Cloning, Characterization, and Expression in *Escherichia coli* of Three Creatine Kinase Muscle Isoenzyme cDNAs from Carp (*Cyprinus carpio*) Striated Muscle

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Hsi-Wen Sun‡§, Cho-Fat Hui¶, and Jen-Leih Wu‡†

From the ‡Laboratory of Marine Molecular Biology and Biotechnology, Institute of Zoology, Academia Sinica, Nankang, Taipei 115, Taiwan, Republic of China and the §Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 117, Taiwan, Republic of China

In vertebrates, the creatine kinase isoenzyme family consists of four types of isofoms: cytosolic muscle type (M-CK), cytosolic brain type (B-CK), mitochondrial ubiquitous, acidic type (Miu-CK), and mitochondrial sarcoplasmic, basic type (Mis-CK). Until recently, the existence of more than one subisoform of CK isoenzyme has been demonstrated only in fishes by starch gel electrophoresis. We report herein the isolation of three full-length cDNAs that correspond to three closely related creatine kinase M-CK genes from common carp (*Cyprinus carpio*), designated the M1-CK, M2-CK, and M3-CK genes. Using oligonucleotide probes that correspond to the same region but with the most variable sequences, different restricted genomic hybridization patterns have been obtained. These Southern blot results indicate that the three cDNAs come from different genes. Northern blot analysis using probes that correspond to the 3′-untranslated regions further show that all three subisoforms are expressed specifically in carp muscle. The deduced amino acid sequences of these three subisoforms of carp M-CK show about 85% identity to mammalian M-CK isoenzyme. Finally, the three cDNAs have been expressed in *Escherichia coli* with a molecular mass of approximately 43,000 Da, and these recombinant proteins exhibit creatine kinase activity. All of these data suggest that the M-CK isoenzymes have at least three subisoforms in carp.

All living organisms require energy to survive and carry out the many tasks that characterize biological activity. Cellular energy demand and supply are generally balanced and tightly regulated for economic and efficient energy use. The enzyme creatine kinase (CK), EC 2.7.3.2, plays a key role in the energy metabolism of cells that have fluctuating energy requirements (for a review, see Ref. 1). Cells contain a number of different CK isoenzymes, which are, in part, compartmentalized specifically at places where energy is produced or utilized, such as in mitochondria, skeletal and cardiac muscle fibers, neurons, electrocytes, photoreceptors, and spermatozoa (for a review, see Ref. 2). Two fundamental types of CKs can be found in vertebrates: cytosolic and mitochondrial CKs (3).

So far, there are four different CK isofoms known in vertebrates; two are found in cytosol, and two are found in mitochondria. The cytosolic forms are called M-CK (muscle) and B-CK (brain) and can dimerize with each other (4–6). They are found in soluble form in the cytosol, but fractions are also associated with the M-line of the sarcomeres, the sarcoplasmic Ca2+-ATPase, or the spermatozoan tail (7–9). MM creatine kinase purified from tissue exists in a single form but upon release into the plasma exhibits three forms in both dogs (MM1, MM2, and MM3) and humans (MM-A, MM-B, and MM-C) (10, 11). The hydrolytic cleavage of a basic amino acid, presumably by carboxypeptidase N, is responsible for conversion of muscle tissue MM1 to MM2 and MM3 (10). In humans, these isofoms are formed by the successive removal of the COOH-terminal lysine residue from one M subunit at a time, resulting in the conversion of MM-A to isoforms MM-B and MM-C (11). In mammals, there is just one isoform of B-CK, and the two B-CK isoforms of chicken are derived from a single gene by alternative splicing of the second exon (12, 13). Additional heterogeneity of B-CK has been shown to be due to alternative initiation of translation or post-translation phosphorylation (14–17).

The four CK isoenzymes of teleost fish are termed CK-A to CK-D and are all of cytoplasmic origin. CK-A, CK-C, and CK-D are expressed predominantly in striated muscle, stomach, and testis, respectively, while CK-B is expressed ubiquitously or is confined to neural tissue (18). In trout, a DNA encoding for a CK named TCK-1 has been demonstrated to show enhanced testicular expression, and an s-CK protein has been purified from sperm (19). Since *Torpedo* electrocytes have been shown by isoenzyme and two-dimensional electrophoresis to contain the same major CK isofoms as muscle, the two *Torpedo* CKs very likely represent the CK-A isoenzyme (20–22). Based on a comparison of the tissue specificity of expression of the various isofoms, it has been hypothesized that CK-II of frogs and CK-A of fish correspond to M-CK of mammals and birds, while CK-IV and CK-C correspond to B-CK (23, 24).

In this study, we report the cloning, sequence analysis, and expression in *Escherichia coli* of three carp M-CK cDNAs. Although the existence of more than one subisoform of each CK isoenzyme has been demonstrated in fish by starch gel electrophoresis, this is the first time that the cloning of more than one M-CK subisoform cDNA in any organism has been reported (25). We have explored the possible existence of CK gene families in teleost fish, and so far, we have cloned three M-CK subisoforms and four B-CK subisoforms. Here, we report the
nucleotide sequences; the predicted amino acid sequences of the three subisoforms of M-CK along with data on their tissue distribution; and the E. coli-expressed recombinant M-CK protein catalytic activities. Also, since the common carp is a poikilothermic fish, we have focused our attention on the catalytic activity of the E. coli-expressed recombinant M-CK proteins of carp at various temperatures.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals**—Common carp (Cyprinus carpio) were maintained in tanks of circulating aerated water at 25 °C for 3 months under a 12:12 light cycle fed a commercial diet.

**Isolation of cDNA Clones**—Poly(A)^+ RNA was prepared from striated muscle of the common carp by an established procedure (26). The double-stranded cDNA was supplied with EcoRI and XhoI linkers and was inserted into the λ-ZAP II vector (Stratagene). The λ library was packaged in the Gigapack II gold packaging extract (Stratagene) and was plated on E. coli XL1-blue MRF^+ cells (27). The probe for screening the cDNA library was synthesized by PCR using carP as a template and the entire 3'-UTR of carp striated muscle cDNA as a template with a sense oligonucleotide primer (CK-5', 5'-CAY AAY CAY ATG GCC AA-3', alignment positions amino acids 26–32 in the M-CK isoenzyme) and an antisense oligonucleotide primer (CK-3', 5'-CAT RTT NCC NCC YTT YTG CAT-3', alignment positions 239–246 in the M-CK isoenzyme). The amplified products were subcloned into pUC 19 (New England Biolabs) and transformed into JM109 cells. Preliminary Northern blot analysis using the PCR product (663 bp) as a probe revealed that it was expressed in carp striated muscle. After in vitro packaging, 1 × 10^6 primary phages were amplified and plated at a density of ~50,000 plaques/plate (15 cm), with 12 plates being screened. The probe was prepared by rediPrime labeling system (Amersham Pharmacia Biotech) with [α-^32P]dATP (3000 Ci/ mmol, Amersham Pharmacia Biotech). Prehybridization and hybridization were carried out in standard hybridization buffer at 42 °C for 16 h (27). Filters were finally washed with 0.1× SSC, 0.1% SDS for 1 h twice at 65 °C. Positive clones were isolated, purified, and sequenced.

**Sequence Analysis and Computer Homology Search**—The nucleotide sequences were determined by double-stranded sequencing according to the dideoxy chain termination method using an ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems). The nucleotide sequences were then analyzed with the Applied Biosystems 377A automated DNA sequencer. A sequence homology search and comparison were performed with computer programs of the GCG sequence analysis system (Amersham Pharmacia Biotech) with [α-^32P]dATP (3000 Ci/ mmol, Amersham Pharmacia Biotech). Prehybridization and hybridization were carried out in standard hybridization buffer at 42 °C for 16 h (27). Filters were finally washed with 0.1× SSC, 0.1% SDS for 1 h twice at 65 °C. Positive clones were isolated, purified, and sequenced.

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could be grouped into three PstI restriction patterns. One clone from each group of the cDNAs, designated M1-CK, M2-CK, and M3-CK was picked, and subjected to nucleotide sequence analysis.

The lengths for these three cDNA inserts, excluding the poly(A), were 1559, 1580, and 1508 bp for M1-CK, M2-CK, and M3-CK, respectively (Fig. 1). The presumptive polyadenylation signal, ATAAA, was located at nucleotides 1528–1532 and 1541–1545 for M1-CK, nucleotides 1549–1553 and 1562–1566 for M2-CK, and nucleotides 1516–1520 for M3-CK. The lengths of the open reading frame were 1146, 1146, and 1143 bp for M1-CK, M2-CK, and M3-CK, respectively. The homology of the

**TABLE I**

Sequence homology among CK isoenzymes within the coding region

| Species     | Cpm1 | Cpm2 | Cpm3 | DgM | MsM | RtM | HuM | ChM | TrM | RtB | RtMi | RtMis |
|-------------|------|------|------|-----|-----|-----|-----|-----|-----|-----|------|-------|
| Cpm1        | 96.3 | 96.0 | 84.3 | 80.2 | 79.5 | 79.3 | 79.9 | 79.9 | 77.8 | 76.7 | 73.4 | 62.2  |
| Cpm2        | 96.3 | 96.0 | 84.3 | 80.2 | 79.5 | 79.3 | 79.9 | 79.9 | 77.8 | 76.7 | 73.4 | 62.2  |
| Cpm3        | 87.1 | 87.1 | 85.3 | 96.4 | 91.2 | 83.1 | 79.9 | 76.3 | 63.4 | 61.7 |      |       |
| DgM         | 86.8 | 87.1 | 85.0 | 96.8 | 91.6 | 83.1 | 80.4 | 75.6 | 63.6 | 61.7 |      |       |
| MsM         | 86.8 | 87.1 | 85.0 | 96.8 | 91.6 | 83.1 | 80.4 | 75.6 | 63.6 | 61.7 |      |       |
| RtM         | 86.4 | 86.9 | 84.8 | 96.3 | 99.6 | 89.1 | 82.6 | 75.8 | 63.7 | 61.1 |      |       |
| HuM         | 84.8 | 85.8 | 83.4 | 94.8 | 97.4 | 94.5 |    |    |    |    |    |       |
| ChM         | 84.5 | 84.5 | 85.0 | 96.8 | 91.2 | 83.1 | 79.9 | 76.3 | 63.4 | 61.7 |      |       |
| TrM         | 80.6 | 80.1 | 78.2 | 86.1 | 85.1 | 83.5 | 83.5 | 83.5 | 77.8 | 74.4 | 61.5 | 59.9 |
| RtMi        | 76.7 | 75.4 | 75.3 | 79.1 | 80.3 | 78.5 | 78.5 | 78.5 | 75.8 | 64.0 | 60.0 |      |
| RtMis       | 61.9 | 62.2 | 61.8 | 65.2 | 65.6 | 65.1 | 65.3 | 65.3 | 65.3 | 63.8 | 70.5 |      |

**Fig. 1.** cDNA sequences of carp M1-CK, M2-CK, and M3-CK. Nucleotides are numbered in the 5’ to 3’ direction starting with the first nucleotide. Dashed lines below the M1-CK sequence denote DNA identical with the M1-CK cDNA. Gaps in the nucleotide sequence alignment are indicated by dots and are not taken into account for nucleotide numbering. Numbers 1–70 and 1217–1568 in M1-CK, 1–86 and 1233–1580 in M2-CK, and 1–61 and 1205–1508 in M3-CK represent the 5’- and 3’-untranslated regions, respectively. The start and stop codons are shaded. Putative polyadenylation signals are underlined in the 3’-untranslated region, and the poly(A) tail is in boldface type. The probe sequences for Southern hybridization are outlined with boxes.
open reading frame nucleotide sequences of M1-CK, M2-CK, and M3-CK was 80% identity to dog and human M-CKs, and this result indicates that these three cDNA inserts represent the muscle form creatine kinase cDNAs (Table I). Nucleotide homology comparison of these carp cDNAs among themselves and with the M-CK, B-CK, Miu-CK, and Mis-CK cDNAs of other organisms is shown in Table I. Nucleotide homology comparison of these carp cDNAs among themselves in the 5'- and 3'-untranslated regions and the open reading frames is shown in Table II.

**Amino Acid Primary Structure of Carp M1-CK, M2-CK, and M3-CK Subisoforms**—The open reading frame sequences could be translated into proteins of 381 amino acids for both M1-CK and M2-CK and 380 amino acids for M3-CK (Fig. 2). Computer alignment and search were carried out using the programs Pileup and Pretty of the GCG software package and protein sequence data banks. The identity between M1-CK and M2-CK proteins is as high as 96% (Fig. 2, Table I). For the M3-CK protein, the identity with the homologous M1-CK and M2-CK subisoforms is somewhat lower, at approximately 87%, due to differences within sequences of different domains, especially at the N terminus (Fig. 2). Analysis of the deduced amino acid sequences of the three carp muscle subisoforms revealed 83–87% identity with human and other species’ creatine kinase muscle isoenzyme (Table I). In mammals and birds, the amino acid identities within each isoenzyme class range from 88 to 99% (24). Evidently, a higher degree of homology can be observed between the B- and the M-CKs (77–82%), while the homology between the cytosolic and the mitochondrial CK isoforms is lower (60–65%) (24).

**TABLE II**

Percentages of identity of different regions of the three carp creatine kinase cDNAs

| cDNA comparison | 5'-UTR | Coding region | 3'-UTR |
|-----------------|--------|---------------|--------|
| M1-CK vs. M2-CK | 97.1   | 96.1          | 92.8   |
| M1-CK vs. M3-CK | 70.5   | 84.3          | 50.0   |
| M2-CK vs. M3-CK | 70.5   | 84.6          | 53.4   |

*a* UTR, untranslated region.

**FIG. 2.** Deduced amino acid sequences of the three carp M-CK isoenzymes with comparisons with chicken, human, and *Torpedo* M-CK isoenzymes. The amino acid sequences of the carp, chicken, human, and *Torpedo* were aligned and arranged using the programs Pileup and Pretty of the GCG software package. Amino acids that are identical to the carp M1-CK are represented by hyphens in the corresponding sequences. Gaps in the nucleotide sequence alignments are indicated by dots. **Boxed** amino acid residues (numbered I to VI) below the amino acid sequences mark the regions with the most pronounced sequence conservation. **Shaded areas** mark Cys-74, Thr-133, Ser-239, Cys-283, Thr-322, and Asp-340. The **underlined regions** (A to I) are either isoform-specific or allow a clear cut distinction between mitochondrial and cytosolic CK isoenzymes.

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A previously defined “CK framework,” consisting of the six most conserved sequence blocks and “diagnostic boxes,” which are characteristic for any creatine kinase isoenzyme and which may serve to distinguish this isoenzyme from all others, could be observed in the protein sequences of the three carp M-CKs (24). The six highly conserved blocks are **boxed** in the carp M-CK consensus sequences in Fig. 2. These conserved sequences are flanked by regions that are less conserved, among them the N and C termini (Fig. 2). The absolutely conserved

**FIG. 2.** Deduced amino acid sequences of the three carp M-CK isoenzymes with comparisons with chicken, human, and *Torpedo* M-CK isoenzymes. The amino acid sequences of the carp, chicken, human, and *Torpedo* were aligned and arranged using the programs Pileup and Pretty of the GCG software package. Amino acids that are identical to the carp M1-CK are represented by hyphens in the corresponding sequences. Gaps in the nucleotide sequence alignments are indicated by dots. **Boxed** amino acid residues (numbered I to VI) below the amino acid sequences mark the regions with the most pronounced sequence conservation. **Shaded areas** mark Cys-74, Thr-133, Ser-239, Cys-283, Thr-322, and Asp-340. The **underlined regions** (A to I) are either isoform-specific or allow a clear cut distinction between mitochondrial and cytosolic CK isoenzymes.
Carp Creatine Kinase Muscle Isoenzymes

Expression of the Three Carp M-CK Recombinant Subisoforms—To express and purify the recombinant carp M-CK proteins, a 1-L culture of BL21 (DE3) pLysS cells harboring the expression plasmid pETM1, pETM2, or pETM3 was grown and harvested. The SDS-polyacrylamide gel depicting the purified recombinant proteins is shown in Fig. 5A. Western analysis showed that they were all immunoreactive with anti-human M-CK antibody (Fig. 5B).

Different Enzyme Catalytic Activities and Kinetic Parameters of Carp M-CK Subisoforms—The specific activities of carp M1-CK, M2-CK, and M3-CK recombinant proteins were 45.1, 54.4, and 20.7 units/mg, respectively (Table III). Specific activities were determined under standard conditions of 20 mM phosphocreatine, 4 mM ADP, and 4 mM MgCl2. Kinetic parameters $K_m$ and $V_{max}$ were calculated from three sets of independent experiments, and in each set of experiments, three independent measurements were taken, with the phosphocreatine concentrations being varied between 0.5 and 15 mM while with constant concentrations of ADP at 4 mM and of MgCl2 at 4 mM. The enzyme amount in our assay was 0.04 mg, and the enzyme activity was linear up to 0.04 μg of enzyme. Also, the enzyme activity was linear up to 30 min under the assay conditions; therefore, the end point creatine value was taken at 30 min for this reaction (data not shown). $K_m$ values of carp M1-CK, M2-CK, and M3-CK recombinant proteins for PCr were 1.4 ± 0.2, 0.8 ± 0.1, and 3.0 ± 0.4 mM, respectively. $V_{max}$ values of the recombinant proteins were similar, while that of the
Comparison of specific activity and kinetic parameters of three carp M-CK isoenzymes

| M-CK subisoform | Specific activity | K\textsubscript{m} for PCr (μM) | V\textsubscript{max} (units/mg) |
|-----------------|------------------|-------------------------------|-------------------------------|
| M1-CK           | 45.1             | 1.4 ± 0.2                     | 103.2 ± 0.9                   |
| M2-CK           | 54.4             | 0.5 ± 0.1                     | 102.7 ± 0.5                   |
| M3-CK           | 20.7             | 3.0 ± 0.4                     | 70.8 ± 0.8                    |

* A unit of enzyme activity is equal to 1 μmol of creatine formed/min at pH 7.0 and 25 °C.

When specific activities were measured at different temperatures, the M1-CK protein was found to exhibit its highest specific activity at 37 °C (64 unit/mg) and then to fall to around 56% at 20 °C, and at 10 °C only 18% activity was retained (Fig. 6). The temperature dependence of the M2-CK protein was very similar to that of human M-CK. The highest specific activity appeared at 37 °C (63 unit/mg), was maintained at 85% activity from 30 to 25 °C, and then was reduced further to 66% activity at 20 °C and finally to 28% activity at 10 °C (Fig. 6). In contrast, the temperature dependence of the M3-CK protein-specific activities was quite different. The highest activity was measured at 25 °C (21 units/mg), and at 37 and 10 °C, 41 and 28% activities were measured, respectively (Fig. 6).

**DISCUSSION**

In this report, we describe the cloning, characterization, and expression of three creatine kinase isoenzymes from common carp striated muscle. According to the nucleotide and amino acid sequence homology comparison, the three carp M-CKs should belong to cytosolic M-CKs. Southern hybridization analysis using oligonucleotides specific to individual cDNA as probes indicates that three carp M-CK cDNAs are encoded by different genes. From Northern blot analysis, mRNAs for M-CKs were detected in striated muscle (red, white, and cardiac muscle), and the expression levels varied in each muscle. However, with our probe design we could not tell whether there is any quantitative difference in expression levels of each enzyme. The Northern signal is faint in carp heart, and we speculate that there is the possibility that a mixture of MB and BB forms exists in heart (25). Another more interesting possibility is that another heart-specific M-CK subisform exists whose DNA sequence in the corresponding position might be sufficiently different so that it only weakly cross-hybridized with our probes. All of these results indicate that these three carp Cks are muscle creatine kinase subisforms.

In the experiments where the M-CK expression plasmids were transformed and expressed in *E. coli* cells, we detected the expressed recombinant proteins by immunoblot analysis with human muscle creatine kinase antibody. The three recombinant proteins exhibited somewhat different K\textsubscript{m} values, despite the amino acid homology between M1-CK and M2-CK being as high as 96%. As for V\textsubscript{max} values, M1-CK and M2-CK showed similar values, while that of M3-CK was 30% lower. The K\textsubscript{m} of carp M-CK has previously been found to be around 2.84 mM for PCr, and in light of our present findings, it is possible that this previous value was a measurement of a mixture of M-CKs (46). Finally, in the temperature dependence-specific activity studies, the decreasing trends of carp M1-CK and M2-CK enzyme activity when temperatures decreased from 37 to 10 °C were somewhat similar. Yet, more interestingly, the specific activities of M3-CK recombinant protein showed a plateau between 30 and 20 °C. All of these results suggest that these carp muscle creatine kinase subisforms possess quite different enzyme properties.
Carp Creatine Kinase Muscle Isoenzymes

Taken together, the three carp M-CKs are a new combination of cytosolic CK subisoforms that may have overlapping but not necessarily redundant physiological enzyme functions. Whether their mRNA expression patterns are similar or different at different developmental stages or at different environmental temperatures is an interesting question. Likewise, it is important to learn the different mechanisms or physiological roles played by the three carp M-CKs, if any, in energy homeostasis during environmental adaptation in ectothermic animals. Since there are multiple subisoforms of M-CK in carp, it would be important to learn whether heterodimers of M-CKs exist in a single carp muscle cell and whether there are differential subcellular localizations of these subisoforms.

Cold is a major environmental problem for all living organisms. Piscifilar animals respond adaptively to chronic cold by a suite of cellular responses that compensate for varying extents for the rate-depressing effects of cooling. In ectothermic fish, body temperature is totally dependent on ambient temperature, and yet, even at low temperatures, carp have evolved strategies to maintain growth and swimming ability. Various review articles concerning possible acclimation mechanisms have been published (47, 48). Hazel and Prosser (49) suggested that acclimation might involve a temperature-dependent synthesis of new proteins, which would require the expression of different sets of temperature-specific isoenzyme genes. Also, the nature and significance of changes in enzymic myosin ATPase activity and the recruitment of different muscle fiber types in relation to acclimation and environmental temperature in carp have been reviewed (50, 51). It has been reported that there are different myosin heavy chain isoform genes that are expressed at warm and cold environmental temperatures (52, 53). Myofibrillar creatine kinase and myosin ATPase are associated with the same microenvironment and provide and utilize ATP for muscle contraction. Since M3-CK exhibited a specific activity peak at 25 °C, we could imagine that some cold-specific subisoform carp M-CKs should exist to maintain normal muscle activity at lower environmental temperatures.

In conclusion, we have cloned three carp M-CK cDNAs encoded by different genes. The three M-CK subisoforms are not due to alternative initiation sites, alternative splicing, post-translational glycosylation, phosphorylation, or proteolytic modification. These results suggest that multiple genes give rise to creatine kinase heterogeneity in carp.

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