Differential Scanning Calorimetry and CD Spectrometry of Acclimation Temperature-Associated Types of Carp Light Meromyosin[†]

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ABSTRACT: Differential scanning calorimetry and CD spectrometry were employed to study the thermal unfolding of light meromyosin (LMM) prepared from carp acclimated to different temperatures. The transition temperatures given by the major peaks at pH 8.0 in 0.6 M KCl for LMM from carp acclimated to 10 °C were 32.5 and 39.5 °C with the calorimetric enthalpies (ΔH_{cal}) of 269 and 52 kcal/mol, respectively. LMM from carp acclimated to 20 °C exhibited three peaks of transition temperatures at 34.5, 40.2, and 46.9 with ΔH_{cal} of 152, 20, and 10 kcal/mol, respectively. On the other hand, LMM from carp acclimated to 30 °C showed two different patterns. The first experiment gave two transition temperatures at 39.2 and 47.3 °C with ΔH_{cal} of 231 and 39 kcal/mol, respectively. The second series of experiments resulted in showing three peaks of 34.4, 39.5, and 47.5 °C with ΔH_{cal} of 117, 123, and 28 kcal/mol, respectively. N-terminal amino acid sequence analysis revealed that LMM at the second series of experiments with the 30 °C-acclimated carp contained component(s) predominant in the 20 °C-acclimated carp. Thermal unfolding responsible for these transition temperatures was well explained by melting of α-helices which could be determined by far-ultraviolet CD spectroscopy. These results clearly demonstrate that the 30 °C-acclimated carp contained the most thermostable LMM.

Sarcomeric myosins of a hexameric protein contain two heavy chain and four light chain subunits. The heavy chain consists of an N-terminal globular head, subfragment-1, and a C-terminal rod-like tail which forms a coiled-coil of α-helices wound each other from the two heavy chains (*1*). The rod portion responsible for the assembly of myosin to form the functional thick filaments is split by limited proteolysis into subfragment-2 and light meromysin (LMM) which are N- and C-terminal halves of mysoin rod, respectively.

Differential scanning calorimetry (DSC) has remarkable advantages in demonstrating cooperative domain structures and domain interaction in proteins (*2*,*3*). DSC measurements provide information on the thermodynamic behavior of proteins such as regarding the progress of the unfolding reaction and the calorimetric heat associated with the reaction. The heat capacity obtained by DSC is closely related to the thermal unfolding of complexed multidomain proteins. Regarding such advantages in studying thermal stability of proteins containing multiple domains, DSC has been employed for examining thermal unfolding of rabbit skeletal myosin (*4*,*5*). It has been demonstrated that the thermal stability of the myosin molecule is remarkably sensitive to pH and to the ionic strength of the solution, whereas the rod part contains the least thermostable part at the LMM/S2 junction (*6*).

In the previous study, we also employed DSC for studying the thermal unfolding of myosin and its rod part prepared from carp acclimated to 10 and 30 °C (*7*). Differences in the thermal stability reflecting structural properties were clearly demonstrated by the DSC data. For example, the transition temperatures (T_m) of myosin rod given by the major peaks from the 10 °C-acclimated carp were 32.9, 33.4, and 44.1 °C with the calorimetric enthalpies (ΔH_{cal}) of 86, 146, and 69 kcal/mol. On the other hand, myosin rod from the 30 °C-acclimated carp showed T_m at 34.5, 39.7, and 46.7 °C with ΔH_{cal} of 95, 115, and 159 kcal/mol, respectively. Thermal unfolding responsible for these endotherms was mostly explained by melting of α-helices which could be determined by far-ultraviolet CD spectroscopy.

The objective of this study was to examine whether differences on DSC and CD analyses observed with myosin rod from carp acclimated to different temperatures could be attributable to those in LMM.

MATERIALS AND METHODS

Materials. Carp *Cyprinus carpio* (0.6−0.8 kg in body weight) were acclimated to 10, 20, or 30 °C for a minimum of 5 weeks, and their dorsal fast skeletal muscles were used for protein preparations.

Protein Preparations. Myosin was prepared as reported previously (*8*), whereas myosin rod prepared by using DEAE-Toyopearl 650M column chromatography as described before (*7*). LMM was prepared from myosin rod according to Kato and Konno (*9*). Briefly, purified myosin rod was dialyzed against 20 mM Tris (pH 7.5) containing 0.5 M KCl and subsequently digested at 20 °C for 10 min

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using *N*-tosyl-L-lysine chloromethyl ketone-treated α-chymotrypsin at an enzyme-to-rod weight ratio of 1:200. Digestion was stopped by the addition of phenylmethylsulfonyl fluoride to a final concentration of 0.5 mM. The digest thus obtained was subjected to centrifugation at 35000*g* for 30 min, and the resulting pellet was dissolved in and dialyzed against 20 mM sodium pyrophosphate (pH 7.5). After centrifugation at 35000*g* for 30 min, the resulting supernatant was applied to a DEAE-Toyopearl 650M column (1.5 × 7.5 cm) equilibrated with the same buffer as described above. Absorbed proteins were then eluted with a linear gradient from 20 to 50 mM sodium pyrophosphate. LMM fractions were collected and concentrated by salting out with ammonium sulfate.

Rabbit fast skeletal LMM was also prepared by the same method after isolation of myosin according to Lowey et al. (*10*).

Protein concentrations were determined by the biuret method using bovine serum albumin as the standard (*11*).

Analytical Methods. SDS−PAGE was carried out by the method of Laemmli (*12*), using 7.5−12.5% polyacrylamide gradient slab gels containing 0.1% SDS. Molecular weight markers (Sigma) were myosin heavy chain (205 kDa), *β*-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). 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 sequences were determined essentially according to Matsudaira (*13*). Proteins in the SDS−PAGE gels were electroblotted to Millipore polyvinylidene difluoride membranes. Protein bands were cut and put onto a Blott cartridge block and analyzed for the sequence with a Perkin-Elmer/Applied Biosystems model 476A protein sequencer.

DSC was performed with a MicroCal model MC2 differential scanning microcalorimeter essentially as described before (*7*). The solvent used was 20 mM Tris (pH 8.0) containing 0.6 M KCl, 5 mM $MgCl_2$, and 0.1 mM dithiothereitol (DTT). DSC scans were performed at a rate of 45 °C/h in the temperature range from 5 to 60 °C. DSC data were analyzed using a software package, Origin, developed by MicroCal. The subunit molecular weight of LMM was assumed to be 65 000 (*10*, *14*−*16*). After minimization by appropriate computer programs, the values for the thermal transition, i.e., the transition temperature (T_m), calorimeter enthalpy (ΔH_{cal}), van't Hoff enthalpy (ΔH_{vh}), and molar excess heat capacity (ΔC_p), could be obtained.

CD spectra were measured at various temperatures ranging from 4 to 60 °C in the same buffer as in DSC measurements with a JASCO J-600 spectropolarimeter.

RESULTS

Protein Characterization. LMM isolated from the 10 °C-acclimated carp (10-LMM) gave three bands in SDS−PAGE with apparent molecular masses of 69, 66, and 62 kDa, while LMM from the 30 °C-acclimated carp (30-LMM) showed four bands of 74, 69, 66, and 62 kDa (Figure 1), in good agreement with data reported previously (*17*). Rabbit fast skeletal LMM also exhibited a few bands with approximately the same molecular masses.

Imai et al. (*16*) isolated three cDNA clones encoding carp fast skeletal LMM. The deduced amino acid sequence from

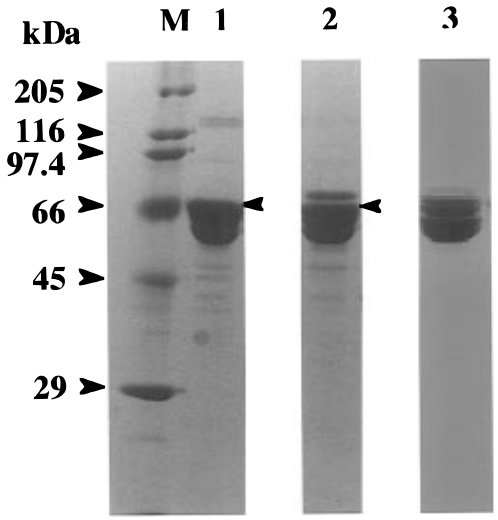

FIGURE 1: SDS−PAGE patterns of light meromyosins from carp acclimated to 10 and 30 °C, together with that of rabbit. LMM was prepared from fast skeletal muscle of carp acclimated to 10 °C (lane 1) and to 30 °C (lane 2) and from rabbit (lane 3) by DEAE-Toyopearl 650M ion-exchange chromatography. Proteins of 10 *μ*g/lane were applied to the 7.5−12.5% gradient polyacrylamide slab gels and stained with Coomassie Brilliant Blue R-250. Arrowheads indicate the major bands subjected to N-terminal amino acid sequence analysis. Molecular mass markers (M) used are myosin heavy chain (205 kDa), *β*-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

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                    5         10        15        20
Carp10      RAKYE TDAIQ RTEEL EESKK
10-LMM      ..... ..... ..... .....

Carp I      RAKYE TDAIQ RTEEL EEAKK
20-LMM      ..... ..... ..... .....

Carp30      RTKYE TDAIQ RTEEL EEAKK
30-LMM      ..... ..... ..... .....
30-LMM'     .T... ..... ..... .....
            (A)
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FIGURE 2: Comparison of N-terminal amino acid sequences of light meromyosin from carp acclimated to 10, 20, and 30 °C, with sequences deduced from cDNA clones encoding carp light meromyosin isoforms. The "Carp10" and "Carp30" represent amino acid sequences deduced from DNA nucleotide sequences for the 10 °C and 30 °C type LMM isoforms, respectively, whereas "Carp I" represents the intermediate-type LMM isoform (*16*). The 10-, 20-, and 30-LMMs denote typical LMM preparations purified from carp acclimated to 10, 20, and 30 °C, respectively, by DEAE-Toyopearl 650M ion-exchange chromatography, whereas the 30-LMM′ denotes another preparation from carp acclimated to 30 °C having mixed N-terminal amino acid sequences.

one of three clones, the 10 °C type, predominating in carp acclimated to 10 °C, contained alanine at the second residue and serine at the 18th residue from the N terminus (Figure 2). The second clone, the 30 °C type, predominating in carp acclimated to 30 °C, encoded threonine and alanine at corresponding positions. The last one, the intermediate type, showed an intermediate feature between the 10 and 30 °C types in both cDNA nucleotide and deduced amino acid sequences, having alanine residues at both the second and the 18th positions from the N terminus. Therefore, we determined N-terminal amino acid sequences for major bands each of the 10, 20, and 30 °C-acclimated carp LMMs. The N-terminal amino acid sequence of the major band for the 10-LMM indicated with an arrowhead in Figure 1 was identical with that of the 10 °C type LMM (Figure 2). One LMM preparation from the 30 °C-acclimated carp (30-

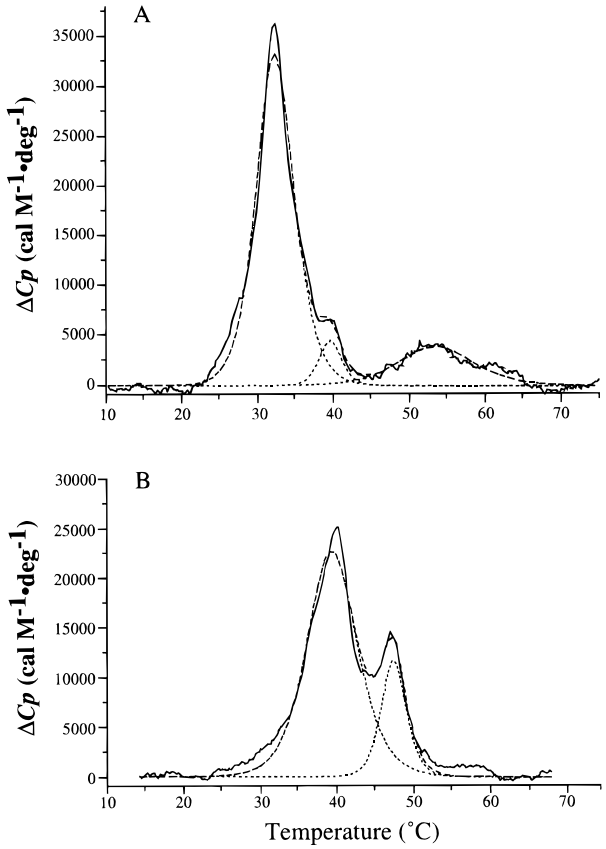

FIGURE 3: DSC scans of light meromyosin preparations from carp acclimated to 10 and 30 °C, together with their computer-calculated differential endotherms by the convolution analysis (dotted lines). The 10-LMM and 30-LMM shown in Figure 2 were used. DSC scans were performed in 50 mM Tris (pH 8.0) containing 0.6 M KCl, 5 mM $MgCl_2$, and 0.1 mM DTT at protein concentrations of 0.62 (A) and 0.79 (B) mg/mL. The scan rate was 45 °C/h, and data were collected every 15 s. ΔC_p represents excess molar heat capacity.

LMM), gave the same sequence as that of the 30 °C type for the major band shown in Figure 1 with an arrowhead. The other LMM preparation from the 30 °C-acclimated carp (30-LMM′) contained an additional component with a different sequence which was the same as the intermediate type, considering the presence of alanine at the second residue but no serine at the 18th residue. Although SDS−PAGE patterns are not shown, the LMM preparation from the 20 °C-acclimated carp (20-LMM) was found to contain the intermediate-type LMM, together with the 10 and 30 °C types, since the second residue was mixed with threonine and the 18th residue was mixed with serine.

DSC Scans. Results of DSC for the 10 and the 30 °C-acclimated carp LMMs are shown in Figure 3. The ordinate of this figure represents ΔC_p for the 65 000 kDa LMM subunit (see Materials and Methods). Two prominent endotherms having T_m at 32.5 and 39.5 °C with ΔH_{cal} of 269 and 52 kcal/mol, respectively, were found for the 10-LMM (Table 1). Myosin rod from the 10 °C-acclimated carp had roughly two endotherms of 33.0 and 44.0 °C as reported in our previous paper (*7*). It was noted that T_m of the 10-LMM at higher temperature was considerably lower than that of its rod counterpart, whereas T_m at lower temperature was comparable for both LMM and rod. The 10-LMM also exhibited a broad peak above 50 °C. However, this peak was not satisfactorily interpreted at present, since myosin rod from the 10 °C-acclimated carp had no peak in this temperature area (*7*).

Table 1: Thermodynamic Parameters on the Thermal Unfolding of Light Meromyosins from Carp Acclimated to 10, 20, and 30 °C, Together with Those of Rabbit Light Meromyosin

sample	T_m (°C)	ΔH_{cal} (kcal/mol)
10 °C-acclimated carp	32.5	269 (0.4)[a]
(10-LMM)[b]	39.5	52 (2.5)
20 °C-acclimated carp	34.5	150 (0.6)
(20-LMM)	40.2	20 (16.1)
	46.9	10 (30.4)
30 °C-acclimated carp[c]	39.2	231 (0.4)
(30-LMM)	47.3	39 (5.8)
30 °C-acclimated carp[d]	34.4	117 (1.0)
(30-LMM′)	39.5	123 (0.7)
	47.5	28 (8.9)
rabbit	42.5	184 (0.7)
	53.0	76 (2.2)

[a] Values in parentheses are ratios of ΔH_{vh} to ΔH_{cal}. [b] Letters in parentheses are abbreviations for carp LMM preparations used in Figure 2. [c] Values obtained from Figure 3B. [d] Values obtained from Figure 4.

The 30-LMM showed two peaks at 39.2 and 47.3 °C with ΔH_{cal} of 231 and 39, respectively (Figure 3 and Table 1). The ΔH_{cal} at 39.2 °C of the 30-LMM was comparable to that at 32.5 °C of the 10-LMM, whereas the ΔH_{cal} at 47.3 °C of the 30-LMM was comparable to that at 39.5 °C of the 10-LMM. Therefore, the T_m values of the two peaks from the 30-LMM were about 7 °C higher than those of the corresponding peaks from the 10-LMM. These results suggest that LMM preparations, whether from the 10 or 30 °C-acclimated carp, consist of two regions susceptible to thermal unfolding and that the α-helix of the 30-LMM is much more thermostable than that of the 10-LMM. In our previous study, myosin rod from the 30 °C-acclimated carp showed three endotherms at 34.5, 39.7, and 46.7 °C with ΔH_{cal} of 95, 115, and 159 kcal/mol, respectively (*7*). The higher two T_m values are comparable to the observed two T_m values of the 30-LMM at 39.2 and 47.3 °C as described before, although the ΔH_{cal} values are somewhat different between corresponding endotherms of myosin rod and LMM from the 30°C-acclimated carp.

When the other LMM preparation from the 30 °C-acclimated carp (30-LMM′) was subjected to DSC scans, four endotherms were observed (Figure 4). One endotherm at around 20 °C has not been observed in myosin or its rod at such a low-temperature area (*7*). However, three endotherms of 34.4, 39.7, and 47.5 °C for the 30 °C-acclimated carp LMM′ were roughly consistent with those of 34.5, 39.7, and 46.7 °C for myosin rod from the 30 °C-acclimated carp reported previously (*7*). Two endotherms of 39.5 and 47.5 °C for the 30-LMM′ were also comparable to those of 39.2 and 47.3 °C for the 30-LMM. Therefore, the lowest T_m at 34.4 °C was specific to the 30-LMM′.

Since the 30-LMM′ was suspected to be contaminated with the intermediate-type LMM from N-terminal amino acid sequence analysis (Figure 2), the 20-LMM was subjected to DSC scans in comparison with those of LMM preparations from carp acclimated to 10 and 30 °C (Figure 5). The 20-LMM showed an intermediate DSC pattern between the 10- and 30-LMMs showing three endotherms of 34.5, 40.2, and 46.9 °C with ΔH_{cal} of 152, 20, and 10 kcal/mol, respectively (Figure 5). Three T_m values were almost consistent with those obtained with the 30-LMM′ (see Figure 4). Since the ΔH_{cal} ratio of the 34.5 °C peak to the 40.2 °C peak for the 20-LMM was 7.6 and markedly higher than the ratio of peaks at 34.4 °C and 39.5 °C for the 30-LMM′ (1.0), it is likely

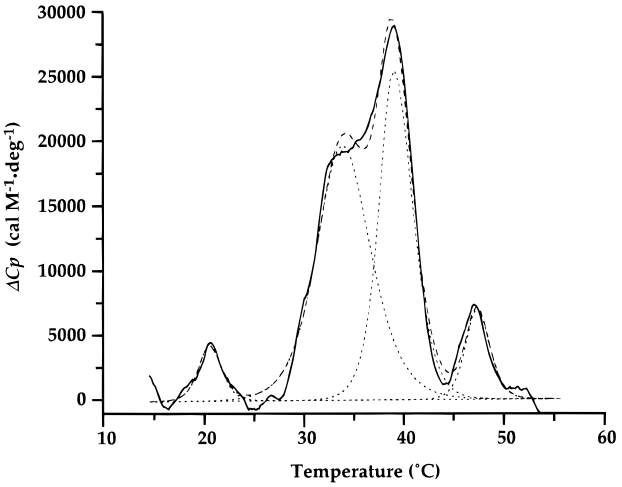

FIGURE 4: DSC scan of light meromyosin preparation from carp acclimated to 30 °C having mixed N-terminal amino acid sequences, together with its computer-calculated differential endotherms by the convolution analysis (dotted lines). The 30-LMM′ shown in Figure 2 was used for analysis at a protein concentration of 1.09 mg/mL. Refer to the legend of Figure 3 for other experimental conditions.

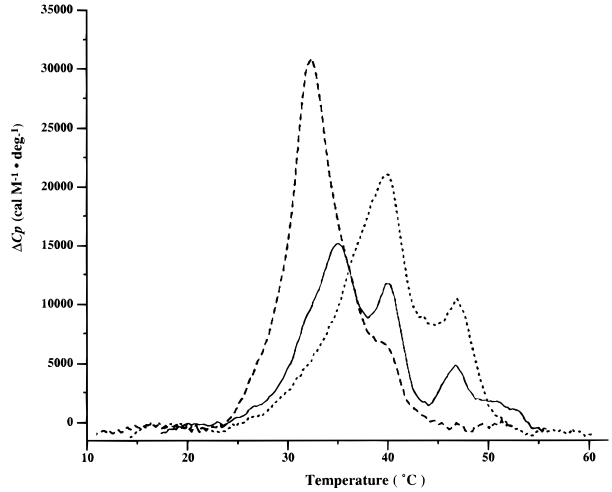

FIGURE 5: Comparison of DSC patterns for light meromyosins from carp acclimated to 10, 20, and 30 °C. Protein concentrations used were 0.62 mg/mL for the 10-LMM (dashed line), 1.10 mg/mL for the 20-LMM (solid line), and 0.79 mg/mL for the 30-LMM (dotted line). Refer to the legends of Figures 2 and 3 for abbreviations and other experimental conditions.

that the 34.4 °C-peak of the 30-LMM′ was derived from contaminating intermediate-type LMM which is expressed in a temperature range from 10 to 30 °C (*16*). On the other hand, the 46.9 °C peak of the 20-LMM seems to be specific to the 30 °C-type LMM. However, the lowest T_m of the 20-LMM at 34.5 °C was different from either a lower (32.5 °C) or a higher (39.5 °C) T_m value of the 10-LMM. Some unidentified types of LMM isoforms may be further present in the 20-LMM.

It was noted that the 20-LMM showed relatively small total enthalpy values compared with those of the 10- and 30-LMMs. However, these values were somewhat variable from 190 to 320 kcal/mol depending on samples prepared (data not shown), suggesting that the lower value for the 20-LMM in Figure 5 is probably not so much critical for comparison in the T_m value among three temperature-acclimated carp LMM preparations. In Table 1 are shown the typical results in order to compare T_m values and ΔH_{cal} ratios for different LMM preparations.

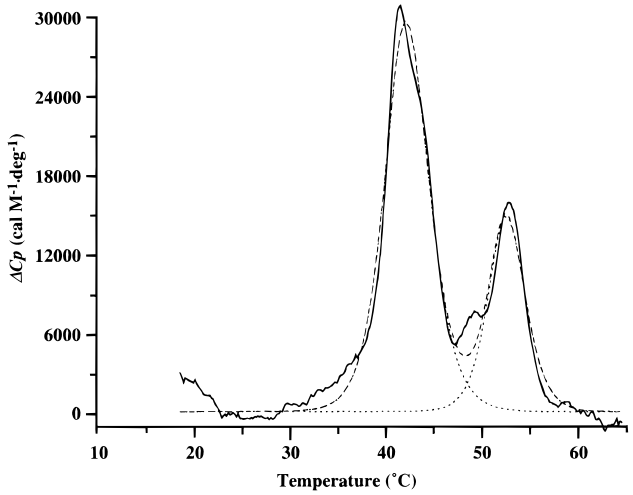

FIGURE 6: DSC scan of light meromyosin from rabbit fast skeletal muscle, together with its computer-calculated differential endotherms by the convolution analysis (dotted lines). The protein concentration was 0.8 mg/mL. Refer to the legend of Figure 3 for other experimental conditions.

To ascertain a possible intrinsic feature that LMM has two endotherms under our analytical conditions, LMM from rabbit fast skeletal muscle was subjected to DSC run. Rabbit LMM also exhibited two endotherms at 42.5 and 53.0 °C with ΔH_{cal} of 184 and 76 kcal/mol, respectively (Figure 6). Higher T_m values for both endotherms of rabbit LMM compared to those of corresponding endotherms of carp LMM even from the 30 °C-acclimated fish suggest that rabbit LMM is highly thermostable. It was noted that the ΔH_{cal} value of a lower T_m in rabbit was markedly larger than that of a higher T_m as in the cases of the 10- and 30-LMMs. Bertazzon and Tsong (*6*) obtained endotherms for rabbit LMM at 42.4, 48.8, 49.0, 53.7, and 54.8 with ΔH_{cal} of 165, 70, 112, 63, and 203 kcal/mol in 20 mM potassium phosphate buffer (pH 7.03) containing 0.5 M KCl. Since we observed similar DSC patterns in both carp myosin and its rod with 0.6 M KCl in 20 mM Tris at pH 8.0 and in 20 mM potassium phosphate at pH 6.5 (*7*), differences in DSC patterns for rabbit LMM between data reported by Bertazzon and Tsong (*6*) and from the present study are probably caused by differences in the deconvolution computer analysis adopted.

CD Spectroscopy. In our previous study, it was demonstrated that T_m values in DSC runs for carp myosin and its rod were almost consistent with temperatures for unfolding of α-helix (*7*). In the present study, two preparations of carp LMM, the 10-LMM and the 30-LMM′, were subjected to CD spectrometry. Typical CD spectra are shown in Figure 7. When measured at 21 °C, both the 10-LMM and the 30-LMM′ exhibited a typical pattern of α-helix having two minima at 222 and 208 nm. Assuming that 100% of helical content corresponds to a $[\theta]$ value of $-36\,000$ deg cm^{-2} dmol at 222 nm (*18*), both carp LMM preparations were calculated to have α-helical content over 90%. It has been reported that rabbit skeletal LMM contains 90% α-helix (*10*). According to the increase of measuring temperatures, the minimum at 222 nm was gradually decreased, suggesting a temperature-dependent unfolding of α-helix. No minimum at 222 nm was observed over 50 °C. Once treated at 52.6 °C and subsequently returned to 19.0 °C, the 10-LMM again showed a typical pattern of α-helix, recovering its content to about 70%.

Plots of α-helical content against measuring temperatures for the 10-LMM and the 30-LMM′ are shown in Figure 8.

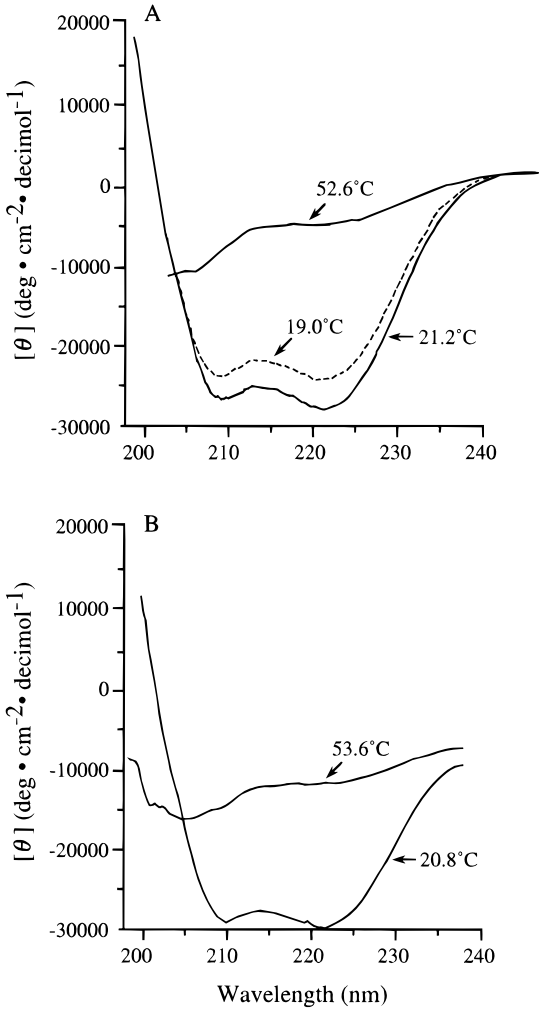

FIGURE 7: CD spectra of light meromyosins from carp acclimated to 10 and 30 °C. CD spectrometry was performed at various temperatures with the 10-LMM and 30-LMM′ (see Figures 3 and 4, respectively) at protein concentrations of 0.62 mg/mL (A) and 1.09 mg/mL (B), respectively, in 50 mM Tris (pH 8.0) containing 0.6 M KCl, 5 mM $MgCl_2$, and 1 mM DTT. Typical results at about 20 and 50 °C are shown. Reversibility was examined for the 10-LMM once heated at 52.6 °C and subsequently measured at 19.0 °C (dotted line). [θ] represents the mean residue ellipticity.

Decreasing rate derivatives of α-helical content against temperatures for both carp LMM preparations are also superimposed in Figure 8. The decreasing rate maxima were observed at 31 and 37 °C with the 10-LMM, whereas the 30-LMM′ showed rate maxima at 30, 33, 37, and 47 °C. Except for the peak at 30 °C of the decreasing rate derivative for the 30-LMM′, all other temperatures showing the rate maxima for the 10-LMM and the 30-LMM′ were fairly consistent with the T_m values in DSC runs (Table 1). These results suggest that endotherms of LMM mainly result from unfolding of α-helix.

DISCUSSION

Carp expresses different myosin heavy chain isoforms of fast skeletal muscle in association with temperature acclimation (*8*, *19*−*21*). Such changes of carp myosin are regulated at the genetic level (*16*, *17*, *22*, *23*). Guo et al. (*21*) demonstrated that at least three heavy chains of myosin subfragment-1 with different enzymatic properties could be separated by ion-exchange column chromatography.

Imai et al. (*16*) cloned three different cDNAs which encoded carp myosin heavy chain. Three clones were

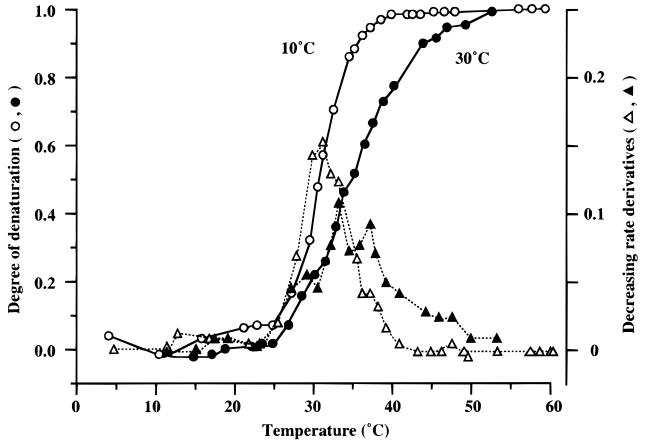

FIGURE 8: Comparison of the decreasing rate derivatives of the mean residue ellipticity, [θ], against measuring temperatures for light meromyosins from carp acclimated to 10 (△) and 30 °C (▲). The decreasing rate derivatives were calculated from the increment of [θ] at 222 nm per unit change of temperature (°C) for the 10-LMM (○) in Figure 3 and for the 30-LMM′ (●) in Figure 4.

identified to be the 10 and 30 °C types, together with the intermediate type having an intermediate feature between the former two types, according to the alignment of deduced amino acid sequences to those determined by N-terminal amino acid sequence analyses for LMM isolated from thermally acclimated carp (*17*). The 10 and 30 °C types mean dominant myosin heavy chain types in the 10 and 30 °C-acclimated carp, respectively, although exact compositions of different myosin heavy chain types in carp acclimated to different water temperatures have remained unknown.

In the preceding paper, different DSC patterns between the 10 and 30 °C-acclimated carp were observed with myosin and its rod (*7*). For example, two endotherms were observed with myosin rod from the 10 °C-acclimated carp, whereas three endotherms were observed with rod from the 30 °C-acclimated fish. In this study, LMM, a C-terminal half of myosin rod, was prepared from thermally acclimated carp and subjected to DSC analysis. A subunit molecular weight of 65 000 was employed to show ΔC_p values for one α-helical molecule. It is well-known that LMM is composed of a coiled-coil structure of two α-helices. The 10-LMM showed two endotherms with the T_m values roughly corresponding to those of myosin rod from the 10 °C-acclimated carp, suggesting that endotherms of myosin rod are mainly attributable to those of LMM. Therefore, it seems that the structure−function relationship of LMM would be much easier to understand in terms of thermodynamic properties, since the primary structures of LMM isoforms from thermally acclimated carp are available (*16*). However, LMM from the 30 °C-acclimated carp showed different patterns according to preparations. One LMM preparation, the 30-LMM′, had three endotherms as in the case of myosin rod from the 30 °C-acclimated carp, and the other, the 30-LMM, had only two endotherms as the case of the 10-LMM. DSC runs on 20-LMM explained well the lowest T_m in the 30-LMM′ which was assigned to be that of the intermediate-type LMM. Different patterns for LMM preparations from the 30 °C-acclimated carp might be caused by slightly different feeding conditions on carp unconsciously adopted. It has been reported that temperature acclimation-associated changes in myofibrillar ATPase are not observed in starved carp (*24*). Alternatively, different degrees of α-chymotryptic digestion

might lead to such different patterns, since carp myosin rod has multiple proteolytic cleavage sites both in N- and C-terminal regions of LMM. As shown in Figure 1, LMM preparations from carp fast skeletal muscle were not homogeneous in terms of the molecular weight. It has been claimed that LMM fragments with different molecular weights from carp fast skeletal muscle have differences in the C-terminal length (*17*). Additional differences probably result from multiple proteolytic cleavage sites in the LMM: subfragment-2 junction of myosin rod as in the case of rabbit fast skeletal muscle (*25*). In relation to these facts, we have observed that myosin rods prepared from the 10 and 30 °C-acclimated carp show no difference in α-chymotryptic susceptibility (*26*). Nevertheless, differences in the T_m value among LMM preparations from the 10, 20, and 30 °C-acclimated carp were clear, suggesting that these values are inherent to respective LMM isoforms expressed in an acclimation temperature-dependent manner, possibly from the 10 °C, intermediate-, and 30 °C type genes. It has been demonstrated that these isoforms are encoded by different genes in carp (*16*, *17*, *22*).

DSC patterns with two endotherms were typical under our experimental conditions for not only carp but also rabbit skeletal LMM. Therefore, it is likely that the T_m values of 32.5 and 39.5 °C with ΔH_{cal} of 269 and 52 kcal/mol, respectively, are intrinsic to the 10 °C type LMM, whereas those of 39.2 and 47.3 °C with ΔH_{cal} of 231 and 39 kcal/mol, respectively, to the 30 °C type LMM. Differences in the T_m values of about 7 °C for corresponding endotherms between the 10 °C and 30 °C type LMMs were due to differences in thermal stability of α-helix, which are probably reflected by different primary structures. LMM has a coiled-coil structure of α-helix. Its primary structure has been well established and contains heptad repeats of amino acid residues, a, b, c, d, e, f, and g, where positions of a and d are occupied by hydrophobic amino acid residues (*27*). In addition, heptad repeats further construct 28-residue units often containing skip residues for the best alignment (*27*−*29*). We have also determined the primary structures of the 10 °C and 30 °C type LMMs by deducing cDNA nucleotide sequences (*16*). Amino acid sequence identity between the two types was very high, accounting for 95.6%. However, variations distributed to the whole LMM molecule, suggesting that some region(s) of the molecule or even some amino acid residue(s) are responsible for differences in T_m values of about 7 °C between the two types of carp LMM. The variation of thermal stability of myosin rod was shown earlier for various species living at widely different temperatures (*30*). Since the variation in amino acid sequence is only 4.4% between the 10 and 30 °C type carp LMMs as described before, it seems to be a good model for investigating the relationship between the structure and thermostability of LMM. It has been shown by techniques of molecular biology and electron microscopy that LMM is extremely important for the formation of myosin filaments (*31*−*33*). Bacterial expression of carp LMM and site-directed mutagenesis are currently being carried out to identify regions responsible for different thermal stabilities which possibly compensate for seasonal fluctuations of water temperature that carp encounters in natural habitat.

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