ADP and ATP were obtained from Boehringer Mannheim (Federal Republic of Germany), Cr from Sigma, PCr from Calbiochem (Lucerne, Switzerland), guanidine hydrochloride from Fluka (Buchs, Switzerland), and urea (ultra pure) from ICN Biomedicals, Inc. (Eschwege, FRG). All other chemicals were at least of reagent grade. The Blue Sepharose CL-6B affinity matrix was obtained from Pharmacia (Dübendorf, Switzerland).

Purification of Chicken Brain Mi-CK—All purification steps were performed at 4 °C. 74 g of brain tissue from 4-week-old chicken were homogenized with a Polytron mixer (Kinematica, Kriens, Switzerland) in 520 ml of buffer A (220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 0.2 mM EDTA, 1 mM BME, 1 mM NaN_3, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). The supernatant of a first centrifugation at 650 × *g* for 10 min was centrifuged at 27,500 × *g* for 30 min. After resuspension of the pellet with a Sorvall Omnimixer (Du Pont Instruments, Bad Nauheim, FRG) in 220 ml of buffer A, another two centrifugations were performed at 650 × *g* for 10 min and at 27,500 × *g* for 40 min, respectively. The mitochondria were then swollen by two consecutive incubations in 115 ml of bidistilled water for 15 min, followed by centrifugations at 27,500 × *g* for 40 and 60 min, respectively. From the mitoplast pellet thus obtained, Mi_a-CK was extracted for 15 min in a total volume of 335 ml of hypotonic phosphate buffer (10 mM sodium phosphate, 5 mM BME) at pH 8.75. After ultracentrifugation at 38,000 × *g* for 4 h, the supernatant containing most of the CK activity was adjusted to the buffer composition of buffer B (50 mM sodium phosphate, 0.2 mM EDTA, 1 mM $MgCl_2$, 1 mM BME, 1 mM NaN_3) by addition of the pure substances. During these additions, the pH of the mixture was always kept above pH 6.75 with 10 M NaOH, and at the end it was adjusted to pH 6.80. This material was loaded with a flow rate of 2–3 ml/min on a Blue Sepharose CL-6B column (1 mg of protein/ml of column matrix) preequilibrated with 10 column volumes of buffer B, pH 6.75 (Fig. 1, fractions 1–18). The column was then washed with 2.5 volumes of buffer B, pH 6.95 (fractions 19–41) which released, among other proteins, all the B-CK from the affinity matrix. Mi_a-CK was specifically eluted by three volumes of buffer C (37.5 mM sodium phosphate, 25 mM ADP, 0.2 mM EDTA, 1 mM $MgCl_2$, 1 mM BME, 1 mM NaN_3, pH 6.95; fractions 42–61). An additional wash of the column with buffer B supplemented with 1 M NaCl (pH 7.05) (fractions 62–83) did not result in a further release of CK activity, suggesting that the CK loaded had been recovered almost completely in the two peaks containing CK activity. Fractions 49–55 containing most of the Mi-CK activity were pooled, concentrated by ultrafiltration (Amicon, Grace AG, Wallisellen, Switzerland) to a protein concentration of 8 mg/ml (1.7 ml final volume), and dialyzed extensively against buffer D (10 mM sodium phosphate, 50 mM NaCl, 2 mM BME, 0.2 mM EDTA, pH 6.5). About 3 mg of the dialyzed protein/run were then applied on a Mono S-HR 5/5 column (FPLC, Pharmacia) preequilibrated with 10 column volumes of buffer D. Separation of the bound proteins was achieved by a step gradient from 50 to 480 mM NaCl (Fig. 2). Mi_a-CK was eluted at a NaCl concentration of 265 mM.

Generation of Heterodimeric CK Molecules—Heterodimer formation experiments with guanidine hydrochloride (GdnHCl) as denaturing agent were performed as follows: Purified Mi_a-, Mi_b-, M-, and B-CK, as well as all possible combinations of two out of the four isoenzymes were denatured at a protein concentration of 0.1 mg/ml and at 25 °C for 30 min in buffer E supplemented with 4 M GdnHCl (buffer E: 50mM sodium phosphate, 150 mM NaCl, 0.2 mM EDTA, 2 mM BME, 1 mM NaN_3, pH 7.2). After this incubation, the samples were immediately transferred to a BRL microdialysis system (Gibco/BRL AG, Basel, Switzerland) and dialyzed twice for 4 h at 4 °C against buffer E supplemented with 4 mM ATP and 5 mM $MgCl_2$.

Heterodimer formation experiments with urea as denaturing agent were performed in a similar way with the following exceptions: 6 M urea was used instead of 4 M GdnHCl, and the samples were dialyzed twice against buffer E supplemented with 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM KNO_3. For the immunodetection of Mi_aM-CK (Fig. 5), denaturation and renaturation were performed as described for the heterodimer formation experiments with 6 M urea, except that 8 M urea was used in order to generate a larger amount of this Mi_aM-CK heterodimer.

Generation of Heterooctameric Mi-CK Molecules—For the experiment shown in Fig. 6, pure Mi_a- and Mi_b-CK as well as a mixture of equal amounts of both isoenzymes were incubated at fixed Mi-CK concentrations for 24 h at 4 °C in buffer E supplemented with 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM KNO_3 (pH 7.2). Then, the ratio of Mi-CK octamers to dimers was determined by gel permeation chromatography on a FPLC Superose 12 column (Pharmacia).

For the experiment shown in Fig. 7, a mixture of equal amounts of Mi_a- and Mi_b-CK was completely dimerized by incubation for 24 h at 4 °C in buffer E supplemented with 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM KNO_3 (pH 7.2; at a Mi-CK concentration of 0.1 mg/ml, total volume 1.08 ml). Subsequently, this mixture was concentrated with a Centricon™ 10 microconcentrator (Amicon) to 40 μl, washed once with 1 ml of buffer E, and concentrated again to 70 μl. The sample thus obtained was analyzed by FPLC gel permeation chromatography (Superose 12, Pharmacia) and by cellulose polyacetate electrophoresis. After storage at 4 °C for 3 months, this same sample was dimerized again by incubation in buffer E supplemented with 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM KNO_3 (pH 7.2) and analyzed by cellulose polyacetate electrophoresis.

Cellulose Polyacetate Electrophoresis—Cellulose polyacetate electrophoresis was performed at room temperature on Cellogel strips (No. 01A42, Biotec-Fischer, Reiskirchen, FRG) in an electrophoresis tank purchased from the same company, with buffer F (11.7 mM barbiturate, 52.4 mM diethylbarbiturate, 1.4 mM BME, pH 8.6) as running buffer. The CK isoenzymes were separated at room temperature at a constant voltage of 145 V for 40 min (Fig. 4), at 100 V for 135 min (Fig. 5), or at 100 V for 90 min (Fig. 7). After electrophoresis, the CK bands were visualized by the overlay gel technique as described in Ref. 31.

For the immunodetection of the CK bands, the proteins from the Cellogel strips were semi-dry-blotted in an SDS-containing transfer buffer at a constant current of 1 mA/cm^2 for 105 min onto nitrocellulose membranes (50). Incubation with the primary and secondary antibody as well as the peroxidase staining with chloronaphthol as substrate were performed as described in Ref. 10. Rabbit anti-chicken Mi_a-, Mi_b-, and M-CK sera diluted 1:50 with blocking buffer (10% horse serum in phosphate-buffered saline, 1 mM NaN_3, pH 7.2–7.4) were used as primary antibodies. Peroxidase-labeled goat anti-rabbit IgG (Nordic, Biogenzia Lemania AG, Lausanne, Switzerland) diluted 1:1000 with blocking buffer was used as secondary antibody.

Peptide Isolation and Sequencing—Purified Mi_a-CK (~100 μg) was reduced using 1 mM dithiothreitol in 6 M GdnHCl under nitrogen for 4 h in the dark. The cysteines were then alkylated using a 2-fold excess of iodoacetic acid over dithiothreitol for 4 h. The reaction was stopped by addition of dithiothreitol to a final concentration of 5 mM. The protein was precipitated with trichloroacetic acid (final concentration 10%) and washed twice with 10% trichloroacetic acid. The precipitate was taken up in 100 μl of 70% formic acid in water and digested with a hundredfold excess of CNBr over methionine under nitrogen in the dark for 24 h.

The solution was diluted to 1 ml with water and then dried under a stream of dry nitrogen at 55 °C. This was repeated three times. The peptides were then taken up in 100 μl of 0.1% trifluoroacetic acid in water and injected onto the HPLC. The first separation was done using a gradient of 0.1% trifluoroacetic acid in water (pH 2.0) to 100% *n*-propanol, 0.065% trifluoroacetic acid on a Brownlee 300 Å, C-4, 7 μm, 2.1 × 30-mm reverse phase column (Paul Bucher, Basel, Switzerland). Detection was at 206 nm and fractions were collected by hand. The peaks were rechromatographed on a Brownlee 300 Å, C-18, 7 μm, 2.1 × 30-mm reverse phase column using a gradient of 0.1% trifluoroacetic acid in water to 70% acetonitrile in water, 0.09% trifluoroacetic acid.

Purified peptides and the N terminus of the pure protein were sequenced on an Applied Biosystems 470A Sequencer with on-line microbore phenylthiohydantoin detection. Amino acid sequences were aligned according to the method of Needleman and Wunsch (51).

Other Methods—pH optima and CK activities in general were determined in both reaction directions by the pH-Stat method as described in Ref. 52 at pH 7.00 (ATP synthesis) or 8.00 (PCr synthesis) and at 25 or 42 °C, respectively, with either 20 mM HCl or 20 mM NaOH as titrant, but without BSA in the assay mixture. 1 IU corresponds to 1 μmol of PCr transphosphorylated per min at pH 7.0 and 25 °C. When enzyme activities were measured at other temperatures, enzyme units (EU) are used instead of IU.

After SDS-PAGE performed according to Ref. 53, proteins were visualized either by Coomassie Brilliant Blue R-250 or by silver staining (54). The isoelectric points of Mi_a-and Mi_b-CK were determined by isoelectric focusing on IEF 3–9 gels with the PhastSystem™ of Pharmacia and with the IEF calibration kit "broad" (Pharmacia) as reference. Except otherwise stated, protein concentrations were determined according to Bradford (55) using the Bio-Rad reagents and BSA as standard. Scanning transmission electron microscopy of glutardialdehyde-fixed Mi_a-CK octamers with tobacco mosaic virus as standard as well as ultracentrifugation experiments were performed as described in Ref. 41. Gel permeation chromatography was performed on a Superose 12 column (FPLC, Pharmacia) as described in (33) with ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), chicken BB-CK (80 kDa), and α-chymotrypsinogen (24.5 kDa) as marker proteins. Optical densities at 280 nm ($A_{280\,nm}$) were measured with a Beckman DU-7 spectrophotometer.

RESULTS AND DISCUSSION

Purification of Mi_a-CK from Chicken Brain—In Table I, the results of a typical purification of Mi_a-CK starting with 74 g of chicken brain are depicted. A four-step purification yielded a fraction highly enriched in Mi_a-CK. In a first step, mitoplasts were generated by homogenization of the brain tissue, differential centrifugation, and subsequent swelling of the mitochondria in bidistilled water. The specific CK activity determined for these mitoplasts (0.95 IU/mg protein) corresponds well to the values found for chicken heart (0.6 IU/mg, Ref. 33, 1.5 EU/mg at 30 °C, Ref. 32).

In a second step, Mi_a-CK was extracted from the mitoplasts by hypotonic phosphate treatment (10 mM sodium phosphate, 5 mM BME) at pH 8.75. For some reason, the indicated yield of the extraction (48%) as well as the specific CK activity of the mitochondrial extract of this particular preparation (5.0 IU/mg protein) were significantly lower than the values typically obtained in other Mi_a-CK purifications which generally were in the range of 70–83% and 10.2–14 IU/mg protein, respectively.

As a third step, affinity chromatography on Blue Sepharose CL-6B matrix was performed. The elution profile (Fig. 1) shows that CK activity was eluted in two peaks. As checked by cellulose polyacetate electrophoresis, B-CK was specifically eluted in the first and Mi_a-CK in the second peak, therefore proving that by this affinity chromatography step, the two CK isoenzymes can effectively be separated as already shown for chicken heart Mi- and B-CK (31).

After the fractions from the affinity chromatography containing Mi-CK activity had been pooled, concentrated, and dialyzed against buffer D, the specific activity of Mi_a-CK for all purifications performed was in the range of 24–36 IU/mg protein. Finally, this material was loaded onto a Mono S cation exchange column and eluted by a step gradient with increasing NaCl concentrations (Fig. 2). Again, two peaks with CK activity were observed. Gel permeation chromatography on a FPLC Superose 12 column (Pharmacia) showed that in the breakthrough fractions dimeric and with 265 mM NaCl octameric Mi_a-CK was eluted, indicating that the two oligomeric forms of Mi_a-CK behaved very differently on the cation exchanger. Mi_a-CK octamers eluting in the second peak had a specific activity of 50–150 IU/mg protein. The specific activity was generally dependent on the presence of BME. In all our experiments, no differences between the specific activities of dimeric and octameric Mi_a-CK were evident, in accordance to the results for Mi_b-CK (33), but in contrast to the results published by Fedosov and Belousova (44) for bovine heart Mi-CK.

In Fig. 3, the protein composition of fractions of the different purification steps is shown by SDS-PAGE. Mi_a-CK was highly enriched by this purification procedure, the final fraction containing only very small amounts of protein contaminations of unknown identity.

As compared to the published purification method for Mi_b-CK from chicken heart (33), some important differences are clearly apparent. In the case of Mi_b-CK, the extraction was followed by ammonium sulfate fractionation increasing the

TABLE I

Purification of mitochondrial creatine kinase from chicken brain

Protein concentrations were determined by the Bio-Rad method with BSA (Pierce Chemical Co.) as standard. CK activity measurements were performed by the pH-Stat method in the direction of ATP synthesis at 42 °C. In brackets, the values calculated for the enzymatic activity at 25 °C are also indicated. In the case of Mi_a-CK, CK activity has been determined to be 5.38 times higher at 42 °C than at 25 °C (see Table II).

	Protein	Total CK activity	Specific CK activity	Yield of activity
	mg	*EU (IU)*	*EU (IU)/mg*	%
Total homogenate	5,910	46,450 (8,634)	7.9 (1.46)	
Extraction mixture	1,350	6,940 (1,290)	5.1 (0.95)	100.0
Supernatant of ultracentrifugation	123.0	3,330 (613)	27.1 (5.0)	47.6
Mi_a-CK fractions after Blue Sepharose chromatography	14.3	2,200 (409)	153.8 (28.6)	31.7
Mi_a-CK fractions after Mono S chromatography	2.2	670 (125)	304.5 (56.8)	9.7

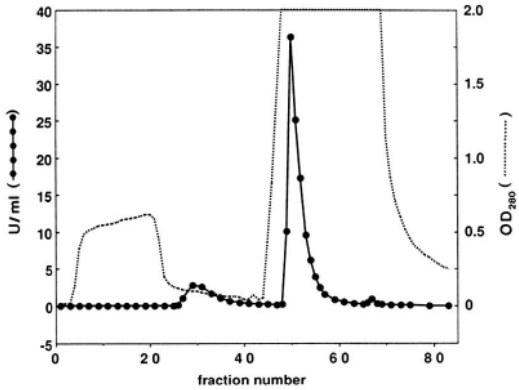

FIG. 1. **Elution profile of the Blue Sepharose-affinity chromatography.** Affinity chromatography on a Blue Sepharose CL-6B column was performed as described under "Experimental Procedures." (–●–●–), CK activity in U/ml; enzyme activity measurements were performed at 42 °C; (- - - -), $A_{280\,nm}$. Fractions of 18.5 ml were collected. Note that the smaller first peak continued exclusively BB-CK, whereas in the larger second peak, Mi_a-CK was specifically eluted.

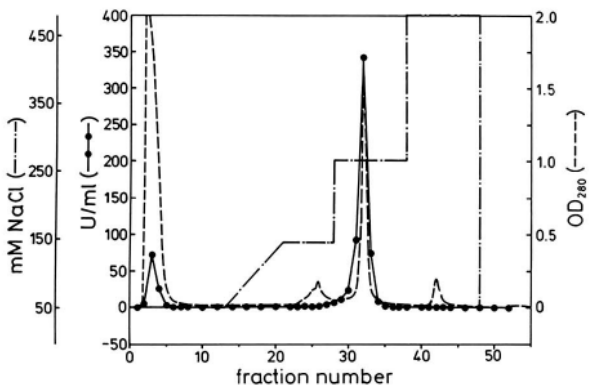

FIG. 2. **Elution profile of the Mono S cation exchange chromatography.** Cation exchange chromatography on a Mono S-HR 5/5-FPLC column (Pharmacia) was performed as described under "Experimental Procedures." (–●–●–), CK activity in units/ml; enzyme activity measurements were performed at 42 °C; (- - - -), $A_{280\,nm}$; (–·–·–), NaCl concentration in mM. Fractions of 0.5 ml were collected.

specific CK activity from 1.6 to 19 IU/mg protein. The fact that for Mi_a-CK a specific CK activity of 5–14 IU/mg protein was already achieved by only extracting the enzyme from the inner mitochondrial membrane, together with the observed deleterious effect of ammonium sulfate on Mi_a-CK activity, led to the omission of the ammonium sulfate fractionation step from the purification scheme for Mi_a-CK.

The most serious problem, however, emerged from the significantly faster dissociation of Mi_a-CK octamers into dimers as compared to Mi_b-CK octamers. Due to this fact, the Blue Sepharose column could not be washed at pH 8.0 to remove contaminating proteins, since at this elevated pH, octamer dissociation was induced, and Mi_a-CK dimers leaked continuously from the column. Since the relative proportion of Mi_a-CK octamers to dimers is strongly dependent on the protein concentration (28, 44, 45), care had also to be taken to load only highly concentrated Mi_a-CK samples (≥1 mg of protein/ml) onto the Mono S column to favor the octameric form, because under the buffer conditions used only the octamer did bind to the column.

The fact that Mi_a-CK could not be purified following the protocol developed for Mi_b-CK (different conditions necessary for binding and elution of Mi_a-CK in affinity and ion exchange

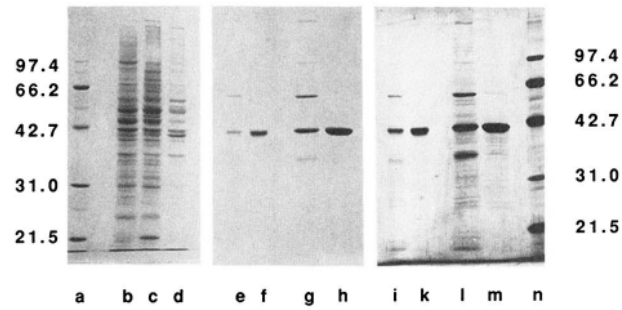

FIG. 3. **SDS-PAGE analysis of protein samples from the different purification steps.** Samples of the different fractions were loaded onto a SDS-polyacrylamide gel with a 4% stacking and a 10% running gel. Proteins were visualized either by Coomassie Brilliant Blue R-250 (*lanes a–h*) or by silver staining (*lanes i–n*). *Lanes a* and *n*, low molecular weight standard of Bio-Rad (2 μg of protein/band). *Lane b*, total brain homogenate, 40 μg of protein. *Lane c*, extraction mixture, 40 μg. *Lane d*, supernatant of the ultracentrifugation, 20 μg. *Lanes e* and *i*, Mi_a-CK fraction after Blue Sepharose chromatography, 1 μg. *Lanes f* and *k*, Mi_a-CK fraction after Mono S chromatography, 1 μg. *Lanes g* and *l*, Mi_a-CK fraction after Blue Sepharose chromatography, 4 μg. *Lanes h* and *m*, Mi_a-CK fraction after Mono S chromatography, 4 μg.

chromatography) as well as the lower kinetic stability of the Mi_a-CK octamer structure as compared to the Mi_b-CK octamer are further proof that Mi_a- and Mi_b-CK are indeed two different isoenzymes (see also below).

Biophysical Characterization of Mi_a-CK—In Table II, the biophysical data for Mi_a-CK are compiled and compared with the corresponding values for Mi_b-CK, some of which have been published (10, 33, 41).

Although Mi_a- and Mi_b-CK are two different isoenzymes, the overall three-dimensional structure of these enzymes seems to be very similar, for electron microscopy of both negatively stained Mi_a- and Mi_b-CK octamers had revealed a cube-like structure with a central, strain-filled cavity (10). To further prove the similarity between the two isoenzymes, the size of the different oligomeric forms of Mi_a-CK (monomer, dimer, and octamer) was determined by a variety of different techniques. SDS-PAGE revealed a molecular mass of 41.5 kDa for the monomer, gel permeation chromatography and analytical ultracentrifugation a molecular mass of 85 kDa for the dimer, and gel permeation chromatography, analytical ultracentrifugation as well as scanning transmission electron microscopy a molecular mass of 306–352 kDa for the octamer. The variance in values obtained for the size of the octamer can be explained in part by the different standards used for the different techniques (*i.e.* marker proteins for gel permeation chromatography and tobacco mosaic virus for STEM) and by the fact that the molecular mass values determined by STEM were not corrected for the protein-bound glutardialdehyde, which typically amounts to approximately 10% of the total mass (41). However, comparison of the molecular masses of Mi_a- and Mi_b-CK points to a very similar size of Mi_a- and Mi_b-CK monomers, dimers, and octamers, Mi_a-CK being perhaps somewhat smaller or more densely packed than Mi_b-CK. The above values are also in the same range as already published values for Mi-CK from chicken (10, 32, 33), dog (34), human (36), pig (37), rabbit (37), bovine (44, 56), and rat cardiac muscle (38), as well as from pigeon breast muscle (39), which were in the range of 42–44 kDa for the monomer, 75–87 kDa for the dimer, and 240–380 kDa for the high molecular mass oligomer. The value of about 240 kDa reported for the rat heart Mi-CK octamer (38) may be explained by dissociation of the octamer during conventional gel filtration,

TABLE II

Biophysical characterization of mitochondrial creatine kinase from chicken brain

		Mi_a-CK	Mi_b-CK
Mass determined by SDS-PAGE		41.5 ± 1.1 kDa	41.8 ± 0.5 kDa
Mass determined by gel permeation chromatography	Dimer	85 ± 8 kDa	86 ± 10 kDa[a]
	Octamer	352 ± 20 kDa[b]	364 ± 25 kDa[b]
Mass determined by STEM[c] (INTARE program)	Fixed octamers	339 ± 69 kDa	378 ± 60 kDa[d]
Mass determined by analytical ultracentrifugation	Dimer	85 ± 8 kDa	83 ± 8 kDa[d]
	Octamer	306[e]–322[f] ± 25 kDa	321[e]–328[f] ± 25 kDa[d]
Sedimentation constants $s_{20,w}$	Dimer	4.9–5.3	~5.0[d]
	Octamer	11.6–12.0	12.8–13.5[d]
Isoelectric point	Dimer	8.4–8.5[g]	9.3[g]
	Octamer	8.7–9.0[g]	9.4–9.5[g]
pH optima determined at 42 °C (25 °C) in the direction of	ATP synthesis	6.25 (6.0)	6.25–6.50 (6.7)[h]
	PCr synthesis	8.00–8.25	7.50–7.75
Temperature optimum in the direction of	ATP synthesis (pH 7.0)	42–45 °C	42 °C
	PCr synthesis (pH 8.0)	42 °C	42 °C
CK activity at 43 °C/CK activity at 25 °C in the direction of	ATP synthesis (pH 7.0)	5.38	4.72
	PCr synthesis (pH 8.0)	3.23	1.93
K_m value for PCr		0.4 mM[b]	1.6 mM[b]
Specific activities (25 °C, pH 7.0) in the direction of	ATP synthesis	50–150 IU/mg	130–140 IU/mg
$A_{280\,nm}$ value for 1 mg of Mi-CK/ml[i]		1.01/cm	0.88/cm

[a] Ref. 33.
[b] Ref. 10.
[c] Direct mass measurement of glutardialdehyde-fixed Mi-CK octamers. Values include mass of protein-bound glutardialdehyde.
[d] Ref. 41.
[e] Determined at a protein concentration of 0.6 mg/ml.
[f] Determined at a protein concentration of 0.2 mg/ml.
[g] Ref. 43.
[h] B. Zurbriggen, unpublished results.
[i] The protein concentrations of the Mi_a- and Mi_b-CK samples used for this experiment were determined according to Bradford (54) and according to Smith *et al.* (69) with BSA as standard. The protein concentration used for the calculation of $A_{280\,nm}$ was obtained by averaging three values each of both protein determinations.

since FPLC gel permeation chromatography (performed within 0.5 h) of rat heart Mi-CK kindly provided by Dr. D. Cheneval revealed a molecular mass of 341 kDa for the octamer. Thus, the high molecular mass forms of all avian and mammalian Mi-CK isoenzymes seem to have a $M_r > 300$ kDa, therefore favoring the interpretation that this form of Mi-CK is indeed an octamer. In no experiment was there any indication for an intermediate hexameric form of chicken Mi_a- and Mi_b-CK as suggested by Fedosov and Belousova for bovine heart Mi-CK (44).

Isoelectric focusing revealed a higher isoelectric point for the octamer than for the dimer for both Mi_a- and Mi_b-CK (43). Due to the marked tendency of the Mi_a-CK octamer to dissociate into dimers during isoelectric focusing, its pI is indicated in Table II with a somewhat larger variability. For both Mi_a- and Mi_b-CK, the pI values are in accordance to the findings of Dr. C. Vial's group (57, 58) which reported a significantly higher pI for the rabbit heart Mi-CK octamer (8.83) than for the dimer (8.24). Since other studies indicated that the interaction of Mi-CK with the inner mitochondrial membrane is at least in part of ionic nature (59–62), it has been assumed that this higher pI value may be responsible for the preferential binding of the octamer to the inner mitochondrial membrane (43, 58). Nevertheless, it should be kept in mind that, in contrast to all these results, Lipskaya *et al.* (63) have published a higher pI value for the pigeon breast muscle and bovine heart Mi-CK dimer than for the octamer.

The enzymatic activities of Mi_a- and Mi_b-CK are highly temperature-dependent, with the dependence being more pronounced for Mi_a-CK. For both Mi-CK isoenzymes as well as for both directions of the enzymatic reaction, the temperature optima were essentially the same and correspond well to the body temperature of the chicken (40–43 °C). Above 51 °C, both isoenzymes were rapidly inactivated probably by denaturation of the protein. The similarity between the $A_{280\,nm}$ values of Mi_a- and Mi_b-CK (1.01 *versus* 0.88/cm) was further corroborated by the value obtained for Mi_b-CK, when protein determination was performed by amino acid analysis (1.2/cm).[2] In contrast to the V_{max} values in the direction of ATP synthesis, which were also rather similar for Mi_a- and Mi_b-CK, significant differences in the pH optima of the two isoenzymes were found; taken together with the pI values (see above) and with the already published K_m values for PCr of 0.4 mM for Mi_a- and 1.6 mM for Mi_b-CK (10), these results point to clear functional differences between the two isoenzymes.

In Table III, the sequences of six Mi_a-CK peptides obtained by cyanogen bromide digestion are shown and compared with the cDNA-derived sequences for chicken leg muscle Mi-CK (which is thought to be identical to chicken heart Mi_b-CK, Ref. 9) and human placenta Mi-CK (64). Whereas the very N-terminal part of the molecule is completely different between Mi_a- and Mi_b-CK (nine amino acid changes among the 14 N-terminal amino acids), the other parts of the Mi_a-CK sequence shown here are very similar to the corresponding ones of Mi_b-CK (six amino acid changes among 93 amino acid residues determined). This high degree of homology is not surprising, for chicken leg muscle Mi-CK (9) and human placenta Mi-CK (64) display a homology of 84%, chicken and human M-CK one of 89%, and chicken and human B-CK one of 88%. Compared to that, homologies between either chicken or human Mi-, and M-, and B-CK are considerably smaller (68–81% in the case of the chicken CK isoenzymes, 65–77% in the case of the human isoenzymes). The fact that placenta

[2] M. Rojo, unpublished data.

TABLE III

Amino acid sequences of mitochondrial creatine kinase from chicken brain

The amino acid sequences of six Mi_a-CK peptides obtained from cyanogen bromide digestion (a) are aligned with the cDNA-derived amino acid sequences for Mi-CK from chicken leg muscle (c) and human placenta (e) as well as with the N-terminal sequences determined for chicken Mi_a-CK (b), chicken Mi_b-CK (d), human heart Mi-CK (f), and rat heart Mi-CK (g).

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                                10                  20                  30                  40                  50
                                v                   v                   v                   v                   v
Mia-CK[a]       G E R Q R R R - Y P P S A Q Y P D L R K           A S C L T P A I Y A R L R D K X T P N
Mia-CK[b]       G E R Q R R R - Y P P S A E Y P D L R K H N N X M A S X L T
leg muscle Mi-CK[c] T V H E K R K L F P P S A D Y P D L R K H N N C M A E C L T P A I Y A K L R D K L T P N G Y S L D Q
Mib-CK[d]       T V H E K R K L F P P S A D Y P D L R K H N N C M A E C L T
human plac. Mi-CK[e] A A S E R R R L Y P P S A E Y P D L R K H N N C M A S H L T P A V Y A R L C D K T T P T G W T L D Q
human heart Mi-CK[f] E V R E Q P R L F P P S A D Y P D L R K H N N C M A
rat heart Mi-CK[g] D A R E Q C K L F P P H A D Y P D L R K H N N C M A E C L T P I K Y A K L N C G

                                60                  70                  80                  90                  100
                                v                   v                   v                   v                   v
Mia-CK[a]                                           V A G D E E T Y E V F A E L F D P V I Q
leg muscle Mi-CK[c] C I Q T G V D N P G H P F I K T V G M V A G D E E S Y E V F A E I F D P V I K A R H N G Y D P R T M
human plac. Mi-CK[e] C I Q T G V D N P G H P F I K T V G M V A G D E E T Y E V F A D L F D P V I Q E R H N G Y D P R T M

                                110                 120                 130                 140                 150
                                v                   v                   v                   v                   v
Mia-CK[a]       K H X T D L D A S K I X X G(E)F D E R
leg muscle Mi-CK[c] K H H T D L D A S K I T H G Q F D E R Y V L S S R V R T G R S I R G L S L P P A C S R A E R R E V E
human plac. Mi-CK[e] K H T T D L D A S K I R S G Y F D E R Y V L S S R V R T G R S I R G L S L P P A C T R A E R R E V E

                                160                 170                 180                 190                 200
                                v                   v                   v                   v                   v
leg muscle Mi-CK[c] N V V V T A L A G L K G D L S G K Y Y S L T N M S E R D Q Q Q L I D D H F L F D K P V S P L L T C A
human plac. Mi-CK[e] R V V V D A L S G L K G D L A G R Y Y R L S E M T E A E Q Q Q L I D D H F L F D K P V S P L L T A A

                                210                 220                 230                 240                 250
                                v                   v                   v                   v                   v
Mia-CK[a]         A R D X(P)D A R G I X H N N D X T                                       K R V F E R F(C)R
leg muscle Mi-CK[c] G M A R D W P D A R G I W H N N D K T F L V W I N E E D H T R V I S M E K G G N M K R V F E R F C R
human plac. Mi-CK[e] G M A R D W P D A R G I W H N N E K S F L I W V N E E D H T R V I S M E K G G N M K R V F E R F C R

                                260                 270                 280                 290                 300
                                v                   v                   v                   v                   v
Mia-CK[a]       G L K E V E
leg muscle Mi-CK[c] G L K E V E R L I K E R G W E F M W N E R L G Y V L T C P S N L G T G L R A G V H V K L P R L S K D
human plac. Mi-CK[e] G L K E V E R L I Q E R G W E F M W N E R L G Y I L T C P S N L G T G L R A G V H I K L P L L S K D

                                310                 320                 330                 340                 350
                                v                   v                   v                   v                   v
leg muscle Mi-CK[c] P R F P K I L E N L R L Q K R G T G G V D T A A V A D V Y D I S N L D R M G R S E V E L V Q I V I D
human plac. Mi-CK[e] S R F P K I L E N L R L Q K R G T G G V D T A A T G G V F D I S N L D R L G K S E V E L V Q L V I D

                                360                 370                 380
                                v                   v                   v
leg muscle Mi-CK[c] G V N Y L V D C E K K L E K G Q D I K V P P P L P Q F G R K
human plac. Mi-CK[e] G V N Y L I D C E R R L E R G Q D I R I P T P V I H T K H
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[a] This study.
[b] Refs. 9 and 10.
[c] Ref. 9.
[d] Refs. 9 and 32.
[e] Ref. 64.
[f] Ref. 64.
[g] Ref. 38.

contains the B-CK isoenzyme (65) and that the peptide sequences determined for chicken brain Mi_a-CK are more similar to human placenta Mi-CK than to chicken heart Mi_b-CK make it very likely that human brain contains the same Mi-CK isoenzyme as human placenta.

Generation of Heterodimeric CK Molecules—To investigate the ability of different creatine kinase subunit isoforms to form heterodimeric molecules, either pure isoenzymes or mixtures of two out of the four known chicken CK isoenzymes (Mi_a-, Mi_b-, M-, and B-CK) were denatured at a CK concentration of 0.1 mg/ml in 4 M GdnHCl and renatured in a buffer containing 4 mM ATP and 5 mM $MgCl_2$. These latter substrates were added to favor renaturation and to avoid octamerization of the mitochondrial CK isoenzymes.[3] The differ-

[3] M. Wyss, unpublished results.

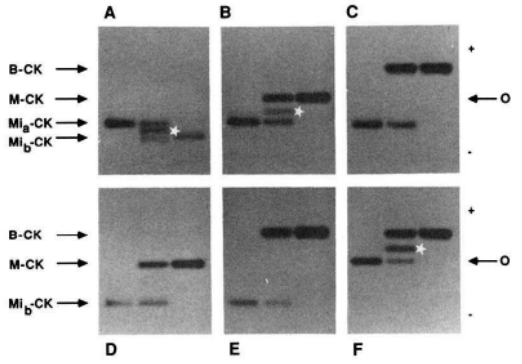

FIG. 4. **Cellulose polyacetate electrophoresis of samples from the heterodimer formation experiments.** Formation of heterodimers out of mixtures of purified chicken CK isoenzymes as well as cellulose polyacetate electrophoresis were performed as described under "Experimental Procedures." In each panel (*A–F*), the results of a heterodimer formation experiment between two particular CK isoenzymes (Mi$_a$-, Mi$_b$-, M-, and B-CK) are shown. The electrophoretic patterns of the respective denatured and subsequently renatured pure isoenzymes (*left* and *right lane* of each panel) are compared with the pattern obtained with a mixture of both isoenzymes treated identically (*middle lane*). Additional bands due to CK heterodimer formation are marked with a star (*). *A*, Mi$_a$- and Mi$_b$-CK. *B*, Mi$_a$- and M-CK. *C*, Mi$_a$- and B-CK. *D*, Mi$_b$- and M-CK. *E*, Mi$_b$- and B-CK. *F*, M- and B-CK. *O*, origin.

ent samples were then analyzed by cellulose polyacetate electrophoresis (Fig. 4) and by enzyme activity measurements. The recoveries of the enzymatic activity were in the range of 26–82%, with Mi$_a$-CK displaying the lowest and M-CK the highest degree of recovery.

In the case of the renatured pure homodimeric isoenzymes (Fig. 4, *A–F, left* and *right lane* of each panel), cellulose polyacetate electrophoresis revealed one single band each exactly at the same positions as the nondenatured isoenzymes, indicating that no additional bands were created by the denaturation procedure itself. Analysis of the mixtures of two heterologous isoenzymes treated identically (Fig. 4, *A–F, middle lane*) revealed in three cases an additional band that was apparently due to the formation of heterodimeric molecules between Mi$_a$- and Mi$_b$-CK (*panel A*), Mi$_a$- and M-CK (*panel B*), and M- and B-CK (*panel F*). No additional bands, however, were observed for the other three combinations (*panels C–E*) even on heavily overloaded cellulose polyacetate strips (not shown). This was definitely not due to insufficient denaturation of the homodimers, since the formation of MB- and Mi$_a$Mi$_b$-CK heterodimers as an internal control unambiguously proved that all four CK isoenzymes were monomerized and did reassemble again into dimers during renaturation. The ratio of heterodimers to homodimers could therefore be used as a reliable measure for the strength of subunit interaction in the respective heterodimers.

The Mi$_a$Mi$_b$-CK and MB-CK heterodimer bands in the respective samples made up a large proportion of the total CK activity, indicating that the subunit interaction strength in these heterodimers is almost the same as in the original homodimers. In contrast, Mi$_a$- and B-CK, Mi$_b$- and B-CK, and Mi$_b$- and M-CK did not form heterodimers implying that the two different subunit isoforms failed to interact strongly enough with each other. However, quite surprisingly, a small amount of Mi$_a$M-CK heterodimers was also formed as shown in Fig. 4*B*.

Heterodimer formation experiments similar to those shown in Fig. 4 were also performed by using 6 M urea instead of 4 M GdnHCl as denaturing agent. Essentially the same results were obtained, except that in all cases the ratio of heterodimers to homodimers formed upon renaturation was smaller. The recoveries of the enzymatic activity were in the range of 58–93%. Since up to now no unequivocal evidence for a heterodimer between a mitochondrial and a cytoplasmic CK isoenzyme has been presented, cellulose polyacetate strips as the ones shown in Fig. 4 were directly semi-dry-blotted onto nitrocellulose membranes. Subsequently, Mi$_a$- and M-CK subunits were immunodetected by polyclonal rabbit anti-Mi$_a$- and anti-M-CK antibodies (Fig. 5). The two antibodies proved to be very specific, for the anti-Mi$_a$-CK antibody did not cross-react with M-CK, and vice versa. Both antibodies, however, stained the intermediate band between the Mi$_a$-CK and the M-CK homodimer bands, thereby strongly supporting the conclusion that this band is due to Mi$_a$M-CK heterodimers.

Even though the relative proportion of the Mi$_a$M-CK heterodimer was rather small, one might ask about its physiological significance. A hypothesis shall be given here. Mi-CK, M-CK, and B-CK are all coded for by nuclear genes (9, 64, 66). In tissues containing CK activity and PCr, at least one mitochondrial and one cytoplasmic CK isoenzyme are generally coexpressed. The cytosolic isoenzyme remains in the cytosol, whereas Mi-CK has to be transported to the mitochondria and translocated into the intermembrane space. If heterodimer formation between the mitochondrial and the cytoplasmic CK isoenzyme were allowed, Mi-CK would be trapped in the cytosol due to the surplus of the cytosolic CK isoenzyme. For "economical" reasons, therefore, heterodimer formation between the coexpressed mitochondrial and cytoplasmic CK isoenzymes must be suppressed. The formation of a Mi$_a$M-CK heterodimer is allowed because Mi$_a$-CK (found in chicken brain and retina) and M-CK (found in chicken skeletal muscle) probably are never coexpressed *in vivo* within the same cell.

To support this hypothesis, and to get more information about the physiological significance of the Mi$_a$Mi$_b$-CK heterodimer, the tissue distribution of all CK homo- and heterodimers has to be studied in detail.

Generation of Heterooctameric Mi-CK Molecules—Two different experiments were performed to analyze the ability of Mi$_a$- and Mi$_b$-CK homodimers to form heterooctameric molecules (Figs. 6 and 7). We did not perform any heterooctamer formation experiments starting with Mi$_a$Mi$_b$-CK heterodimers.

Fig. 6 shows the dependences of octamer proportion *versus* Mi-CK concentration for Mi$_a$-CK (●), Mi$_b$-CK (□), and mixtures of equal amounts of Mi$_a$- and Mi$_b$-CK (▲), all in the presence of 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM

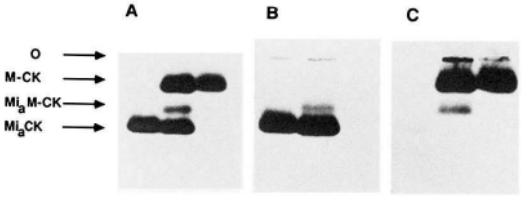

FIG. 5. **Immunodetection of the Mi$_a$M-CK heterodimer.** A mixture of equal amounts of Mi$_a$- and M-CK at a protein concentration of 0.1 mg/ml was denatured by 8 M urea, renatured, and separated by cellulose polyacetate electrophoresis. *A* shows an activity staining of a cellulose polyacetate strip. Immunodetection with anti-Mi$_a$-CK (*B*) and anti-M-CK antibodies (*C*) was performed with identically treated cellulose polyacetate strips blotted onto nitrocellulose membranes. In the middle lane of each panel, the electrophoretic pattern of the sample from a heterodimer formation experiment is shown and compared to those of pure Mi$_a$-CK (*first lane* of each panel) and of pure M-CK homodimers (*third lane* of each panel). *O*, origin.

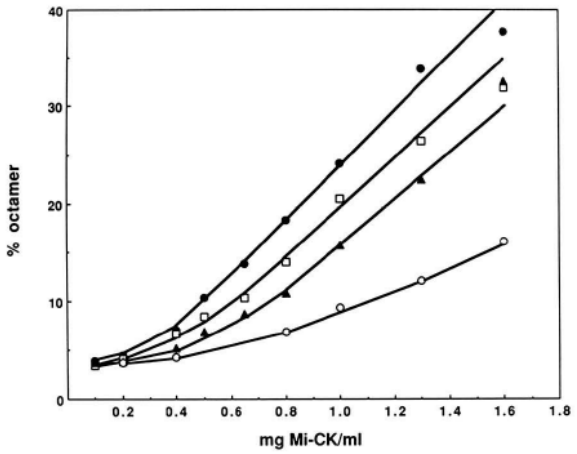

FIG. 6. **Dependence of the equilibrium between dimeric and octameric Mi-CK on the concentration of Mi-CK (in the presence of reagents inducing a transition state-analogue complex).** The experiment was performed as described under "Experimental Procedures." ●, Mi_a-CK; □, Mi_b-CK; ▲, mixture of equal amounts of Mi_a- and Mi_b-CK; ○, curve calculated for a mixture of equal amounts of Mi_a- and Mi_b-CK under the assumption that heterooctamer formation between Mi_a- and Mi_b-CK homodimers is not possible. The calculation was based on the values obtained for pure Mi_a- and Mi_b-CK.

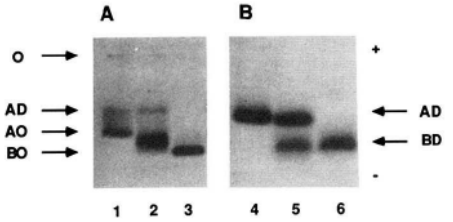

FIG. 7. **Cellulose polyacetate electrophoresis of Mi_aMi_bCK heterooctamers.** *A*, the experiment was performed as described under "Experimental Procedures." The sample of the heterooctamer formation experiment is shown in *lane 2* and compared to the electrophoretic behavior of pure Mi_a-CK (*lane 1*) and Mi_b-CK octamers (*lane 3*). *B*, re-dimerization of Mi_aMi_b-CK heterooctamers after prolonged storage. The sample of the heterooctamer formation experiment (*A, lane 2*) was completely dimerized again as described under "Experimental Procedures" and analyzed by cellulose polyacetate electrophoresis (*lane 5*). The electrophoretic behaviors of pure Mi_a-CK and Mi_b-CK dimers are shown in *lanes 4* and *6*. *O*, origin; *AD*, Mi_a-CK dimer position; *AO*, Mi_a-CK octamer position; *BD*, Mi_b-CK dimer position; *BO*, Mi_b-CK octamer position.

KNO_3 (pH 7.2). This experimental set-up was chosen for the following reasons: The equilibrium between dimeric and octameric Mi-CK was shown to be highly dependent on the concentration of Mi-CK (28, 44, 45). In addition, this equilibrium can be shifted in the direction of dimeric Mi-CK by forming a transition state-analogue complex of the enzyme with MgADP, Cr, and NO_3^- (67). It was a definite advantage that in the presence of the latter substances, the equilibrium between octameric and dimeric Mi-CK was already reached after a few hours, whereas in their absence, it was reached with Mi_b-CK only after several months (68).

If Mi_a- and Mi_b-CK homodimers were not able to interact with each other to form heterooctameric molecules, the effective concentration of both isoenzymes responsible for the ratio of Mi-CK octamers to dimers would only be half the total Mi-CK concentration. In this case, the ratio of octameric to dimeric Mi-CK would be expected to be considerably lower than if both Mi-CK isoenzymes were to participate in this equilibrium reaction. For this same case, one can calculate the dependence of octamer proportion *versus* Mi-CK concentration from the values obtained with pure Mi_a- and Mi_b-CK. The corresponding curve is also shown in Fig. 6 (○). The intermediate position of the octamer/dimer equilibrium curve obtained experimentally for the mixture of Mi_a- and Mi_b-CK (▲) between the curves for the pure isoenzymes (Mi_a-CK, ●; Mi_b-CK, □) and the curve calculated for the case as if no heterooctamer formation between Mi_a- and Mi_b-CK homodimers were possible (○) strongly indicates that (i) Mi_a- and Mi_b-CK homodimers indeed can interact with each other and form heterooctameric molecules and that (ii) in the heterooctamers, the interaction between the homodimers is somewhat weaker than in the homooctamers.

In a second experiment, a mixture of both Mi-CK isoenzymes was completely dimerized by incubation with 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM KNO_3, pH 7.2. Reoctamerization was then achieved by removing the substrates and by concentrating the sample. The Mi-CK mixture thus obtained consisted of 68% octameric and 32% dimeric Mi-CK as checked by FPLC gel permeation chromatography. Cellulose polyacetate electrophoresis of this mixture (Fig. 7*A*, *lane 2*) followed by staining for CK activity revealed prominent, broad staining between the positions of pure octameric Mi_a- (*lane 1*) and Mi_b-CK (*lane 3*), but no discrete CK bands. The lack of discrete heterooctamer bands is probably due to the limited resolution of this separation method and to partial dimerization of Mi_a-CK octamers during electrophoresis, a phenomenon that was always observed (see also *lanes 1* and *3* of Fig. 7). Therefore, it is evident from both experiments described above (Figs. 6 and 7) that homodimers of the two Mi-CK isoenzymes can combine to give different heterooctamer species.

After storage for 3 months at 4 °C, the Mi-CK heterooctamer mixture (Fig. 7*A*, *lane 2*) was dimerized again by incubation with 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM KNO_3 (pH 7.2). Cellulose polyacetate electrophoresis (Fig. 7*B*) revealed almost no Mi_aMi_b-CK heterodimers indicating that Mi-CK octamers are composed of discrete Mi-CK dimers, and that in the octameric Mi-CK molecule, no subunit exchange between these discrete dimers takes place. The much more pronounced stability of Mi-CK dimers is further corroborated by the finding that Mi-CK dimers, in contrast to octamers, were remarkably stable to up to 2 M urea or GdnHCl (41). Thus, it can be concluded that, within the Mi-CK octamers, discrete dimers and not the monomers are the fundamental building blocks. Despite the pronounced dimer stability, formation of Mi_aMi_b-CK heterodimers is possible at least *in vitro* after complete dissociation of the Mi-CK dimers by urea of GdnHCl (Fig. 4). Future studies will focus on the natural occurrence of Mi_aMi_b-CK heterodimers and heterooctamers.

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