Protease-susceptible sites and properties of fragments of aortic smooth-muscle myosin

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We have examined the protease susceptibility of aortic myosin, the thermal unfolding profiles of myosin rod and light meromyosin (LMM) and the solubility properties of the LMM fragments. Two major protease-susceptible sites were found, located at the head–rod junction and the heavy meromyosin (HMM)–LMM junction. Both tryptic and chymotryptic digestion of aortic myosin rod produced the LMM (80–85 kDa) and short subfragment 2 (S-2) (40–45 kDa) segments, which were similar to those of gizzard myosin rod and differed from the short LMM (70 kDa) and long S-2 (58 kDa) segments produced from skeletal-muscle rod. The thermal unfolding profile of aortic myosin rods exhibited three helix-unfolding transitions, at 47.5, 51 and 54 °C, similar to those of gizzard rods yet different from those of skeletal-muscle rods. There was a dramatic difference in the solubility of aortic LMM fragments of various molecular mass, as for gizzard smooth-muscle LMM and rabbit skeletal-muscle LMM. LMM fragments of molecular mass 77 kDa or more were completely insoluble in low-ionic-strength buffer, whereas LMM fragments of molecular mass 73 kDa or less were completely soluble in low-ionic-strength buffer. Proteolytic digestion patterns of LMM showed two additional protease-susceptible sites located 13 and 30 kDa from the ends of the LMM molecule. This suggests the existence of flexible regions within the LMM molecule, which may be responsible for the folded form of aortic myosin.

INTRODUCTION

Smooth-muscle myosin, like striated-muscle myosin, is comprised of two heavy chains. The N-terminal region of each heavy chain folds together with light chains to form a globular head region called subfragment 1 (S-1). Each head contains a site for ATP binding or hydrolysis and an actin-binding site. The rest of the two heavy chains wrap around each other to form a coiled-coil of α-helices referred to as a rod. This region contains the binding sites for assembling myosin into thick filaments, the functional form of myosin in muscle. The rod portion of myosin molecules from either striated muscle or gizzard smooth muscle can be separated from the head by limited papain digestion [1] or chymotrypsin proteolysis in the absence of bivalent cations [2]. Subsequent digestion of the rod with either trypsin or chymotrypsin results in two subfragments: subfragment 2 (S-2), which is the soluble portion immediately adjacent to the head, and light meromyosin (LMM), the C-terminal half of the rod which is thought to account for the rod's insolubility [1].

Vertebrate smooth-muscle and non-muscle myosins share a distinct property that their actin-activated ATPase activities are primarily regulated through the phosphorylation of the 20 kDa light chains [3,4]. In spite of the remarkable similarity between aortic and gizzard myosin, recent experimental evidence has demonstrated significant differences between myosin molecules from these two smooth muscles. Gizzard myosin, but not aortic myosin, contains a seven-amino acid insert near the ATP-binding site in the head, which appears to account for the higher velocity of actin filament movement *in vitro* and the higher actin-activated Mg-ATPase activity of gizzard myosin when compared with aortic myosin [5]. In addition, the actin-activated ATPase activity of the unphosphorylated aortic myosin but not that of the unphosphorylated gizzard myosin can be stimulated by skeletal-muscle tropomyosin, implying that differences exist between these myosin molecules [6,7]. In this study, we examined the protease susceptibility of aortic myosin, rod and LMM, and the solubility properties of their proteolytic fragments. The protease-susceptible sites of aortic myosin appear to be the same as those of gizzard myosin, indicating that they have similar proteolytic domains. The solubility comparison of LMM and its subfragments indicates that only a small portion (about 5 kDa) of LMM is critical for the insolubility of LMM in low-ionic-strength buffer, suggesting that this small segment plays an essential role in myosin filament assembly. This property resembles that of LMM from gizzard smooth muscle [8–10] and rabbit skeletal muscle [11].

Skeletal-muscle myosin rods exihibit multiple thermal- and denaturant-induced unfolding transitions involving regions of different stability [12,13]. Unfolding profiles of gizzard smooth-muscle myosin rod also show two thermal- and denaturant-induced co-operative transitions, indicating the existence of two major structural domains [14]. In this study, we determined the number and size of unfolding domains of aortic rod and LMM and their relative stabilities against temperature by CD measurements. The thermal unfolding profile of aortic myosin rods is slightly different from that of gizzard rods and is distinct from those of skeletal-muscle rods, suggesting that these proteins differ in their co-operative structural domains and domain stabilities.

Myosin from smooth muscle can exist in two monomeric forms, an extended form that sediments at 6S and a folded form that sediments at 10S. Myosin in the 6S form possesses an extended tail which is able to form filaments. In contrast, myosin in the 10S form folds its 150 nm tail at sites approximately 50 and 100 nm from the heads, and the heads are bent towards the tail [15,16]. The 6S myosin appears to be the functional form whereas

Abbreviations used: HMM, heavy meromyosin; LMM, light meromyosin; S-1, subfragment 1; S-2, subfragment 2; DTT, dithiothreitol.

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the 10S myosin has no Mg-ATPase activity [17]. Phosphorylation of the regulatory light chains, which activates interaction between actin and myosin filaments in smooth muscle, induces the transition of the folded monomer into the extended monomer at physiological ionic strength [18]. The regulated formation of the 10S myosin in this system probably couples force generation to myosin filament assembly. Observations of the folded myosin conformation suggest that, in addition to the head–rod junction and the heavy meromyosin (HMM)–LMM junction, a third flexible region must exist to allow the tail to fold back to the head. In this paper, we demonstrate the existence of protease-susceptible sites located in the LMM region, which probably correspond to the flexible regions in the folded (or 10S) conformation of the smooth-muscle myosin molecule.

MATERIALS AND METHODS

Bovine aortic myosin was purified by the method of Chacko [19] with modifications. Briefly, minced bovine aorta without intima was homogenized with a blender in washing buffer containing 60 mM KCl, 20 mM Mops (pH 6.8), 1 mM $MgSO_4$, 1 mM dithiothreitol (DTT) and 100 nM aprotinin. The washed pellet was then extracted with 3 vol. of extraction buffer containing 0.6 M KCl, 20 mM Mops (pH 7.5), 2 mM EGTA, 1 mM EDTA, 1 mM DTT and 100 nM aprotinin. $MgCl_2$ was added to the muscle extract to a final concentration of 10 mM. After the addition of 10 mM ATP (pH 7.4), the muscle extract was fractionated immediately into 0–35% and 35–70% fractions by adding saturated $(NH_4)_2SO_4$ solution containing 10 mM EDTA (pH 7.0). The 35–70%-satd. $(NH_4)_2SO_4$ fraction was dissolved in a solution containing 0.8 M KCl, 20 mM Tris/HCl (pH 7.5), 1 mM EDTA and 1 mM DTT, then dialysed thoroughly against a low-salt buffer containing 40 mM KCl, 20 mM Mops (pH 7.4), 1 mM EDTA and 1 mM DTT to remove $(NH_4)_2SO_4$. The pellet produced on centrifugation contained about 95% myosin.

Chicken gizzard myosin was prepared by the method of Ebashi [20] with modifications. $(NH_4)_2SO_4$ fractionation and column chromatography were substituted for the repeated high-salt/low-salt polymerization cycles. Rabbit skeletal-muscle myosin was prepared by the standard procedures of Sreter et al. [21] followed by dialysis and column chromatography to remove titin and other impurities [22].

Myosin (5 mg/ml) in buffer A (0.6 M NaCl, 1 mM EDTA, 1 mM DTT and 50 mM sodium phosphate, pH 7.3) was digested into S-1 fragments and rods with 0.01 mg/ml papain at room temperature, the reaction being terminated with iodoacetic acid at a final concentration of 5 mM. Papain (1 mg/ml) was activated for 1 h at 37 °C in a solution containing 50 mM freshly dissolved cysteine, 10 mM EDTA and 10 mM Tris/HCl, pH 8.0. Myosin rods were obtained from the insoluble fraction of a 50 min digest of myosin after dialysis against low-ionic-strength buffer (a solution containing 40 mM NaCl, 20 mM sodium phosphate, 1 mM EDTA and 1 mM DTT, pH 6.5). The pellet obtained after centrifugation at 18000 ***g*** for 30 min was redissolved in a solution containing 0.6 M NaCl, 50 mM sodium phosphate and 1 mM EDTA, pH 7.0, and chromatographed on a Bio-Gel A-5m (Bio-Rad) column equilibrated in the same buffer. Both chymotrypsin and trypsin digestion studies were also carried out in buffer A at room temperature. The chymotrypsin reaction was terminated with PMSF at 5 mM final concentration. Trypsin digestion was terminated by adding soybean trypsin inhibitor to a final concentration of 0.2–1 mg/ml. The results of these digestion studies were analysed by SDS/PAGE.

CD measurements were made with an Aviv 60 DS spectropolarimeter (Lakewood, NJ, U.S.A.) containing a Hewlett–Packard 89100A temperature controller which provided programmable sample temperature changes with 0.1 °C resolution. Ellipticity values at 222 nm were obtained from 25 to 70 °C using solutions contained in a stoppered standard 1 cm Spectrosil quartz cuvette into which the temperature probe of the unit was placed. The solution was stirred with a magnetic bar placed below the light path, enabling rapid temperature equilibration to be obtained. Data were collected automatically in 0.2 °C steps using an equilibrium time of 0.4 min and a data averaging time of 10 s at each temperature step.

RESULTS AND DISCUSSION

Thermal unfolding of rod and LMM

There were three major thermal unfolding transitions for aortic myosin rod. The first and second transitions were broad, with transition midpoints at 47.5 and 51 °C respectively, whereas the third transition was very sharp, and had a midpoint at 54 °C. The first and second transitions corresponded to approximately 40% of total helix loss and the third transition accounted for the remaining 60% of total helix loss (Figure 1). Approximately 17% of the helix remained at 70 °C. The recovery of the helix after cooling the system back to 25 °C was about 94%. The thermal unfolding profiles of different myosin rods under the same buffer and measuring conditions are shown in Figure 2. Gizzard myosin rod showed transition midpoints of 48 and 52 °C. The 48 °C transition corresponded to about 30% total helix loss and the 52 °C transition corresponded to about 70% helix loss. Rabbit skeletal myosin rod unfolds with helix-unfolding transition midpoints at 43, 47 and 53 °C. The first and second transitions corresponded to about 75% total helix loss

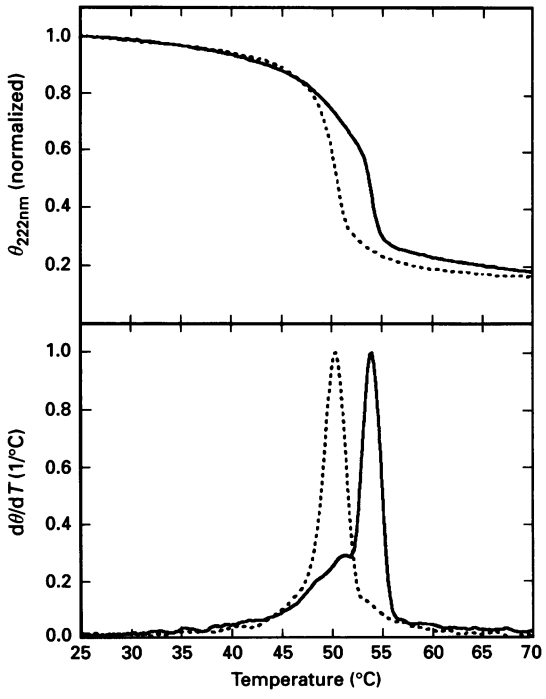

Figure 1 Thermal unfolding profiles of aortic myosin rod and LMM

Top: normalized thermal helix-unfolding profiles of aortic myosin rod (—) and LMM (....) in 50 mM sodium phosphate solution, pH 7.1, containing 0.6 M NaCl, 1 mM EDTA and 1 mM DTT. Bottom: normalized derivatives of thermal unfolding profile of aortic rod (—) and LMM (....).

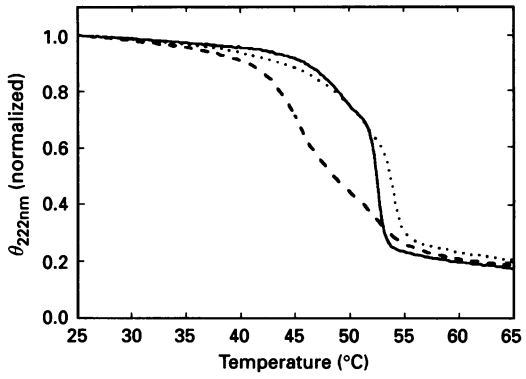

Figure 2 Comparison of the thermal unfolding profiles of different myosin rods

Normalized thermal helix-unfolding profiles of aortic myosin rod (....), gizzard myosin rod (—) and rabbit skeletal-muscle myosin rod (----) in 50 mM sodium phosphate buffer, pH 7.1, containing 0.6 M NaCl, 1 mM EDTA and 1 mM DTT.

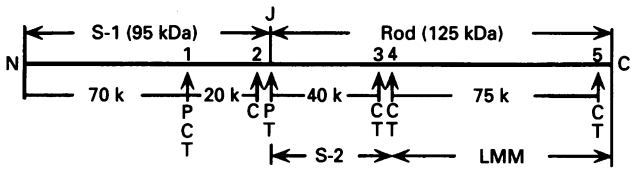

Figure 3 Summary of the major proteolytic fragments of aortic myosin

P, papain-susceptible site; C, chymotrypsin-susceptible site; T, trypsin-susceptible site; k, kDa. The sites in LMM are not marked in the diagram.

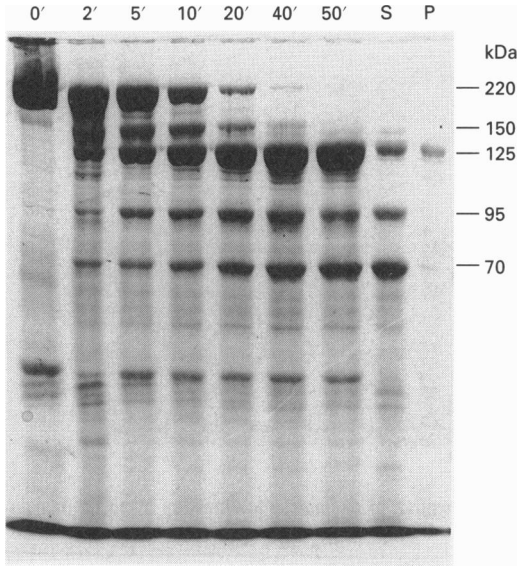

Figure 4 Time course of papain digestion of aortic myosin

Aortic myosin (5 mg/ml) was allowed to react with 0.01 mg/ml papain in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (7.5% gel). Lanes S and P indicate the soluble and insoluble fractions (in low-ionic-strength buffer consisting of 40 mM NaCl and 20 mM sodium phosphate) of 50 min digest respectively.

and the third transition accounts for about 25% total helix loss. The obvious difference between the thermal unfolding profiles of these proteins suggests that the proteins differ in their co-operative structural domains and domain stabilities. In general, the helix structures of smooth-muscle myosin rods appear to be thermally more stable than those of skeletal-muscle myosin rods: the major transition of both aortic and gizzard rods started above 51 °C whereas the major transition of both chicken [22] and rabbit skeletal-muscle rods had finished at that temperature.

Three thermal unfolding transitions were also observed for aortic LMM. The transition midpoints were 46, 50.5 and 54 °C respectively. The first transition corresponded to about 15% total helix loss. The second transition was sharp, corresponding to about 70% total helix loss. The third transition corresponded to about 15% total helix loss (Figure 1). Similarly to results for the rod, about 16% of the helix remained at 70 °C and about 95% of the helix was recovered after cooling to 25 °C. Comparison between the unfolding profiles of rod and LMM indicates that the 54 °C transition is mainly located in the S-2 region, assuming that no interaction occurs between S-2 and LMM regions. Therefore most of the S-2 region of aortic myosin rod is likely to be more stable than the LMM region in high-ionic-strength buffer.

Proteolytic digestion of aortic myosin

The major proteolytic fragments of aortic myosin are described in Figure 3. Papain digestion of aortic myosin produced 150, 125, 95 and 70 kDa species. The 150 kDa fragment was gradually degraded to the 125 kDa fragment as digestion time increased (Figure 4). The 125 kDa fragment was relatively insoluble in low-ionic-strength buffer (40 mM NaCl, 20 mM sodium phosphate) (lane P of Figure 4), corresponding to the rod portion. The soluble 95 and 70 kDa fragments (lane S of Figure 4) correspond to the S-1 portion. The production of 125 and 95 kDa species indicates a susceptible site (site J) located at the head–rod junction. There is a lysine residue next to the proline residue at the head–rod junction in all the available myosin sequences, which may correspond to the papain cleavage site. The number of this lysine residue is 842 in the amino acid sequence of rabbit uterus myosin. The reason for selecting rabbit uterus myosin sequence instead of gizzard myosin sequence as the reference for predicting the proteolytic cleavage sites of aortic myosin is twofold: (1) there is an insert of seven amino acids in S-1 of gizzard myosin [5], resulting in different numbering of amino acid residues in this sequence; (2) the available 501 C-terminal amino acids of rabbit aortic myosin [23] are the same as those of rabbit uterus myosin except for two amino acid residues, whereas the amino acid sequence of chicken gizzard myosin differs by approximately 15% from that of rabbit uterus myosin.

The production of 150 and 70 kDa fragments indicates an additional protease-susceptible site (site 1) located at the head portion 25 kDa from the head–rod junction. Three lysine residues are located in this region (Lys-633, -644, -646 in the rabbit uterus myosin sequence) and their positions in the sequence are well conserved in all available smooth, striated and non-muscle sequences. One of these lysine residues may provide the peptide bond that is cleaved. The 25 kDa fragment was more clearly demonstrated in the 12% polyacrylamide gel of Figure 7(b). An additional 26 kDa band appeared at earlier stages of digestion, whereas the 25 kDa band became the major species at later stages of digestion.

Limited chymotrypsin digestion of aortic myosin produced

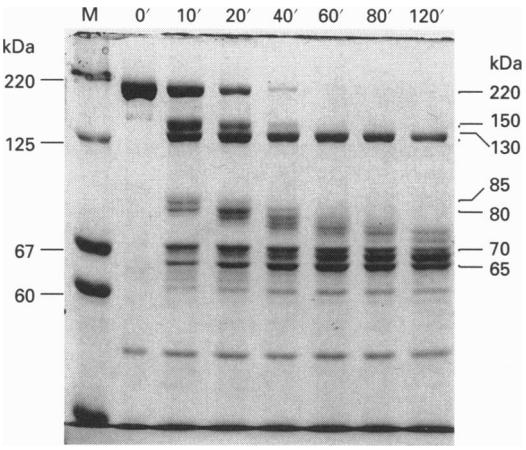

Figure 5 Time course of chymotryptic digestion of aortic myosin

Myosin (1.5 mg/ml) was allowed to react with 0.075 mg/ml chymotrypsin in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (7.5% gel). Lane M, molecular-mass markers.

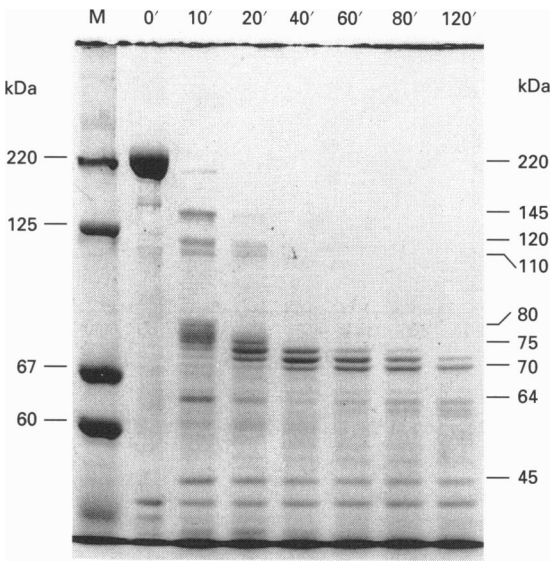

Figure 6 Time course of tryptic digestion of aortic myosin

Myosin (1.5 mg/ml) was allowed to react with 0.075 mg/ml trypsin in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (7% gel). Lane M, molecular-mass markers.

150, 130, 85, 80 and 65–70 kDa species (Figure 5). The appearance of 150 and 70 kDa species indicates a chymotrypsin-susceptible site about 70 kDa from the N-terminus, near site 1 defined by papain digestion. The production of a 130 kDa fragment indicates a site (site 2) located at the head portion, 5 kDa from the head–rod junction. Site 2 may be located in the region between Phe-793 and Phe-806 (FQAMCRGYLARKAF) which contains several aromatic and large hydrophobic amino acid residues as potential chymotryptic cleavage sites. The 150 kDa fragment was gradually degraded to 130 kDa, and the 85–80 kDa fragments were gradually degraded to one of less than 75 kDa as digestion time increased (Figure 5). The degradation pattern of the 85–80 kDa species resembled that observed for rod digestion (see Figure 9). It suggests that these species may come from the rod portion instead of the head portion, by cleavage at sites 3 and 4 respectively. The lack of 95 kDa fragment S-1 may be attributed to its liability to protease digestion, resulting in further degradation to 70 kDa and smaller fragments.

Limited trypsin digestion of aortic myosin produced 145, 120–110, 80–75, 64 and 45 kDa species. All the 145, 120–110 and 80–75 kDa fragments were degraded to 70 kDa as digestion time increased (Figure 6). The formation of 145 and 120 kDa species suggests the existence of two trypsin-susceptible sites located at the same position as those for papain digestion, plus an additional cleavage site (site 5) located 5 kDa from the C-terminus of the rod. Site 5 must be close to the chymotryptic cleavage site identified between Phe-1943 and Ala-1944 [24] of the gizzard myosin sequence which is equivalent to Phe-1936 and Val-1937 of the rabbit uterus myosin sequence. The trypsin-cleavage site is therefore predicted to be located in the region between Lys-1925 and Arg-1930 (KSKLRR) which contains several lysine and arginine residues as potential cleavage sites. Those fragments from the S-1 portion of myosin (i.e. 70 and 95 kDa fragments) appear to be readily degraded and thus are not observed on the gel. The formation and degradation of 80–75 kDa species exhibited a pattern similar to that observed during tryptic digestion of the rod (see Figure 10), suggesting that these fragments probably come from the rod portion.

Apparently papain digestion of aortic myosin produces S-1 and the rod, whereas limited chymotrypsin and trypsin digestion yields both a head–rod pair and an HMM–LMM pair. These results indicate that two major protease-susceptible sites are located at the head–rod junction and the HMM–LMM junction, which resemble those of striated-muscle myosin [1,2] and gizzard smooth-muscle myosin [25].

The digestion work was performed simultaneously with skeletal-muscle, gizzard and aortic myosin for direct comparison. The papain digestion patterns of these myosins are shown in Figure 7(a). The gizzard myosin exhibited exactly the same digestion pattern and size of fragments as those of aortic myosin. However, the rabbit skeletal-muscle myosin showed a different pattern. The 95 kDa fragment S-1 was not clearly observed, suggesting that it was degraded into smaller fragments. Two major fragments (72 and 75 kDa) were slightly longer than that of smooth-muscle myosin (70 kDa) and an additional 58 kDa fragment appeared in the digestion pattern.

The chymotryptic digestion pattern of gizzard myosin resembles that of aortic myosin (Figure 8a), especially in the following two respects: (1) the S-1 fragment was not observed, probably because of its immediate degradation to 70 kDa and smaller subfragments; (2) the LMM (85 kDa) gradually decreased in size as digestion time increased. The smallest fragment of this ladder pattern of gizzard LMM is about 80 kDa whereas that of aortic LMM is less than 75 kDa. The rather limited degradation of gizzard LMM agrees with the result of Tashiro et al. [8]. However, the chymotryptic digestion pattern of rabbit skeletal-muscle myosin (Figure 8b) is dramatically different from those of smooth-muscle myosins. There is a well-preserved 90 kDa fragment identified as S-1 because it did not appear in the digestion pattern of rabbit skeletal-muscle myosin rod (Figure 8c). In addition, the size of LMM is no more than 70 kDa at the earliest stage of digestion, indicating a chymotrypsin-susceptible site between the 'long S-2' and 'short LMM' segments. In contrast, chymotryptic digestion of both gizzard and aortic myosin produced an 85 kDa 'long LMM' at the early stage of digestion.

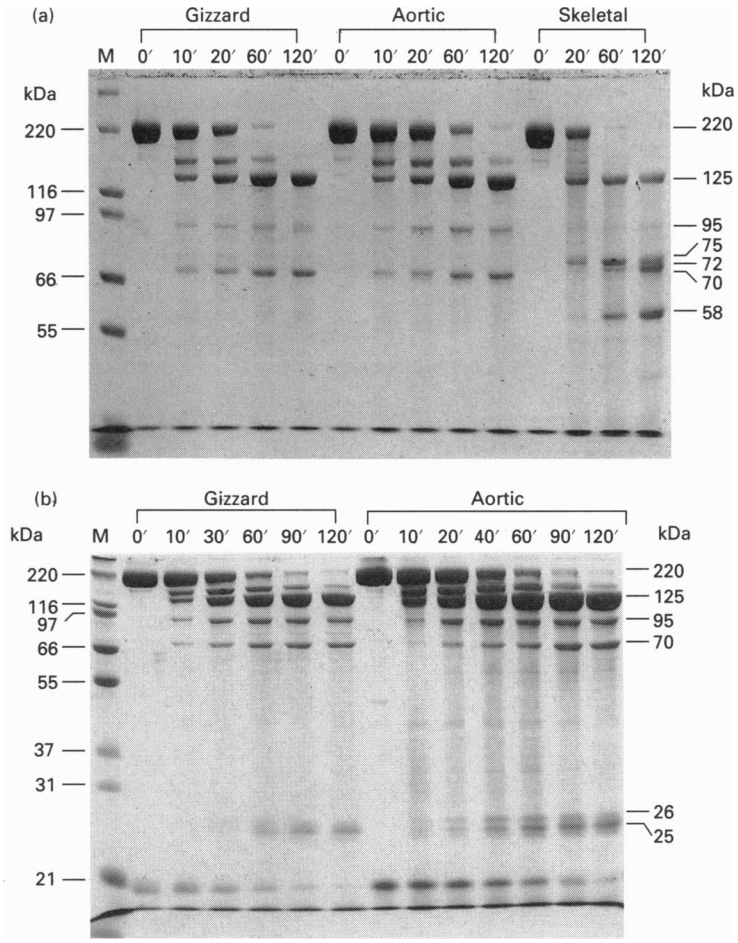

Figure 7 Comparison of papain digestion patterns of gizzard, aortic and skeletal-muscle myosins

(**a**) Myosin (0.7 mg/ml) from chicken gizzard, bovine aortic and rabbit skeletal muscles was allowed to react with 0.0017 mg/ml papain in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (7.5% gel). (**b**) Gizzard myosin and aortic myosin (0.5 mg/ml) were allowed to react with 0.001 mg/ml papain in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (12% gel).

Proteolytic digestion of aortic rod

Limited chymotrypsin digestion (myosin rod, 0.2 mg/ml; chymotrypsin, 0.02 mg/ml) of the aortic myosin rod (125 kDa) produced 80 and 45 kDa fragments, indicating that the rod was cut at site 4 at the very beginning of digestion by chymotrypsin (Figure 9a). The 80 kDa fragment was gradually degraded to 79, 77 and 75 kDa species step by step. The 75–80 kDa species were insoluble in low-ionic-strength buffer (lane P of Figure 9a), corresponding to the LMM portion of the myosin rod, whereas the soluble 45 kDa fraction (lane S of Figure 9a) corresponds to the S-2 portion of the myosin rod. The resulting 45 kDa S-2 species is larger than skeletal-muscle short S-2 (34 kDa) and smaller than skeletal-muscle long S-2 (58 kDa). This result resembles that of the chymotryptic digestion of gizzard rod [26].

Limited tryptic digestion of the aortic myosin rod (125 kDa) also produced 80 kDa (LMM) and 45 kDa (S-2) species (Figure 10). However, the 45 kDa species was degraded to a 40 kDa fragment as reaction time increased, indicating that an additional proteolytic site is located 5 kDa from the end of S-2 (site 3). This site might correspond to the protease-susceptible site reported for the myosin rod of chicken gizzard [8,27] and pig stomach [27] smooth-muscle myosin. The fact that only the 40 kDa species was observed in those studies might be attributed to the stronger digestion conditions used than in this study.

The S-2 segments obtained from aortic myosin rod are similar to those of chicken gizzard rod and pig stomach rod. In contrast, chymotryptic digestion of rabbit skeletal-muscle rod under the same conditions produces the shorter LMM (70 kDa) and long S-2 (58 kDa) segments at the early stage of digestion (Figure 8c). The long S-2 segment of skeletal-muscle myosin was not observed in smooth-muscle myosin rod even under the mild digestion conditions used in this study. Thus the shorter S-2–LMM junction hinge region appears to be a common property of vertebrate smooth-muscle myosin rod, which is distinct from the longer hinge segment of the skeletal-muscle myosin rod. Whether the differences in the length of hinge affect the contractile properties of the cross-bridges in these muscles remains to be determined.

Solubility properties of LMM fragments

The 80 kDa LMM produced by tryptic digestion of myosin rod was gradually degraded to a 70 kDa fragment as the digestion time increased (Figure 10). The 70 kDa fragment was further

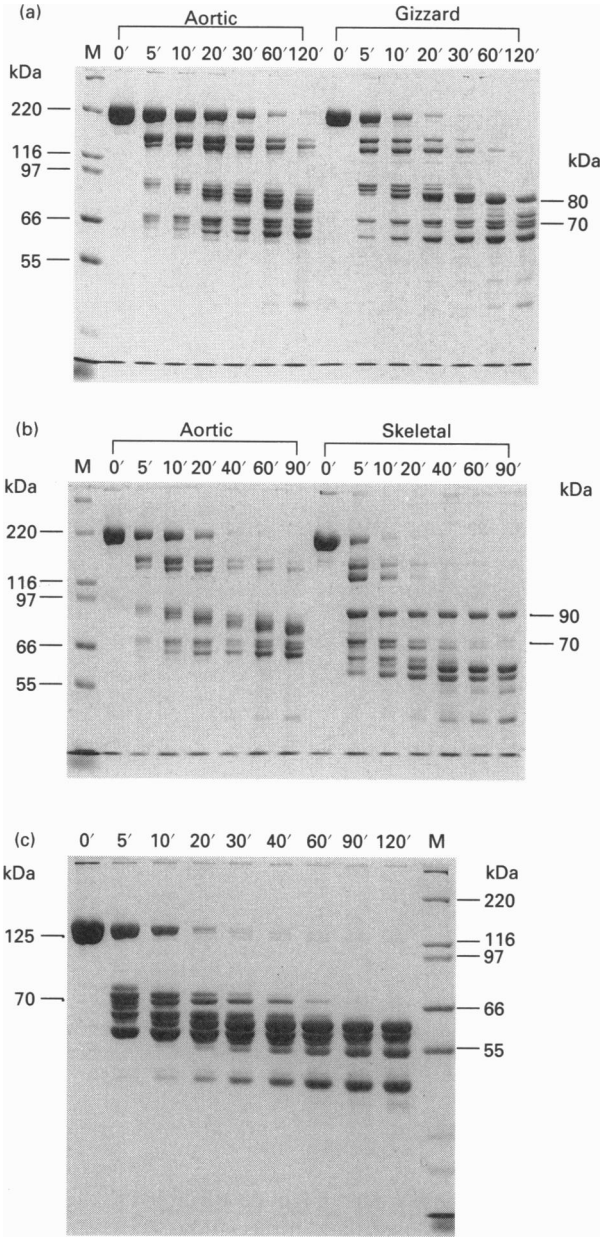

Figure 8 Comparison of chymotryptic digestion of aortic, gizzard and skeletal-muscle myosins

(**a**) Myosin (0.7 mg/ml) from bovine aorta and chicken gizzard was allowed to react with 0.11 and 0.18 mg/ml chymotrypsin respectively in buffer A at room temperature. (**b**) Aortic myosin and rabbit skeletal-muscle myosin (0.7 mg/ml) were allowed to react with 0.18 and 0.11 mg/ml chymotrypsin in buffer A at room temperature respectively. (**c**) Rabbit skeletal-muscle rod (2 mg/ml) was allowed to react with 0.22 mg/ml chymotrypsin in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (7.5% gel for **a** and **b**; 8% gel for **c**). Lane M, molecular-mass markers.

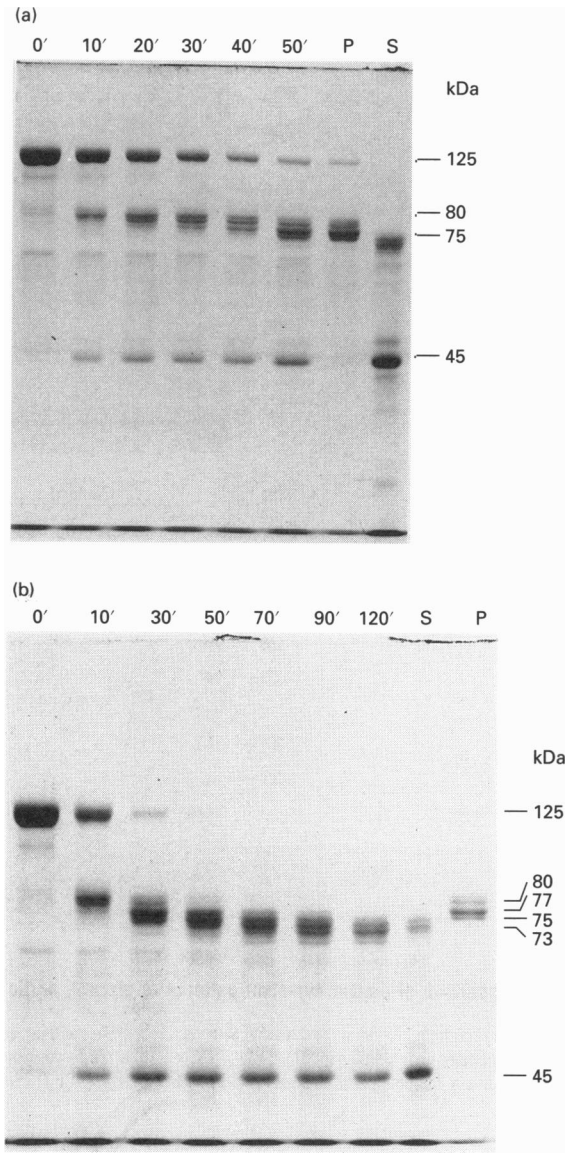

Figure 9 Time course of chymotryptic digestion of aortic myosin rod

(**a**) Rod (0.2 mg/ml) was allowed to react with 0.02 mg/ml chymotrypsin in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (7.5% gel). Lanes P and S indicate the insoluble and soluble fractions (in low-ionic-strength buffer) of 50 min digest respectively. (**b**) Rod (0.4 mg/ml) was allowed to react with 0.04 mg/ml chymotrypsin in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (7% gel). Lane S, soluble fraction (in low-ionic-strength buffer) of 120 min digest; lane P, insoluble fraction from experiment in (**a**).

degraded to a 65 kDa fragment during the dialysis time span. In contrast with the 80 kDa LMM, the 65 kDa fragment of LMM was soluble in low-ionic-strength buffer (40 mM NaCl, 20 mM sodium phosphate), suggesting that only a small portion of LMM is critical for the insolubility of myosin in low-ionic-strength buffer.

The 75 kDa LMM produced by chymotryptic digestion shown in Figure 9(a) was further degraded to 73 and 70 kDa species as the concentrations of myosin rod and chymotrypsin were increased. Figure 9(b) illustrates the pattern produced by digestion of 0.4 mg/ml myosin rod with 0.04 mg/ml chymotrypsin. After the reaction had proceeded for 1 h, LMM fragments of molecular mass less than 75 kDa gradually appeared. At about 2 h, all the LMM fragments were degraded to species of molecular mass no more than 75 kDa. These fragments of LMM were soluble in low-ionic-strength buffer (40 mM NaCl, 20 mM sodium phosphate) (lane S of Figure 9b). In contrast, LMM fragments greater than 75 kDa were insoluble (lane P of Figure 9b). The fact that 73 kDa LMM is completely soluble and 77 kDa LMM is completely insoluble in low-ionic-strength buffer suggests that

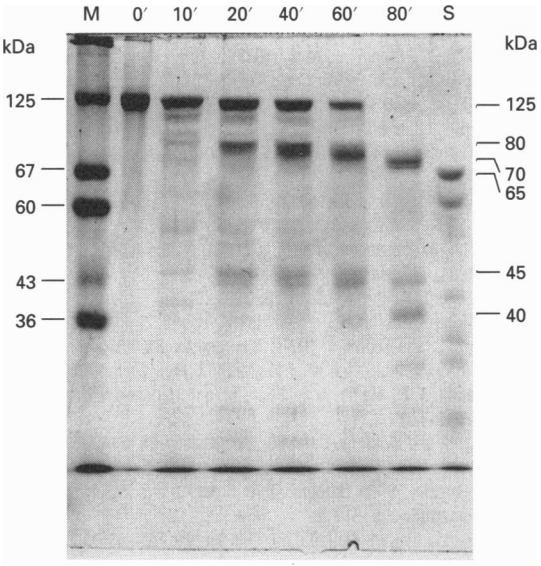

Figure 10 Time course of tryptic digestion of aortic myosin rod

Rod (0.8 mg/ml) was allowed to react with 0.02 mg/ml trypsin in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (8% gel). Lane S, soluble fraction (in low-ionic-strength buffer) of 80 min digest; lane M, molecular-mass markers.

only a small portion (about 4 kDa) of LMM is critical for filament formation. This result is in good agreement with previous reports showing that a 5 kDa short segment of LMM is critical for the insolubility of both rabbit skeletal-muscle myosin and gizzard smooth-muscle myosin in low-ionic-strength buffer [8–11]. Sequence analysis indicates that this fragment is located near the C-terminus of rabbit skeletal myosin [11]. Experiments

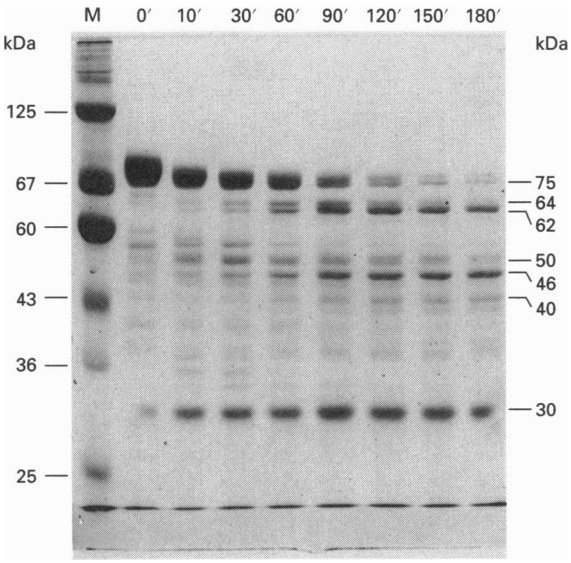

Figure 11 Time course of chymotryptic digestion of aortic LMM

LMM (1.1 mg/ml) was allowed to react with 0.55 mg/ml chymotrypsin in buffer A at room temperature. Digests were sampled at the time points indicated and subjected to SDS/PAGE (8.5% gel). Lane M, molecular-mass markers.

using myosin with partially deleted sequences provide further evidence that deletion of a short peptide at the C-terminus significantly increases its solubility in low-ionic-strength buffer [28], decreases aggregation [29] and increases by 50-fold the critical concentration for myosin rod assembly [30]. In sharp contrast, deleting a peptide of similar length at the N-terminus of the rod or LMM has only minor effects [28,30]. Although we are not able to determine the position of this 4 kDa fragment along the LMM sequence at present, it is tempting to speculate that it is located near the C-terminus, similarly to myosin from skeletal muscle or non-muscle.

The dramatic solubility difference between 77 kDa and 73 kDa LMM fragments raises an interesting question: do the interactions between myosin molecules in the thick filament take place in a segment that accounts for less than 2% of the molecular mass of a myosin molecule? Hodge et al. [30] provide models to explain the role of the tailpiece of the myosin rod in the filament-assembly process, which may be applicable to the interpretation of our results.

Protease-susceptible sites in LMM

Limited chymotrypsin digestion of the 75–80 kDa LMM species generated from chymotrypsin digestion of myosin rod produced 50 and 30 kDa species (Figure 11). The 50 kDa fragment was gradually degraded to 46 kDa as reaction time increased. This result indicates that there is a chymotrypsin-susceptible site located 30 kDa from one terminus of LMM. In addition, the 75–77 kDa LMM is gradually degraded to 62–64 kDa species. This result suggests that the other chymotrypsin-susceptible site is located 13 kDa from the other terminus of LMM. This site was also observed in chicken gizzard LMM [10], which was considered to be a solubility-determining domain located at the C-terminus of myosin. The peptide bond between Phe-1812 and Lys-1813 may correspond to this cleavage site.

It has been suggested that regions in the myosin molecule that exhibit high susceptibility to proteolytic enzymes correspond to the hinge regions. We have demonstrated that protease-susceptible sites exist in both the head–rod junction and the HMM–LMM junction, similarly to all other types of myosin. Moreover, our results have identified additional protease-susceptible sites within the LMM region which would permit the myosin tail to fold back to the head region. Thus these protease-susceptible sites within LMM provide a structural basis for the formation of the folded myosin conformation observed by electron microscopy [15,16]. The existence of more than one protease-susceptible site within LMM makes the bending of the rod possible from the structural point of view. However, the flexibility of the molecule may create thousands of different conformations. How does the myosin molecule lock itself into two specific conformations: the 6S and 10S forms? These two forms must be the most stable forms in their respective environments (ionic strength, pH, temperature etc.). Intermolecular interaction of myosin in filaments may stabilize the 6S conformation to maintain the myosin molecule in its functional form in muscle. What is the reason for the high stability of the folded 10S conformation? Morphological studies reveal contact of the folded tail with the head–rod junction region. Conceivably, certain interactions (e.g. ionic) exist in this contact region to stabilize the folded structure. Indeed, it has been demonstrated that cleavage of the 4 kDa segment at the C-terminus of gizzard myosin by chymotrypsin significantly modified ATPase activities of the myosin head [24]. It is known that dephosphorylation of myosin regulatory light chains favours the folded form of smooth myosin. The site of phosphorylation is located in the

neck region of myosin close to the head–rod junction [31,32]. The charge on the phosphate group may play a crucial role in the interaction between the tail and head in this region, thus regulating the transition between 10S and 6S conformations. Precise identification of these contact regions on both head and tail, and further investigation of the nature of their interaction, is both necessary and feasible.

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REFERENCES

1 Lowey, S., Slayter, H. S. Weeds, A. G. and Baker, H. (1969) J. Mol. Biol. **42**, 1–29
2 Weeds A. G., and Pope, B. (1977) J. Mol. Biol. **111**, 129–157
3 Adelstein, R. S. and Eisenberg, E. (1980) Annu. Rev. Biochem. **49**, 921–956
4 Hartshorne, D. J. and Siemankowski, R. F. (1981) Annu. Rev. Physiol. **43**, 519–530
5 Kelley, C. A., Yakahashi, M., Yu, J. H. and Adelstein, R. S. (1993) J. Biol. Chem. **268**, 12848–12854
6 Wagner, P. D. and Vu, N. D. (1986) J. Biol. Chem. **261**, 7778–7783
7 Wagner, P. D. and Vu, N. D. (1987) J. Biol. Chem. **262**, 15556–15562
8 Tashiro, Y., Kumon, A., Yasuda, S., Murakami, N. and Matsumura, S. (1985) Eur. J. Biochem. **148**, 521–528
9 Kumon, A., Kuba, M., Murakami, N. et al. (1986) Eur. J. Biochem. **160**, 499–506
10 Cross, R. A. and Vandekerckhove, J. (1986) FEBS Lett. **200**, 355–360
11 Nyitray, L., Mocz, G., Szilagyi, L. et al. (1983) J. Biol. Chem. **258**, 13213–13220
12 Privalov, P. L. (1982) Adv. Protein Chem. **35**, 1–104
13 King, L. and Lehrer, S. S. (1989) Biochemistry **28**, 3498–3502
14 King, L., Seidel, J. C. and Lehrer, S. S. (1995) Biochemistry **34**, 6770–6774
15 Trybus, K. M., Huiatt, T. W. and Lowey, S. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 6151–6155
16 Onishi, H. and Wakabayashi, T. (1982) J. Biochem. (Tokyo) **92**, 871–879
17 Cross, R. A. (1988) J. Muscle Res. Cell Motil. **9**, 108–110
18 Craig, R., Smith, R. and Kendrick-Jones, J. (1983) Nature (London) **302**, 436–439
19 Chacko, S. (1981) Biochemistry **20**, 702–707
20 Ebashi, S. (1976) J. Biochem. (Tokyo) **79**, 229–231
21 Sreter, F. A., Holtzer, S., Gergely, J. and Holtzer, H. (1972) J. Cell Biol. **55**, 586–594
22 King, L., Liu, C. C. and Lee, R.-F. (1994) Biochemistry **33**, 5570–5580
23 Kuro-o, M., Nagai, R., Nakahara, K. et al. (1991) J. Biol. Chem. **266**, 3768–3773
24 Ikebe, M., Hewett, T. E., Martin, A. F., Chen, M. and Hartshorne, D. J. (1991) J. Biol. Chem. **266**, 7030–7036
25 Nath, N., Nag, S. and Seidel, J. C. (1986) Biochemistry **25**, 6169–6176
26 Suzuki, H., Wong, A., Lu, R. C. and Seidel, J. C. (1984) Biophys. J. **42**, 43a
27 Cross, R. A., Bardsley, R. G., Ledward, D. A., Small, J. V. and Sobieszek, A. (1984) Eur. J. Biochem. **145**, 305–310
28 Atkinson, S. J. and Stewart, M. (1991) J. Cell Sci. **99**, 823–836
29 Maeda, K., Rosch, A., Maeda, Y., Kalbitzer, H. R. and Wittinghofer, A. (1991) FEBS Lett. **281**, 23–26
30 Hodge, T. P., Cross, R. and Kendrick-Jones, J. (1992) J. Cell Biol. **118**, 1085–1095
31 Katayama, E. and Wakabayashi, T. (1981) J. Biochem. (Tokyo) **90**, 703–714
32 Flicker, P., Wallimann, T. and Vibert, P. (1981) Biophys. J. **33**, 279a

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