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Mitochondrial Creatine Kinase from Cardiac Muscle and Brain Are Two Distinct Isoenzymes but Both Form Octameric Molecules*

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Mitochondrial creatine kinase (Mi-CK) from chicken cardiac muscle and brain, recently shown to differ in their N-terminal amino acid sequences and to be encoded by multiple mRNAs (Hossle, H. P., Schlegel, J., Wegmann, G., Wyss, M., Böhlen, P., Eppenberger, H. M., Wallimann, T., and Perriard, J. C. (1988) *Biochim. Biophys. Res. Commun.* 151, 408–416) were separated on two-dimensional nonequilibrium pH-gradient electrophoresis gels and visualized as two distinct protein spots by immunoblotting. Analysis of the two proteins purified by specific elution from Blue-Sepharose with ADP (Wallimann, T., Zurbriggen, B., and Eppenberger, H. M. (1985) *Enzyme* 33, 226–231) followed by fast protein liquid chromatography cation exchange chromatography showed obvious differences in peptide maps, in immunological cross-reactivity with monoclonal antibodies, and in kinetic parameters. However, even though the two proteins were different, tissue-specific mitochondrial isoforms, both formed regularly-sized, perforated cube-like octameric structures with M_r of 364,000 ± 25,000 and 352,000 ± 20,000 for the cardiac and brain isoform, respectively.

Electron microscopy of cardiac and brain Mi-CK octamers revealed cube-like molecules with a central cavity or transverse channel filled by negative stain. The octameric molecular structure of Mi-CK isoforms differs from the generally accepted dimeric arrangement of "cytosolic" muscle MM- and brain BB-CK.

In mammals and birds the following two tissue-specific "cytosolic" creatine kinase isoforms (CK,[1] EC 2.7.3.2) can be distinguished: an ubiquitous BB-CK form, expressed at high levels in brain, and a muscle-specific form, MM-CK expressed at high levels in striated muscle (1, 2; for review see 3). A further CK isoenzyme called Mi-CK is specifically associated with mitochondria, presumably with the inner mitochondrial membrane (4–6) and seems to be functionally coupled to the ATP-ADP-translocase (7; for review see 8). It is thought to play a crucial role in the energy supply within tissues of high, sudden energy demand, *e.g.* cardiac and skeletal muscle (9–11), brain (12, 13), spermatozoa (14, 15), and photoreceptor cells of the retina (16). In these tissues or cells Mi-CK is always coexpressed with one of the cytosolic CK-isoforms, MM-CK or BB-CK. Some fractions of the latter CK isoforms, however, have been shown to be specifically bound at subcellular sites of high ATP demand, *e.g.* at the myofibrillar M-band (17) functionally coupled to the myosin ATPase (18), at the sarcoplasmic reticulum (10) coupled to the Ca^{2+}-ATPase, at the sarcolemma (10) coupled to the Na^+/K^+-ATPase, at the postsynaptic acetylcholine receptor-rich membrane (19), in outer segments of photoreceptor cells (61) coupled to a variety of ATP-dependent membrane functions. In spermatozoa BB-CK and Mi-CK were also found to be segregated and restricted to the tail and mid-piece region, respectively (15). These facts led to the postulation of the CP shuttle model (10, 11, 17, 20–24, 61). This concept is gaining increasing support from recent ^{31}P NMR data in muscle (25–29). Additionally, the computer-assisted analysis of sea urchin sperm-tail oscillation clearly revealed that existing diffusional restraints between the spermatozoan mid-piece, where energy (ATP/CP) is generated in the mitochondria, and the tail region, where energy is utilized by the dynein ATPase, are overcome by a CP shuttle working in a similar way in spermatozoa (14) as in muscle (11). A progressive loss of sperm-tail oscillation starting at the distal end of the sperm tails is observed if the functionally segregated CK isoenzyme populations are inactivated stepwise by dinitrofluorobenzene (30).

The fact that Mi-CK monomers do not hybridize with B- or M-type subunits (31) and antibodies against Mi-CK do not cross-react with the latter two (32) was indicative for possible differences in primary structure between Mi-CK and the other CK-isoforms. This has recently been confirmed by N-terminal amino acid sequencing (33) as well as molecular cloning and DNA sequencing (34). Nucleic acid hybridization experiments at low stringency as well as N-terminal sequence analysis of the purified Mi-CK isoenzymes furthermore demonstrated the existence of a brain Mi-CK isoprotein encoded by a different mRNA, distinct in primary structure from both the heart and skeletal muscle Mi-CK isoproteins (34).

Assuming a central role of Mi-CK for energy supply, and considering a number of conflicting reports about its oligomeric structure (several high M_r "aggregates" of Mi-CK have been described ranging in M_r from 150,000–350,000; for review see (Ref. 35), we have undertaken to purify to homogeneity Mi-CK from mitochondria of chicken cardiac muscle (36, 37) and brain by a novel method and have studied their biochemical and structural properties. Two interconvertible forms of Mi-CK, dimers and octamers, have recently been characterized in detail for cardiac Mi-CK (37–40), but no information was so far available for brain Mi-CK. Two sets of information

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[1] The abbreviations used are: CK, creatine kinase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; 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 the subunits of the dimeric muscle type MM-CK and brain-type BB-CK isoforms, respectively. C, creatine; CP, phosphorylcreatine; mAb, monoclonal antibodies; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay.

are being presented here: (*a*) the two tissue-specific mitochondrial Mi-CK isoenzymes from heart and brain display extensive dissimilarities by protein-chemical, immunological, and kinetic criteria, and (*b*) both isoproteins form homogeneous populations of regularly sized octameric molecules with a 4-fold symmetry. Parts of this work have been presented as abstracts (38, 39).

EXPERIMENTAL PROCEDURES

Purification of Mi-CK from Chicken Cardiac Muscle and Brain—Mi-CK from chicken cardiac muscle and brain was extracted from fractions enriched for mitoplasts by 25 mM phosphate at pH 8.3 and 10 mM phosphate at pH. 8.8, respectively. After adsorption onto Blue-Sepharose matrix (at pH 6.5 for cardiac and pH. 6.8 for brain Mi-CK), cardiac and brain Mi-CK were specifically eluted by 10 mM ADP at pH 8.0 (see Ref. 36) and 25 mM ADP at pH 7.1, respectively. Final purification of the isoforms to greater than 99.5% purity as judged by silver-stained SDS-polyacrylamide gels was achieved by cation exchange chromatography on FPLC Mono-S column (at pH 7.0 for cardiac and at pH 6.5 for brain Mi-CK) and elution by different salt gradients. The procedure for the purification and characterization of the two Mi-CK isoforms are described in detail elsewhere (37).[2]

Gel Permeation Chromatography—Gel filtration experiments with purified Mi-CK on FPLC Superose-12 (Pharmacia LKB, Biotechnology Inc.), calibrated by the M_r standards indicated, were performed in a buffer containing 50 mM NaP_i, 150 mM NaCl, 0.2 mM EDTA, 2 mM β-mercaptoethanol, and 1 mM NaN_3 at pH 7.2.

Peptide Maps Obtained by Limited Proteolysis—Purified cardiac and brain Mi-CK (at 0.3 mg/ml each) were dissolved in 0.2 M NaP_i buffer, pH 7.0, containing 0.1% SDS and digested at 20 °C at a ratio of 5% (w/w) trypsin (Difco) to Mi-CK protein for different times varying between 3 min to 4 h. Mi-CK or trypsin incubated individually at 20 °C for the respective times were used as controls. Aliquots were taken at the time points indicated, SDS-polyacrylamide gels run together with M_r markers, and the polypeptide bands were stained by silver staining.

Monoclonal Antibodies—BALB/c mice were immunized intraperitoneally with 100 μg of purified chicken cardiac Mi-CK emulsified in complete Freund's adjuvant, boosted 4 weeks later with the same amount of protein, but in incomplete adjuvant. After 4 weeks three consecutive daily boosts with 50 μg of Mi-CK in incomplete adjuvant plus 50 μg of Mi-CK in phosphate-buffered saline each were given intraperitoneally. Two days later spleen cells were isolated and fused (41) by 50% (v/v) PEG-4000 (Merck, 9727) in GKN (137 mM NaCl, 5.36 mM KCl, 10 mM Na_2HPO_4, 5 mM NaH_2PO_4, 11.1 mM glucose, 10 mg/ml phenol red) with a mouse myeloma cell line derived from P-3 × 63-Ag8 PAI cells (3.5×10^7 PAI cells with 1.5×10^8 spleen cells). Hybrid clones were grown and selected in RPMI 1640 medium (Amimed) supplemented with 10% neonatal calf serum (Seralab), 2 mM glutamine (GIBCO), 0.1 mM hypoxanthine, 0.4 μM aminopterin, 1.6 μM thymidine (42). Hybridomas selected by ELISA were subcloned twice by limited dilution and then grown in the above HAT-free medium. Subclasses of secreted mAbs were determined by dot blots on nitrocellulose using subclass-specific antibodies (Nordic).

Solid-phase Immunoassay—Enzyme-linked immunoassay (ELISA) was used for screening hybridoma supernatants. Microtiter plates (Greiner, 655161) were coated with 100 ng/ml of purified brain or cardiac Mi-CK isoforms in 15 mM Na_2CO_3, 35 mM $NaHCO_3$ buffer at pH 9.6. mAbs were added for 2 h, followed by peroxidase-conjugated rabbit anti-mouse IgG (RaM-horseradish peroxidase, Dakopatts) for 2 h. Bound antibody was visualized by horseradish peroxidase staining with *o*-phenylenediamine (4 μM, Merck) and H_2O_2 (0.015%) as substrates, and the absorbance read at 490 nm with a Microelisa Autoreader (Dynatech, Alexandria, VA).

Immunoblotting of Two-dimensional Nonequilibrium pH-gradient Electrophoresis Gels—Nonequilibrium pH-gradient electrophoresis with mitochondria and mitochondrial extracts was performed according to O'Farell *et al.* (43) with an ampholyte mixture covering the range of pH 3.0 to 10.0 and a limited running time of 6 h at 400 V. Proteins resolved by two-dimensional gel electrophoresis were electrophoretically transferred (44). After blocking unspecific protein-binding sites for 2 h at 20 °C with 10% horse serum in phosphate-buffered saline, incubation with polyclonal anti-chicken cardiac Mi-CK antibody (at 1:100 dilution, in 3% BSA in phosphate-buffered saline), washing several times, incubation for 2–4 h at 20 °C with second antibody (goat anti-rabbit IgG horseradish peroxidase-conjugated and diluted 1:2000 × in phosphate-buffered saline containing 10 mg/ml BSA and 0.02% Nonidet P-40) and washing again, the peroxidase reaction was developed with 0.03% chloronaphtol and 1 μl/ml of 30% H_2O_2 and stopped by washing with H_2O.

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 β-mercaptoethanol (1–100 mM). Drops of the diluted enzyme solution were put on parafilm and glow-discharged carbon-coated 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 15 s. Some specimens were fixed with 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 Philips 420 electron microscope equipped with an anticontamination device at an acceleration voltage of 100 kV.

Other Methods—Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and immunoblotting was performed as described (44) and the protein bands stained by Coomassie Brilliant Blue R-250 or by silver staining (Bio-Rad method derived from Merril *et al.* (45)).

Protein concentrations were determined by the BCA-protein reagent method (Pierce) (46) or according to the Bio-Rad method (47) using a BSA standard (2 mg/ml, Pierce).

CK activity was measured by the pH-stat (Radiometer RTD 822 autoburette) method (18) at 25 °C and pH 7.0 using 20 mM HCl as titrant, but omitting BSA from the assay mix.

RESULTS

Cardiac and Brain Mi-CK Are Different, Tissue-specific Mitochondrial Isoproteins—Attempts to purify Mi-CK from brain by the same procedure that was developed for cardiac Mi-CK (37, 38) failed until some modifications of the affinity chromatography step on Blue-Sepharose and the ion exchange chromatography step on FPLC Mono-S were introduced. The necessary changes indicated a somewhat lower isoelectric point for brain *versus* cardiac Mi-CK. This was verified by two-dimensional nonequilibrium pH-gradient electrophoresis gels (Fig. 1) and by isoelectric focusing on Phast-System isoelectric focusing pH 3–9 gels (Pharmacia, Sweden) (not shown). On two-dimensional gels, Mi-CK from brain displayed a lower isoelectric point but a somewhat higher electrophoretic mobility in the second dimension (SDS) compared to cardiac Mi-CK. The two mitochondrial CK isoforms were resolved as two distinct spots (see Fig. 1) when phosphate extracts from cardiac and brain mitochondria adjusted to contain equal amounts of CK activity were coelectrophoresed. They were blotted by electrophoretic transfer onto nitrocellulose and stained by monospecific polyclonal rabbit anti-chicken cardiac Mi-CK antibodies which were characterized in this laboratory (37). The somewhat weaker immunostaining of brain Mi-CK by anti-cardiac Mi-CK antibodies indicated a quantitative difference in immunoreactivity of the two proteins with the same antibody. The isoelectric points for native cardiac and brain Mi-CK were determined by isoelectric focusing on Phast isoelectric focusing gels pH 3–9 to be approximately pH 9.3–9.5 and 8.5–8.9, respectively.

Monoclonal Antibodies against Cardiac Mi-CK: Specificity and Cross-reactivity with Brain Mi-CK, M-, and B-CK—Twenty hybridoma clones produced anti-cardiac Mi-CK mAbs which were characterized by ELISA and immunoblotting. Their cross-reactivities with Mi-CK from brain as well as with muscle-type MM-CK and brain-type BB-CK were determined (Table I). Monoclonal anti-cardiac Mi-CK antibodies were specific for Mi-CK and generally showed a 10^3–10^5 times weaker reactivity with M- or B-CK in ELISA tests where the majority of epitopes are most probably exposed in

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

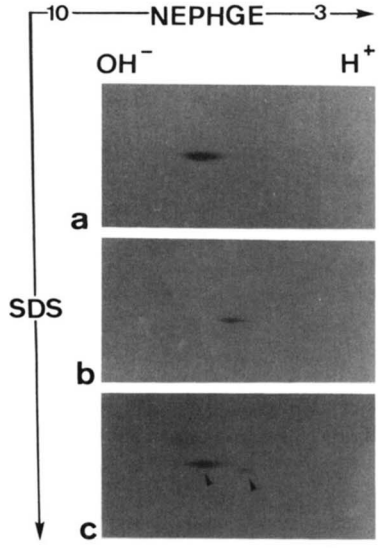

FIG. 1. **Differences in isoelectric point and apparent M_r between cardiac and brain Mi-CK subunits.** Nonequilibrium pH gradient gel electrophoresis (NEPHGE) of phosphate extracts from cardiac (*a*) and brain (*b*) mitochondria containing equal amounts of CK activity either loaded on separate gels (*a* and *b*) or coelectrophoresed (*c*) after electrophoretic transfer onto nitrocellulose and immunostaining with polyclonal rabbit anti-chicken cardiac Mi-CK antibody followed by horseradish peroxidase-conjugated second antibody. Note the lower isoelectric point and higher electrophoretic mobility of brain *versus* cardiac Mi-CK and the weaker immunoreactivity of brain Mi-CK with the anti-cardiac Mi-CK antibody (*b*) compared to the reaction in the homologous system (*a*).

a native conformation. The isoenzyme specificity was somewhat less pronounced on immunoblot tests under condition of denaturation (Table I). This is in accordance with earlier observations obtained with polyclonal antibodies using less sensitive and only semiquantitative methods (32). However, considering the extensive amino acid sequence homology between all CK isoforms sequenced so far (48) including cardiac Mi-CK (34), the high specificity of anti-cardiac Mi-CK even with denatured antigens is surprising. It seems therefore that the isotype-specific domains within the different CK isoforms are the most antigenic ones. In ELISA tests 2 of 20 mAbs (clones 7 and 8) and on immunoblots only three clones (clones 3, 7, and 8) showed significant cross-reaction with heterotypic B-CK (Table I, log of cross-reactivity index = −1). Only one mAb (clone 26) cross-reacted with M-CK, but only on immunoblots and not by ELISA. Even more surprising was the fact that mAbs generated against cardiac Mi-CK would clearly discriminate between cardiac and brain Mi-CK. According to the ELISA tests (shown in Table I), there was a broad spectrum in specificity and cross-reactivity of anti-cardiac Mi-CK mAbs with homologous Mi-CK from heart and brain mitochondria. Some of them were very specific for native cardiac Mi-CK (clones 4 and 41) showing no cross-reaction with brain Mi-CK at all and some did not discriminate between the two Mi-CK isoforms (clone 35 and to some extent also clone 34, Table I). On immunoblots, a number of mAbs showed strong cross-reaction with denatured brain Mi-CK (clones 4, 35, 41, 43, see also Fig. 2), some showed intermediate (clones 3, 5, 11, 31, 36) and some nondetectable cross-reaction (clones 14–25, 30, see also Fig. 2). Cross-reactivity behavior of individual mAbs differed depending on the assay conditions (ELISA or immunoblots) most likely as a function of epitope presentation, *e.g.* mAb of clone 4 did not cross-react with brain Mi-CK in ELISAs but showed strong reaction on immunoblots. A preliminary epitope mapping using cardiac Mi-CK fragments, obtained by limited proteolysis, revealed at least nine different epitopes recognized by a total of 20 mAbs.

TABLE I

Characterization of monoclonal anti-chicken cardiac Mi-CK antibodies and their cross-reactivity with the brain Mi-CK isoform and with cytosolic M- and B-CK

Immunological cross-reactivity determined by ELISA and immunoblotting of 20 mAbs produced by the hybridoma clones indicated (1–43) with their subclasses specified. Cross-reactivity of anti-cardiac Mi-CK mAbs with brain Mi-CK, M-, and B-CK is expressed on a log-scale ranging from 0 to −5 with respect to their reactivity with homologous cardiac Mi-CK, *e.g.* antibodies of clone 30 had to be diluted 10^5 times to produce in the homologous system with cardiac Mi-CK the same weak signal that was obtained with identical amounts of B-CK coated to the microtiter plate wells using undiluted mAb 30. Cross-reactivity by immunoblot analysis is indicated by a subjective scale ranging from undetectable (−), weak (±), medium (+), to strong (++) horseradish peroxidase-staining (relative to the staining with homologous cardiac Mi-CK) of nitrocellulose blots with equal amounts of cardiac Mi-CK, brain Mi-CK, muscle type M-CK, and brain-type B-CK, incubated and developed under identical conditions with anti-cardiac Mi-CK mAbs and horseradish peroxidase-conjugated second antibody (see also Fig. 2).

Anti-cardiac Mi-CK		Log of cross-reactivity by ELISA with			Cross-reactivity by immunoblotting with		
mAb	Clones	Brain Mi-CK	M-CK	B-CK	Brain Mi-CK	M-CK	B-CK
3	IgG1	−2	−4	−4	+	−	+
4	IgG1	−4	−4	−4	++	−	−
5	IgG2b	−3	−4	−4	+	−	−
7	IgG2a	−2	−4	−1	ND	−	+
8	IgG2a	−2	−3	−1	ND	−	+
10	IgG2a	−3	−4	−4	±	−	−
11	IgG2a	−3	−4	−4	+	−	−
13	IgG2a	−3	−4	−4	ND	−	−
14	IgG2a	−3	−3	−4	−	−	−
16	IgG2a	−2	−4	−4	−	−	−
17	IgG2a	−3	−4	−4	−	−	−
25	IgG2a	−3	−4	−4	−	−	−
26	IgG2a	−3	−2	−4	ND	+	−
30	IgG2a	−3	−4	−5	−	−	−
31	IgG2b	−2	−4	−5	+	−	−
34	IgG1	−1	−3	−3	±	−	−
35	IgG1	0	−3	−3	++	−	−
36	IgG1	−2	−4	−4	+	−	−
41	IgG1	−4	−5	−5	++	−	−
43	IgG1	−2	−4	−4	++	−	−

The differential cross-reactivity of anti-cardiac mAbs with Mi-CK from brain indicated that immunological differences between cardiac and brain Mi-CK isoproteins exist and that brain Mi-CK shares some common antigenic epitopes with cardiac Mi-CK, but, on the other hand, is lacking some of the antigenic features specific for cardiac Mi-CK.

All polyclonal anti-cardiac Mi-CK antibodies recognized brain Mi-CK although to a somewhat lesser extent than cardiac Mi-CK (see Fig. 1) but were rather specific for Mi-CK and did not significantly cross-react with native B- or M-CK (not shown). However, by immunoblotting, depending on the conditions used, some cross-reactivity with B- and M-CK was often seen with polyclonal antibodies.

Different Peptide Maps by Limited Proteolysis—A remarkable stability of both Mi-CK isoforms toward digestion by trypsin was noted even in the presence of small amounts (0.1%) of SDS required to generate the lower M_r fragments seen in Fig. 3.

Limited digestion of the two highly purified Mi-CK isoforms in the presence of 0.1% SDS shown in Fig. 3 (*lane a* = cardiac Mi-CK; *lane a′* = brain Mi-CK, both silver-stained) clearly resulted in rather different tryptic peptide products revealed on the basis of differential electrophoretic mobility

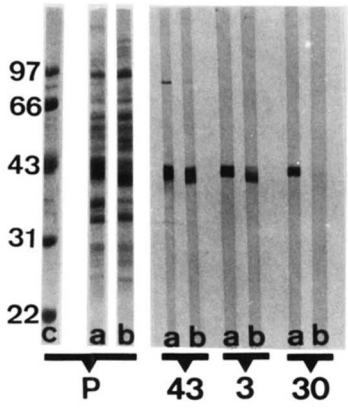

FIG. 2. **Differential immunoreactivity of monoclonal anti-cardiac Mi-CK antibodies with brain Mi-CK.** Phosphate extracts of cardiac (*a*) and brain (*b*) mitochondria after SDS-polyacrylamide gel electrophoresis and electrophoretic transfer to nitrocellulose stained for protein (*P*) with Ponceau red (*lanes Pa* and *Pb*). Corresponding blots after staining with monoclonal anti-cardiac Mi-CK antibodies (mAb clones 43, 3, 30) all showing strong reactivity with homologous cardiac Mi-CK (*lanes 43a, 3a, 30a*) and strong (43b), intermediate (3b), and undetectable cross-reactivity (30b) with Mi-CK from brain. The same molecular mass markers indicated in kDa (*lane Pc*) were used as in Fig. 3.

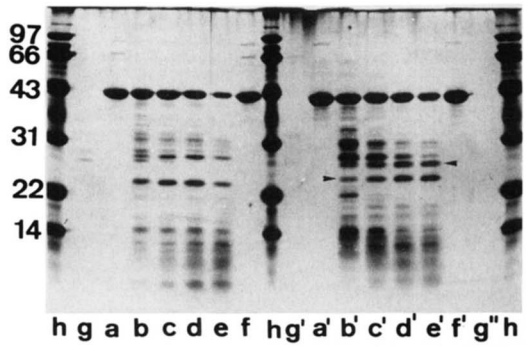

FIG. 3. **Different peptide patterns obtained by limited proteolysis of brain and cardiac Mi-CK.** Tryptic peptide maps of purified cardiac (*a–f*) and brain (*a′–f′*) Mi-CK resolved on 15% polyacrylamide gels in the presence of SDS after staining for protein by silver staining. Purified cardiac (*a*) and brain (*a′*) Mi-CK before digestion; after 3 min (*b* and *b′*), 15 min (*c* and *c′*), 1 h (*d* and *d′*), 4 h (*e* and *e′*) digestion time with 5% (w/w) of trypsin to protein. Purified cardiac (*f*) and brain (*f′*) Mi-CK after 4 h of incubation under the same conditions, but in the absence of trypsin; and trypsin alone after 3 min (*g*), 15 min (*g′*), and 4 h (*g″*) as controls. Molecular mass markers indicated in kDa (*h*): phosphorylase *b*, 97,400; BSA, 66,200; ovalbumin, 42,700; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 22,000; lysozyme, 14,000. Note: some clearly different peptides are indicated by *arrowheads*.

on SDS gels (compare *lanes a–e* from cardiac with *lanes a′–e′* from brain Mi-CK). These results demonstrating differences in the primary structure of the two mitochondrial isoforms allow the conclusion that the two Mi-CKs differ not only by their N termini as has been demonstrated recently but are probably quite different throughout their primary structures as had been suggested by the lack of hybridization of brain RNA with full length heart Mi-CK cDNA (34).

Differences in N-terminal Amino Acid Sequences—The N-terminal sequences derived by direct protein sequencing of purified Mi-CK from chicken brain and cardiac muscle showed that the two proteins are clearly distinct isoproteins differing in 13 of 30 N-terminal amino acids, the very 5 N-terminal amino acids being completely different (for sequence information see Ref. 34), thus indicating that the two tissue-specific mitochondrial CK isoforms arise by a genetic mechanism.

Kinetic Differences between Cardiac and Brain Mi-CKs—The linearization plot (modified Eadie-Hofstee plots) of (V/V_{max}) *versus* ($V/V_{max} \cdot S$) resulted in different slopes, their numerical values indicating the K_m, for Mi-CK from cardiac muscle (Fig. 4, *filled circles*) and brain (Fig. 4, *open circles*). The average K_m for cardiac Mi-CK was 1.6 mM CP while the value for brain Mi-CK (0.4 mM CP) was significantly lower. In addition, a plot of log ($V/V_{max} - V$) *versus* log (S) also resulted in different slopes for the two mitochondrial CK isoforms (Hill plot analysis, not shown). In the latter case the slopes are numerically identical to a term called cooperativity which is an index of inter- and intrasubunit communication within proteins during reaction catalysis. For cardiac Mi-CK the average value was 1.2, while brain Mi-CK gave a slightly smaller value of 1.0 indicating no cooperativity. Thus, the two mitochondrial CK isoforms are not only different by immunological and biochemical criteria, but also displayed distinct, tissue-specific kinetic properties that may be relevant for proper functioning of the respective isoforms in their different physiological environments, namely within brain and cardiac cells.

Both Mi-CK Isoenzymes Form Octamers of Similar Structure—A new, rapid isolation procedure for cardiac Mi-CK involving specific elution of the enzyme from Blue-Sepharose by ADP (36, 37) made possible the isolation of Mi-CK as a distinct monodisperse octameric form with a M_r of 320,000–360,000 (37, 38, 40). The existence of the octamer was verified by gel permeation chromatography, analytical ultracentrifugation, and direct mass measurement of single Mi-CK molecules by scanning transmission electron microscopy (40).

Cardiac Mi-CK octamers were slowly converted into dimers by dilution of the enzyme to lower than 0.5 mg/ml, but rapid and complete transformation into dimers was achieved by the addition of a mixture of Mg^{2+}-ADP, NO_3^-, and creatine (37, 49) which is inducing a transition-state analogue complex (50). The cardiac Mi-CK enzyme had been characterized in detail by electron microscopy where the octameric structure had been demonstrated to be a cube-like particle with side lengths of 10 nm and a central negative stain-filled cavity or channel (37, 40). Here, we compared freshly isolated Mi-CK from brain with Mi-CK from cardiac muscle by gel filtration on a FPLC Superose-12 column (37) and by negative staining of single molecules and observation by electron microscopy.

Comparison of the two isoenzymes by gel permeation chromatography (Fig. 5) revealed that brain Mi-CK, like cardiac

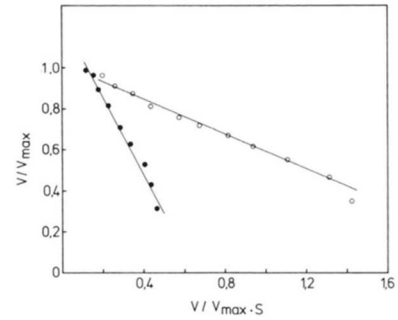

FIG. 4. **Kinetic differences of cardiac and brain Mi-CK isoforms.** Modified Eadie-Hofstee linearization plot of (V/V_{max}) against ($V/V_{max} \cdot S$). (V is the experimentally determined enzyme velocity at various limited CP concentrations, V_{max} is the maximal enzyme velocity in the presence of excess CP, and S is the CP concentration in mM). The plotted data for purified cardiac Mi-CK (●) and brain Mi-CK (○) fitted by two straight lines show different negative slopes indicating differences in K_m for CP between the two Mi-CK isoforms.

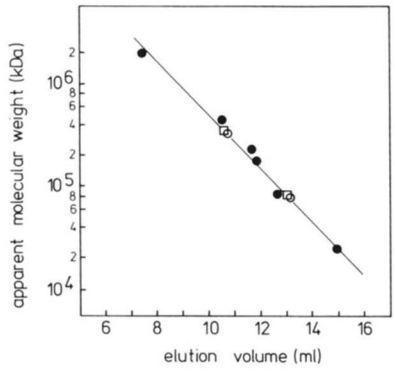

FIG. 5. **Apparent molecular weights of cardiac and brain Mi-CK isoforms: an octameric and dimeric species each.** Gel permeation chromatography by FPLC with Superose-12 column of purified cardiac (□) and brain (○) Mi-CK. Major protein peaks at 364 ± 25 and 352 ± 25 kDa corresponding to cardiac and brain Mi-CK octamers, respectively (see Ref. 37 for actual column profiles for cardiac Mi-CK); and minor protein peaks at 84 ± 5 and 82 ± 5 kDa corresponding to cardiac and brain Mi-CK dimers, respectively.

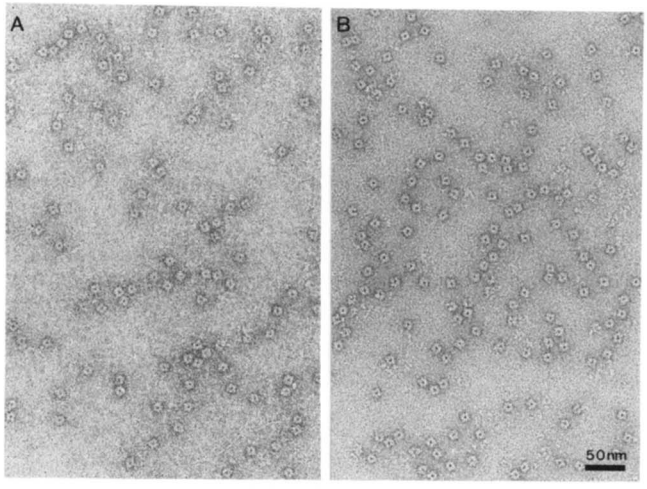

FIG. 6. **Negative staining of isolated Mi-CK isoforms: cardiac and brain Mi-CK octamers.** Negatively stained preparations of Mi-CK purified to homogeneity from chicken cardiac muscle (*A*) and brain (*B*). Homogeneous populations of cube-like single molecules with 4-fold symmetry and a central cavity or channel filled by negative stain are seen in both cardiac (*A*) and brain (*B*) Mi-CK preparations. (*bar* = 50 nm).

Mi-CK, also formed octameric molecules, albeit of somewhat lower M_r (352,000 ± 20,000 compared to 364,000 ± 25,000 for cardiac Mi-CK), and that brain Mi-CK octamers, like cardiac Mi-CK, could be reversibly converted into dimers as revealed by the variable proportions of Mi-CK dimer present in brain Mi-CK preparation. The kinetics of spontaneous dissociation of brain Mi-CK octamers into dimers was faster (within days) than that of cardiac Mi-CK (within months), but in both cases the dissociation into dimers was slower by several orders of magnitude than the octamerization of both types of Mi-CK dimers which was immediate (within minutes) when induced by simply concentrating Mi-CK dimer solutions. Electron microscopy of freshly isolated brain Mi-CK revealed the presence of a high percentage of homogeneously sized "cube-like" molecules with a central cavity or transverse channel filled by negative stain (Fig. 6*B*) which were very similar to cardiac Mi-CK prepared for electron microscopy under the same conditions (Fig. 6*A*), both showing 4-fold symmetry. Thus, even though the two isoforms differed significantly in protein-chemical and kinetic parameters, they both formed regularly shaped octameric structures which could be reversibly converted into dimers as shown for cardiac Mi-CK (37). In addition the occurrence of the octameric form of Mi-CK is not restricted to the chicken species, but has recently been identified in cardiac muscle from rat as well (37) and hence may represent a general structural feature of the Mi-CK isoform.

DISCUSSION

The finding that Mi-CK from cardiac and brain mitochondria are two distinctly different, tissue-specific isoproteins which not only differ in their amino-terminal sequence as had been shown recently (34), but possibly also throughout their entire primary structure, as indicated by nucleic acid hybridization analysis (34) and tryptic peptide maps, is new. Only a few examples of tissue-specific, mitochondrial isoproteins, expressed within the same species, have been described so far: NADP-dependent isocitrate dehydrogenase (51), mitochondrial ATP synthase (52), ATP-ADP-translocase (53), and cytochrome *c* oxidase (54). The mitochondrial-encoded catalytic subunit of the latter is probably identical in all rat tissues, but all nine nuclear-encoded subunits of the same enzyme showed immunological differences between tissues which of course could also be due to modification.

N-terminal amino acid sequence analysis showed that cardiac and brain Mi-CK differed in 13 of 30 N-terminal amino acids. The absence of hybridization at low stringency of a full length cardiac Mi-CK cDNA with brain RNA allows the conclusion that the two Mi-CK isoforms are derived from at least two different mRNAs possibly transcribed from different genes. However, from a comparison of the recently completed sequence of cardiac Mi-CK with published sequences of the brain-type B-CK and muscle-type M-CK, it became obvious that all these sequences contained a CK-specific sequence framework of remarkable homology and with isotype-specific variable segments interspersed throughout their sequences (34). The fact that Mi-CK from brain, assumed to show even higher sequence homology to cardiac Mi-CK than to B- or M-CK, was rather different in tryptic peptide maps from heart Mi-CK and also was immunologically distinguishable by mAbs from cardiac Mi-CK, confirmed our conclusion obtained from the hybridization experiments and the partial amino acid sequences (34). The unusually high isoenzyme specificity of antibodies raised against different CK isoenzymes, despite the extensive amino acid sequence homologies (34, 48), may be explained by assuming that the isoform-specific sequences, especially those at the amino-terminal ends and some additional ones that are interspersed throughout the sequence, must be dominant epitopes and thus responsible for induction of isoform-specific antibodies. This is compatible with the idea that hydrophilic domains of high segmental and thermal mobility, *e.g.* N-terminal floppy ends, are to be considered most antigenic (55). However, since isoform-specific sequences seem to be rather highly conserved between different species (34, 48) it is surprising that anti-CK isoenzyme antibodies generally show very high species specificity with native CK isoenzymes. An answer to these questions may be obtained by detailed epitope mapping in combination with solving the structure of all CK isoforms. The specificity of some of the anti-cardiac mAbs for cardiac Mi-CK and their lack of cross-reactivity with brain Mi-CK is remarkable and indicates that the two isoforms share some common epitopes, but also possess some isoprotein-specific epitopes unique to the respective Mi-CK isoforms. These discriminating mAbs may be very useful tools for further studies of Mi-CK function in cardiac muscle and brain and

may also be of clinical diagnostic value.

The finding that the brain Mi-CK form, like cardiac Mi-CK, also forms highly ordered octameric structures of similar size and 4-fold symmetry with a central cavity or channel is new and indicates that the octameric nature of Mi-CK is not restricted to cardiac Mi-CK, but is likely to be a general structural feature of Mi-CK. The structure-function relationship postulated for cardiac Mi-CK (37, 40) connecting ATP-ADP translocators of the inner mitochondrial membrane to the cytoplasm via interaction with the outer mitochondrial membrane at those sites where the two membranes are in close contact (37, 40, 56, 62) seems to be relevant also for brain Mi-CK. To fulfill the common task within the mitochondrial side of the CP shuttle (11, 37, 40), severe restraints are probably imposed on the structure of Mi-CK so that different Mi-CK isoforms have to meet common structural and functional criteria, *e.g.* for the interaction with the inner mitochondrial membrane and for the reversible octamer-dimer interconversion (37). As far as catalytic properties are concerned, the structurally similar Mi-CK isoforms seem to meet the physiological criteria needed for proper function within their respective metabolic environment, *e.g.* brain or cardiac cells. The different kinetic properties between brain and cardiac Mi-CK, the former showing a significantly lower K_m for the substrate (CP), may be related to the significantly lower total C and CP concentrations in brain (approximately 3–5 mM) *versus* cardiac and skeletal muscle (20–40 mM) (57). We propose that the formation of octameric structures is a general feature of mitochondrial CK and is physiologically relevant for the proper working of the CP shuttle (11) which has recently gained support also from *in vivo* ^{31}P NMR studies with normal and ischemic heart (25, 26, 29, 59, 60), by kinetic studies comparing oxygen consumption and CP levels in muscle (24, 58), and by inactivation of CK in spermatozoa (14, 30). In spermatozoa as well as in retina photoreceptor cells Mi-CK is expressed together with cytosolic brain-type B-CK (15, 16). Both of these cells represent polar cell types with high, sudden energy requirements, where a CP shuttle is thought to greatly facilitate energy transport and to connect mitochondrial respiration to sperm motility (14, 15) or visual transduction (16, 61).

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