J. Biochem. 120, 788-791 (1996)

Lower Activation Energy for Sliding of F-Actin on a Less Thermostable Isoform of Carp Myosin

Shigeru Chaen,*[,1] Misako Nakaya,[†] Xiao-Feng Guo,[†] and Shugo Watabe[†]

Department of Physiology, School of Medicine, Teikyo University, Itabashi-ku, Tokyo 173; and †Laboratory of Aquatic Molecular Biology and Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113

Received for publication, May 27, 1996

We have examined the temperature-dependence of sliding velocity of fluorescent F-actin on myosins isolated from 10°C- and 30°C-acclimated carp. Activation energies for the sliding of F-actin were 63 and 111 kJ/mol for the 10°C- and 30°C-acclimated carp myosins, respectively. Arrhenius plots of the sliding velocity from 10°C- and 30°C-acclimated carp myosin were shown to intersect at high temperature (about 30°C). The thermostability estimated by measuring the Ca^{2+}-ATPase activity was less for myosin from 10°C- than 30°C-acclimated carp. We suggest that a less thermostable structure in cold-acclimated carp myosin results in a reduced activation energy for the contractile process, which allows the F-actin to slide fast even at low temperatures.

Key words: activation energy, carp myosin, *in vitro* motility assay, temperature acclimation, thermostability.

Changes in physiological and biochemical properties induced by environmental temperature change have been extensively studied in eurythermal temperate fish such as carp and goldfish. These fish experience a change in the environmental temperatures from 0-4°C in winter to above 30°C in summer. But even at low temperature in winter, they show the same locomotor activity as that at high temperature in summer. Maximum cruising speed of goldfish acclimated to cold temperature is increased at low temperature compared with the warm-acclimated goldfish (*1*). Changes in the fish myofibrillar Mg^{2+}-ATPase activity and its thermostability have been shown to be involved in the temperature adaptation: the acclimation to cold temperature led to an increase in the myofibrillar Mg^{2+}-ATPase activity and a decrease in the thermostability of the myofibrillar ATPase activity (*2, 3*). Physiological properties such as maximum shortening velocity and isometric force development of the skinned muscle fibers were also increased in the muscle from cold-acclimated as compared with warm-acclimated carp (*4*). In addition, changes in the regulatory proteins (*5, 6*), myosin light chain ratio (*7*) and Ca^{2+}-ATPase activity of sarcoplasmic reticulum (*8, 9*) were reported to be involved. According to Gerlach *et al.* (*10*), different MHC isoform genes were expressed in carp muscle at warm and cold environmental temperatures.

Fish skeletal myosins have similar structures to those of mammalian myosins, consisting of four light chains with an approximate molecular weight of 20 kDa and two heavy chains of about 200 kDa (*11-13*). Hwang *et al.* (*14, 15*) have shown that specific isoforms of the myosin are expressed in association with cold- and warm-temperature acclimation, and myosins from the cold-acclimated carp increase their ATPase activities with a concomitant decrease in their thermostability in comparison with the myosin from the warm-acclimated carp.

In the present study, in order to elucidate the molecular mechanism that maintains locomotor ability even at low temperature for the cold-acclimated carp, we focused on the properties of the isolated myosin isoforms from cold- and warm acclimated carp by measuring the velocity of fluorescent F-actin sliding on the isoforms, of which the thermostabilities are greatly different. The thermostability of enzymes has been reported to correlate with the flexibility of the protein molecule (*16-18*). The more thermostable structure the protein has, the less flexible motion it shows. Thus, this study was designed to examine the correlation between the thermostability of the carp myosin isoform and its functional activity. We demonstrate that the activation energy for the sliding of fluorescent F-actin on myosin from cold-acclimated carp is much less than that on myosin from warm-acclimated carp, suggesting that the low activation barrier for the F-actin sliding on the myosin isoform expressed at low temperature is one of the evolved strategies for sustaining the locomotor activity.

EXPERIMENTAL PROCEDURES

Materials—Carp, *Cyprinus carpio* (0.6-0.8 kg in body weight) were acclimated to either 10 or 30°C for a minimum of 5 weeks, and their dorsal fast muscles were used for preparation of myosin. Myosin isoforms from 10°C- and 30°C-acclimated carp were prepared according to the method previously described (*14*). Carp myosin isoforms (15 mg/ml) in a solution containing 0.5 M KCl, 20 mM Tris-maleate (pH 7.5), 0.1 mM DTT, and 10% sucrose

[1] To whom correspondence should be addressed. Tel: +81-3-3964-1211 (Ext. 2155), Fax: +81-3-5375-8789, E-mail: chaen@med.teikyo-u.ac.jp

Abbreviations: MHC, myosin heavy chain; K_D, rate constant for thermal inactivation.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

were stored at −80°C after quick freezing in liquid nitrogen. Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt (*19*). ATP, BSA, glucose oxidase, catalase, and methyl cellulose were from Sigma. Other chemicals were of analytical grade.

Measurement of Thermostability—Thermostability of carp myosin was determined by measuring the remaining Ca^{2+}-ATPase activity of the myosin after heat treatment at 30°C for 0-20 min in a medium containing 20 mM Tris-maleate, pH 7.5, 0.5 M KCl, and 0.1 mM DTT (*20*). The Ca^{2+}-ATPase activity was measured in a reaction mixture containing 25 mM Tris-maleate, pH 7.0, 0.5 M KCl, 5 mM $CaCl_2$, 1 mM ATP, and 0.5 mg/ml myosin at 20°C. The ATPase reaction was initiated by the addition of ATP and stopped with 5% trichloroacetic acid. Liberated inorganic phosphate was measured by the method of Fiske and Subbarow (*21*). As a parameter of the thermostability, the inactivation rate constant K_D (*20*), defined as the rate of inactivation (s^{-1}), was calculated from the remaining activity *versus* incubation time.

Measurement of the Sliding Velocity of F-Actin on Carp Myosin—Carp myosin (100 μg/ml) in a high salt solution (0.5 M KCl, 20 mM PIPES, pH 7.0, 2 mM $MgCl_2$, and 0.1 mM DTT) was perfused into a flow cell made of a nitrocellulose-coated glass coverslip. After 5 min, the flow cell was rinsed with the high salt solution including 1 mg/ml BSA, then left for 5 min, and finally 10 nM F-actin filaments labeled with rhodamine-conjugated phalloidin (Molecular Probe, Eugene, OR) in a motility buffer (25 mM KCl, 25 mM imidazole, pH 7.5, 2 mM ATP, 4 mM $MgCl_2$, 1 mM EGTA, 10 mM DTT, 1 mg/ml BSA, 0.2% methyl cellulose, 1% β-mercaptoethanol, 4.5 mg/ml glucose, 210 μg/ml glucose oxidase, and 35 μg/ml catalase) were perfused into the flow cell. All these treatments were performed at 0°C on ice. The flow cell was then equilibrated for 5 min between the temperature-controlled slide carrier and the temperature-jacketed objective of an inverted microscope. Methyl cellulose was required to observe the F-actin sliding on both isoform preparations. Fluorescent F-actin filaments were observed with the inverted microscope (Axiovert 35; Zeiss, Germany) equipped with epifluorescence optics, a Zeiss Neofluoar ×100 objective (N.A. 1.3) and SIT camera (C2400-08; Hamamatsu Photonics, Hamamatsu), and recorded on S-VHS videotape (AG7355; Panasonic, Osaka). Selected video frames were digitized with a frame grabber (LG-3; Scion, Frederick, MD) and NIH Image (public domain program for the image analysis, written by Wayne Rasband, NIH) on a personal computer (Power Macintosh 8100/80AV; Apple Computer Japan, Tokyo). Sliding velocity of fluorescent F-actin was calculated by measuring the displacement of the filament between successive video frames using a macro program for tracking (written by K. Anderson, R. Cross, and C.R. Bagshaw) in NIH Image. Before the experiment, temperatures were checked by inserting a fine thermo-couple needle (Hayashi Denko, Tokyo) into the flow cell.

Experiments were carried out at 3, 8, 13, 18, and 23± 0.5°C using a temperature-controlled slide carrier and temperature-jacketed objective.

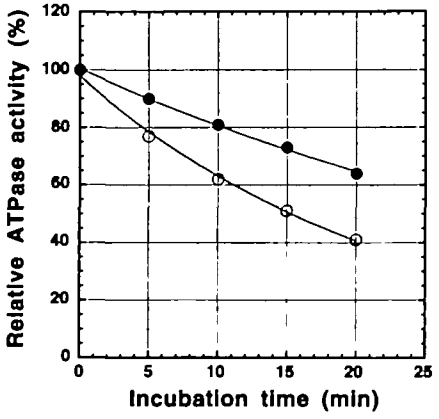

Fig. 1. **Remaining myosin Ca^{2+}-ATPase activity from carp acclimated to 10°C (open circles) and 30°C (closed circles).** The Ca^{2+}-ATPase activities were measured after incubating myosin isoforms at 30°C for 0-20 min. Lines are regression lines drawn to fit the data.

RESULTS

As a measure of the thermostability of the carp myosin isoform, the remaining Ca^{2+}-ATPase activity of the myosin after heat treatment at 30°C was examined. Figure 1 shows the remaining Ca^{2+}-ATPase activity, after incubation at 30°C for 0-20 min for 10°C, and 30°C-acclimated carp myosin. The Ca^{2+}-ATPase activity of 10°C-acclimated carp myosin was inactivated faster than that of 30°C-acclimated carp myosin. The inactivation rate constant (K_D) was calculated to be 7.5×10^{-4} and 3.7×10^{-4} s^{-1} for 10°C- and 30°C-acclimated carp myosin isoforms, respectively.

The sliding velocities of F-actin on 10°C- and 30°C-acclimated carp myosin were measured at various temperatures (Table I and Fig. 2). Video recording of F-actin sliding at 3, 8, 13, 18, and 23°C was done for about 17, 17, 10, 6, and 4 min, respectively. In each experiment at various temperatures for both myosin isoforms, there was little change in the velocity between the record taken at the beginning and at the end of the observation period. Figure 2 shows histograms of the sliding velocities of fluorescent F-actin filaments on both carp myosin isoforms at 3, 8, 13, 18, and 23°C. Broadness of the distribution, *i.e.*, the standard deviation of the data was compatible with that using rabbit skeletal myosin and heavy meromyosin (*22-24*). Figure 3 shows the Arrhenius plot of the sliding velocity of fluorescent F-actin on 10°C- and 30°C-acclimated carp myosin. The data from both myosin preparations were fitted to a regression line without an inflection point. The sliding

TABLE I. **F-Actin filament velocity on myosin from carp acclimated to 10°C and 30°C at various temperatures.** Conditions and methods for measuring the velocity of fluorescent F-actin filament sliding on carp myosin isoforms were described in the "EXPERIMENTAL PROCEDURES." The velocities were expressed as the average and standard deviation (SD) with the number of velocity data (n) calculated between the video frames, and from the number of filaments (N_f).

Temp. (°C)	Filament velocity, μm/s					
	10°C-acclimated myosin, Mean±SD	n	(N_f)	30°C-acclimated myosin Mean±SD	n	(N_f)
3	0.49±0.18	178	(20)	0.088±0.043	235	(23)
8	0.71±0.24	202	(25)	0.24 ±0.096	191	(35)
13	1.14±0.32	114	(21)	0.50 ±0.23	154	(42)
18	1.83±0.52	136	(34)	1.19 ±0.43	108	(36)
23	3.32±1.06	99	(35)	2.25 ±0.74	104	(45)

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

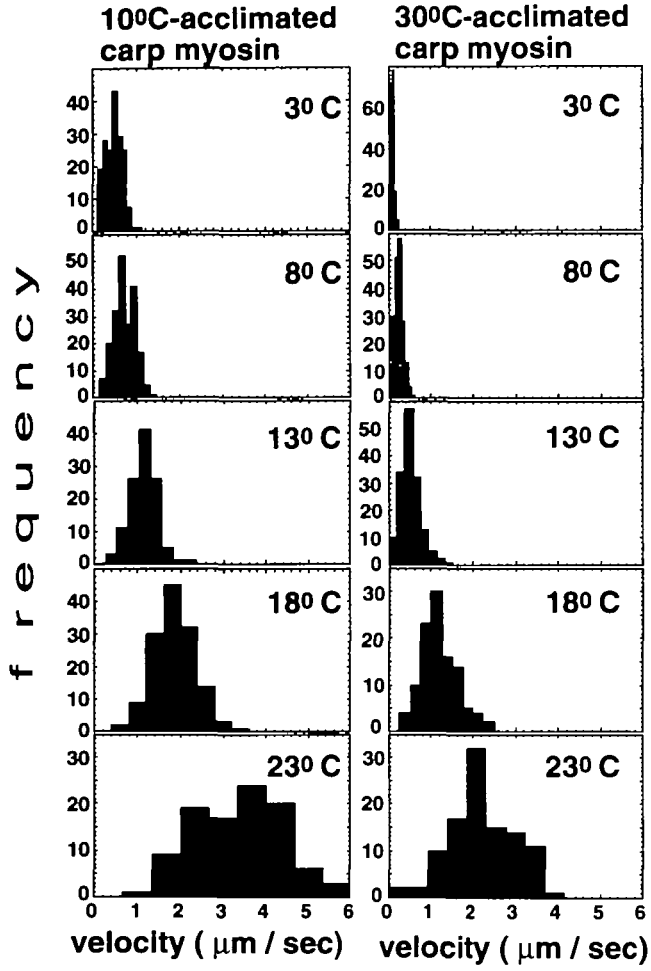

Fig. 2. **Histograms comparing the sliding velocities of fluorescent F-actin filaments over 10°C- (left five panels) and 30°C-acclimated carp myosin (right five panels), at 3, 8, 13, 18, and 23°C (from upper to bottom panels).** The number of classes in each histogram (N) was chosen by use of the equation, $N \cong 1+3.3 \log n$, where n is the number of data for each experiment.

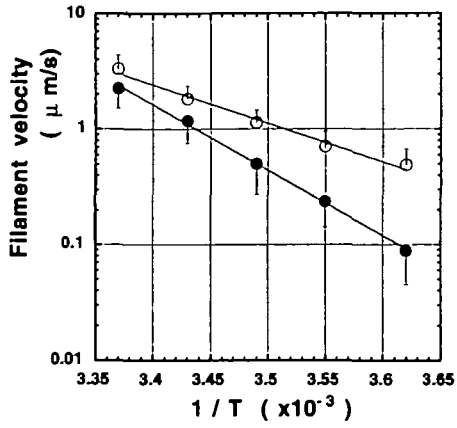

Fig. 3. **Arrhenius plot of the sliding velocity of fluorescent F-actin on myosin preparations from carp acclimated to 10°C (open circle) and 30°C (closed circle).** Each data point represents the average and standard deviation. Lines are regression lines drawn to fit the data.

velocity at 3°C for the 10°C-acclimated carp myosin approximately corresponds to that at 13°C for the 30°C-acclimated carp myosin. At 8°C, the filament velocity was 3 times higher for myosin from 10°C- than 30°C-acclimated carp. The point at which the Arrhenius plots from the 10°C- and 30°C-acclimated carp myosin intersect was calculated to be about 4 μm/s at about 30°C. The value of activation energy for the sliding velocity of F-actin on the 10°C- and 30°C-acclimated carp myosin were calculated to be 63 and 111 kJ/mol, respectively.

DISCUSSION

The present study was undertaken to obtain an insight into the correlation between the thermostability of myosin and the rate of its mechanical process, using myosin isoforms from carp acclimated to different environmental temperatures. It has been shown that myosin from cold-acclimated carp has higher ATPase activity and reduced thermostability (*13, 14*).

The data presented show that the energy barrier for the sliding of F-actin on 10°C-acclimated carp myosin is much lower than that on 30°C-acclimated carp myosin, and the thermostability of the 10°C-acclimated carp myosin is decreased in comparison with that of the 30°C-acclimated carp myosin. Inactivation of 10°C-acclimated carp myosin during the experiment at higher temperatures could explain the lower velocity at high temperature than expected if the activation energy is assumed to be the same as that of 30°C-acclimated carp myosin. However, from the result that there was little change in the velocity between the beginning and the end of the observation period, and the report that addition of ATP and F-actin reduced the thermal denaturation of myosin (*20, 25-28*), it seems unlikely that sufficient inactivation occurred to influence the sliding velocity. Johnston *et al.* (*2*) reported the value of the activation energy for goldfish myofibrillar Mg^{2+}-ATPase activity to be 14.3 and 21.9 kcal/mol for cold- and warm-acclimated goldfish, respectively. Based on these data, Johnston (*5*) has concluded that regulatory proteins in the muscle have properties responsible for the change in the activation energy for Mg^{2+}-ATPase activity, since there was no difference in the activation energy between the two actomyosin preparations without regulatory proteins. However, the difference in the activation energy for F-actin sliding between cold- and warm-acclimated myosin in the absence of regulatory proteins in this study is similar to that of goldfish myofibrillar Mg^{2+}-ATPase activity. Since myosin isoforms from thermally acclimated carp have different actin-activated Mg^{2+}-ATPase activities (*14, 29*), our data strongly suggest that differences in the enzymatic properties between carp myosin isoforms are responsible for the change in the activation energy for mechanical processes.

The dynamics of the structural fluctuation of the protein molecule and its functional activities are closely related (*30-33*). It has also been observed that a less thermostable protein has a more flexible structure (*16-18*). Varley and Pain (*18*) have shown that at a given temperature a thermophilic enzyme is more stable, and the activation energy for acrylamide quenching of tryptophan fluorescence is lower for a mesophilic enzyme than that for a thermophilic enzyme. While the conformational dynamics

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

of the carp myosin isoforms remain to be determined by an appropriate method, *e.g.*, hydrogen-deuterium exchange, fluorescence quenching, and so on, it would not be surprising if the less thermostable carp myosin isoform has a more flexible structure. In cold-acclimated carp myosin, a more flexible structure related to the lower thermostability would be a possible reason for the lower activation energy for the sliding process. This low energy barrier for the sliding process would make it possible for carp living at low temperature to swim efficiently.

Recently, Nakaya *et al.* (*34*) reported that a differential scanning calorimetric study of a rod portion prepared from a cold-acclimated carp myosin isoform shows less thermostability than that from a warm-acclimated myosin isoform. Therefore, the structures responsible for the changes in thermostability between the isoforms are considered to involve the entire region of the myosin molecule, although the contributions of restricted regions of the molecule to the changes in the thermostability may vary. Studies on determination of the differences in the primary structure between the two isoforms are now in progress.

We wish to thank Professor Haruo Sugi (Teikyo University, Tokyo) for his support and encouragement, Dr. Ian A. Johnston (University of St. Andrews, St. Andrews) for his valuable comments on the manuscript, and Dr. Clive R. Bagshaw (University of Leicester, Leicester) for help with the macro program for tracking F-actin on NIH Image.

REFERENCES

1. Fry, F.E.J. and Hart, J.S. (1948) Cruising speed of goldfish in relation to water temperature. *J. Fish Res. Bd. Canada* **7**, 169-175
2. Johnston, I.A., Davidson, W., and Goldspink, G. (1975) Adaptation in Mg^{2+}-activated myofibrillar ATPase activity induced by temperature acclimation. *FEBS Lett.* **50**, 293-295
3. Heap, S.P., Watt, P.W., and Goldspink, G. (1985) Consequences of thermal change on the myofibrilar ATPase of five freshwater teleosts. *J. Fish Biol.* **26**, 733-753
4. Johnston, I.A., Sidell B.D., and Driedzic, W.R. (1985) Force-velocity characteristics and metabolism of carp muscle fibers following temperature acclimation. *J. Exp. Biol.* **119**, 239-249
5. Johnston, I.A. (1979) Calcium regulatory proteins and temperature acclimation of actomyosin ATPase from a eurythermal teleost (*Carassius auratus* L.) *J. Comp. Physiol.* **B129**, 163-167
6. Penney, R.K. and Goldspink, G. (1981) Regulatory protein and thermostability of myofibrillar ATPase in acclimated goldfish. *Comp. Biochem. Physiol.* **69B**, 577-583
7. Crockford, T. and Johnston, I.A. (1990) Temperature acclimation and the expression of contractile protein isoforms in the skeletal muscles of the common carp (*Carassius auratus* L.). *J. Comp. Physiol. B* **160**, 23-30
8. Fleming, J.R., Crockford, T., Altringham, J.D., and Johnston, I.A. (1990) Effects of temperature acclimation on muscle relaxation in the carp: A mechanical, biochemical, and ultrastructural study. *J. Exp. Zool.* **255**, 286-295
9. Ushio, H. and Watabe, S. (1993) Effects of temperature acclimation on Ca^{2+}-ATPase of the carp sarcoplasmic reticulum. *J. Exp. Zool.* **265**, 9-17
10. Gerlach, G.G., 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.* **259**, R237-R244
11. Connell, J.J. (1958) Studies on the proteins of fish skeletal muscle. 5. Molecular weight and shape of cold myofibrillar proteins. *Biochem. J.* **70**, 81-91
12. Huriaux, F. and Focant, B. (1978) Effect of some factors on the molecular weight determination of a light chain (LC3) of carp (*Carassius auratus* L.) skeletal muscle myosin by SDS-polyacrylamide gel electrophoresis. *Comp. Biochem. Physiol.* **61B**, 195-198
13. Watabe, S. and Hashimoto, K. (1980) Myosins from white and dark muscles of mackerel—Some physico-chemical and enzymatic properties. *J. Biochem.* **82**, 1491-1499
14. Hwang, G.-C., Watabe, S., and Hashimoto, K. (1990) Changes in carp myosin ATPase induced by temperature acclimation. *J. Comp. Physiol. B* **160**, 233-239
15. Hwang, G.-C., Ochiai, Y., Watabe, S., and Hashimoto, K. (1991) Changes of carp myosin subfragment-1 induced by temperature acclimation. *J. Comp. Physiol. B* **161**, 141-146
16. Delpierre, M., Dopson, C.M., Selvarajah, S., and Weldin, R.E. (1983) Correlation of hydrogen exchange behaviour and thermal stability of lysozyme. *J. Mol. Biol.* **168**, 687-692
17. Wrba, A., Schweiger, A., Schultes, V., Jaenicke, R., and Závodszky, P. (1990) Extremely thermostable-glyceraldehyde-3-phosphate dehydrogenase from the eubacterium *Thermotoga maritima*. *Biochemistry* **29**, 7584-7592
18. Varley, P.G. and Pain, R.H. (1991) Relation between stability, dynamics and enzyme activity in 3-phosphoglycerate kinase from yeast and *Thermus thermophilus*. *J. Mol. Biol.* **220**, 531-538
19. Spudich, J.A. and Watt, S. (1971) The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **246**, 4866-4871
20. Yasui, T., Kawakami, H., and Morita, F. (1968) Thermal inactivation of myosin A-adenosine triphosphatase in the presence of F-actin. *Agric. Biol. Chem.* **32**, 225-233
21. Fiske, C.H. and Subbarow, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**, 375-400
22. Kron, S.J. and Spudich, J.A. (1986) Fluorescent actin filaments move on myosin fixed to a glass surface. *Proc. Natl. Acad. Sci. USA* **83**, 6272-6276
23. Toyoshima, Y.Y., Kron, S.J., McNally, E.M., Niebling, K.R., Toyoshima, C., and Spudich, J.A. (1987) Myosin subfragment-1 is sufficient to move actin filaments in vitro. *Nature* **328**, 536-539
24. Homsher, E., Wang, F., and Sellers, J.R. (1992) Factors affecting movement of F-actin filaments propelled by skeletal muscle heavy meromyosin. *J. Am. Physiol.* **262**, C714-C723
25. Ouellet, L., Laidler, K.J., and Morales, M.F. (1952) Molecular kinetics of muscle adenosine triphosphatase. *Arch. Biochem. Biophys.* **32**, 37-50
26. Lowey, S. and Holtzer, A. (1959) The aggregation of myosin. *J. Am. Chem. Soc.* **81**, 1378-1383
27. Blum, J.J. (1960) Interaction between myosin and its substrates. *Arch. Biochem. Biophys.* **87**, 104-119
28. Mackie, I.M. (1966) The effect of adenosine triphosphate, inorganic triphosphate on the stability of cod myosin. *Biochim. Biophys. Acta* **115**, 160-172
29. Guo, X.-F., Nakaya, M., and Watabe, S. (1994) Myosin subfragment-1 isoforms having different heavy chain structures from fast skeletal muscle of thermally acclimated carp. *J. Biochem.* **116**, 728-735
30. Huber, R. (1979) Conformational flexibility and its functional significance in some protein molecule. *Trends Biochem. Sci.* **4**, 271-274
31. Welch, G.R., Somogyi, B., and Damjanovich, S. (1982) The role of protein fluctuation in enzyme action: A review. *Prog. Biophys. Mol. Biol.* **39**, 109-146
32. Karplus, M. and McCammon, J.A. (1983) Dynamics of proteins: Elements and function. *Annu. Rev. Biochem.* **53**, 263-300
33. Brooks, C.L., III, Karplus, M., and Pettitt, B.M. (1988) *Proteins: A Theoretical Perspective of Dynamics, Structure, and Thermodynamics*, pp. 1-21, John Wiley and Sons, New York
34. Nakaya, M., Watabe, S., and Ooi, T. (1995) Differences in the thermal stability of acclimation temperature-associated types of carp myosin and its rod on differential scanning calorimetry. *Biochemistry* **34**, 3114-3120

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012