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Role of Neck Region in the Thermal Aggregation of Myosin

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Carp dorsal myosin formed oligomers that retained ATPase activity upon heating. Cleavage of the oligomeric myosin at subfragment-1 (S-1)/rod junction released monomeric S-1 and rod, indicating that ATPase retaining myosin associated near the S-1/rod junction. The digest also contained rod oligomers. Heating a mixture of S-1 and rod generated neither ATPase retaining S-1 oligomers nor rod oligomers. Electron microscopic observation of the heated myosin revealed that some oligomers were formed by associating at the S-1/rod joining region, exhibiting a recognized double head, probably ATPase retaining oligomers. No myosin oligomers associated at the tail region were observed, thus, rod aggregation would be formed at its very restricted region near the S-1/rod junction. Based on the findings, we proposed that the neck structure is important in the thermal oligomerization process of myosin.

KEYWORDS: Myosin; denaturation; aggregation; gelation; carp

INTRODUCTION

Myosin comprises two unique characteristic structures: a globular double-head, termed subfragment-1 (S-1); and a coiled-coil long flexible tail, termed rod. Connection of these two structures forms a region termed "neck". S-1 contains actin and ATP binding sites, and rod is responsible for the filament formation of myosin molecule under physiological conditions. Myosin is selectively cleaved into these two subfragments by digestion with chymotrypsin in the absence of divalent cations (*1*).

It is generally accepted that fish myosin is much more unstable than mammal myosin (*2*, *3*). This is a consequence of cold-adaptation to its low habitat temperature. Carp survives at a wide range of temperatures by expressing different myosin isoforms with different thermal stabilities (*4*−*6*). Complete amino acids sequences for these myosin heavy chains have been determined by deduction from the cDNA sequences (*4*). However, a clear explanation has not been made regarding which residues determine the thermal stability.

We have reported that fish myosin S-1 is more easily denatured than that of rabbit; upon heating, releasing light chain components and producing large aggregates by S-1 heavy chain (*7*). Thermal unfolding experiments with rod from various species of animals with different habitat temperatures demonstrated that rod from Antarctic fish easily unfolded at a lower temperature than that for mammal (*8*). This was confirmed by Ogawa et al. (*9*), and by us using urea denaturation (*10*). Although information on the thermal denaturation of rod and S-1 in isolated forms has been accumulated, there is little information regarding how the head and rod portions affect each other in the thermal denaturation process.

Myosin is the protein responsible for the thermal gelation of fish meat (*11*, *12*), and the thermal aggregation of myosin molecules is a crucial process for developing the elastic gel. In the process, it is believed that the rod portion is important for the thermal gelation. In this paper, we investigated the thermal aggregation process of carp myosin, and we propose that the neck region connecting the head and tail portions is important in the thermal association process by myosin molecule.

MATERIALS AND METHODS

Myosin was prepared from the dorsal muscle of carp (*Cyprinus carpio*) by using ammonium sulfate fractionation in the presence of Mg-ATP (*13*). Myosin collected as a pellet between 40 and 50% saturation was dissolved and dialyzed against 0.5 M KCl and 20 mM Tris−HCl (pH 7.5), and the supernatant after centrifugation at 100,000 *g* for 60 min was used. Myosin was used within a few days because of its unstable nature.

The thermal denaturation process of myosin at 30 °C in the above medium was investigated by monitoring its Ca-ATPase inactivation and oligomerization as studied on Sepharose CL 4B gel filtration (1.6 × 90 cm, 0.5 M KCl, 20 mM Tris−HCl (pH 7.5)). Ca-ATPase was routinely assayed at 25 °C in a medium of 0.5 M KCl, 25 mM Tris−maleate (pH 7.0), 5 mM $CaCl_2$, and 1 mM ATP by colorimetrically measuring the liberated inorganic phosphate.

Chymotryptic digestion of myosin was conducted at 20 °C using 1/200 (w/w) of chymotrypsin in a medium of 0.05 M KCl, 20 mM Tris−maleate (pH 7.0), and 1 mM EDTA so as to cleave myosin into

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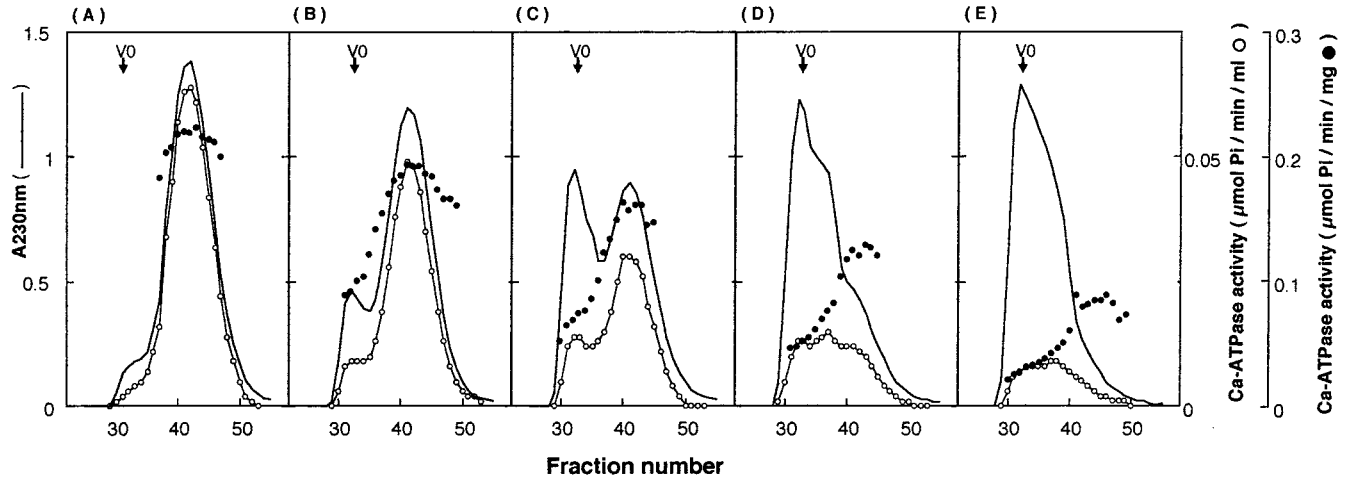

Figure 1. Thermal oligomerization and ATPase inactivation of carp myosin upon heating: Carp myosin heated at 30 °C was applied to the Sepharose CL-4B column (1.6 × 90 cm) equilibrated with 0.5 M NaCl and 20 mM Tris–HCl (pH 7.5). Vo is a void volume. The elution profile of myosin (—), Ca-ATPase activity (μmol Pi/min/mL, open circles), and specific activity (μmol Pi/min/mg, closed circles) were shown. Myosin samples heated for 0 min (A), 5 min (B), 10 min (C), 20 min (D), and 30 min (E) were analyzed.

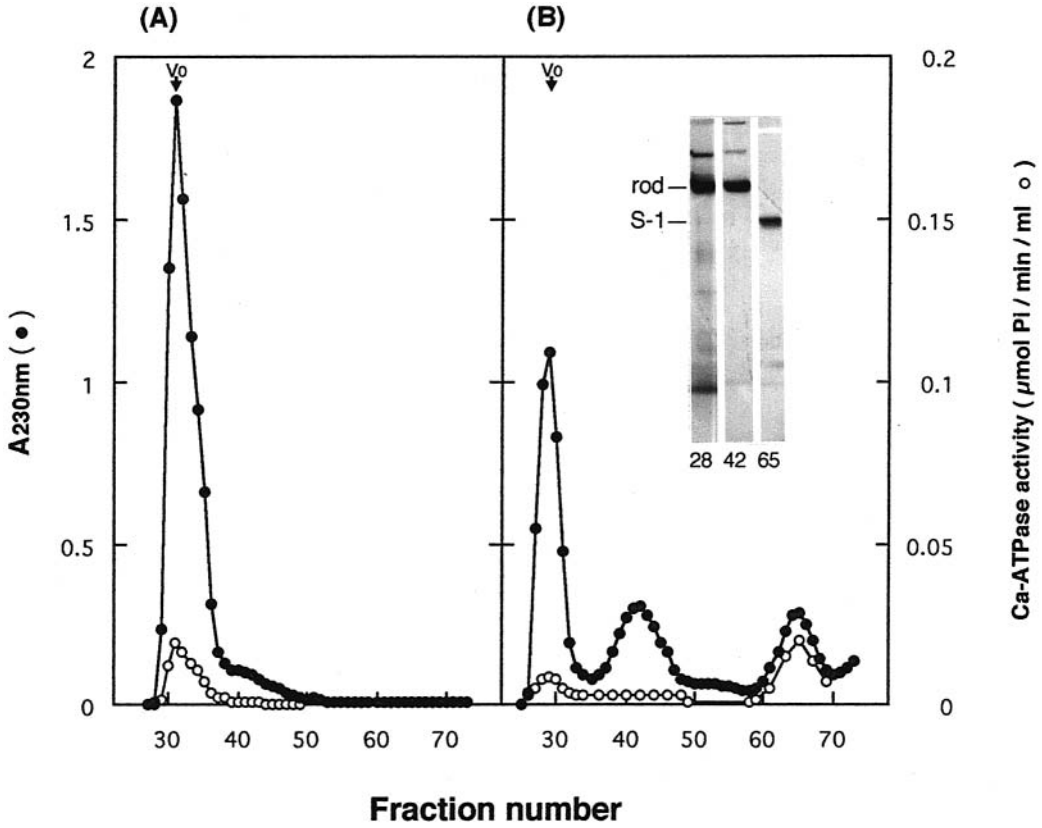

Figure 2. Analysis of the oligomer myosin by chymotryptic digestion: Myosin oligomer (A) was prepared from 10-min-heated myosin under the same conditions as in Figure 1, and its chymotryptic digestion (B) was applied to the Sepharose CL-4B column as in Figure 1. The inserted figures in (B) are the SDS–PAGE patterns of the fractions separated with fraction numbers. Rod and S-1 are rod and subfragment-1 heavy chain, respectively.

S-1 and rod (*1*). Chymotryptic fragments derived from the heated myosin were characterized by using the following two methods: Western blotting by using acti-carp S-1 heavy chain to examine whether the fragment contained a part of the S-1 portion; and amino terminal sequencing of the fragments to identify the cleavage site on myosin. Both were conducted routinely as described in the previous papers (*14*, *15*). SDS−PAGE was carried out as Laemmli described (*16*).

The morphological change of the myosin molecule upon heating was studied by observing it under an electron microscope (Hitachi H-800) at 75 kV. The procedures for the sample preparation and rotary shadowing were as Katoh and Morita (*17*) have described.

RESULTS AND DISCUSSION

Oligomerization of Carp Myosin upon Heating. We first examined the oligomerization of carp myosin upon heating. Myosin was heated at 30 °C up to 30 min in 0.5 M KCl and 20 mM Tris−HCl (pH 7.5). Myosin association upon heating was analyzed by Sepharose CL 4B gel filtration (Figure 1). Heated myosin solution was neither turbid nor sedimentable by the centrifugation at 100,000*g* for 60 min. We referred to the process as oligomerization rather than aggregation because no large aggregate was formed. Unheated myosin was eluted as a

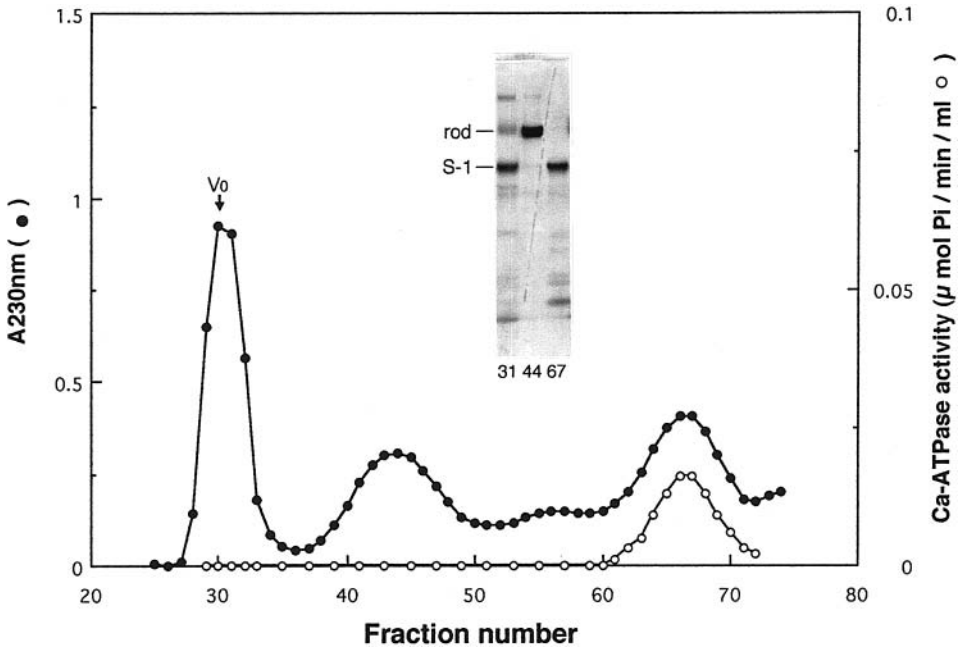

Figure 3. Effect of cleavage at the S-1/rod junction on the aggregation process of myosin: Myosin was digested into S-1 and rod as in Figure 2, and was heated at 30 °C for 10 min. The heated digest was centrifuged to remove insoluble particles, and was applied to the Sepharose CL-4B column as in Figure 2. Inserted SDS–PAGE patterns are of the three peaks with fraction numbers.

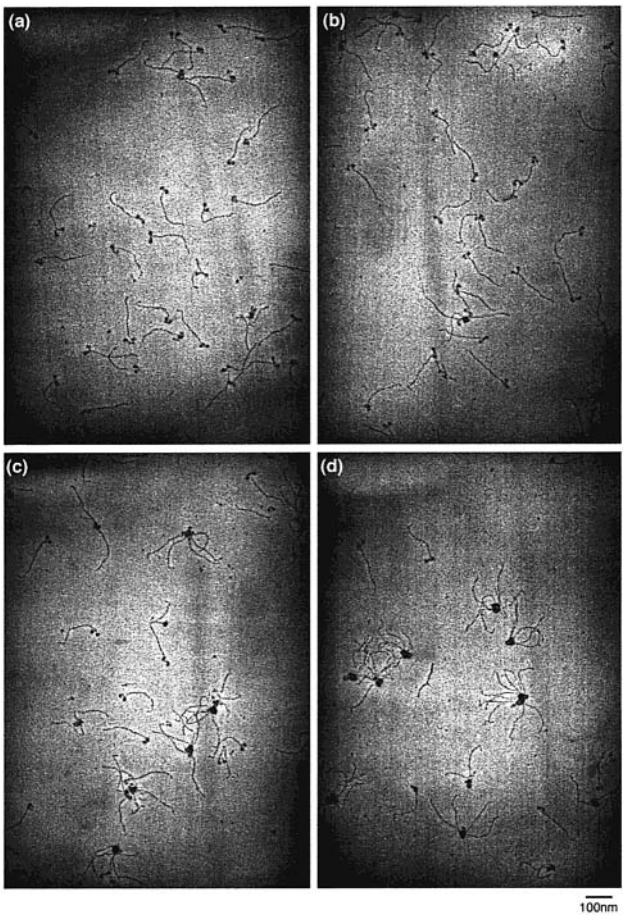

Figure 4. Morphological study on the heated myosin molecule on electron microscope: Myosin heated at 30 °C for 0 min (a), 5 min (b), 15 min (c), and 30 min (d) were rotary shadowed, and observed. Magnification was × 60,000, and the scale bar indicates 100 nm.

symmetrical peak at fraction number 41 (Figure 1A). When heated myosin was applied to the column, a new peak at 31 (void volume, Vo) appeared as a result of oligomer formation. The Vo peak became higher with duration (Figs. 1B, C, D, and E). Although the 30-min-heated myosin still retained 40% of ATPase activity, the monomer peak almost disappeared from the elution profile indicating that the oligomerization was a faster event than the inactivation. To examine how ATPase inactivation and oligomerization related, ATPase activity for the separated fractions was measured. The activity was reasonably detected in the monomer peak. In addition, the activity was clearly detected for the oligomers eluted at Vo fractions. It is certain that myosin that retained ATPase activity formed oligomer. The specific activity for the Vo fraction for the 10-min-heated myosin was about 1/4 that of the monomer peak for the unheated myosin, although Vo was not well separated from monomer. A majority of myosin that constructed the oligomers was inactivated. We also noticed that a specific activity for the monomer peak for the heated myosin was lower than that for unheated monomer. Supposing that the oligomerization is slower than the inactivation, inactive monomer would be generated; however, this does not explain the generation of active oligomers. There seemed to be two pathways in the oligomerization process of myosin upon heating, oligomerization was unnecessarily the following step of inactivation.

We next characterized the myosin oligomers that contained active myosin. We could not separate active oligomers from inactivated ones, however, myosin oligomers were successfully separated from monomers by fractionation at 40% saturated ammonium sulfate (40% AS) as pellet. The elution profile for the oligomers collected from the heated myosin for 10 min at 30 °C as above showed a high peak at Vo containing practically no monomer peak (Figure 2A). It has been reported that release of light chain components induces the association of myosin at the neck region where the light chain bonds (*18, 19*). We examined whether light chain was detached from the myosin in oligomer by analyzing its light chain composition on SDS–PAGE. Oligomer contained light chains with the same quantity (data not shown). Moreover, light chain release was not detected by analysis of the elution profiles of the heated myosin in Figure

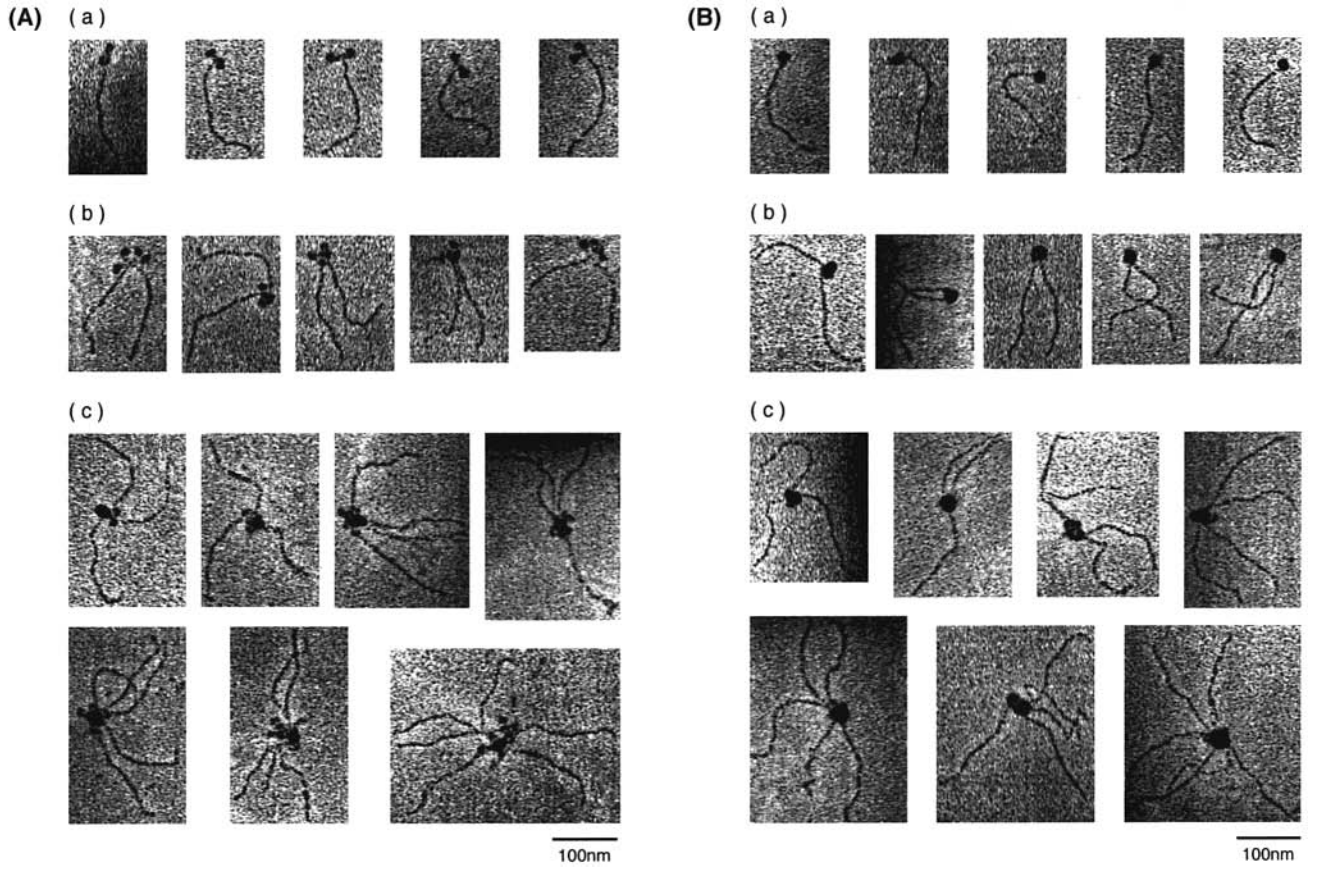

Figure 5. Gallery of myosin molecules observed: Molecules whose double head was recognizable were collected in (A), and those whose double head was packed into one were collected in (B). Myosin monomer (a), dimer (b), and trimer and oligomer (c) are separately shown. The scale bar indicates 100 nm.

1. Therefore, light chain dissociation was not the reason for the active myosin oligomer formation. The oligomers were digested by chymotrypsin into S-1 and rod and applied to the same column. The elution profile is shown in Figure 2B. Three peaks appeared at the fraction numbers 28 (Vo), 42, and 65. ATPase activity was recovered in the third S-1 monomer peak as revealed by SDS−PAGE. We have already demonstrated that S-1 was produced only from myosin that retained ATPase activity, and inactivated S-1 was degraded into short fragments as a consequence of its increased susceptibility to chymotrypsin (*20*). Thus, S-1 must be generated from the active myosin that constructed the oligomers. SDS−PAGE analysis also showed the second peak of monomer rod. As the protein eluted at Vo fraction was rod as shown in the inserted SDS−PAGE pattern, some rods produced were present as oligomers in the digest. The easiest explanation for the generation of active oligomers was that myosin formed oligomers at the rod portion without losing the activity. Although the data are not presented, we studied the oligomerization of rod upon heating by analyzing the chymotryptic digest of the heated myosin on Sepharose CL 4B. It was showed that rod aggregation proceeded more slowly than the inactivation; heating for 30 min induced 60% inactivation, but only 30% of rod was oligomers. Thus, the association of active myosin at the rod region seemed improbable. Active myosin oligomer would also be explained by assuming the independent inactivation in two heads of myosin, that is, one inactivated head is involved in the oligomerization and the another head of the same molecule is still active. Such an oligomer should generate no rod oligomer, but the chymotryptic digestion of the active oligomers generate a large quantity of rod oligomer. Therefore, that kind of oligomerization seemed improbable.

Thermal Denaturation of S-1 and Rod Mixture. As a small possibility still remained, we examined whether the rod itself forms oligomers under the same heating conditions. To study this, myosin was cleaved into S-1 and rod with chymotrypsin in advance, and the digest, a mixture of S-1 and rod, was heated in 0.5 M KCl and 20 mM Tris−HCl (pH 7.5). Oligomerization by S-1 and by rod was studied on Sepharose CL 4B gel. Being different from the case of parent myosin, the heated digest was turbid, and the turbid components were removed by a light centrifugation at 20,000*g* for 15 min. SDS−PAGE analysis of the pellet showed the aggregates formation by S-1 (data not shown). Turbid aggregates formed by S-1 upon heating were the same as have been reported with carp S-1 (*7*). The supernatant was applied to the column. As shown in Figure 3, the supernatant gave three peaks, monomeric S-1 (fraction number 67), monomeric rod (44), and Vo (31). As shown in the inserted SDS−PAGE pattern, Vo was of aggregated S-1. As no ATPase activity was detected for the Vo, the aggregates were formed by inactivated S-1. ATPase activity was detected only for the S-1 monomer peak. As the specific activity for the fraction was the same as that for control S-1, it was confirmed that active S-1 never forms oligomers. This is in good agreement with our previous report (*21*). Moreover, rod in a separated form formed no oligomer under the heating conditions. It was suggested that active myosin associates at a different region rather than rod or S-1. It seemed essential for the active oligomer and rod oligomer formation that S-1 and rod were connected. These results suggested that the myosin association is at the

connecting region of these two portions, the "neck". It was thus demonstrated that the oligomerization process by myosin molecule was completely different from a simple sum of that by individual components of S-1 and rod.

Electron Microscopic Observation of the Heated Myosin Oligomers. Myosin oligomers formed by the thermal treatment were visualized by electron microscopy (Figure 4). Observing unheated myosin, almost all molecule was dispersed as monomer, and it showed the conventional two-headed structure; separated two heads were connected to the long flexible tail (Figure 4A). Carp myosin molecule was proved to be morphologically indistinguishable from rabbit skeletal myosin. This would be the first microscopic observation to show that fish myosin molecule is morphologically identical to mammal myosin. When the heated samples were observed (Figures 4B, C, and D), the monomer population decreased and oligomeric forms gradually increased in the field. Almost all oligomers were formed around the head portion, and no oligomer which associated at the tail portion was seen in the field. Although a majority of heads in oligomers were packed into one particle, there were some molecules whose two heads were recognized even in the oligomers. We classified the myosin molecules by their head structure whether a double-head structure was recognized or not. A gallery of molecules classified into the above two categories is shown in Figure 5A and B. Double headed myosin were found in dimers, trimers, and in oligomers, as well as in monomers (Figure 5A). These species seemed to associate at the neck region connecting between S-1 and rod. It has been demonstrated that inactivated carp S-1 immediately forms aggregates with no inactivated S-1 monomer. Because the S-1 structure was kept intact morphologically in these oligomers, these species would be the ones that retained ATPase activity as assumed in Figure 1. Oligomers with packed heads, probably inactivated oligomers, were abundant in the field (Figure 5B). We also noticed that there were monomers whose two heads were packed together, exhibiting a single headed structure. These would probably be inactivated monomer as we also assumed in Figure 1 and assumed with carp S-1 (*21*).

We quantitatively analyzed the oligomers by counting the number of myosin molecules in oligomers. Myosin retaining two-headed structures and fused-head structures were counted separately. The histogram shown in Figure 6 was made by counting about 250 to 390 molecules per sample. Unheated myosin was 93.6% monomeric, containing two-headed species of 90.3%. With duration, the percentage of monomer gradually decreased and in turn the amount of oligomers increased. With 5-min-heated myosin, monomer was still the major portion (85.5%) with a small amount of oligomers up to hexamers. With 15-min-heated myosin, the monomer population decreased to 51.1% containing 42.7% double-headed structure. Oligomers with less than six molecules were the major species for the sample. With the 30-min-heated sample, the monomer population decreased to 22.3% with double-headed species of 16.0%. Oligomers formed by a variety of numbers of molecules were found. The largest oligomer observed was a decamer. Yamamoto (*22*) has reported a formation of the daisy-wheel-shaped myosin oligomers upon heating of rabbit myosin. Our oligomerization was somewhat different from that reported by Yamamoto. The first difference was that some of our oligomers still retained a double-head structure, and the magnitude of association was much smaller in our case. The largest oligomer formed in our sample was decamer, while oligomers of more than 30 molecules were not rare in Yamamoto's case. We have no clear explanation for it; however, this difference could be due to the different pH for the heating medium: pH 7.5 in this paper and pH 6.0 in Yamamoto's case. Myosin S-1 readily denatures and associates at acidic pH, resulting in formation of large aggregates. The difference would not be due to the species of myosin used, because carp S-1 much more easily forms aggregates than rabbit S-1 does (*7*).

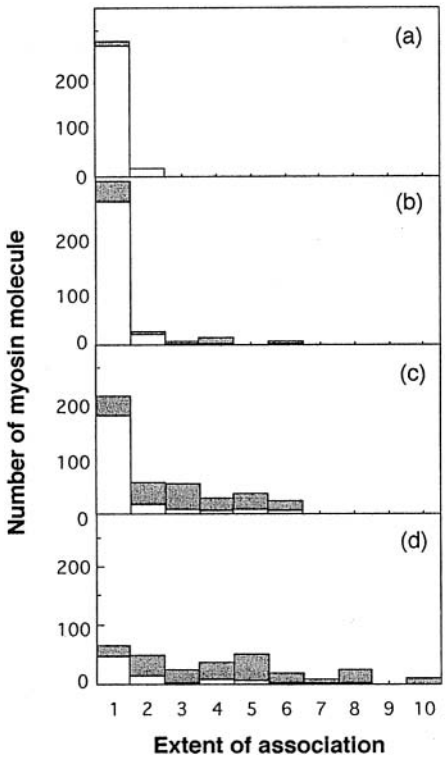

Figure 6. Population of the double-headed myosin in oligomers: Myosin heated for 0 min (a, $n = 249$), 5 min (b, $n = 330$), 15 min (c, $n = 393$), and 30 min (d, $n = 287$) was analyzed. The number of molecules analyzed is represented by n. Molecules whose double head was recognizable (open bar) and those whose double head was packed into one (shaded bar) were separately counted.

Characterization of Rod Oligomers. The oligomer structure revealed by electron microscope suggested that rod oligomers were generated by associating myosin molecules at the very amino terminal region of rod. We analyzed the oligomerization process by rod in myosin oligomers. To understand how rod oligomers were formed, we carefully analyzed the chymotryptic digestion pattern for the heated myosin (Figure 7A). Several new fragments migrating between myosin heavy chain and rod in addition to S-1 and rod were obvious in an early phase of the digestion. As no such fragment was produced from the unheated myosin, these were characteristic fragments derived from the heated myosin. New fragments were referred to as fragments-a, -b, and -c. These fragments gradually disappeared and seemed to be converted into rod with digestion, suggesting that these were intermediate products of rod. Gel filtration analysis of the partially digested product revealed that these intermediate fragments were all eluted at Vo as oligomers (data not shown). Rod oligomers observed in Figure 2B would be produced by a further cleavage at S-1/junction within the intermediates. Western blotting analysis of the fragments demonstrated that all three of the fragments contained a part of S-1 (Figure 7A), indicating that these were produced by being cleaved within the S-1 portion. As active S-1 will never be

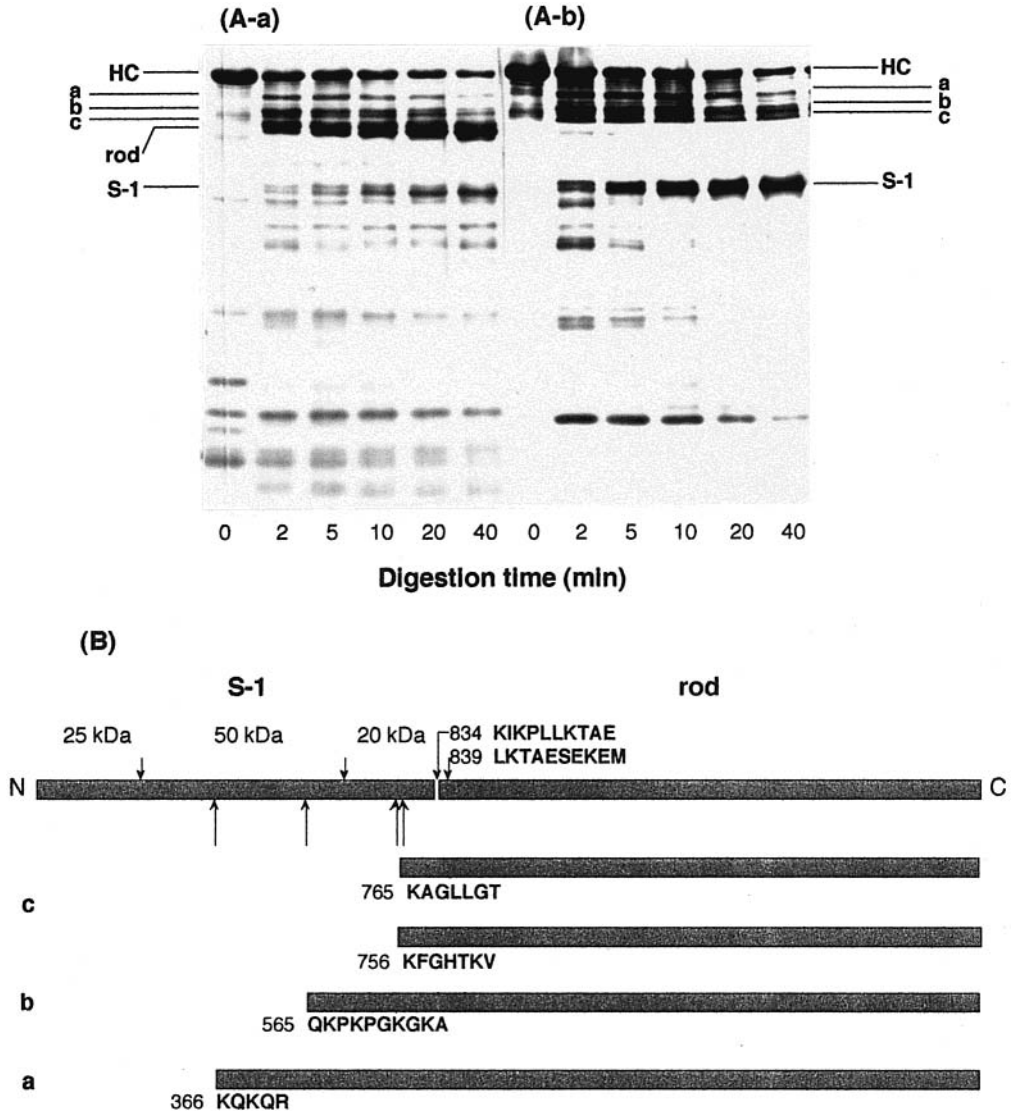

Figure 7. Characterization of the rod oligomers: (A) Chymotryptic digest of the heated myosin (30 °C, 10 min) was allowed to react with anti-carp S-1 heavy chain. HC, rod, and S-1 are myosin heavy chain, rod, and subfragment-1 heavy chain, respectively. Newly generated fragments are designated a, b, and c. (A-a) and (A-b) are protein staining with Coomassie brilliant blue and immunostaining, respectively. (B) N-terminal sequences for the fragments a, b, and c were determined after transferring to PVDF membrane. Determined sequences were arranged in myosin sequence. 25 kDa, 50 kDa, 20 kDa are three tryptic domains of S-1 arranged from its amino terminal end.

cleaved by chymotrypsin, the intermediate fragments were generated from inactive myosin oligomers. In addition to these three fragments and S-1, three positive bands migrating faster than S-1 were detected in an early phase of digestion (Figure 7A-b). These bands disappeared gradually from the pattern probably due to the further degradation into much shorter fragments which are not reactive to the antibody. It seemed reasonable to think that these fragments were counterparts of three S-1-containing intermediate fragments of rod, fragments-a, -b, and -c. We identified the cleavage sites for generation of these fragments by sequencing their amino terminal region. Three isoforms were reported for carp myosin heavy chain (*4*), but the difference did not disturb the identification of the cleavage sites. The determined sequences are shown in Figure 7B; residue numbers were taken from the complete sequence of carp myosin heavy chain (*4*). Amino ends of fragments-a, -b, and -c were Lys-366, Gln-565, and a mixture of Lys-756 and Lys-765, respectively. The former two sites were within the 50 kilodalton (kDa) tryptic domain of S-1, and the site to generate the shortest fragment, fragment-c, was within the 20 kDa carboxyl terminal tryptic fragment of S-1. As the amino end of rod was Leu-839, the shortest fragment, fragment-c, stretched 83 residues within S-1. Assuming that carp myosin S-1 has the same 3D structure as published with chicken S-1 (*23*), all three sites were on the surface. The carboxyl terminal region of S-1 forms an extended structure named "lever arm". The shortest fragment, fragment-c, spans this whole extended region. It seemed very probable to associate myosin molecule at the extended structure when the thermal movement of the myosin molecule was enhanced upon heating.

We proposed that the neck structure formed by the carboxyl terminal region of S-1 and the connecting rod is the structure essential for the oligomerization of carp myosin upon heating. Elevated temperature enhances the thermal movement of myosin molecule. The tail portion moves more freely than the head portion, causing a tangling at the neck region or lever arm to produce the active oligomer. In the inactivated myosin oligomers, a flexible long tail would rotate around the fused head particle having a large mass, resulting in an irreversible association at rod and forming rod oligomers.

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