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Temperature Acclimation Induces Light Meromyosin Isoforms with Different Primary Structures in Carp Fast Skeletal Muscle*

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Summary: Carp acclimated to 10°C gave 69k, 66k, and 62kDa light meromyosin (LMM) fragments in SDS-PAGE, while fish acclimated to 30°C gave 74k, 69k, 66k, and 62kDa fragments. The microsequence analysis revealed that the 69k and 66kDa components from the 10°C-acclimated carp contained an N-terminal amino acid sequence different from that of 62kDa. The four fragments from the 30°C-acclimated carp showed the same sequence as that of the 69k and 66kDa components from the 10°C-acclimated carp, except that the 2nd amino acid, Ala, of the 10°C-acclimated LMM was replaced by Thr. DNA fragments encoding an N-terminal region of LMM were amplified by PCR or reverse transcriptase-PCR, demonstrating that the two acclimated groups further contained several amino acids substituted.

Water temperature is the most critical for aquatic poikilotherms which change their body temeprature in association with fluctuation of water temperature (1). To cope with such changes in temperature, eurythermal temperate fish such as carp and goldfish changes in myofibrillar ATPases in an acclimation temperature-dependent manner (2 - 5). Acclimation to low temperature results in an increase in ATPase activity. It has recently been shown that different myosin heavy chain isoform genes are expressed in carp at warm and cold temperatures (6), and that different myosin isoforms are responsible for such changes of myofibrillar ATPase activity (7 - 9). It is well known that myosin is a complex multimeric protein which consists of two functionally different halves, a rodlike tail and a pair of globular head called subfragment-1 (S1) (10). Cross-bridge head, myosin subfragment-1 (S1), provided with actin binding and ATPase sites, is the force generator for muscle contraction. Carp expresses specific S1 isoforms in association

*The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession numbers: D43699 and D43700, for mRNAs from carp acclimated to 10 and 30°C, respectively.

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with cold and warm temperature acclimation (11,12). Changes in the myosin molecule prevail not only on S1, but also on the rod portion having the filament-forming ability (9). Differences between the 10- and 30°C-acclimated myosin rod were clearly detected in peptide maps and in apparent sizes of light meromyosin (LMM). However, it is umbiguous which parts are responsible for such changes at a level of the primary structure.

The objective of this study was to reveal differences in the N-terminal amino acid sequence of LMM, a C-terminal domain produced by proteolytic digestion of myosin rod, from carp acclimated to cold and warm water temepratures.

MATERIALS AND METHODS

Fish - Carp, *Cyprinus carpio* (0.5 - 0.8 kg in body weight), were acclimated to either 10 or 30°C for a minimum of 5 weeks (4), and their dorsal fast skeletal muscles were used for protein preparations and cDNA cloning as described below.

Protein Preparations - Carp myosin was prepared as reported previously (7). Myosin rod was prepared from myosin by digesting 10 - 15 mg/ml myosin at 10°C for 30 min in 20 mM phosphate buffer (pH 7.0) containing 0.12 M NaCl, 1 mM EDTA, and 0.1 mM dithiothreitol (DTT), using *T*-tosyl-L-lysine chloromethyl ketone-treated a-chymotrypsin at an enzyme-to-myosin weight ratio of 1 : 130. Rod fraction obtained as precipitate after centrifugaion at 100,000 x g for 1 h was dialyzed against 20 mM sodium PPi (pH 7.5) containing 0.1 mM DTT, and further purified with a DEAE-Toyopearl 650M column (2 x 9 cm) equilibrated with the same buffer, essentially according to Kato and Konno (13). The proteins adsorbed were eluted with a linear gradient from 20 to 50 mM PPi (pH 7.5) containing 0.1 mM DTT. Myosin rod thus purified was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 0.5 M KCl and susequently digested at 20°C for 10 min using TPCK-treated a-chymotrypsin at an enzyme-to-rod weight ratio of 1 : 250. LMM was obtained as precipitate in a low-ionic-strength buffer and further purified by the same DEAE-column chromatography. Protein concentrations were determined by the biuret method using bovine serum albumin as the standard (14).

SDS-PAGE - SDS-PAGE was carried out by the method of Laemmli (15), using 7.5 - 12.5% polyacrylamide gradient slab gels containing 0.1% SDS. Molecular weight markers (Sigma) were myosin heavy chain (205kDa), b-galactosidase (116kDa), phosphorylase b (97.4kDa), bovine serum albumin (66kDa), ovalbumin (45kDa), and carbonic anhydrase (29kDa). Gels were stained with Coomassie brilliant blue R-250 and destained with a solution containing 25% methanol and 7% glacial acetic acid.

N-Terminal Amino Acid Sequence Analysis - N-terminal amino acid sequences were determined essentially according to Matsudaira (16). Proteins in the SDS-PAGE gels were electroblotted to Millipore polyvinylidene difluoride (PVDF) membranes using a Biocraft model BE300 horizontal semi-dry blotting apparatus in a buffer containing 10 mM 3-cyclohexylamino-1-propanesulfonate buffer (pH 11) containing 10% methanol. Proteins blotted onto PVDF membranes were stained with Coomassie brilliant blue and stained protein bands were excised with scissors. Three or four excised sheets were put onto a blott cartridge block and analyzed for the N-terminal amino acid sequence with an Applied Biosystems model 476A protein sequencer equipped with an on-line data processor system model 610A.

cDNA Cloning - Total RNA for preparation of mRNA was prepared from the dorsal skeletal muscle of freshly killed carp acclimated to either 10 or 30°C by the method of Chomczynski et al.(17). Poly $(A)^+$ mRNA was isolated from total RNA using an oligo $d(T)^+$ cellulose column (Pharmacia). A carp cDNA library was constructed using isolated mRNA in the phage vector lgt11 accoding the supplier's instructions (Stratagene). Five mg of mRNA were used for double-stranded cDNA synthesis which was carried out using Pharmacia cDNA synthesis kits. The PCR amplifications (18) were carried out for 1 min at 93°C for denaturation, 1.5 min at 53 °C for annealing, and 1.5 min at 72°C for extension, using a DNA thermal cycler model 9600 and GeneAmp kits (Perkin Elmer Cetus Instruments). This procedure was performed for 30 cycles. The 100ml reaction buffer contained 200 mM dNTPs, 100 pmol of forward and reverse primers, 2 units Taq DNA polymerase, and 50 ng of phage DNA. DNA primers for PCR were synthesized with an Applied Biosystems model 373A DNA/RNA synthesizer. Alternatively, reverse transcriptase (RT)-PCR was performed using random hexamers for the RT reaction and then a primer synthesized based upon the data obtained in this study and a dT primer. DNA sequencing

was carried out with Taq dye deoxy terminator cycle sequencing kits using a model 373A DNA sequencer with an on-line model 672 GENESCAN data processor system (Perkin Elmer, Applied Biosystems Division) to verify the PCR products.

Sequence Analysis - Sequence entry and analysis were performed using an Applied Biosystems model 6701 INHERIT sequence analysis system with suites of computer programs. The sequences used for comparison are available from either SwissProt or GenBank databases.

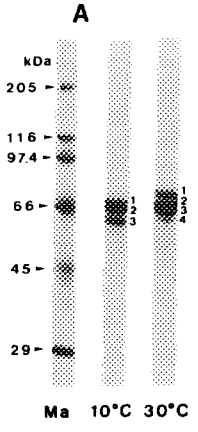
RESULTS

The isolated LMM fraction from the 10°C-acclimated carp gave three bands in SDS-PAGE with apparent molecular weights of 69k, 66k, and 62k, while LMM from the 30°C-acclimated carp showed four bands of 74k, 69k, 66k, and 62kDa (Fig. 1A).Different LMM bands separated in SDS-PAGE were transferred electrophoretically onto PVDF membranes, cut into sheets, and subjected to N-terminal amino acid sequence analysis using a blot cartridge block.

Both the 69k and 66kDa LMM components from the 10°C-acclimated carp contained two sequences (Fig. 1B). A predominating sequence started at Arg as an N-terminal amino acid, while a minor component gave a sequence starting at Glu, the 5th amino acid from the N-terminus of the predominant component. On the other hand, the 62kDa component from the 10°C-acclimated carp showed a sequence completely different from those obtained for other 10°C-acclimated LMM fragments.

All the 30°C-acclimated LMM fragments showed the same sequence as far as 10 amino acids from the N-terminus were concerned. When compared with the sequence of the 69k and 66kDa LMM fragments from the 10°C-acclimated carp, the 30°C-acclimated LMM fragments exhibited general resemblance. However, it was noted that the 2nd amino acid, Ala, in the 10°C-acclimated LMM was replaced by Thr in the 30°C-acclimated LMM.

LMM fragments of 69k and 66kDa from the 10°C-acclimated carp as well as all those from the 30°C-acclimated fish located their N-terminus at Arg-1374 of chicken myosin heavy chain, while



B

LMM 10-1	(69k)	RAKYETDAIQRTE ETDAIQRTEELEE
-2	(66k)	RAKYETDAIQ ETDAIQRTEE
-3	(62k)	AANLDKKQRNLDKVLA
LMM 30-1	(74k)	RTKYETDAIQRTEELEEAK
-2	(69k)	RTKYETDAIQRTEELEEAK
-3	(66k)	RTKYETDAIQRTEELEEAK
-4	(62k)	RTKYETDAIQ

FIG. 1. **SDS-PAGE patterns (A) and N-terminal amino acid sequences (B) of LMM fragments from carp acclimated to 10 and 30°C.** SDS-PAGE was carried out using a 7.5 - 12.5% polyacrylamide gradient slab gel. LMM 10 and 30 represent LMM fragments from carp acclimated 10 (10-1, -2, and -3) and 30°C (30-1, -2, -3, and -4), respectively, with apparent molecular weights in parentheses determined using molecular weight markers (Ma).

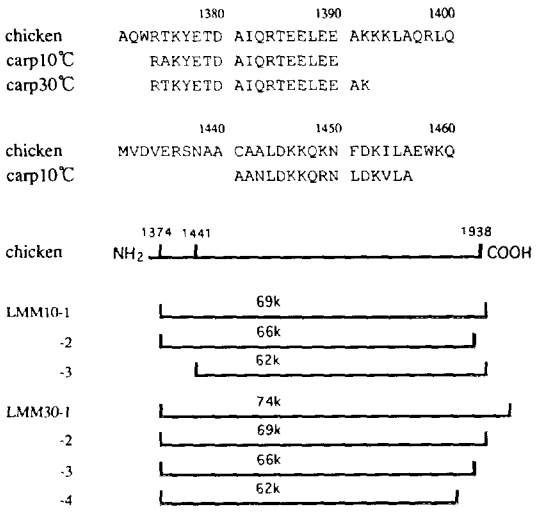


FIG. 2. **Comparison of N-terminal amino acid sequences and molecular masses of LMM fragments from carp acclimated to 10 and 30°C with those of chicken fast skeletal muscle.** The data from chicken were cited from Maita et al. (19). Numerals shown above amino acid residues and schematic representatives of LMM molecules are residue numbers from the N-termimus of chicken myosin heavy chain. Refer to the legend of Figure 1 for abbreviations.

the N-terminal amino acid sequence of the 62kDa fragment from the 10°C-acclimated carp corresponded to that of chicken starting at Cys-1441 (19) (Fig. 2). These results suggest that LMM from the 10°C-acclimated carp has a sequence different from that of the 30°C-acclimated carp near the region where the 62kDa fragment of the 10°C-acclimated LMM was sequenced. Different molecular weights between 69k and 66kDa fragments of the 10°C-acclimated LMM and among four fragments of the 30°C-acclimated LMM suggest differences in the C-terminal length, in the assumption that the remaining sequence undetermined is the same among the LMM components from the same temperature acclimation group.

Since two amino acid sequences located at different sites in the N-terminal region of the 10°C-acclimated LMM were determined, several primers composed of 20 - 26 bases encoding 7 - 9 amino acids were designed and synthesized. DNA sequences reported for LMMs from other myosins (20 - 24) were taken into consideration to construct these primers. When these primers were applied to cDNA library from the 10°C-acclimated carp fast skeletal muscle for PCR amplification, one pair of primers produced the DNA fragment of about 200bp (data not shown). These primers were 5'-AGGGCCAAATATGAGACGGACGCC-3' for 5'-nucleotide site and 5'-TGGACAAGAAGCAGAGGAAC-3' for 3'-nucleotide site. These two DNA nucleotide sequences were derived from the amino acid sequences, Arg-Ala-Lys-Tyr-Glu-Thr-Asp-Ala and Leu-Asp-Lys-Lys-Gln-Arg-Asn, respectively. However, any combination of primers was not able to amplify DNA fragments encoding LMM with cDNA library from the 30°C-acclimated carp.

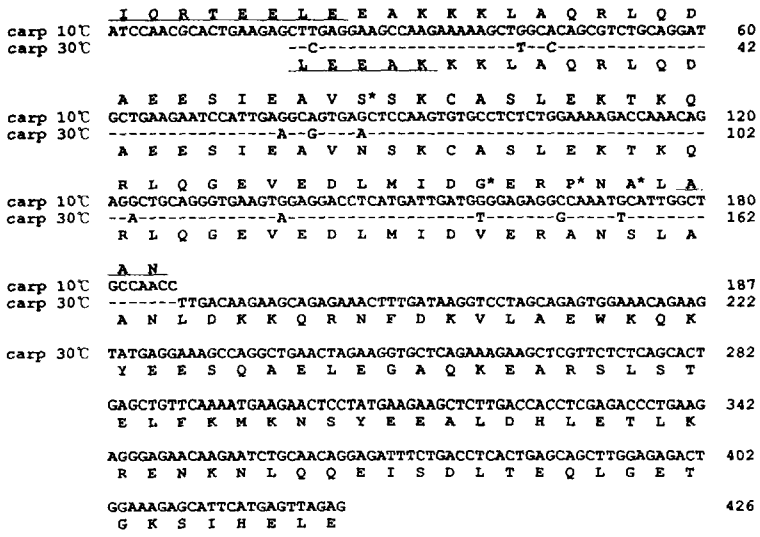


FIG. 3. **Partial DNA nucleotide sequences and deduced amino acid sequences of LMMs from carp acclimated to 10 and 30°C.** Dashed lines represent nucleotides determined for PCR products from the 30°C-acclimated carp, which are identical to those of the 10°C-acclimated fish. Asterisks indicate amino acid differences between the 10- and 30°C-acclimated carp. The sequences determined by microsequence analysis are underlined.

Then, RT-PCR was employed for mRNA from the 30°C-acclimated carp. 3' Primer used was oligo dT, while 5' primer was 5'-ATCCAACGCACTGAAGAG-3' which encodes the peptide consisting of Ile-Gln-Arg-Thr-Glu-Glu of the 10°C-acclimated LMM (Fig. 3). The RT-PCR resulted in amplification of DNA with an approximate size of 2kbp (data not shown), which roughly corresponds to the intact molecular size of LMM referring to the data for other myosins (20 - 24).

Totally 187 and 426 nucleotides were sequenced for the 10- and 30°C-acclimated LMM, respectively. It was impossible to sequence 3'-nucleotide site of the RT-PCR product from the 30°C-acclimated carp. When an overlapping region with 169 nucleotides was compared for the two types of LMM, 11 nucleotides were replaced between them (Fig. 3). This overlapping region encoded 56 amino acids, with four codons expressing different amino acids between the two types of LMM. It was noted that the DNA nucleotide sequence just in the upstream of the region encoding the N-terminal amino acid sequence of the 10°C-acclimated 62kDa LMM component was considerably different from that of the 30°C-acclimated LMM. This acclimation temperature-specific sequence containing 21 bases showed three different amino acids in four between the two types of LMM.

An overlapping amino acid sequence for the 10- and 30°C-acclimated LMM, either determined by microsequence analysis or deduced from cDNA nucleotide sequences, contained 83 amino acids, seven residues being substituted between the two types of carp LMM (Fig. 4). When compared with amino acid sequences reported for homoiotherms (19-24), carp LMMs showed

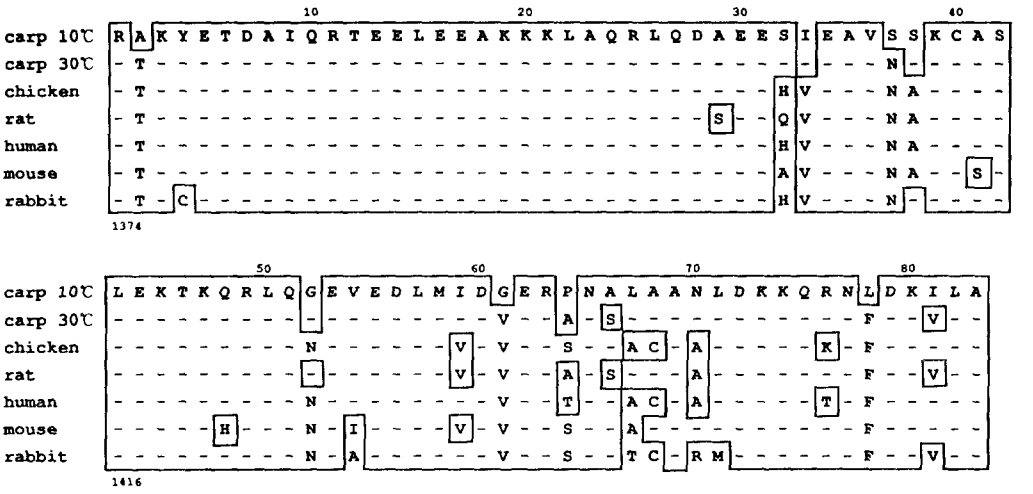


FIG. 4. **N-terminal amino acid sequences of LMMs from carp acclimated to 10 and 30°C in comparison with those of homoiotherms.** Amino acid residues identical to those of the 10°C-acclimated carp are shown by dashed lines. Numerals indicated above and below amino acid residues are residue numbers from the N-terminus of LMMs and those from chicken myosin heavy chain (19), respectively. Sequences other than that of chicken are cited from rat embryonic (24), human (20) and mouse embryonic (23), and rabbit skeletal muscle (21).

different residues at several positions. The sequence in this region is fairly well conserved among homoiotherms. The most striking differences between the two types of carp LMM were located at the 2nd, 37th, 61st, and 78th residues from the N-terminus, where corresponding amino acids in the 30°C-acclimated LMM were identical to those of homoiotherms.

DISCUSSION

The 10- and 30°C-acclimated carp rod produced LMM fragments different from each other by chymotryptic digestion (see Fig. 1). N-terminal amino acid sequences determined with a protein sequencer for LMM from carp acclimated to 10 and 30°C were clearly different from each other. First, the 69k and 66kDa LMM fragments from the 10°C-acclimated carp contained a predominating sequence, together with a minor sequence which was apparently lacking in four N-terminal amino acids of the predominating component. On the other hand, no heterogeneity was observed in the sequence analysis on LMM fragments from the 30°C-acclimated carp. Only the 2nd amino acid residue from the N-terminus was different between the two acclimated groups. The substitution of the 2nd residue from Thr to Ala in the 10°C-acclimated LMM may change its structure slightly susceptible to a-chymotryptic digestion, although no evidence was available to support such idea with suites of computer programs for sequence analysis (data not shown). Second, the N-terminal amino acid sequence of the 62kDa LMM fragment from the 10°C-acclimated carp was not found in any fragment of the 30°C-acclimated carp.

For further comparison, we adopted cDNA cloning for sequence analysis on the two types of carp LMM. In the 10°C-acclimated carp, 51 amino acid residues, which located between the two different N-terminal amino acid sequences, was deduced from the DNA nucleotide sequence (see Fig. 3). This peptide size well agreed with differences in the molecular weight between the 69k and 62kDa components from the 10°C-acclimated carp. The DNA fragment from the 30°C-acclimated carp deduced 137 amino acid residues. In these sequences including the data from microsequence analysis, the overlapping region between the 10- and 30°C-acclimated LMM contained 83 residues (see Fig. 4). Among the seven residues substituted between the 10- and 30°C-acclimated LMM, three residues were located in relatively a narrow region next to the N-terminus of the 62kDa LMM component from the 10°C-acclimated carp. This may be one of the reasons why 3' primer constructed in this study referring to the sequence of the 10°C-acclimated 62kDa component did not work for PCR amplification with cDNA library from the 30°C-acclimated carp. The cleavage in the 10°C-acclimated LMM between the 67th Leu and the 68th Ala may have occurred in loosening the a-helical structure of LMM caused by amino acid substitution in this region.

Another striking differences were clearly observed when the two types of carp LMM were compared in amino acid sequence. The overlapping sequence containing 83 residues showed a general resemblance between the two types of carp (see Fig. 4). This region of the primary structure of LMM is also well conserved among skeletal muscle LMMs so far reported, including mammals (20-24), chicken (19), and carp in this study. However, seven amino acids were substituted between the 10- and 30°C-acclimated LMM as described before. In these substituted amino acids, four from the 30°C-acclimated LMM were identical to those of homoiotherms. It has been demonstrated that myosin from the 10°C-acclimated carp is thermally more unstable than that from the 30°C-acclimated carp, taking an inactivation rate constant of Ca^{2+}-ATPase as a parameter (7 - 9, 25). Polyacrylamide gel electrophoresis in the presence of sodium PPi and the subsequent peptide mapping of separated bands showed that carp expresses different types of S1 heavy chain in an acclimation temperature-dependent manner (11,12). Although the above differences in thermal stability was observed with myosin head, S1, our recent observation in differential scanning microcalorimetry revealed that the temperature for unfolding of a-helix in LMM was clearly lower with the 10- than 30°C-acclimated carp, which will be reported elsewhere.

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