Early Structural Changes in Myosin Rod upon Heating of Carp Myofibrils

Kunihiko Konno,* Takeshi Yamamoto, Masayuki Takahashi, and Sanae Kato

Laboratory of Food Biochemistry, Faculty of Fisheries, Hokkaido University, Hakodate, Hokkaido 041-8611 Japan

Upon heating carp myofibrils at 40 °C, the amount of myosin that is soluble and monomeric dropped very quickly, roughly 5 times faster than the ATPase inactivation. This rapid decrease of solubility was well explained by a rapid denaturation of the rod portion as measured by chymotryptic digestibility. Chymotryptic digestion of heated myofibrils in a low-salt medium with EDTA generated a reduced amount of rod and subfragment-1 (S-1). The decrease of S-1 produced from the heated myofibrils was consistent with the ATPase inactivation. The decrease of rod produced from the heated myofibrils was explained by the increased susceptibility of the heavy meromyosin (HMM)/light meromyosin (LMM) junction to chymotryptic. It was, therefore, concluded that the fastest event occurring in the myosin molecule upon heating of myofibrils is the irreversible exposure of the HMM/LMM junction.

Keywords: *Myosin; denaturation; myofibril; gelation; carp*

INTRODUCTION

The myosin molecule consists of two distinct structures, two globular portions designated subfragment-1 (S-1), and an almost completely α-helical coiled-coil tail, namely the rod. ATP and actin-binding sites locate on S-1, and filament-forming ability, or the property that myosin dissolves in high-salt medium, relies on the rod portion. It is generally believed that myosin rod is much more stable than S-1 because the former is very resistant to treatment with ethanol or urea, or to heating (Rodgers et al., 1987; King and Lehrer, 1989; Kato and Konno, 1993a). This is the conclusion delivered from the study by using isolated two portions of myosin. Recently, solubility of myosin in salt decreased much faster than did ATPase inactivation, when carp myofibrils were heated (Konno and Ueda, 1989), and carp myosin forms aggregates before losing its ATPase activity (Kato et al., 1996). Based on the foregoing, we proposed that the rod portion may be more labile than S-1 when heated as myosin or myofibrils, in connected form with S-1.

Myosin is the protein most responsible for the thermal gelation of fish-muscle protein. It is accepted that myosin denaturation in raw material, such as surimi, leads to deterioration of the quality of the final product of thermal gel (Katoh et al., 1979). In that case, myosin denaturation was monitored by measuring calcium-adenosinetriphosphatase (Ca-ATPase) activity. As the ATPase active site locates on S-1, ATPase inactivation is the indicator for S-1 denaturation, providing no information on rod denaturation.

It is generally believed that dissolving myofibrillar protein into salt prior to heating is essential for developing an elastic thermal gel. However, there is little reported on how rod denaturation proceeded in myofibrils upon heating.

* To whom correspondence should be addressed. E-mail: konno@fish.hokudai.ac.jp.

In this paper, we elucidated how the rod portion and S-1 undergo structural changes when myofibrils were heated to understand the myosin denaturation in raw material. To investigate the rod denaturation in myofibrils, chymotryptic digestion was successfully applied. This technique has been widely employed to detect the structural change of various kind of proteins. The principle is that chymotrypsin specifically cleaves the carboxyl end of hydrophobic residues, and these residues are usually varied in the core of the protein molecule. Protein denaturation would expose these residues, resulting in increased susceptibility.

MATERIALS AND METHODS

Myofibrils were prepared from the dorsal muscle of carp as described by Katoh et al. (1977), and were suspended in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5). The myofibril suspension was incubated at 40 °C for up to 30 min.

The first indicator we employed for detecting structural changes of S-1 and rod upon heating was "chymotryptic susceptibility". The digestion of myofibrils was conducted in a medium of 0.05 M KCl, 20 mM Tris-maleate (pH 7.0), 1 mM EDTA at 20 °C, using 1/250 (w/w) of chymotrypsin, i.e., the condition for cleaving myosin into S-1 and rod. To estimate the amount of S-1 and rod produced from the heated myofibrils, the digestion was continued until myosin heavy chain (HC) completely disappeared from the pattern (usually for 60 min under the conditions just mentioned). The amounts of S-1 and rod were estimated by measuring the staining intensity of S-1 HC and rod separated on sodium dodecyl sulfate−polyacramide gel electrophoresis (SDS−PAGE). The amount of respective product derived from the control myofibrils was taken as 100%. A Shimadzu dual-wavelength flying spot scanning densitometer, model CS 9300PC, was used for the measurement. SDS−PAGE was carried out routinely using 10% polyacrylamide gels containing 0.1% SDS (Laemmli 1970).

Chymotryptic fragments were characterized with respect to their solubility in water and their dissociability from F-actin with ATP. To study these, the digest was centrifuged at 200 000*g* for 20 min to sediment F-actin in a medium containing either 0.05 M KCl or 0.5 M KCl with or without 1 mM ATP−Mg.

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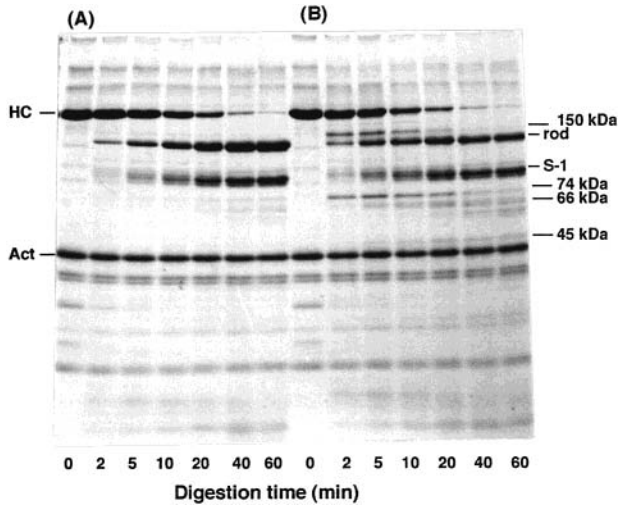

Figure 1. Changes in the chymotryptic digestion pattern of myofibrils upon heating. Carp myofibrils unheated (A), and those heated at 40 °C for 5 min in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) (B) were digested at 20 °C with 1/250 (w/w) of chymotrypsin in 0.05 M KCl, 20 mM Tris-maleate (pH 7.0), and 1 mM EDTA. HC, rod, S-1, and Act denote myosin heavy chain, myosin rod, subfragment-1 heavy chain, and actin band, respectively. Numbers indicate size of the fragments in kDa.

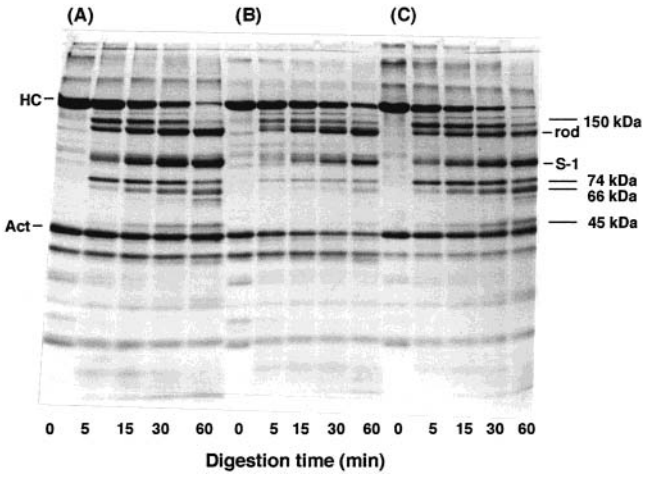

Figure 2. Comparison of the chymotryptic digestion pattern for the salt-soluble and salt-insoluble fractions separated from the heated myofibrils. Carp myofibrils heated for 3 min solubilized with 0.5 M KCl with 1 mM Mg-ATP and centrifuged at 20 000*g* for 15 min. The supernatant (B) and pellet (C) were separately dialyzed against 0.05 M KCl and 20 mM Tris-maleate (pH 7.0), and were digested as in Figure 1. Unfractionated heated myofibrils (A) were also digested.

To identify the chymotryptic fragments, their amino-terminal amino acid sequences were determined. Protein bands separated on SDS-polyacrylamide gel were transferred to poly(vinylidenedifluoride) (PVDF) membranes. Protein bands visualized by staining with CBB were cut, and were sequence-analyzed with a protein sequencer (Perkin-Elmer Applied Biosystems model 473A).

Ca-ATPase inactivation was measured for monitoring myosin denaturation. This activity was assayed in a medium of 0.5 M KCl, 25 mM Tris-maleate (pH 7.0), 5 mM $CaCl_2$, and 1 mM ATP at 25 °C. Loss of solubility in salt was also measured as an indicator for myosin denaturation. As we focused on the structural change occurring in the myosin molecule upon heating, we used the following procedures for measurement of solubility. Myofibrils were dispersed in 0.5 M KCl and 20 mM Tris-HCl (pH 7.5), and left for 30 min in ice; then the mixture was centrifuged at 20 000*g* for 15 min immediately after addition of 1 mM ATP together with 1 mM $MgCl_2$ (Mg-ATP). The solubility was estimated under conditions in which myosin had been detached from F-actin with the aid of 1 mM Mg-ATP to eliminate the effect of F-actin on myosin solubility. It is still possible that soluble myosin contains oligomeric myosin. The amount of monomeric myosin was then measured (Koseki et al., 1993). This index was proposed based on the finding that ammonium sulfate at 40% saturation selectively sedimented oligomeric myosin which is still soluble in 0.5 M KCl (Kato and Konno, 1993b). Briefly, to the myofibrils dispersed in 0.5 M KCl and 20 mM Tris-HCl (pH 7.5) with 1 mM Mg-ATP, saturated ammonium sulfate was added to give 40% saturation; then the mixture was centrifuged at 20 000*g* for 15 min. Myosin content in the supernatant relative to that in myofibrils was estimated by measuring the staining intensity of myosin HC appearing in the SDS−PAGE pattern.

RESULTS AND DISCUSSION

Altered Chymotryptic Digestion Profile of Myosin upon Heating Myofibrils. The structural changes occurring in the S-1 and rod regions caused by heating myofibrils were investigated by the chymotryptic digestion technique. The digestion profiles for the control and for heated (5 min) myofibrils are in Figure 1. The latter retained nearly 65% of their Ca-ATPase, but retained only 30% of solubility (see Figure 6 below). With the control myofibrils (Figure 1A), myosin was selectively cleaved into S-1 and rod as expected (Kato and Konno, 1990). The digestion pattern for the heated myofibrils (Figure 1B) was more complicated and clearly distinguished from that for the control myofibrils. In an early phase of digestion, two new bands migrating above rod and below S-1 HC appeared. Their sizes were 150 and 74 kDa, respectively. The 150 kDa fragment gradually disappeared upon prolonging the digestion. The 74 kDa fragment seemed to be gradually converted into a 66 kDa fragment. In a later phase of digestion, a band of size 45 kDa migrating above the actin band (42 kDa) became obvious; this band accumulated during the digestion period. We noticed that the rod band (130 kDa) generated by the heated myofibrils was less dense than that in the control digest. The appearance of these new fragments, and the decreased production of rod, indicated the exposure of new cleavage sites on the myosin rod upon heating.

As the heating of myofibril for a short period altered the chymotryptic digestion pattern and reduced the solubility of myosin, we then studied the correlation between chymotryptic susceptibility of myosin and the solubility. The idea was that heated myofibrils were first separated into salt-soluble and insoluble fractions, then their chymotryptic digestibility was compared. For this purpose, we employed 3-min-heated myofibrils at 40 °C, in which solubility dropped by roughly 50%, but which retained roughly 85% of their ATPase (see Figure 6 below). The soluble- and the insoluble-fractions were separately dialyzed against 0.05 M KCl and 20 mM Tris-maleate (pH 7.0), and were digested in the presence of EDTA. The digestion patterns for the unfractionated myofibrils, and those for the soluble and insoluble fractions are shown in Figure 2. The digestion profile for the unfractionated myofibrils (Figure 2A) was practically the same as that in Figure 1B. The soluble fraction (Figure 2B) generated S-1 and rod as major products with a small amount of 150 and 74 kDa fragments. On the other hand, the insoluble fraction (Figure 2C) generated the 150 kDa and 74 kDa fragments as prominent components with a small amount of rod. It was therefore concluded that myosin which

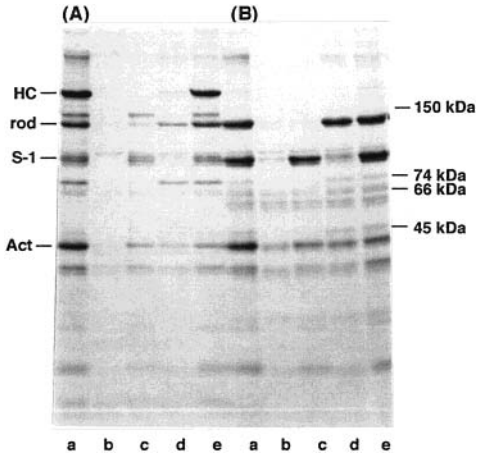

Figure 3. Characterization of chymotryptic digestion products. Carp myofibrils heated for 5 min as in Figure 1 were digested with chymotrypsin either for 5 min (A) or 60 min (B). The digests were centrifuged at 200 000*g* for 20 min to sediment F-actin in the presence of either 0.05 M (b, c) or 0.5 M KCl (d, e), all buffered with 20 mM Tris-maleate (pH 7.0). Centrifugation was conducted without (b, d) or with (c, e) 1 mM Mg-ATP. (a) is digests before centrifugation. Abbreviations are the same as in Figure 1.

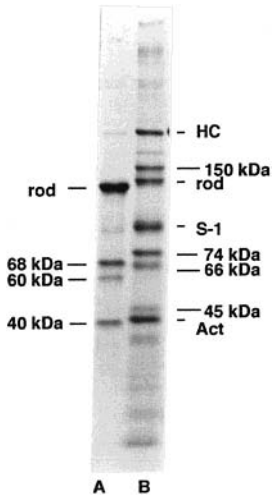

Figure 4. Comparison of LMM-like fragments generated from the heated myofibrils with control LMMs. (A), Insoluble fraction of the chymotryptic digest of the rod. Parent rod and three LMM bands of 68k LMM, 60k LMM, and 40k LMM. (B), Chymotryptic digest of the insoluble fraction of the heated myofibrils in Figure 2. Abbreviations are the same as in Figure 1.

lost salt-solubility was preferentially cleaved into 150 and 74 kDa fragments rather than S-1 and rod.

Characterization of the Chymotryptic Fragments. The new fragments generated by the digestion of the heated myofibrils were characterized. The digest was centrifuged under conditions where F-actin was sedimented (200 000*g* for 20 min). The centrifugation was performed with or without 1 mM Mg-ATP to distinguish the fragments with F-actin-binding ability. The centrifugation medium also contained either 0.05 or 0.5 M KCl to distinguish the water-soluble fragments from salt-soluble fragments. Figure 3 shows the results. With a sample digested for 5 min (Figure 3A), centrifugation at 0.05 M KCl without ATP yielded a very small amount of fragment. A faint band was 95 kDa subfragment-2 (95 k S-2) (Kato and Konno, 1993b). ATP included in the centrifugation medium yielded most of the 150 kDa fragment, a very small amount of the 130 kDa fragment, and several bands near 95 kDa of S-1 HC. These S-1 HC bands migrate a little faster than the 95 kDa S-2. The 150 kDa fragment was thus shown to be the water-soluble fragment that is dissociable from F-actin upon ATP addition (as is S-1). A part of the 130 kDa fragment behaved like the 150 kDa, although the majority of the band was water-insoluble. The 130 kDa band seemed to contain two species of fragment, i.e., a water-soluble fragment with F-actin-binding ability and a water-insoluble fragment. Neither the 74 kDa, 66 kDa, nor the 45 kDa fragment was recovered in a low-salt medium. Almost all of these fragments were recovered in the supernatant when centrifuged at 0.5 M KCl, even if the medium lacked ATP. Thus, the 74, 66, and 45 kDa fragments were shown to be salt-soluble fragments having no interaction with F-actin. Centrifugation in 0.5 M KCl with ATP yielded myosin in addition to S-1 and 150 kDa fragment. Therefore, it was concluded that the 150 kDa fragment was a heavy meromyosin (HMM)-like fragment, and the 74, 66, and 45 kDa fragments were all light meromyosin (LMM)-like fragments. In the sample digested for 60 min (Figure 3B), in which no myosin HC or 150 kDa fragment were detectable, S-1 and rod (130 kDa) behaved as expected; S-1 was water-soluble with actin-binding ability, and rod was water-insoluble with no actin-binding ability. The 74, 66, and 45 kDa fragments were again shown to be water-insoluble. The bands below the 66 kDa fragment were recovered in 0.05 M KCl even in the absence of ATP, suggesting that these were S-2 derived from the HMM-like fragment by a further cleavage at the S-1/rod junction.

Identification of the Newly Exposed Cleavage Sites on Rod. First, LMM-like fragments generated from the heated myofibrils was compared with control LMM on SDS−PAGE. We used the digest of the water-insoluble fraction of heated myofibrils that is shown in Figure 2C. Figure 4 demonstrated that none of the LMM-like fragments showed a mobility identical to those of three control LMM, which gave bands of sizes 68, 60, and 40 kDa. The 40-kDa LMM is the shortest filament-forming domain of myosin that we found in carp myosin (Kato and Konno, 1993b). In a careful comparison of the mobility of the LMM-like fragments with that of the control LMM, we noticed that the LMM-like fragments are all longer than the control LMM fragments by 5 or 6 kDa, namely 74 to 68 kDa, 66 to 60 kDa, and 45 to 40 kDa. It is certain that the LMM-like fragments derived from the heated myofibrils contained the extra short fragment with a mass of *5* or 6 kDa at either the amino or carboxyl end of the control LMM.

To identify which end of the LMM has the extra short fragment in its structure, we determined the partial sequences of the three LMM-like fragments from the amino termini. The determined amino acid sequences for the LMM-like fragments are shown in Figure 5, along with those for the control LMMs. In the same figure, residue numbers identified in the amino acid sequence for carp myosin HC are also shown (Imai et al., 1997). Clearly, three LMM-like fragments were found to be generated by cleavage at exactly the same three sites for producing the three LMM species. Therefore, it was concluded that LMM-like fragments all have a short fragment at the carboxyl terminus. It has been reported, with chicken skeletal myosin, that chymotryptic cleavage at the S-1/rod junction is always

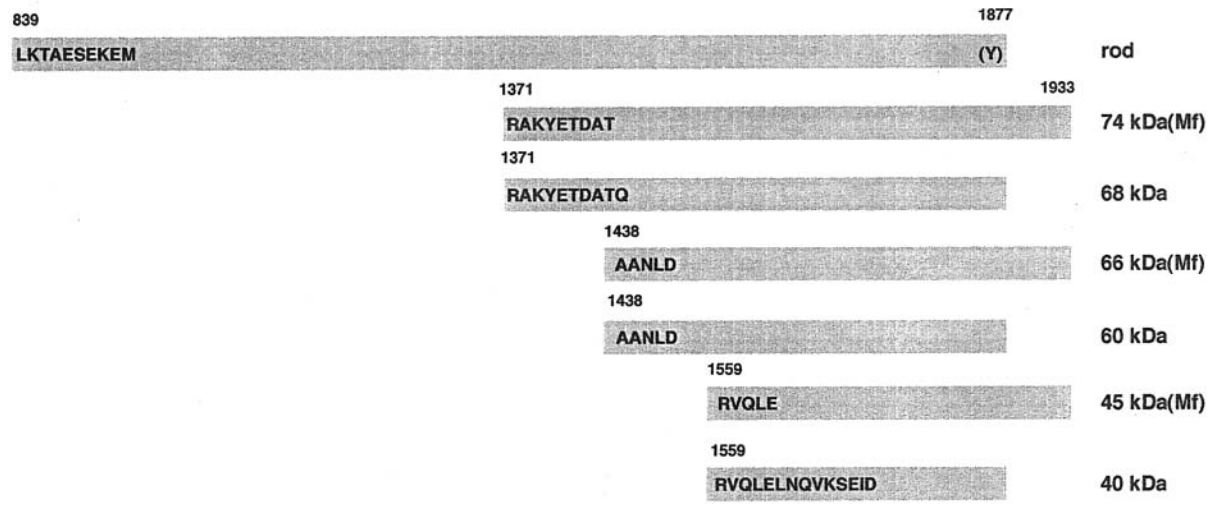

Figure 5. Comparison of the amino terminal sequence for the LMM-like fragments derived from heated myofibrils with control LMMs. Three LMM-like fragments as well as control LMMs were partially sequenced. Abbreviations are the same as in Figures 1 and 4.

accompanied by the tearing off of a short fragment, termed "tailpiece", with a mass of about 5 or 6 kDa, consisting of 58 residues from the carboxyl end of the myosin HC. In other words, rod lacks the fragment (Maita et al., 1991). Although the carboxyl termini of the LMM-like fragments were not determined, it is reasonable to think that LMM generated from the heated myofibrils still retained the tailpiece at the carboxyl end. The carboxyl end of the chicken rod is reported to be Tyr1880 after losing 58 residues (Maita et al., 1991). A homology search between carp and chicken myosin HC revealed that Tyr1880 in chicken myosin corresponds to Tyr1877 in carp myosin. Assuming that the carp myosin rod was produced by cleavage at a site homologous to that in chicken myosin, the carp rod would lose 54 residues in the digestion process. Although the cleavage sites at the HMM/LMM junction were exposed upon heating, the site near the carboxyl end was found to remain protected from chymotryptic digestion.

Comparison of S-1 and Rod Denaturation Rates. Denaturation rates of S-1 and rod were compared. Heated myofibrils (various periods) were digested for 60 min, and the amounts of S-1 and rod generated from the myofibrils were estimated by using SDS−PAGE and densitometer. As has been described in Figure 1, digestion for 60 min was long enough to convert myosin into subfragments almost completely. With duration at 40 °C, the amounts of S-1 and rod generated gradually decreased, however, the decrease of rod was much faster than that of S-1 (Figure 6). The half-lives for S-1 and rod were about 10 and 2 min, respectively, demonstrating that structural change occurring in the rod was much faster than that in S-1 by a factor of roughly 5 as measured by the increased susceptibility. We reported that three domains of fish myosin S-1 become susceptible to the tryptic attack upon thermal denaturation and resulted in a degradation into small pieces (Hamai and Konno, 1990). Thus, the reduced amount of S-1 in the digest may also be explained by the increased susceptibility of denatured S-1 HC to the chymotryptic attack. The decrease in amounts of rod was explained by an additional cleavage at the exposed HMM/LMM junction as described in Figures 3, 4, and 5. Although the data are not shown, ammonium sulfate at 40% saturation, in the presence of 1 mM Mg-ATP, sedimented practically no S-1 or rod, demonstrating that these two subfragments remained monomeric irrespective of their amounts.

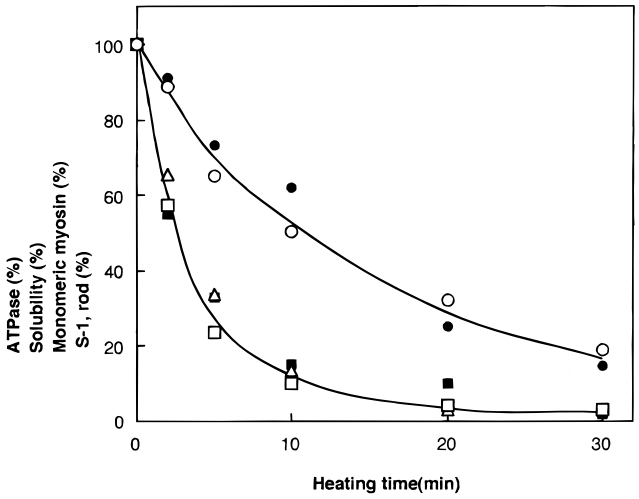

Figure 6. Decreases in the amounts of S-1 and rod generated from the heated myofibrils by their chymotryptic digestion. Carp myofibrils heated at 40 °C were subjected to digestion for 60 min under the same conditions as in Figure 1. The amounts of S-1 (●) and rod (■) produced were estimated by using SDS−PAGE and densitometry. Decreases in the Ca-ATPase activity (○), solubility of myosin (□), and amount of monomeric myosin (△) are shown.

As the ATPase active site is located on S-1, the Ca-ATPase inactivation was compared with the decrease of S-1 produced. As shown in Figure 6, the decrease of S-1 was closely correlated with ATPase inactivation. Thus, the amount of S-1 produced reflected the ATPase activity remaining. In other words, S-1 was generated only from myosin that retained the ATPase activity. As the salt-solubility of myosin depends on rod, solubility of myosin and monomeric myosin remaining were compared with rod produced. Amount of rod was well correlated with the solubility decrease and monomeric myosin content. This indicated that rod was generated only from myosin that retained solubility or HMM/LMM junction for the insoluble myosin was exposed to chymotrypsin. It should be noted that the soluble and monomeric myosin decreased in the same manner; the

soluble myosin was proven to be all monomeric when the myofibrils were heated under the conditions used in this paper.

In this paper, we demonstrated that the rod portion of the myosin molecule preferentially denatures upon heating as detected by an increased susceptibility at the HMM/LMM junction, and this fast denaturation of rod resulted in a rapid loss of solubility of myosin in salt solution. As the solubilization of myosin prior to heating is an essential step for developing the elastic gel formation, denaturation of rod in raw material accompanied by a loss of solubility would affect severely the gel-forming ability of fish muscle.

Heating process of the salted fish-meat paste would involve myosin denaturation, too. Papers on the role of rod denaturation in this process have been accumulated. Samejima et al. (1981) proposed that the myosin head and tail portion separately participate in a gel network structure upon heating. The importance of the tail portion of myosin in thermal gel formation was also pointed out by Sano et al. (1982), who studied the dynamic viscoelastic behavior upon heating, and by Gill and Conway (1989), who studied chemical cross-linking reactions of myosin fragments. However, how the rod portion of myosin undergoes structural change during the process is uncertain. A further study on the denaturation process of myosin S-1 and rod after dissolving into salt would be necessary to understand the molecular mechanism of the thermal gelation of fish muscle. The study is in progress.

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