The carp muscle-specific sub-isoenzymes of creatine kinase form distinct dimers at different temperatures

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We have previously cloned three muscle-specific sub-isoforms of creatine kinase (CK, EC 2.7.3.2) from the common carp (*Cyprinus carpio*), designated M1-CK, M2-CK, and M3-CK. The enzyme has a key role in maintaining the energy homeostasis of cells with fluctuating energy requirements. In the present paper, we report that all three M-CKs in the red and white muscle of different temperature-acclimatized carp were ubiquitously distributed in the cytosol and along membranes. In addition, the expression levels of these isoforms were not significantly altered in response to the temperature acclimatization. Interestingly, our studies showed that the formation of distinct homo- or heterodimers among these three M-CKs was found at various temperatures. At higher temperature, the M1M1-CK and M2M2-CK homodimers, and the M1M3-CK heterodimer are the predominant MM-CKs, whereas the M3M3-CK achieves its homodimeric state at lower temperature. We postulated testable homology models to investigate the chemical properties of these

dimeric interfaces. M1M1-CK was used as a reference to compare the structural differences with the M3M3-CK dimer. The calculated solvent accessible surface area that was buried in the contact interfaces of the M1M1-CK and M3M3-CK dimers showed an overall decrease of 12% in the M3M3-CK interface. The modelling analysis also suggested a net decrease of twelve hydrophobic residues and a Phe³ → Lys substitution in the M3M3-CK interface. An increase in thermolability of the M3M3-CK homodimer might be due to the decrease in subunit ion pairs and buried surface area in this dimer. Based on our findings, we propose that the carp-muscle-specific CK isoenzymes could undergo shuffling to form distinct M-CK homo- or heterodimers in acclimatization to environmental temperatures.

Key words: creatine kinase, dimerization, isoenzyme, temperature.

INTRODUCTION

Water temperature is a major factor that bears a spatial and temporal influence on ectothermic (i.e. with variable body temperature) animals, such as fish. In general, body temperatures of fish depart little from ambient water temperatures [1]. Environmental temperature change takes place over weeks; the physiological reorganization that compensates for such changes is often referred to as an acclimatization response. Therefore fish need to regulate their metabolic rate to maintain their energy requirements for survival at varying temperatures [2]. Over varying temperatures, enzymes from both the central pathway of aerobic metabolism and that of fatty acid oxidation need to be more effective in cells [3]. The common carp (*Cyprinus carpio*) is acclimatized to a broad temperature range. It would be interesting to learn how their metabolic enzymes could vary functionally with such temperature acclimatization.

Different isoenzymes can be expressed in different cell types, where they contribute to specialized properties. Creatine kinase (CK; EC 2.7.3.2) enzymes catalyse the reversible phosphoryl transfer from phosphocreatine to ADP (reviewed in [4]). CK isoenzymes are present in tissues with high-energy demands, for example, skeletal and cardiac muscle, neurons, photoreceptor cells, spermatozoa and electrocytes, where they regenerate ATP from phosphocreatine during cellular work (reviewed in [5]). Isoenzymes of CKs have certain different appearances for the maintenance of cellular energy homeostasis [4,6–9]. The first

level strategy is ‘tissue specificity’: four isoforms of CK are expressed in a tissue-specific manner. M-CK (muscle form of CK) is rather specific for differentiated muscle, whereas B-CK (brain form of CK) is found in the brain and in a variety of other tissues [6]. In most cases, M-CK coupled with Mis-CK (mitochondrial sarcomeric form of CK), and B-CK coupled with Miu-CK (mitochondrial ubiquitous form of CK) are co-expressed in a tissue-specific fashion [7]. The second level strategy is ‘different cell compartmentalization’: cytosolic CKs (M-CK and B-CK) are localized in the cytoplasm and the mitochondrial CKs (Mis-CK and Miu-CK) are localized in the mitochondrial inner membrane matrix [8]. The third level strategy is ‘dimerization or octamerization’ where cytosolic isoenzymes exist in three functional homo- or heterodimeric forms (MM-CK, BB-CK and MB-CK), and mitochondrial CKs occur as dimers or octamers. The cytosolic and mitochondrial isoforms of CK are thought to constitute an intricate energy buffering and transport system that connects sites of high-energy phosphate production to sites of energy consumption [9]. We have previously cloned three muscle-specific sub-isoforms of CK (M1-CK, M2-CK, and M3-CK) from the common carp [10]. The amino acid sequences show homology in the critical shared elements (i.e. activity sites). The isoenzymes are even more closely related, showing homology in excess of 80% with very similar protein structures. Refinement of protein structure during evolution, however, has led to the subtle specialization of function, and the isoenzymes appear to occupy different niches. Since there are

Abbreviations used: B-CK, brain form of CK; CK, creatine kinase; GST, glutathione S-transferase; IVTnT, *in vitro* transcription and translation; M-CK, muscle CK sub-isoform; Mis-CK, mitochondrial sarcomeric form of CK; Miu-CK, mitochondrial ubiquitous form of CK; MM-CK, dimeric muscle (CK) isoenzyme; TBS, Tris-buffered saline.

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multiple sub-isoforms of M-CK in carp, it would be fascinating to know if there is any correlation between the differential temperature expression and the cellular localizations of these sub-isoenzymes, as well as the presence of different M-CKs dimers in carp muscle cells.

There is limited understanding of the functional differences between the sub-isoenzymes. This is largely caused by the difficulty in unravelling the mysteries of their biochemistry and physiology, which are still out of bounds of current molecular genetics. In the present study, we examined the effect of temperature changes on the temporal and spatial expressions of these three M-CK isoenzymes. All three M-CKs had similar expression patterns and cellular localizations in the red and white muscle of the 30, 20, or 10 °C-acclimatized carp. These results indicated that these CK sub-isoenzymes might interact with each other. Our result, using *in vitro* transcription and translation (IVTnT) assays, followed by glutathione *S*-transferase (GST) pull-down assays did indeed show an interaction between the different sub-isoenzymes of M-CK. At 30 °C, M1M1-CK and M2M2-CK homodimers, and M1M2-CK and M1M3-CK heterodimers were formed, whereas M3M3-CK homodimers were preferentially formed at lower temperature. And then, we used a homology modelling approach to explain the difference in thermal stability by comparing the dimeric interface structures of the M1M1-CK and M3M3-CK with the available X-ray crystallographic data at the RCSB Protein Data Bank (1QH4, at 1.4 Å resolution) as a template [11]. In summary, we provided both the experimental and theoretical evidence supporting the physiological role of carp M-CK sub-isoenzymes in temperature acclimatization. The distinct dimerization of these isoenzymes is a novel regulatory mechanism of cold-acclimatized (or adapted) enzymes.

EXPERIMENTAL

Experimental animals

Carp were maintained in tanks of circulating aerated water at 25 °C for 3 months with a 12 h day length and were fed a commercial diet (Pond fish feed, Fwusow Industry Co. Ltd., Taiwan).

Sample preparation

Carp were separately acclimatized at 30, 20, or 10 °C in aquaria for at least 8 weeks. Minced carp muscle, taken from the dorsal part of the white or red muscle, was homogenized with 10 volumes of extraction buffer [250 mM sucrose, 20 mM 2-mercaptoethanol, 50 units/ml heparin, 100 mM Tris/HCl, pH 7.4, and complete protease inhibitor cocktail tablets (Roche)] and centrifuged at 12000 *g* for 30 min. The supernatant muscle protein was stored at −86 °C until use.

Antibodies

The use of synthetic peptides and carrier protein in antibody production was performed as described in [12]. The peptides used for immunization of rabbit or mouse to obtain the sub-isoform-specific antibodies included: M1 + 2 peptide, MPFGNTHNN-FKLNYSVD and M3 peptide, MTKNCNNDYKLLKFSLEE. The synthetic peptides were chemically coupled with multiple antigenic peptides (MAPs) that have eight peptide arms branching out from a lysine core matrix (Applied Biosystems). The sera obtained were named CKM1 + 2 and CKM3 antibodies respectively and an ELISA was carried out to determine peptide reactivity and to characterize native antigens.

ELISA

ELISA was performed essentially as described in [13]. Muscle tissue samples were added to microtitre plates (Nunc) and incubated for 2 h at 37 °C. The remaining binding sites were blocked with 0.25 % BSA and 0.05 % Tween 20 in PBS. CKM1 + 2 (1:2000 dilution) and CKM3 antibodies (1:2000 dilution) were used as the primary antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution) was the secondary antibody (Zymed Laboratories) and ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; Pierce] was the substrate. The reaction was terminated by the addition of 2 M H₂SO₄ and the absorbance at 405 nm was used to measure the amounts of the M-CK proteins in muscle tissues using the purified recombinant M-CK proteins as standards.

SDS/PAGE and immunoblot analysis

Carp muscle total proteins were separated on 10 % polyacrylamide gels as described by Laemmli [14]. Separated proteins were electrotransferred onto a PVDF membrane [15]. The membrane was blocked with 3 % non-fat dried milk in Tris-buffered saline (TBS) (20 mM Tris/HCl, pH 7.6 and 137 mM NaCl) for 2 h at room temperature (25 °C) with agitation, followed by a 1 h incubation in the presence of each primary antibody (as for ELISA; 1:5000 dilution of each). After washing

Figure 1 Expression of the three carp M-CK sub-isoforms at different temperatures

(A) CKM1 + 2 antibody-stained M-CK recombinant proteins and crude muscle tissue lysates. Lane 1 (M) contained molecular size markers. Lanes 2–4 contained M1-CK, M2-CK and M3-CK recombinant proteins respectively, and lanes 5–10 contained total proteins of red muscle (RM) or white muscle (WM) of carp acclimatized to 30, 20, or 10 °C. (B) CKM3 antibody-stained results, with lanes as in (A).

Figure 2 Cellular localization of M-CK isoenzymes in carp muscle tissues

(A) Localization of M1-CK and M2-CK isoenzymes. Recognition of M1-CK and M2-CK in longitudinal section (LS) and cross-section (CS) of red (RM) or white (WM) muscle tissue of 20 °C-acclimatized carp using mouse CKM1 + 2 antibody (Ab) and stained with Texas Red-conjugated donkey anti-mouse IgG. (B) Localization of M3-CK isoenzyme. Recognition of M3-CK in longitudinal section (LS) and cross-section (CS) of red or white muscle tissue of 20 °C-acclimatized carp using rabbit CKM3 antibody, and stained with FITC-conjugated donkey anti-rabbit IgG. (C) Co-localization of the M-CK isoenzymes. The results of the double staining with CKM1 + 2 and CKM3 antibodies were superimposed by confocal microscopy.

in TBST [TBS buffer with 0.1 % (v/v) Tween 20], a secondary antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase (1:5000 dilution, Zymed Laboratories) was added for 30 min at room temperature (25 °C), followed by development with AP Development Buffer (Pierce).

Immunohistochemistry

Immunohistochemistry of frozen tissue sections was carried out by techniques described in [16]. The carp muscles were taken from the dorsal part of the white or red muscle and these tissues were cut into approx. 0.5 cm³ pieces. They were fixed with 4 % (w/v) paraformaldehyde in PBS for 30 min at 4 °C and then washed with PBS. OCT embedding compound (Tissue-Tek, Milan, Italy) was applied to the tissue fragment and frozen at -20 °C. In cryosection, muscle tissues were cut to 15 µm in thickness on a cryostat and adhered onto a poly-L-lysine-coated slide. These samples were washed with two changes of 0.1 % (v/v) Triton X-100 in PBS. The non-specific binding sites were blocked with 5 % horse serum in PBS for 30 min. In immunostaining, the samples were incubated with the primary antibodies (as for ELISA; 1:50 dilution of each) for 2 h in a moist chamber at 4 °C. In the double antibody staining, Texas Red-conjugated

donkey anti-mouse IgG for mouse CKM1+2 antibody (1:50 dilution; Jackson Immunoresearch Laboratories) and FITC-conjugated goat anti-rabbit IgG for rabbit CKM3 antibody (1:50 dilution, Jackson Immunoresearch Laboratories) were added. All incubations were washed with two changes of PBS for 10 min. Samples that were incubated with the single antibody staining or the secondary antibody alone were used as controls. The confocal system consisted of a Leica DMRE fluorescence microscope, a Leica TCS NT confocal scanner and workstation.

Immunoprecipitation

Carp red and white muscle tissue lysates were used to immunoprecipitate different MM-CK isoenzymes. This experiment was based on a protocol in [17]. Tissue extracts (50 µg of total protein) were pre-cleared with pre-immune rabbit antisera and incubated at 4 °C overnight with CKM1 + 2 or CKM3 antibodies (1 µg of antibody) coupled to Protein A-Sepharose (Amersham Biosciences). Immune complexes were recovered by centrifugation (12000 *g* for 10 min at 4 °C), washed extensively with PBST buffer [PBS and 0.5 % (v/v) Tween 20], separated by SDS/PAGE (10 % gels) and transferred onto PVDF membranes. The membranes were probed with either rabbit CKM1 + 2 or

Figure 3 Existence of heterodimers of MM-CK isoenzymes in carp muscle tissues

(A) The co-immunoprecipitation results using CKM1 + 2 antibody. Lanes 1–3 contained recombinant proteins and lanes 4–9 contained proteins precipitated from red muscle (RM) or white muscle (WM) of 30, 20, or 10 °C-acclimatized carp. Lane 10 contained protein precipitated from carp liver extract. Immunoblotting assays were performed with CKM1 + 2 Ab against M1-CK and M2-CK proteins (upper bands) or CKM3 Ab against M3-CK protein (lower bands). (B) The co-immunoprecipitation result using CKM3 Ab. Precipitation of the same muscle tissue extracts from carp as in (A), using CKM3 Ab, followed by immunodetection with CKM1 + 2 Ab (upper bands) or CKM3 Ab (lower bands). Arrows indicate the positions of the M-CK sub-isoforms. The additional band in all tissue lanes was due to strong cross-reaction of the rabbit IgG heavy chain with the anti-rabbit secondary antibody.

CKM3 antibody (1:5000 dilution), followed by goat anti-rabbit IgG alkaline phosphatase-conjugated antibody as a secondary antibody (1:5000 dilution), and stained and developed with AP development buffer.

Coupled IVTnT and GST pull-down assay

An *Xba*I and *Nde*I restriction fragment containing the GST was generated from the plasmid pGEX-4T-1 (Amersham Biosciences) by PCR. The pET-GST-M1-CK, pET-GST-M2-CK, and pET-GST-M3-CK were obtained by inserting GST as an *Xba*I and *Nde*I restriction fragment into the corresponding sites of pET-M1-CK, pET-M2-CK and pET-M3-CK respectively. The constructions of the pET-M-CKs have been described in [10]. The pET-GST was generated by subcloning the *Xba*I and *Bam*HI restriction fragment of GST into the corresponding sites of pET28a (+) (Novagen). The co-IVTnT of one each of the GST-M-CK and M-CK proteins were co-generated from one each of the pET-GST-M-CK and pET-M-CK using the TNT® T7 Quick Coupled System (Promega) following the system protocols (Promega). These various constructs derived from pET (100 ng) were added to 50 µl of TNT® Quick Master Mix in the presence of 20 µCi of [³⁵S]methionine (Amersham Biosciences) and nuclease-free water (Promega) for 1 h at 30 °C and then followed by a 2 h incubation and a GST pull-down assay, carried out at same temperature. In the GST pull-down assay, samples were bound with glutathione-Sepharose beads (Amersham Biosciences) in PBS containing 1 % (v/v) Triton X-100 for 2 h at 30 °C [18]. The samples were washed four times in PBS containing 0.1 % (v/v) Triton X-100. For further experiments on GST-M3-CK interaction with M2-CK or M3-CK at 30, 25, or 15 °C, the co-IVTnTs were incubated at 30 °C, as mentioned above, and the products were incubated further for 2 h at 30, 25, or 15 °C, respectively. The GST pull-down assay was then carried out in PBS containing 0.1 % (v/v) Triton X-100 for 2 h at the specific temperatures. These samples were also washed four times in the

same buffer at the specific temperatures. Samples were then electrophoresed by 10 % SDS/PAGE. The fluorographic signal was amplified for 15 min by Amplify (NAMP 100, Amersham Biosciences) and then underwent fluorography with an intensifying screen overnight at –86 °C.

Molecular modelling

The three-dimensional structures of CK protein (RCSB Protein Data Bank entries 1CRK, 1QH4, 1QK1 and 2CRK) were used as templates for the homology-based model construction of the carp M1-CK, M2-CK and M3-CK monomers. The carp MM-CK dimers were modelled from the 1QH4 homodimer [11]. Amino acid sequence alignment of the CK proteins and generation of their models were carried out using the Swiss-PDB Viewer v.3.1 [19]. Energy minimizations of these models were geometrically optimized by SWISS-MODEL [20]. The dimeric interface of different carp MM-CK dimers were evaluated by the CCP4 v.4.0 [21]. The solvent accessible surface area was calculated on a Silicon Graphics O₂ workstation using the program Homology (ProStat/Access Surf) within the Insight II package (BIOSYM/MSI, San Diego, CA).

RESULTS

Expression of the M-CK sub-isoforms at different temperatures

The attempt to raise M1-CK-specific antibodies that would not cross-react with M2-CK protein and vice versa was unsuccessful. The specificity of the CKM1+2 and CKM3 antibodies was determined using purified M1-CK, M2-CK, and M3-CK recombinant proteins (Figure 1). Using ELISA, we confirmed that the CKM1+2 antibodies recognized M1-CK and M2-CK proteins almost equally (results not shown). These immunoblotting analyses exhibit similar steady-state expression patterns of the three M-CK isoenzymes in the red and white muscle collected

Figure 4 Interactions of the three M-CK sub-isoenzymes

The GST-M-CKs or M-CKs proteins were the IVTnT products at 30 °C, as described in the Experimental section. **(A)** Control experiment. Lanes 1–3 are co-IVTnT results and lanes 4–6 are GST pull-down with co-IVTnT products. **(B)** GST-M1-CK interaction with M1-CK, M2-CK or M3-CK. **(C)** and **(D)** GST-M2-CK or GST-M3-CK interacted with M1-CK, M2-CK or M3-CK respectively. Arrows indicate the positions of the GST-M-CKs or M-CKs protein products. The M1/2/ or 3-CK bands represent the M1-CK, M2-CK or M3-CK proteins that interacted with GST-M1-CK, GST-M2-CK or GST-M3-CK respectively, which were bound by the glutathione-Sepharose beads.

from 30, 20, or 10 °C-acclimatized carp (Figures 1A and 1B). The three carp M-CK isoenzymes appeared at a broad range of temperatures; the percentages of M1-CK, M2-CK and M3-CK differed only slightly in the different temperature-acclimatized carp muscle tissues. This expression pattern was very different from other cold-acclimatized isoenzymes.

Cellular localization of the M-CK isoenzymes

We examined further another possibility: the cellular localization of the three M-CK isoenzymes in the red and white muscle. The immunohistochemical staining results of the three M-CKs in both muscle tissues from carp were very similar (results not

shown). On the other hand, these M-CKs of carp that had acclimatized at 30, 20, or 10 °C also exhibited similar cellular localization, therefore only the 20 °C results were shown. In double-staining experiments, the staining pattern with CKM1 + 2 antibody showed that both M1-CK and M2-CK isoenzymes were present in both muscle tissues (Figure 2A). The fluorescence signal could be seen throughout the cytosol with the membrane for all acclimatization temperatures. No signal was detected in the negative control where primary antibody was absent (results not shown). The CKM3 antibody also gave a similar picture (Figure 2B). When the phase-contrast pictures of two different fluorescence pictures merged, the red-staining CKM1 + 2 antibody overlapping with the green-staining CKM3 antibody gave

an orange or yellow signal in the same area (Figure 2C). Consistent with this result, double-staining results also showed that these M-CK isoenzymes were localized in the same cellular compartment. This somewhat unexpected result might have other physiological meaning as described in the following sections.

Existence of heterodimers of MM-CK isoenzymes

Since all three carp M-CKs were distributed throughout the cytoplasm and along the membrane of both muscle tissues, it was reasonable to speculate that the M-CK sub-isoenzymes could form homo- and heterodimers. To examine the possible existence of heterodimers, total proteins that were extracted from both muscle types of acclimatized carp were used in a co-immunoprecipitation experiment. The MM-CK proteins were precipitated by CKM1+2 or CKM3 antisera. The immunoprecipitated complexes were subjected to immunoblotting analysis using either CKM1+2 or CKM3 antisera. In both muscle types of carp that were acclimatized at different temperatures, M3-CK appeared in the complexes precipitated by the CKM1+2 antibodies, whereas both M1-CK and M2-CK were co-precipitated by CKM3 antibodies (Figure 3). These results demonstrated that M1-CK, M2-CK and M3-CK could interact with each other. Although the CKM1+2 antibody could not selectively differentiate between the M1-CK and M2-CK at this stage, our results suggested that, in both carp muscle types, M1-CK, M2-CK and M3-CK could form distinct dimers. The finding of more than one of the same types of isoenzyme interaction in the same compartment was new to our experience. Interestingly, we also discovered the formation of homo- or heterodimers among these three M-CKs.

Interaction among the three M-CK isoenzymes

Different combinations of GST-fused M-CK and M-CK expression constructs were co-expressed by an IVTnT system at 30 °C with [³⁵S]methionine labelling. In rabbit reticulocyte lysates, pET-GST-M-CK and pET-M-CK constructs (pET-GST-M1-CK with pET-M1-CK, pET-M2-CK or pET-M3-CK; pET-GST-M2-CK with pET-M1-CK, pET-M2-CK or pET-M3-CK; and pET-GST-M3-CK with pET-M1-CK, pET-M2-CK or pET-M3-CK) were co-transcribed and co-translated into proteins of the predicted sizes (Figures 4B–4D). The co-expressed protein products were separately incubated with glutathione-Sepharose beads at 30 °C, and the pull-down complexes were analysed by electrophoresis and fluorography. The tendency that different molecules could associate and dimerize led us to investigate further whether or not the M-CK homodimers or heterodimers formation could be affected by temperature. In the control experiment, GST alone did not interact with the M1-CK, M2-CK or M3-CK proteins (Figure 4A). The coupled IVTnT and GST pull-down assay showed that M1-CK interacted strongly with itself and M3-CK, but weakly with M2-CK (Figure 4B). These results demonstrated the formation of the M1M1-CK homodimer and the M1M2-CK and M1M3-CK heterodimers. Moreover, the pull-down assay showed that M2-CK could dimerize and also form heterodimers, weakly with M1-CK and very weakly with M3-CK respectively (Figure 4C). Yet the most interesting result was that M3-CK also showed almost no interaction with itself at 30 °C (Figure 4D). Subsequently, the M3-CK interaction with M2-CK or M3-CK at 25 and 15 °C was studied further. The formation of the M3-CK dimer was strongly favoured at lower temperatures, but the formation of the M2M3-CK heterodimer did not seem to have been favoured by these

Figure 5 Dimerization of M3-CK with M2-CK or M3-CK at different temperatures

The co-IVTnT experiments of pET-GST-M3-CK with pET-M2-CK or pET-M3-CK expressed at 30 °C, and the IVTnT protein products, were incubated further and bound to glutathione-Sepharose beads at 30, 25 or 15 °C respectively. The coupled co-IVTnT with GST pull-down assay result shows the GST-M3-CK interaction with M2-CK or M3-CK at 30, 25 or 15 °C. The M2-CK or M3-CK bands represent the M2-CK or M3-CK isoenzymes that interacted with GST-M3-CK, which was bound by glutathione-Sepharose beads. The results show that the M3-CK interacted with M3-CK at lower temperatures. Arrows indicate the positions of GST-M3-CK and M2-CK or M3-CK proteins.

lower temperatures (Figure 5). The dynamic homo- and heterodimerization among M1-CK, M2-CK and M3-CK could be a novel mechanism of the creatine kinase isoenzymes in temperature acclimatization.

The structural comparison between M1M1-CK and M3M3-CK models

Our results show that M1M1-CK, M2M2-CK, M1M2-CK and M1M3-CK dimers, but not M3M3-CK, were formed at 30 °C. On the contrary, M3-CK homodimerized at lower temperatures. Cytosolic CK isoenzymes are known to form functional dimers that can be dissociated into monomers under different conditions [22]. The structural dimerization differences of those dimeric interfaces are of particular interest. In the present study, we used M1M1-CK as a reference to compare different chemical properties of the M3M3-CK dimers. In order to find a possible reason for different MM-CK dimer thermal stability, we constructed protein models, using 1QH4 (1.4 Å) as a template, to see if any molecular difference could be found at the dimeric interfaces [11].

After energy minimization, unfavourable chemical geometry was not observed in these models. The carp MM-CK models maintained a similar 'banana-shape', as seen in the rabbit MM-CK [23] and the chicken BB-CK [11]. The primary structures of M1-CK and M3-CK proteins have sequence identities of 87 %. Therefore it is not surprising that the two MM-CKs are highly similar in both secondary and tertiary structures. The active sites of the two enzymes showed considerable similarity. The dimeric interface was located at the N-terminal region and this region was located at the surface of the dimer (Figure 6).

We first calculated the buried protein surface areas of the M1M1-CK and M3M3-CK dimeric interfaces by subtracting the solvent accessible-surface area of the dimer from that of the two

Figure 6 Diagram of the M1M1-CK and M3M3-CK dimeric interfaces

(A) N-terminal sequence alignment and amino acid residues involved in the dimeric interfaces (black frame) of M1M1-CK and M3M3-CK isoenzymes. Amino acid residues in M3-CK that are identical to those in M1-CK are represented by hyphens in the corresponding positions. A gap is indicated by a dot. Asterisks indicate residues that contribute to putative hydrogen bonds between monomers in the dimeric interfaces. The putative α -helices are drawn as helices. (B) Comparison of M1M1-CK (upper panel) and M3M3-CK (lower panel) dimeric interfaces. The interfaces are shown as space-filling representations. The negatively and positively charged residues that form salt-bridges are coloured red and blue respectively. Hydrophobic residues are coloured yellow. Hydrophobicity of these dimers was calculated and displayed with the Insight II package. The rest of each protein monomer is shown by tube model.

Table 1 Total solvent-accessible surface areas (ASAs)* and dimeric buried ASAs of the M1M1-CK and M3M3-CK dimer

	M1M1-CK (Å ²)	M3M3-CK (Å ²)
Total ASAs (dimer)	33 806	34 093
Buried ASAs†	3320	2920
Polar‡	1760	1698
Non-polar‡	1560	1222

* ASA values are calculated according to Lee and Richards [39].

† The buried ASA at the dimeric interface was estimated by subtracting the value computed for dimer from the sum of the values computed for monomers.

‡ The buried ASA at the dimeric interface was calculated separately as polar and non-polar. The values of the polar and non-polar areas inside buried ASAs are shown.

monomers (Table 1) [24,25]. Interestingly, the contribution of non-polar amino acid residues to the decrease in buried interface area from M1M1-CK to M3M3-CK was quite large (21.7%), and that of the polar residues was relatively very small (3.5%). The overall buried interface area decrease in M3M3-CK was about 12% (Table 1).

Then, in the carp MM-CK dimeric interfaces, three main contact domains in M1-CK and M3-CK were found. The first domain was from residues 1 to 20 (M3-CK, 1–19), the second domain from 48 to 50 (M3-CK, 47–49), and the third domain from 145 to 152 (M3-CK, 144–151). These regions were the most polymorphic between these two isoforms (Figure 6A). The dimeric interfaces of the two carp MM-CK protein models were shown as a space-filling model with CPK colour code (Figure 6B). The major differences in the interfaces of these two CK dimers lay in the numbers of salt-bridges and hydrophobic residues. There were seven salt-bridges in the M1M1-CK (Asp⁵⁴:OD1 – Arg¹⁴⁸:NH₂, Asp⁵⁵:OD2 – His⁷:NE₂, Gln⁵⁸:NE₂ – Asp²¹⁰:OD1, Arg¹⁴⁸:NH₂ – Asp⁵⁴:OD1, Glu¹⁵⁰:OE1 – Asn⁸:ND2, Lys¹⁷⁷:NZ – Asp²²:OD2 and Asp²¹⁰:OD1 – Gln⁵⁸:NE₂), while only five were present in M3M3-CK (Asp⁵³:OD1 – Arg¹⁴⁷:NH₂, Gln⁵⁷:NE₂ – Asp²⁰⁹:OD1, Arg¹⁴⁷:NH₂ – Asp⁵³:OD1, Lys¹⁷⁶:NZ – Asp²¹:OD2, and Asp²⁰⁹:OD1 – Gln⁵⁷:NE₂) (Figure 6B, blue and red colours). The number of hydrophobic residues in the interfaces of the donor M1-CK was 14 (Phe³, 10, 20, 51, Val¹⁶, 61, Ile⁵⁷, Ala¹⁴¹, 153, 204, 205, 208, Leu²⁰³ and Met²⁰⁷), 11 for acceptor M1-CK (Phe³, 10, 20, 51, Leu¹², Val¹⁶, 61, Pro²¹, Ile⁵⁷ and Ala¹⁴¹, 153), seven for donor M3-CK (Phe¹³, 50, 139, Ile⁵⁶, Val⁶⁰ and Ala¹⁵², 207) and six for acceptor M3-CK (Leu¹¹, Phe¹³, 139, Ile⁵⁶, Val⁶⁰ and Ala²⁰⁷) (Figure 6A, green colour; Figure 6B, yellow colour). These differences could be accounted for by the decrease in the interface area of the M3M3-CK dimer. Kinetic studies on denatured rabbit MM-CK suggest that proper refolding of the enzyme involves a rapid association of the two already folded monomers, followed by a slow rearrangement of the dimer and it has been suggested that the initial driving force of dimer formation is hydrophobic interaction [26,27]. The decrease in hydrophobicity in the region of the M3-CK protein that becomes the M3M3-CK interface causes a decrease in subsequent interface area and that, in turn, causes the M3M3-CK to become thermolabile.

DISCUSSION

Based on their living environmental temperature, fish can be divided into polar (below 0 °C), temperate and tropical classes. The carp is acclimatized to the seasonal variation every year. In physiological definition, temperature shock (over a day), acclimatization (over weeks to months) and adaptation (over a

longer period still) have different response timetables and strategies. Carp does not have to adapt to extreme temperatures, unlike fish of the Antarctic and Arctic Seas (as low as –1.8 °C), yet they acclimatized between 35 to 10 °C. They have different physiological strategies for acclimatizing or adapting to environmental temperature.

In a previous study [10], we analysed the differences of the three M-CK isoenzymes, and the results suggested that the three isoenzymes possess different enzyme properties at various temperatures. The original prediction of the M-CK sub-isoforms possibly having individual functions at different temperatures was based on findings in carp myosin isoforms. Warm, intermediate and cold forms of myosin, which were expressed at 30, 20, and 10 °C respectively, have been isolated [28,29]. However, our experiments with the specific CKM1+2 and CKM3 antibodies showed that all three M-CKs were detected throughout the cytosol and along the membrane of red and white muscle of all temperature acclimatized carp. Moreover, the three carp CK isoenzymes did not show separate expression patterns at different temperatures as other cytoskeleton proteins do (e.g. myosin). On the other hand, the protein family often exists in tissue- or even cell compartment-specific isoforms. While essentially fulfilling the similar structural and/or catalytic role, these isoenzymes differ in order to respond more efficiently to the specialized metabolic requirements of their surrounding environments. The three M-CK isoenzymes were previously considered to be localized in their respective cytoplasmic compartments where energy production (the glycolysis site) or energy consumption (the cellular ATPase site) are involved. However, the double-staining analysis indicated that they are generally localized in the same location.

Further immunoprecipitation experiments with specific antibodies showed that, in both muscle types, M1-CK, M2-CK and M3-CK isoenzymes could interact to form homo- and heterodimers. In an IVTnT coupled GST pull-down assay performed at 30 °C, M1-CK and M2-CK interacted well to form their respective homodimers. Moreover, the M1-CK could dimerize with either M2-CK or M3-CK, but M2-CK and M3-CK showed weak interaction with each other. Nevertheless, M3-CK itself was able to dimerize at lower temperatures. Our findings demonstrate the formation of the temperature-dependent distinct homo- or heterodimers among these three M-CKs. This is a new regulatory strategy of the CK isoenzyme families that has been found. This independent assortment of different M-CKs molecules also suggested a novel mechanism of energy conservation of the creatine kinase isoenzymes in temperature acclimatization.

There are several levels of difficulty that cold-acclimatized enzymes have to overcome in order to catalyse its function. In the first level, and in the case of multimeric enzymes, dissociation of the quaternary structure, which is often a characteristic of cold denaturation, has to be prevented. This dissociation is thought to result from the interactions between the non-polar side chains in proteins and water, which become thermodynamically more favourable at low temperatures [30,31]. It seems reasonable that hydrophobic interactions might be less important or need to be decreased in cold-acclimatized enzymes. Yet results from recent studies on two non-enzymic proteins, haemoglobin and tubulin in the Antarctic fishes have been shown to the contrary. Haemoglobins from two Antarctic fish were calculated to yield a larger hydrophobic surface area buried at the $\alpha 1$ – $\beta 2$ interface upon association of dimers into tetramers [32]. In the tubulins of the Antarctic fish, lateral substitution specific to the α -chain (Met³⁰² → Leu or Met³⁰² → Phe) may increase the hydrophobicity of the interprotofilament interaction, and two hydrophobic

Figure 7 Comparison of the electrostatic potential of M1M1-CK and M3M3-CK dimeric interfaces

The dimeric interfaces are shown as space-filling models. **(A)** The electrostatic potential of the dimeric interface of the M1-CK acceptor (left) and the M1-CK donor (right). **(B)** The electrostatic potential of the dimeric interface of the M3-CK acceptor (left) and the M3-CK donor (right). The positive potential is in blue and the negative potential is in red. The electrostatic potential is calculated for each molecule in the dimeric interface region. Figures were prepared with WebLab ViewerPro (MSI, San Diego, CA).

substitutions (α : Ser¹⁸⁷ → Ala in helix H5 and β : Tyr²⁰² → Phe in sheet S6) may act to stabilize the monomers in conformations favourable to polymerization [33].

However, in this study, there were twelve net decreases in non-polar residues in the three contact domains of the carp M3M3-CK dimer. This reduction in hydrophobic residues may cause the calculated interface surface area that is buried in the formation of a dimer to decrease by 12% in M3M3-CK. Indeed, M3M3-CK dimer was weakly associated at temperatures at 25 °C and above. The properties of cold-acclimatization proteins should be in contrast with those of thermophilic proteins, for example, an increase of the entropy of unfolding and flexibility and a decrease of compactness and hydrophobicity [34]. The clustering of hydrophobic side chains within the protein core is a major driving force in the stability of the folded conformation. Attempts to evaluate this effect on the basis of structure have been performed, using the hydrophobic index of individual amino acids forming the protein core, or using hydrophobic cluster analysis. Many psychrophilic enzymes have been found to display substitutions

within the hydrophobic core clusters that are accompanied by a sharp decrease of the hydrophobicity index when compared with mesophilic homologues [35–37].

In the stability of molecular contacts, besides the hydrophobic surface buried within a given interface, the role of salt-bridges and hydrogen bonds should not be neglected [38]. Another observable difference between the interfaces of M1M1-CK and M3M3-CK is the charge distributions in the centres of the interfaces. The aromatic residue Phe³ of the M1-CK changed to Lys³ of the M3-CK to create a charge interaction (Figures 6A and 7). This Lys³ in the donor M3-CK interacted with the Asp⁵⁴ in the acceptor M3-CK, and resulted in a change in the electrostatic distribution in the centre of the M3M3-CK interface. It has been suggested that, although hydrophobic forces drive dimeric interaction, hydrogen bonds and salt-bridges provide the specificity [26]. The structural shape of the macromolecular interfaces is a component of the complementary factor. This hydrophilic cavity in the centre of the M3M3-CK interface created by Lys³–Asp⁵⁴ electrostatic interaction may contribute to

the dimer interface specificity, but probably not to the initial stage of dimerization.

We combined immunological, cellular biological, and biochemical methods to demonstrate the mutual interaction of the three carp M-CK isoenzymes. By combining our previous data and current discovery, we postulated that the process of homo- and heterodimerization of different MM-CK molecules could be a novel regulatory mechanism of CK isoenzymes in poikilothermic carp muscle. The formation of M-CK homo- or heterodimers might play a key role in metabolic control and energy transfer for thermal acclimatization.

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REFERENCES

- Hazel, J. R. and Prosser, C. L. (1974) Molecular mechanisms of temperature compensation in poikilotherms. *Physiol. Rev.* **54**, 620–670
- Hazel, J. R. (1993) Thermal biology. In *The Physiology of Fishes* (Evans, D. H., ed.), pp. 427–467. CRC Press, Inc., Boca Raton, FL
- Crockett, E. L. and Sidell, B. D. (1990) Some pathways of energy metabolism are cold adapted in Antarctic fishes. *Physiol. Zool.* **63**, 472–678
- Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. and Eppenberger, H. M. (1992) Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem. J.* **281**, 21–40
- Wallimann, T. (1994) Bioenergetics: Dissecting the role of creatine kinase. *Curr. Biol.* **4**, 42–46
- Qin, W., Khuchua, Z., Cheng, J., Boero, J., Payne, R. M. and Strauss, A. W. (1998) Molecular characterization of the creatine kinases and some historical perspectives. *Mol. Cell. Biochem.* **184**, 153–167
- Schlegel, J., Wyss, M., Schürch, U., Schnyder, T., Quest, A., Wegmann, G., Eppenberger, H. M. and Wallimann, T. (1988) Mitochondrial creatine kinase from cardiac muscle and brain are two distinct isoenzymes but both form octameric molecules. *J. Biol. Chem.* **263**, 16963–16969
- Brdiczka, D., Beutner, G., Ruck, A., Dolder, M. and Wallimann, T. (1998) The molecular structure of mitochondrial contact sites. Their role in regulation of energy metabolism and permeability transition. *Biofactors* **8**, 235–242
- Wallimann, T., Dolder, M., Schlattner, U., Eder, M., Hornemann, T., O'Gorman, E., Ruck, A. and Brdiczka, D. (1998) Some new aspects of creatine kinase (CK): compartmentation, structure, function and regulation for cellular and mitochondrial bioenergetics and physiology. *Biofactors* **8**, 229–234
- Sun, H. W., Hui, C. F. and Wu, J. L. (1998) Cloning, characterization, and expression in *Escherichia coli* of three creatine kinase muscle isoenzymes cDNAs from carp (*Cyprinus carpio*) striated muscle. *J. Biol. Chem.* **273**, 33774–33780
- Eder, M., Schlattner, U., Becker, A., Wallimann, T., Kabsch, W. and Fritz-Wolf, K. (1999) Crystal structure of brain-type creatine kinase at 1.41 Å resolution. *Protein Sci.* **8**, 2258–2269
- Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. and Strober, W. (2000) *Current Protocols in Immunology*, John Wiley & Sons, Inc., New York
- Esser, P., Knudsen, H., Nielsen, V. and Petersen, D. (1999) In *Nunc Guide to Solid Phase* (Rowell, V., ed.), pp. 5–29. Nalge Nunc International, Roskilde
- Laemmli, U. K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Burnette, W. M. (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**, 195–203
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (2000) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York
- Harlow, E. and Lane, D. (1998) *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Melcher, K. and Johnston, S. A. (1995) GAL4 interacts with TATA-binding protein and coactivators. *Mol. Cell. Biol.* **15**, 2839–2848
- Guex, N. and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**, 2714–2723
- Guex, N., Diemand, A. and Peitsch, M. C. (1999) Protein modelling for all. *Trends Biochem. Sci.* **24**, 364–367
- Bailey, S. (1994) The CCP4 suite – Programs for protein crystallography. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **50**, 760–763
- Gross, M., Lustig, A., Wallimann, T. and Furter, R. (1995) Multiple-state equilibrium unfolding of guanidino kinases. *Biochemistry* **34**, 10350–10357
- Rao, J. K., Bujacz, G. and Wlodawer, A. (1998) Crystal structure of rabbit muscle creatine kinase. *FEBS Lett.* **439**, 133–137
- Eisenberg, D. and McLachlan, A. D. (1986) Solvation energy in protein folding and binding. *Nature (London)* **319**, 199–203
- Jackson, R. M. and Sternberg, M. J. E. (1993) Protein surface area defined. *Nature (London)* **366**, 638
- Bickerstaff, G. F., Paterson, C. and Price, N. C. (1980) The refolding of denatured rabbit muscle creatine kinase. *Biochim. Biophys. Acta* **621**, 305–314
- Fersht, A. R. (1984) Basis of biological specificity. *Trends Biochem. Sci.* **9**, 145–147
- Gerlach, G. F., Turay, L., Malik, K. T. A., Lida, J., Scutt, A. and Goldspink, G. (1990) Mechanisms of temperature acclimation in the carp: a molecular biology approach. *Am. J. Physiol.* **28**, R237–R244
- Imai, J. I., Hirayama, Y., Kikuchi, K., Kikuchi, K., Kakinuma, M. and Watabe, S. (1997) cDNA cloning of myosin heavy chain isoforms from carp fast skeletal muscle and their gene expression associated with temperature acclimation. *J. Exp. Biol.* **200**, 27–34
- Privalov, P. L. (1990) Cold denaturation of proteins. *Crit. Rev. Biochem. Mol. Biol.* **25**, 281–305
- Marshall, C. J. (1997) Cold-adapted enzymes. *Trends Biotechnol.* **15**, 359–364
- Giangiaco, L., D'Avino, R., di Prisco, G. and Chiancone, E. (2001) Hemoglobin of the Antarctic fishes *Trematomus bernacchii* and *Trematomus newnesi*: structural basis for the increased stability of the liganded tetramer relative to human hemoglobin. *Biochemistry* **40**, 3062–3068
- Detrich, III, H. W., Parker, S. K., Williams, Jr, R. C., Nogales, E. and Downing, K. H. (2000) Cold adaptation of microtubule assembly and dynamics. Structural interpretation of primary sequence changes present in the α - and β -tubulins of Antarctic fishes. *J. Biol. Chem.* **275**, 37038–37047
- Parthasarathy, S. and Murthy, M. R. N. (2000) Protein thermal stability: insights from atomic displacement parameters (B values). *Protein Eng.* **13**, 9–13
- Feller, G. and Gerday, C. (1997) Psychrophilic enzymes: molecular basis of cold adaptation. *Cell. Mol. Life Sci.* **53**, 830–841
- Smalas, A. O., Heimstad, E. S., Hordvik, A., Willassen, N. P. and Male, R. (1994) Cold adaptation of enzymes: structural comparison between salmon and bovine trypsins. *Proteins* **20**, 149–166
- Genicot, S., Rentier-Delrue, F., Edwards, D., VanBeeumen, J. and Gerday, C. (1996) Trypsin and trypsinogen from an Antarctic fish: molecular basis of cold adaptation. *Biochim. Biophys. Acta* **1298**, 45–57
- Vallone, B., Miele, A. E., Vecchini, P., Chiancone, E. and Brunori, M. (1998) Free energy of burying hydrophobic residues in the interface between protein subunits. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6103–6107
- Lee, B. and Richards, F. M. (1971) The interpretation of protein structures: estimation of static accessibility. *J. Mol. Biol.* **55**, 379–400

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