# Proteolysis and Actin-Binding Properties of 10S and 6S Smooth Muscle Myosin: Identification of a Site Protected from Proteolysis in the 10S Conformation and by the Binding of Actin†

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ABSTRACT: It was shown previously [Ikebe, M., & Hartshorne, D. J. (1985) *Biochemistry 24*, 2380–2387] that the conformation of gizzard myosin, either 10S or 6S, influences proteolysis of myosin at two regions designated sites A and B. The studies reported here are focused on site A, which is located approximately 68 000 daltons from the N-terminus of the myosin heavy chain. With papain, *Staphylococcus aureus* protease, and actinidin, it is shown that the formation of 10S myosin reduces proteolysis at site A. Binding of actin to 6S myosin also hinders cleavage at site A for each of these proteases. To investigate binding of actin to 6S and 10S myosins, adenosine 5′-($\beta,\gamma$-imidotriphosphate) (AMPPNP) is used as a substitute for ATP. In the presence of AMPPNP, it is shown that the 6S to 10S transition occurs and that 10S myosin binds actin with lower affinity than 6S myosin. For 6S myosin at high salt (0.35 M KCl) the dissocation constant of actin from the actin–myosin–nucleotide complex ($K_3$) is approximately the same for phosphorylated (1.9 mol of P/mol of myosin) and dephosphorylated myosin, i.e., 1.3–2.4 $\mu$M, respectively. At lower ionic strength (0.17 M KCl) $K_3$ for dephosphorylated myosin (10S myosin) is 42 $\mu$M and $K_3$ for phosphorylated myosin (6S myosin) is 0.3 $\mu$M. These data show that the conformation of myosin influences the actin–myosin interaction. The constant ($K_4$) for the dissociation of nucleotide from the actin–myosin–nucleotide complex varies slightly (in the range of 0.2–1.3 mM), but there is no marked change as a result of either a conformational change or phosphorylation. Since site A is masked, at least partly, by the formation of 10S myosin and by the binding of actin, this may indicate that site A is located at, or close to, the actin-binding site on the myosin heavy chain.

Over the last decade the idea that the phosphorylation of myosin forms part of the regulatory mechanism in smooth muscle has received considerable experimental support [reviews by Adelstein and Eisenberg (1980) and Walsh and Hartshorne (1982)]. Phosphorylation by myosin light chain kinase of serine-19 on each of the two 20 000-dalton light chains of myosin increases the actin-activated ATPase of myosin, and it is assumed that this event corresponds to the initial development of tension in the muscle fiber. It follows, therefore, that phosphorylation of the light chains influences the ATP hydrolysis sites and possibly the actin-binding sites, both of which are located on the myosin heavy chains. It is assumed that phosphorylation alters the conformation of smooth muscle myosin and converts an inactive species to an active species.

Although the details of this transition are not known, some progress has been made in defining the molecular changes induced by phosphorylation. The critical discovery was the realization that monomeric smooth muscle myosin could exist in two conformations, i.e., the folded (10S) and extended (6S) states (Suzuki et al., 1982; Onishi & Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983). It was subsequently shown that the two conformations were characterized by distinct enzymatic properties (Ikebe et al., 1983), and it was suggested that some component of the 10S–6S transition was involved in the regulation of ATPase activity (Ikebe et al., 1983; Ikebe & Hartshorne, 1984). It was shown also that phosphorylation of myosin favors the 6S state (Trybus et al., 1982; Craig et al., 1983; Ikebe et al., 1983; Onishi, et al., 1983; Trybus & Lowey, 1984). An important point is that phosphorylation does not alter enzymatic activity directly but does so via a change in myosin conformation (Ikebe et al., 1983; Ikebe & Hartshorne, 1984). In this sense, the influence of phosphorylation is secondary to the influence of myosin conformation. The relationship between myosin shape and enzymatic activity, therefore, indicated that the biological properties of myosin were influenced by conformational changes and that these changes occurred during the 10S–6S transition. Despite its simplicity this theory raised several problems: (1) The 10S–6S transition involves an extensive conformational change, and even though it is known that the critical change occurs during this transition, there is still the problem of identifying which region of the molecule is altered. (2) It is known that the actin-activated ATPase of heavy meromyosin (HMM)[1] is regulated by phosphorylation (Sellers et al., 1981; Ikebe et al., 1981; 1982) and as HMM cannot form a folded molecule (since the tail region is removed by proteolysis) this raises the question of how important the overall 10S–6S transition is to the proposed regulatory effect. (3) Under normal in vivo conditions myosin is thought to exist only in the filamentous state (Somlyo et al., 1981), and it was unknown whether or not a regulatory conformational change

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[1] Abbreviations: HMM, heavy meromyosin; S1, heavy meromyosin subfragment 1; S2, heavy meromyosin subfragment 2; LMM, light meromyosin; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; AMPPNP, adenosine 5′-($\beta,\gamma$-imidotriphosphate); HPLC, high-performance liquid chromatography; kDa, kilodalton; ATPase, adenosinetriphosphatase; EDTA, ethylenediaminetetraacetic acid.

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could occur in myosin filaments as well as in monomeric myosin.

Information on each of these points was obtained by the use of limited proteolysis of myosin. Initially, it was found that 10S gizzard myosin is more resistant to papain hydrolysis than 6S myosin and that the resistant region of the molecule is in the head–neck junction (Onishi & Watanabe, 1984; Ikebe & Hartshorne, 1984). Other proteases that cleave at the S1–S2 also show this effect, for example, *Staphylococcus aureus* protease and trypsin, but proteolysis by $\alpha$-chymotrypsin at the HMM–LMM junction is not sensitive to conformation (Ikebe & Hartshorne, 1984). By use of papain digestion as a conformational probe, it was found that both HMM and filamentous myosin possess a protease-resistant region similar to that found in 10S myosin (Ikebe & Hartshorne, 1984). These results indicated that part of the 10S–6S transition involves an alteration of the head–neck, or S1–S2, region of the molecule, and it appeared that this more localized conformational change is responsible for the alteration of enzymatic activity. In a more extensive study using *S. aureus* protease this idea was confirmed, and it was shown that two sites on the myosin heavy chain were protected in the 10S conformation (Ikebe & Hartshorne, 1985a). These were designated the A and B sites. The B site is located at the S1–S2 junction, and hydrolysis at this point is responsible for the release of S1. The A site is located approximately 68 kDa from the N-terminus of the heavy chain. In this study it is shown that hydrolysis at the A site is protected also by the presence of actin, suggesting that this region of the molecule may be involved in actin binding. Since this region of the molecule (i.e., the A site) is protected both by actin and by the formation of the 10S conformation, this could indicate that the actin-binding properties of 6S and 10S myosins are different. A more detailed characterization of hydrolysis at the A site and an evaluation of actin-binding affinity to 6S and 10S myosins are presented.

## Materials and Methods

Myosin (Ikebe & Hartshorne, 1985b) and myosin light chain kinase (Walsh et al., 1983) were isolated from frozen turkey gizzards. Calmodulin was prepared from frozen bull testes (Walsh et al., 1983). Actin from rabbit skeletal muscle was prepared as outlined earlier (Driska & Hartshorne, 1975). Actinidin was isolated from *Actinidia chinensis* by the procedure of Carne and Moore (1978). Papain was obtained from Sigma Chemical Co. and *S. aureus* protease from Pierce Chemical Co. The papain and actinidin were activated before use by incubating for 1 h at 35 °C in 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 20 mM dithiothreitol. The conditions for proteolysis of myosin by *S. aureus* protease, papain, and actinidin are given in the figure legends. The separation of S1 and HMM following proteolysis by *S. aureus* protease was carried out as described earlier (Ikebe & Hartshorne, 1985a).

ATPase activities and phosphorylation of myosin were assayed as described previously [Ikebe and Hartshorne (1985a) and Walsh et al. (1983), respectively]. Viscosity was measured at 25 °C in Cannon-Ubbelohde viscometers with water flow times of approximately 25 s (Ikebe et al., 1983). Electrophoresis was carried out on 7.5–20% polyacrylamide gradient slab gels in the presence of 0.1% $NaDodSO_4$ and the discontinuous buffer system of Laemmli (1970). Gels were stained in 0.06% Coomassie Brilliant Blue R250 (Sigma Chemical Co.) and after destaining were scanned with a GS 300 scanning densitometer (Hoefer Scientific Instruments) attached to an LCl-100 laboratory computing integrator (Perkin-Elmer). Molecular and subunit weights were estimated by using the following standards: myosin heavy chain (200 000), $\beta$-galactosidase (116 000), phosphorylase *b* (97 400), bovine serum albumin (66 000), ovalbumin (45 000), actin (42 000), carbonic anhydrase (29 000), and smooth muscle myosin light chains (20 000 and 17 000).

The binding of smooth muscle myosin to skeletal muscle actin in the presence of AMPPNP (Sigma Chemical Co.) was measured at room temperature by sedimenting actin and actin-bound myosin at 178000*g* for 30 min in a Beckman Airfuge and estimating the concentration of myosin in the supernatant by the dye-binding procedure of Spector (1978). Solvent conditions and concentrations of actin and myosin are given in the figure legends. Under the conditions used, approximately 98% of the F-actin was sedimented.

The data were analyzed by assuming that in the presence of AMPPNP the binding of actin to myosin fit the scheme

$$\begin{array}{ccc} A + M + S & \overset{K_2}{\rightleftharpoons} & AM + S \\ K_1 \uparrow\downarrow & & \uparrow\downarrow K_4 \\ A + MS & \underset{K_3}{\rightleftharpoons} & AMS \end{array}$$

where A, M, and S represent actin, myosin, and AMPPNP, respectively. $K_1$–$K_4$ are the dissociation constant for each step. Under the experimental conditions used, there will be essentially no free myosin, since it will be complexed with either actin or AMPPNP. Thus the above scheme can be simplified to

$$A + MS \underset{K_3}{\rightleftharpoons} AMS \underset{K_4}{\rightleftharpoons} AM + S$$

If the data are plotted as $[AM + AMS]/[MS]$ vs. $1/[S]$, the intercept on the ordinate is equal to $[A]/K_3$, where $K_3$ is the dissociation constant of actin from the myosin–AMPPNP complex, and the intercept on the abscissa is equal to $-1/K_4$, where $K_4$ is the dissociation constant of AMPPNP from actomyosin.

## Results

The proteolysis of gizzard myosin by *S. aureus* protease is shown in Figure 1. The $NaDodSO_4$–polyacrylamide gels show time courses of digestion for 10S and 6S myosin and for 6S myosin in the presence and absence of actin. From these gels the areas of the myosin heavy chain and of the 68-kDa peptide were estimated and plotted as a function of time. As shown previously (Ikebe & Hartshorne, 1985a), the digestion of 6S myosin occurs more rapidly than that of 10S myosin, and the pattern of peptides produced from the two conformations also is different (Figure 1). For 6S myosin, the initial products of digestion are peptides of $M_r$ approximately 160 000 and 68 000. (The apparent molecular weights estimated from $NaDodSO_4$–polyacrylamide gels for the $\alpha$-helical coiled-coil fragments of myosin are anomalously high.) With 10S myosin, the initial proteolytic cleavage yields peptides of $M_r$ approximately 130 000 and 94 000. The more rapid hydrolysis of the heavy chain and the generation of the 68-kDa peptide, both with 6S myosin, are illustrated in Figure 1. Another distinguishing feature is that in the later stages of digestion a 26-kDa peptide is derived from 6S myosin but not from 10S myosin. These findings confirm the earlier results of Ikebe and Hartshorne (1985a), who suggested that the 68- and 26-kDa peptides compose S1 and that the 130-kDa peptide is the heavy chain of the HMM derived from 10S myosin.

The digestion by *S. aureus* protease of 6S myosin in the presence and absence of actin is shown also in Figure 1. The overall extent of myosin proteolysis is reduced in the presence

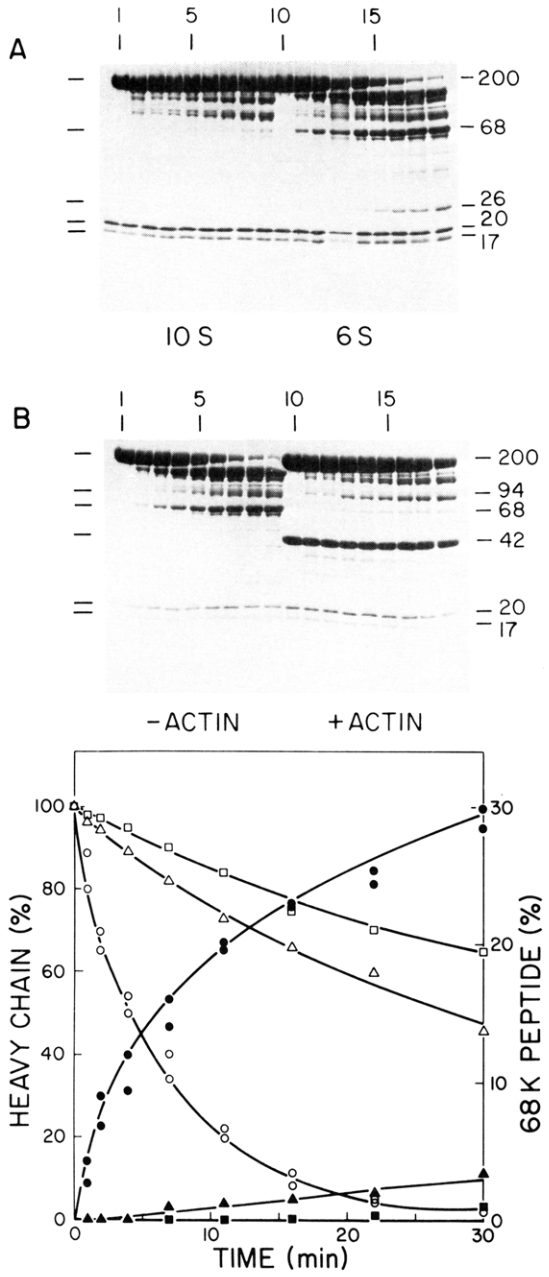


FIGURE 1: Effect of conformational transition and actin binding on proteolysis of gizzard myosin by *S. aureus* protease. Gizzard myosin (2 mg/mL) in 0.2 M NaCl, 1 mM $MgCl_2$, 0.2 mM EGTA, and 30 mM Tris-HCl (pH 7.5), plus or minus 1 mM ATP or skeletal muscle actin (1 mg/mL) was digested at 25 °C with *S. aureus* protease (40 μg/mL). Proteolysis was stopped by the addition of trichloroacetic acid to 5%, and the precipitated protein was collected by centrifugation and dissolved in 1% $NaDodSO_4$ and 25 mM Tris–192 mM glycine (pH 8.6). Electrophoresis and scanning of the gradient gels were carried out as described under Materials and Methods. Gel A: lanes 1–9 show the time course of digestion (0, 1, 2, 4, 7, 11, 16, 22, and 30 min) for 10S myosin in the presence of ATP. Gel A: lanes 10–18 show the same time course of proteolysis for 6S myosin in the absence of ATP. Gel B: lanes 1–9 show the same time course of proteolysis for 6S myosin in the absence of actin and ATP. Gel B: lanes 10–18 show the same time course of proteolysis for 6S myosin in the presence of actin. From the gels the amount of myosin heavy chain (□, △, ○) and 68-kDa peptide (■, ▲, ●) was calculated and plotted vs. time of proteolysis: heavy chain 10S myosin plus ATP minus actin (□), heavy chain 6S myosin minus ATP plus actin (△), and heavy chain 6S myosin minus ATP minus actin (○). Corresponding solid symbols were used for the release of the 68-kDa peptide.

of actin, as indicated by the rate of degradation of the heavy chain, and the pattern of peptide products also is changed. In the presence of actin the initial products of digestion of the 6S myosin are peptides of approximately 94 and 130 kDa, and the production of the 68- and 26-kDa peptides is inhibited. At higher ionic strength (0.35 M NaCl) the protective effect of actin also was observed in the absence of ATP. The addition of ATP caused dissociation of actin, and the proteolysis profile then resembled that of 6S myosin (results not shown).

The proteolysis profiles for 10S myosin and 6S myosin plus actin appear qualitatively similar, as shown by the gel patterns in Figure 1. In both instances, peptides of about 94 and 130 kDa are generated. It was shown previously (Ikebe & Hartshorne, 1985a) that under these conditions the liberation of S1 (i.e., cleavage at site B) from 10S myosin is inhibited and the larger peptide (130 kDa) was identified as HMM. Thus the two major initial products of *S. aureus* protease hydrolysis of 10S myosin would be HMM plus a segment of the myosin rod. In order to determine whether actin protected 6S myosin from proteolysis at site B and to document further the protection at site A, the experiments shown in Figure 2 were carried out. 6S myosin in the presence and absence of actin was digested with *S. aureus* protease, and the products of proteolysis that were soluble at low ionic strength were subject to exclusion chromatography on an HPLC system in order to separate HMM and S1. (Details are given in the legend of Figure 2.) The HPLC elution profiles and Na-$DodSO_4$–polyacrylamide gels of representative fractions are shown in Figure 2. The following conclusions can be drawn from these experiments: (1) The amout of protein contained in the S1 peaks for the two digests is approximately the same. This indicates that proteolysis of 6S myosin at site B is not affected by actin, in contrast to the protection afforded by the transition from 6S to 10S myosin. (2) The peptide compositions of the two S1 preparations in the presence and absence of actin are distinct. For S1 generated in the presence of actin the composite peptides are 94, 68, and 26 kDa. The amount of the 94-kDa peptide in the S1 formed in the absence of actin is reduced considerably (Figure 2). The evidence that the protein eluted at this position is in fact S1 is that the molecular weights and peptide compositions (e.g., the presence of light chains) are reasonable, the protein is soluble at low ionic strength (in contrast to the 94-kDa peptide generated by *S. aureus* protease hydrolysis of 10S myosin), and the peak contains ATPase activity. For both S1 preparations the AT-Pase activity is similar, the $Mg^{2+}$-ATPase of S1 alone was approximately 0.026 $s^{-1}$, and the actin-activated ATPase (at approximately 80-fold molar excess actin) was 0.16 $s^{-1}$. This confirms previous results (Ikebe & Hartshorne, 1985a) that proteolysis at site A does not influence ATPase activity. (3) The amount of HMM liberated in the absence of actin is slightly higher than in the presence of actin. The peptide composition of each preparation of HMM is different. HMM produced in the presence of actin contains a major component of 130 kDa, whereas the two dominant bands found in HMM liberated in the absence of actin are 68 and 62 kDa.

The above results are consistent with the idea that the 94-kDa heavy chain of S1 is degraded into 68- and 26-kDa peptides, and the 130-kDa peptide of HMM is hydrolyzed to products of 68 and 62 kDa. In both instances the site cleaved is about 68 kDa from the N-terminus of the myosin heavy chain, i.e., site A, and in both instances hydrolysis at this site is inhibited by the binding of actin. Proteolysis at this site also is slower for the 10S compared to the 6S conformation (Ikebe & Hartshorne, 1985a). There is no indication that actin protects site B from proteolysis.

For 6S myosin in the absence of actin the most susceptible cleavage site is site A. Thus, it is possible by very brief pro-

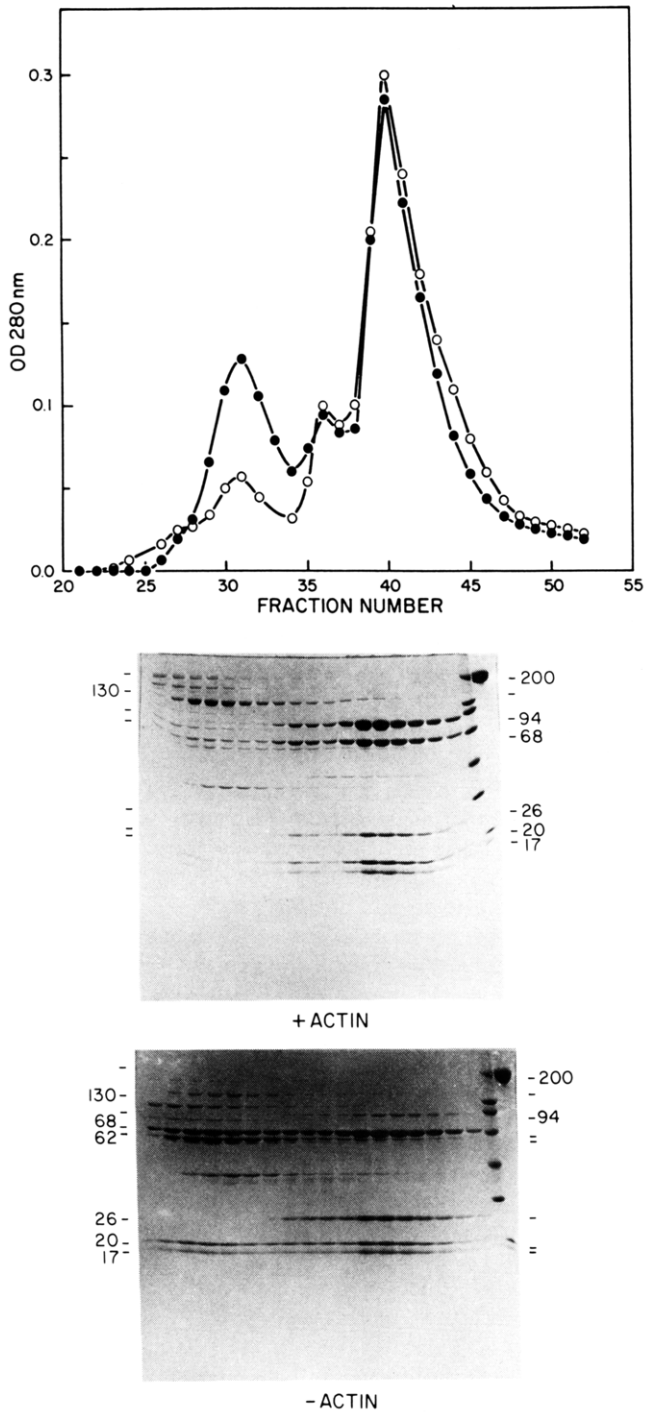


FIGURE 2: HPLC elution profile of the soluble fraction obtained after proteolysis of gizzard myosin by *S. aureus* protease and peptide composition of various fractions. Gizzard myosin (6 mg/mL) was digested at 25 °C for 40 min with *S. aureus* protease (40 μg/mL) in the presence (○) and absence (●) of skeletal muscle actin (1.5 mg/mL) in 0.35 M KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.5), and 1 mM dithiothreitol. Diisopropyl fluorophosphate (stock solution 0.1 M in 2-propanol) was added to 1 mM to stop proteolysis and $MgCl_2$ was added to 15 mM. The mixture was dialyzed (40 min) at 0 °C vs. 30 mM imidazole hydrochloride (pH 6.8), 15 mM $MgCl_2$, and 0.2 mM dithiothreitol; ATP was added to 0.2 mM, and insoluble protein was removed by centrifugation at 180000*g* for 45 min. The supernatant was adjusted to 0.3 M KCl and applied (1 mL) to a TSK G4000 SW column (60 × 0.75 cm) attached to a Perkin-Elmer series 4 system. The flow rate was 1 mL/min. $NaDodSO_4$ gradient gels shown for various fractions were obtained following digestion in the presence of actin (top) and absence of actin (bottom). Lanes 1–18 for each gel are for fractions 27–44. Lanes 19 and 20 are molecular weight standards and myosin, respectively.

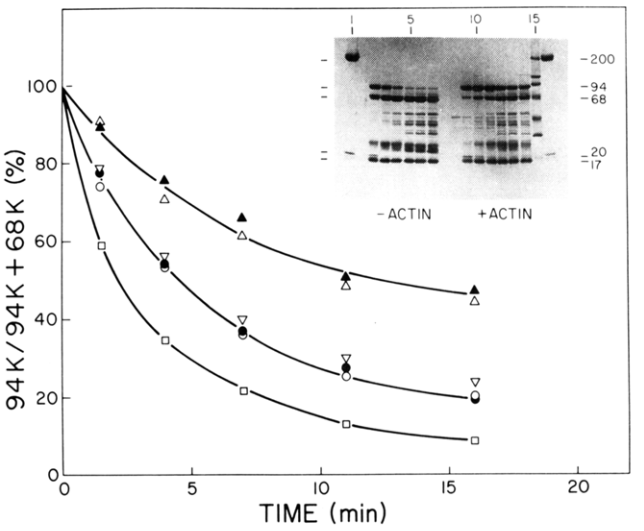


FIGURE 3: Soluble products following papain proteolysis of gizzard myosin. Myosin (4 mg/mL) was digested at 25 °C with papain (16 μg/mL) in the presence (△, ▲, ▽) and absence (○, ●, □) of skeletal muscle actin (1 mg/mL) in 0.35 M KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.5), and 1 mM ATP (○, ▽) or 2 mM AMPPNP (●, ▲). Iodoacetic acid was added to 5 mM to stop proteolysis, and samples were dialyzed vs. 15 mM $MgCl_2$ and 30 mM imidazole hydrochloride (pH 6.8). ATP was added to 0.2 mM, insoluble protein and F-actin were removed by centrifugation at 180000*g* for 45 min, and supernatant was used for $NaDodSO_4$ electrophoresis (see inset). From these and similar gels the amount of the 94- and 68-kDa peptides was estimated (see Materials and Methods). Lanes 1 and 16 are myosin. Lane 15 shows molecular weight standards. Lanes 2–7 show the time course of digestion 1.5, 4, 6, 11, 16, and 21 min) in the absence of ATP and actin. Lanes 9–14 show the same time course of digestion for myosin in the presence of actin minus ATP.

teolysis to hydrolyze only at this site (to produce 68- and 130-kDa fragments). The 68-kDa peptide is not dissociated from the remainder of the molecule and is precipitated at low ionic strength with the rod portion of the molecule. Hydrolysis at site A and not at site B, or at the HMM–LMM junction, would obviously contribute to degradation of the myosin heavy chain as estimated from $NaDodSO_4$–polyacrylamide gels (see Figure1) but would not be reflected in the chromatography profiles of the soluble fraction shown in Figure 2.

The protection against proteolysis in the region of site A by the binding of actin also was demonstrated by using the proteases, papain, and actinidin. Papain cleaves myosin heavy chain predominantly at the S1–S2 junction rather than the HMM–LMM junction. Figure 3 shows the effects of actin and nucleotide (ATP or AMPPNP) on the papain hydrolysis of site A. The inset shows a $NaDodSO_4$–polyacrylamide gel of the fraction soluble at low ionic strength (mainly S1) separated at different times of papain hydrolysis for 6S myosin in the presence of actin and the absence of nucleotide (right side) and for 6S myosin in the absence of both nucleotide and actin. Release of S1 is not influenced by actin as the yield of soluble fraction (after the removal of actin) was similar following papain hydrolysis in the presence and absence of actin. The amount of the intact S1 heavy chain (94 kDa) and the peptide produced following hydrolysis at site A (68 kDa) was estimated from gel scans and plotted as the fraction 94 kDa/94 kDa + 68 kDa vs. time of digestion. Similar analyses also were carried out on the soluble fractions (S1) obtained following digestion with different combinations of actin, ATP, and AMPPNP. Under rigor conditions, i.e., actin minus ATP, the extent of proteolysis of the 94-kDa peptide and the accompanying production of the 68-kDa peptide is suppressed (Figure 3). A similar proteolysis profile is obtained for 6S

myosin in the presence of actin and AMPPNP (2 mM). These results demonstate that papain hydrolysis at site A is reduced by binding of actin and, if this is accepted, that AMPPNP does not dissociate the actomyosin complex. In the absence of actin and nucleotide the loss of the 94-kDa peptide occurs 3.3 times faster than in the presence of actin (calculated from a semilogarithmic plot of the time course data). In addition, it is interesting that the papain proteolysis of 6S myosin is influenced by nucleotide, and in the presence of either ATP or AMPPNP the degradation of the 94-kDa peptide is slightly slower than in the absence of nucleotide (Figure 3). Okamoto et al. (1980) found that ATP protected the $\alpha$-chymotryptic cleavage of a site 71 kDa from the N-terminus of the smooth muscle myosin head.

In contrast to the multiple sites cleaved by *S. aureus* protease and papain, actinidin hydrolyzes myosin predominantly at one location to yield peptides of 160 and 68 kDa. (The apparent increased specificity could be due to the lability of the 68–160 site and a lower protease activity for actinidin.) Both peptides cosedimented at low ionic strength and were eluted with myosin on Sephacryl S-300 chromatography in 0.5 M KCl and 30 mM Tris-HCl (pH 7.5). If the cleavage site for actinidin is located 68 kDa from the C-terminus of the myosin heavy chain, the products of proteolysis would resemble LMM and HMM, and this is not observed. In addition, it has been shown that the 68-kDa fragment can be obtained by actinidin hydrolysis of the 130-kDa heavy chain of HMM. On the basis of this evidence it is suggested that the site of hydrolysis is located approximately 68 kDa from the N-terminus of the heavy chain, i.e., site A. The effect of myosin conformation and the presence and absence of actin on proteolysis by actinidin is shown in Figure 4. As can be seen from the $NaDodSO_4$–polyacrylamide gels and the areas of the myosin heavy chain, the 6S conformation is degraded more rapidly than the 10S conformation, and the binding of actin to 6S myosin also inhibits proteolysis. In the presence of actin plus ATP, the inhibition is not observed. (The band at approximately 29 kDa is actinidin.) Generation of the 68-kDa peptide is effective only with 6S myosin in the absence of actin (Figure 4). Another feature of the digestion with actinidin is that the 20-kDa light chain is protected in the 10S conformation and to a lesser extent by the binding of actin (Figure 4). It was noted previously that the 20-kDa light chain is more resistant to papain digestion in the 10S state (Ikebe & Hartshorne, 1984).

From the above results it is suggested that proteolysis of site A in the myosin head is hindered both by the formation of the 10S conformation and by the binding of actin to 6S myosin. If it is assumed that a change in myosin conformation and the interaction with actin mask site A, then it follows that the 10S–6S transition could also influence actin binding to myosin (see Discussion). In order to test this hypothesis, it is necessary to use a nucleotide that promotes the 6S to 10S transition but is less effective than ATP in dissociating actin and myosin. One possibility is AMPPNP.

In Figure 5 the relative viscosity of phosphorylated (1.9 mol of P/mol of myosin) and dephosphorylated myosin in the presence of ATP or AMPPNP is plotted at varying KCl concentrations. In the presence of ATP the viscosity of dephosphorylated myosin decreases as the KCl concentration is reduced below 0.35 M, and this reflects the formation of 10S myosin (Ikebe et al., 1983). For dephosphorylated myosin in the presence of AMPPNP (20 $\mu$M) the 6S–10S transition also is observed, although this occurs at slightly lower ionic strengths compared to the ATP curve. The amount of

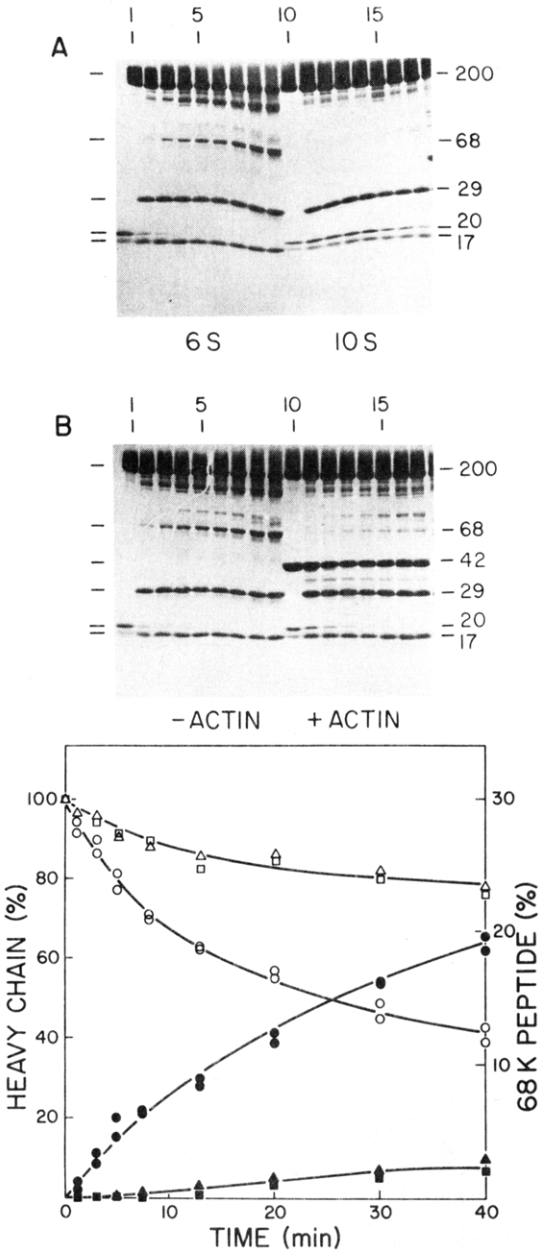


FIGURE 4: Effect of conformational transition and actin binding on proteolysis of gizzard myosin by actinidin. Conditions were as in Figure 1 except that actinidin (0.3 mg/mL) is used. Gel A: lanes 1–9 show the time course of digestion 0, 1, 3, 5, 8, 13, 20, 30, and 40 min) for 6S myosin in the absence of ATP (○, ●). Gel A: lanes 10–17 show the same time course of digestion for 10S myosin in the presence of 1 mM ATP (△, ▲). Gel B: lanes 1–9, are the same as gel A, lanes 1–9. Gel B: lanes 10–17 show the same time course of digestion for 6S myosin in the presence of actin and absence of ATP (□, ■). Myosin heavy chain (○, △, □) and 68-kDa peptide (●, ▲, ■).

AMPPNP necessary to induce the conformational transition is stoichiometric with the myosin head concentration (data not shown). The viscosity of phosphorylated myosin in the presence of AMPPNP is influenced little by a reduction in ionic strength (Figure 5), and over the range of KCl concentrations shown, it remains predominantly in the 6S conformation. Under the same conditions used for the viscosity measurements, the binding of myosin to actin also was estimated. These data are plotted as a ratio of free/bound myosin in Figure 5. For phosphorylated myosin in the presence of AMPPNP essentially all of the myosin is bound to actin over the range of 0.15–0.4 M KCl. Under these conditions phosphorylated myosin is in the 6S conformation. With dephosphorylated myosin over a similar range of ionic strengths and

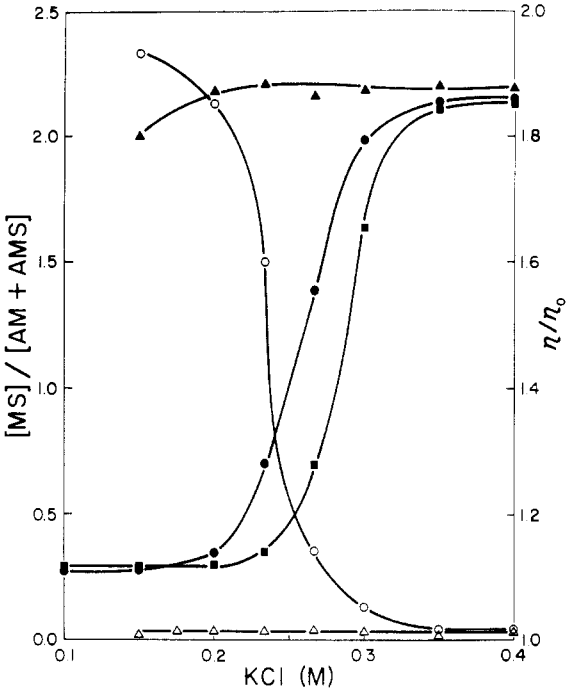


FIGURE 5: KCl dependence of relative viscosity of gizzard myosin in the presence of ATP or AMPPNP and dissociation of actomyosin in the presence of AMPPNP. Myosin (2 mg/mL) was phosphorylated at 25 °C for 15 min in 85 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM [$\gamma$-$^{32}$P]ATP, myosin light chain kinase (7.5 $\mu$g/mL), and calmodulin (10 $\mu$g/mL). Phosphorylated myosin (1.9 mol of P/mol of myosin) was diluted with 5 volumes of 30 mM KCl and 10 mM $MgCl_2$. The insoluble protein was collected by centrifugation, and the pellet was washed 3 times in 30 mM KCl and 10 mM $MgCl_2$. Myosin was dissolved in 0.5 M KCl, 10 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol and diluted with the appropriate solvent for viscosity or dissociation experiments. Conditions for viscosity: 2 mg/mL myosin, 2 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.5), varying KCl concentrations, and either 20 $\mu$M AMPPNP (▲, ●) or 1 mM ATP (■). Conditions for dissociation measurements: myosin (0.12 mg/mL), skeletal muscle actin (0.13 mg/mL), and other conditions the same as viscosity assays. Viscosity of dephosphorylated myosin plus ATP (■), viscosity of dephosphorylated myosin plus AMPPNP (●), viscosity of phosphorylated myosin plus AMPPNP (▲), dissociation of dephosphorylated actomyosin plus AMPPNP (○), and dissociation of phosphorylated actomyosin plus AMPPNP (△).

in the presence of AMPPNP, there is a marked decrease in the amount of bound myosin as the ionic strength is reduced (Figure 5). At KCl concentrations of 0.3 M and above, the conformation of dephosphorylated myosin is 6S, and this myosin is effectively all bound to actin. As the ionic strength is reduced, the 10S conformation is formed and the extent of dissociation of the actomyosin complex increases. For dephosphorylated myosin in the presence of AMPPNP the transition to 10S is complete at 0.15 M KCl, and under these conditions only about 30% of the myosin is bound to actin. The conformational transition and the reduction of actin-binding affinity show a similar KCl dependence (Figure 5).

The AMPPNP dependence of the binding of actin to dephosphorylated and phosphorylated myosin is shown in Figure 6. These experiments were carried out at 0.35 M KCl where both phosphorylated and dephosphorylated myosin form the 6S conformation. The extent of myosin interaction was estimated at three levels of F-actin. The dissociation constant of AMPPNP from actomyosin (i.e., $K_4$) was estimated, from the intercept on the abscissa, to be approximately 0.8 mM. This is the same for the 6S conformation of both phosphorylated and dephosphorylated myosins. The intercepts on the ordinate give values of $[A]/K_3$, and these are plotted vs. actin concentration in the inset of Figure 6. The values of $K_3$ (i.e.,

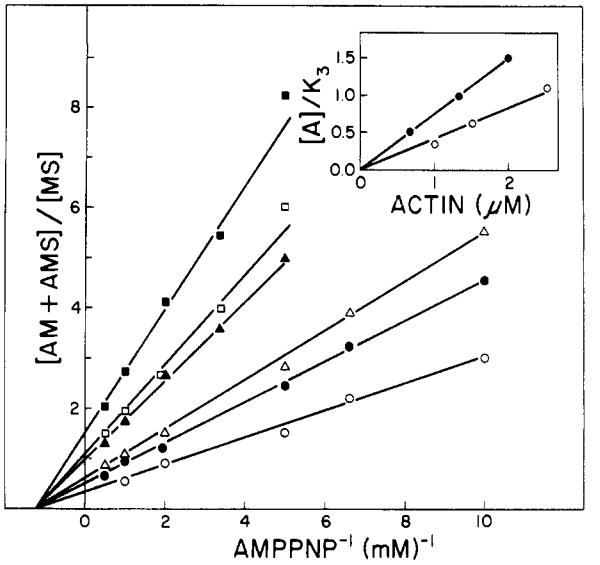


FIGURE 6: Dissociation of 6S myosin and actin at varying concentrations of AMPPNP. Conditions were as in Figure 5 except that 0.35 M KCl was used. Myosin concentration was 0.26 $\mu$M. Dephosphorylated myosin plus 1 $\mu$M actin (○), 1.52 $\mu$M actin (△), and 2.38 $\mu$M actin (□). Phosphorylated myosin plus 0.67 $\mu$M actin (●), 1.33 $\mu$M actin (▲), and 2 $\mu$M actin (■). The inset shows the ordinate intercepts plotted as a function of actin. The values of $K_3$ were obtained from the slopes of these plots.

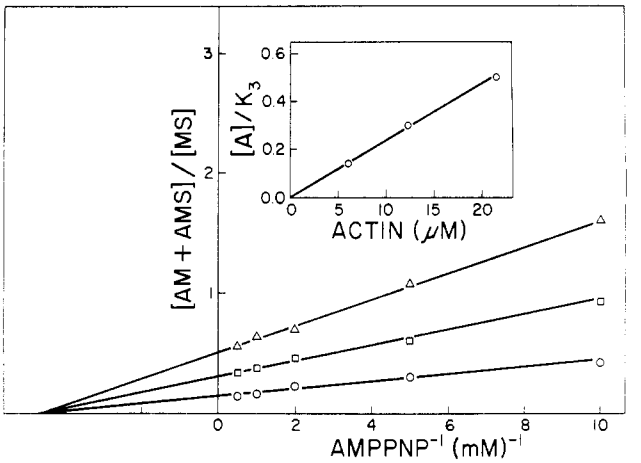


FIGURE 7: Dissociation of 10S dephosphorylated myosin and actin at varying concentrations of AMPPNP. Conditions were as in Figure 5 except that 0.17 M KCl was used. Concentrations of actin: 6 $\mu$M (○), 12.2 $\mu$M (□), and 21.7 $\mu$M (△). The inset shows the ordinate intercepts as a function of actin.

the constant derived for the dissociation of actin from the actomyosin–AMPPNP complex) were estimated to be 2.42 $\mu$M and 1.33 $\mu$M for dephosphorylated and phosphorylated myosin, respectively.

The binding of actin to 10S myosin at different AMPPNP levels also was measured, and the results are shown in Figure 7. For these experiments dephosphorylated myosin was used at a KCl concentration of 0.17 M, i.e., conditions where the 10S conformation is formed. $K_4$ was estimated to be approximately 0.21 mM, and from the inset of Figure 7, a value of 42.5 $\mu$M was obtained for $K_3$. Under identical solvent conditions a similar experiment was carried out with phosphorylated myosin (1.9 mol of P/mol of myosin). It was determined previously (Figure 5) that phosphorylated myosin in 0.17 M KCl and the presence of AMPPNP forms the 6S conformation. As shown in Figure 8, the phosphorylation of myosin affects both $K_3$ and $K_4$ and values of 0.31 $\mu$M and 1.33 mM, respectively, were obtained.

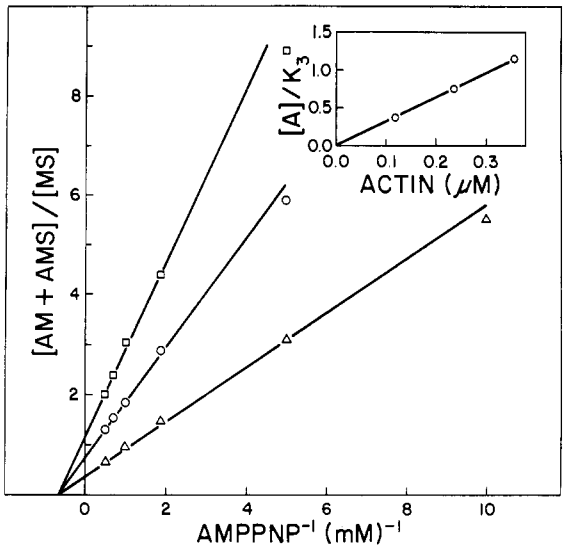


FIGURE 8: Dissociation of 6S phosphorylated myosin and actin at varying concentrations of AMPPNP. Conditions were as in Figure 5 except that 0.17 M KCl was used and the concentration of phosphorylated myosin (1.9 mol of P/mol of myosin) was 0.02 mg/mL (0.04 $\mu$M). Concentrations of actin: 0.12 $\mu$M (△), 0.24 $\mu$M (○), and 0.36 $\mu$M (□). The inset shows the ordinate intercepts as a function of actin.

Table I: Dissociation Constants for the Actomyosin–AMPPNP Complex[a]

| myosin | KCl (M) | conformation of myosin | $K_3$ ($\mu$M) | $K_4$ (mM) |
|---|---|---|---|---|
| dephosphorylated | 0.35 | 6S | 2.42 | 0.80 |
| | 0.17 | 10S | 42.5 | 0.21 |
| phosphorylated | 0.35 | 6S | 1.33 | 0.80 |
| | 0.17 | 6S | 0.31 | 1.33 |

[a] Conditions described in Figures 6–8.

The various dissociation constants obtained under different conditions are summarized in Table I. At high ionic strength (0.35 M KCl) both phosphorylated and dephosphorylated myosins form the 6S conformation and apart from a slight change in the values for $K_3$ an alteration due to phosphorylation is not apparent. At lower ionic strength (0.17 M KCl) phosphorylation of myosin converts the 10S to the 6S conformation. This transition is accompanied by changes in both $K_3$ and $K_4$ values. The largest change is seen with $K_3$ and the affinity of actin for the myosin–AMPPNP complex is considerably higher (over 100-fold) for phosphorylated compared to dephosphorylated myosin.

## Discussion

It was shown previously (Ikebe & Hartshorne, 1985a) that the heavy chain of gizzard myosin S1 (94 kDa) and HMM (130 kDa) can be cleaved by *S. aureus* protease at a point approximately 68 kDa from the N-terminus, and this part of the molecule was termed site A. This study shows that other proteases cleave at site A, including papain and actinidin. A diagram of the S1–S2 region of the myosin molecule is shown in Figure 9, and on this is summarized the cleavage sites for various proteases. For each of the three proteases hydrolysis at site A is inhibited in the 10S conformation. In addition, it was found that the binding of actin to 6S myosin also reduced proteolysis at site A. It has been suggested that the gizzard myosin S1 is composed of a similar substructure (or domain structure) to skeletal muscle S1 and is built up of three fragments of 29, 50, and 26 kDa (Marianne-Pépin et al., 1985) linked by protease-labile regions. The 29-kDa fragment is the N-terminal peptide of the heavy chain. Proteolysis at site A

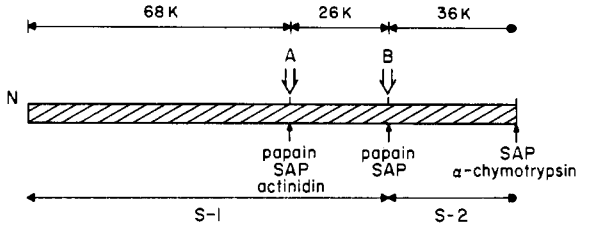


FIGURE 9: Schematic showing sites of hydrolysis of the S1–S2 region of the myosin heavy chain by various proteases. Sites A and B are those influenced by myosin conformation, i.e., 6S or 10S. Site A is also protected by the binding of actin. Cleavage at site B is required to liberate S1. SAP is *Staphylococcus aureus* protease. The values given for the peptide products are in kilodaltons.

with 6S myosin or S1 occurs readily, and it is therefore expected that it is located in one of the two protease-sensitive regions and the most logical choice is the 50–26 junction. [On the basis of the assignment of molecular weights of Marianne-Pépin et al. (1985) proteolysis of S1 at site A would yield fragments of 79 and 26 kDa, and the former value is higher than that obtained in our laboratory.] Our results suggest that the accessibility of this junction to protease attack is altered by the formation of 10S myosin and by the binding of actin. Thus it is possible that site A is located at, or close to, the actin-binding site. In support of this idea are the data of Marianne-Pépin et al. (1985), who showed that actin was cross-linked to the 26-kDa C-terminal fragment and the adduct of actin with the 50-kDa fragment was not obtained. However, in spite of the contention that actin may induce changes in the 50–26-kDa junction, Marianne-Pépin et al. (1985) did not observe protection of this junction against proteolysis (in S1 and myosin) following cross-linking with actin. This is in contrast to our results, and there is no obvious explanation for the discrepancy. Although it is reasonable to conclude that the actin-binding site is close to site A, it cannot be eliminated that actin binds at some distant point on the molecule and influences site A via long-range interactions.

Considerably more is known about the substructure of skeletal myosin S1 [see review by Harrington and Rodgers (1984)], and it has been suggested that the interaction with actin involves both the 50- and 20-kDa fragments (Mornet et al., 1981; Labbé et al., 1982; Sutoh, 1983; Muhlrad & Morales, 1984). A two-site interaction with actin also was proposed by Morita and co-workers and involved a weak-binding site (J-site) close to the 50–20 junction and a strong binding site (S-site) on the 20-kDa fragment between the SH1 and SH2 groups (Katoh & Morita, 1984; Katoh et al., 1984). In addition, it was shown that the isolated 20-kDa fragment binds actin (Muhlrad & Morales, 1984). The binding of actin protects the 50–20 junction from proteolysis (Mornet et al., 1979; Yamamoto & Sekine, 1979a; Chaussepied et al., 1983) and also decreases the rate of proteolysis at the other sensitive region, i.e., the 25–50 junction (Applegate & Reisler, 1983). In addition, it was shown that as the 50–20 junction is cleaved by trypsin, the actin-activated ATPase activity is lost (Mornet et al., 1979; Yamamoto & Sekine, 1979b). This effect is probably due to a decrease in actin-binding affinity since at infinite concentrations of actin ATPase activity is not altered (Botts et al., 1982; Furukawa & Arata, 1984; Katoh & Morita, 1984). Later Mornet et al. (1981) suggested that the loss of ATPase activity at finite actin concentrations was actually due to the degradation of the approximately 2-kDa linker region between the 50- and 20-kDa domains and the initial cleavage at the 50–22 junction did not alter ATPase activity. For smooth muscle myosin (above results; Ikebe & Hartshorne, 1985) and S1 (Marianne-Pépin et al., 1985)

cleavage at site A by itself does not result in the loss of actin-activated ATPase activity. On the basis of preliminary evidence (M. Ikebe and D. J. Hartshorne, unpublished results) we feel that it is unlikely that a linker region (analogous to the 2-kDa peptide of skeletal muscle S1) is important for enzymatic activity with smooth muscle myosin since the tryptic hydrolysis of the 26-kDa fragment to lower molecular weight forms does not eliminate actin-activated ATPase activity.

The actin-binding sites of skeletal and smooth muscle myosins, therefore, are apparently different. A difference in the actin-binding properties has been documented, and it was found by Greene et al. (1983) that the acto–smooth muscle S1 is more resistant to dissociation by increasing ionic strength than the acto–skeletal muscle S1. In the results presented above, it was shown also that the actomyosin complex in the presence of AMPPNP was not dissociated at a KCl concentration as high as 0.4 M.

As mentioned above, one interpretation for the protection of site A against proteolysis by the binding of actin is that site A forms part of, or is close to, the actin-binding site. Since proteolysis of site A is retarded for 10S myosin, it might be inferred that the formation of the folded conformation masks or hinders accessibility of the proteases to the putative actin-binding site. If this is true, then it would be expected that the actin-binding properties of 6S and 10S myosins are different. The simplest interpretation is that the actin-binding site is exposed or available in the 6S conformation but is partially blocked in the 10S conformation. In order to test this hypothesis, it was necessary to assess actin-binding for the two myosin conformations. The presence of nucleotide was required to facilitate the formation of 10S myosin but ATP was not satisfactory since it induced extensive dissociation. For this reason, the use of AMPPNP was investigated. Initially, it was determined that AMPPNP does support the 6S–10S transition, in confirmation of previous results (Onishi, 1982; Suzuki et al., 1984). Also in agreement with the preliminary results of Suzuki et al. (1984) it was shown that the binding of actin to the 10S conformation is weaker than to the 6S conformation. Under conditions where phosphorylation drives the 10S–6S transition (i.e., 0.17 M KCl) the dissociation constant of actin for the actin–myosin–AMPPNP complex (i.e., $K_3$) was over 100-fold higher for dephosphorylated myosin (10S) compared to phosphorylated myosin (6S). This was not a direct effect of phosphorylation since the $K_3$ values of the 6S conformation for both phosphorylated and dephosphorylated myosin (at high ionic strength) were similar (1.33 and 2.42 $\mu$M, respectively). If one compares the $K_3$ values for phosphorylated myosin at 0.17 and 0.35 M KCl (i.e., both in the 6S conformation), there is only a 4-fold increase at the higher ionic strength. The relatively small influence of ionic strength on the actin-binding affinity is in agreement with the results of Greene et al. (1983), who used gizzard S1 and obtained $K_3$ values of 2 and 10 $\mu$M at ionic strengths of 0.023 and 0.43 M, respectively. The finding that myosin in the 6S conformation binds actin more strongly than myosin in the 10S conformation is consistent with the results of Ikebe et al. (1981), who found that phosphorylation of gizzard HMM increased actin affinity about 10-fold. On the other hand, Sellers et al. (1982) reported about a fourfold difference in the actin-binding affinity for phosphorylated and dephosphorylated HMM, and this difference they considered to be inadequate for a significant influence on the regulation of ATPase activity. The constant ($K_4$) for the dissociation of AMPPNP from the ternary complex (i.e., actin–myosin–nucleotide) also was influenced by the myosin conformation. However, the variation was not marked and there was only about a sixfold increase in $K_4$ accompanying the transition of 10S to 6S myosin (0.21–1.33 mM). For 6S myosin there was essentially no effect of phosphorylation on the value of $K_4$ (see Table I). On the basis of data obtained with skeletal muscle S1 it may be assumed that binding of AMPPNP to myosin (or S1) is considerably stronger than binding to the actomyosin (or acto-S1) complex. Dissociation constants of skeletal muscle S1–AMPPNP range from approximately 0.6 $\mu$M (Greene & Eisenberg, 1980) to 20 nM (Konrad & Goody, 1982).

Most of our previous studies have focused on site B since it is suggested that this region of the myosin molecule, located at the S1-S2 junction, is involved in determining enzymatic activity (Ikebe & Hartshorne, 1984, 1985a) and could therefore be an important component of the regulatory mechanism. A role for the region of the molecule incorporating site A is not obvious. We have presented evidence suggesting that site A may form part of the actin-binding site and this is located close to the junction of the 50- and 26-kDa fragments. Further, it was shown that this part of the molecule is altered during the 10S–6S transition and that 10S myosin has a lower affinity for actin than 6S myosin. The obvious question is whether the reduced affinity for actin is an important part of the contraction–relaxation cycle of smooth muscle? As proposed by Sellers et al. (1982), the change in the affinity for actin resulting from the phosphorylation of myosin is unlikely to be responsible for the activation of the myosin ATPase activity. It is more likely that phosphorylation of myosin alters one (or more) of the steps of the kinetic cycle (Sellers et al., 1982). Thus it is difficult to assign the increased affinity for actin of 6S myosin as a primary factor in the initial activation of the contractile apparatus, although it could play a contributory role. During relaxation, myosin is dephosphorylated, and it is our contention that it then forms a conformation analogous to 10S myosin. (It is not necessary to form the complete folded structure, and the conformational change presumably is restricted to only part of the molecule, possibly in the head–neck junction.) This would result in a reduced actin affinity and would facilitate cross-bridge detachment. There would also be a lower possibility of actin–myosin interaction when the muscle is relaxed (i.e., 10S). The lower affinity for actin of the dephosphorylated or 10S myosin does not seem to be compatible with the formation of latch bridges (Aksoy et al., 1982; Dillon et al., 1981; Dillon & Murphy, 1982). It was suggested that the latter are formed with dephosphorylated myosin and maintain tension via slowly cycling attachments to actin. The formation of such attachments would not be favored by a reduction in actin-binding affinity, although obviously they could still occur.

**Registry No.** ATP, 56-65-5; AMPPNP, 25612-73-1.

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