Effects of Phosphorylation, MgATP, and Ionic Strength on the Rates of Papain Degradation of Heavy and Light Chains of Smooth Muscle Heavy Meromyosin at the S1-S2 Junction*

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The effects of ionic strength, MgATP, and phosphorylation on the degradation rates of heavy meromyosin (HMM) by papain have been compared to their effects on the sedimentation coefficient (s_{20,w}) to determine the relationship of the degradation rate to the equilibrium between the flexed and the extended forms (Suzuki, H., Stafford, W. F., Slayer, H. S., and Seidel, J. C. (1985) J. Biol. Chem. 260, 14810–14817). At 0.025 M NaCl, where HMM is predominantly in the flexed form, MgATP, Mg-adenyl imidodiphosphate or MgADP reduce k_L by 80–90%. MgATP exerts its optimal effect at this ionic strength, where at least 70% of HMM is flexed in the presence or absence of MgATP, suggesting that nucleotides reduce k_L by decreasing the proteolytic susceptibility of the flexed form. At 0.5 M NaCl, where HMM is in the extended form, MgATP has no effect on k_L. At low ionic strengths phosphorylation decreases k_L but increases it in the presence of MgATP. Plots of k_L against s_{20,w} determined at various ionic strengths are linear, the data for phosphorylated and dephosphorylated HMM falling on the same line. Thus, raising the ionic strength or phosphorylating the 20-kDa light chain appears to alter k_L by increasing the fraction of HMM in the extended form. The degradation rate of the 20-kDa light chain (k_L) of dephosphorylated HMM responds to changes in ionic strength in essentially the same way as does k_H, suggesting that the response of k_L to changes in ionic strength can also be attributed to conversion of HMM to the extended form. However, k_H for phosphorylated HMM measured in the presence of MgATP exhibits very little dependence on ionic strength.

Myosin from smooth muscle can assume two monomeric forms, a folded form sedimenting in the ultracentrifuge at 10 S or an extended form sedimenting at 6 S (1–6). The extended form has a 5–10-fold higher ATPase activity measured in the absence of actin than does the folded form (1, 5). At ionic strengths between 0.15 and 0.2 M where both forms can exist, MgATP increases the fraction of myosin in the folded form, while phosphorylation or raising the ionic strength favors the extended form (1–6). Light scattering (7) and hydrodynamic measurements (3) indicate a substantial change in conformation when myosin assumes the folded form, without a corresponding change in molecular weight. Two major structural features of the folded form are observed by electron microscopy: folding of the myosin tail at sites approximately 50 and 100 nm from the head-tail junction (3, 4, 6, 8, 9) and reorientation of the heads towards the tail (8, 9). The observation that phosphorylation increases the fraction of myosin in the extended form (1, 2, 4, 6) led to the proposal that the S6–10 S transition or an associated structural change plays a role in the phosphorylation-dependent regulation of actin-activated ATPase activity (5).

Heavy meromyosin, which lacks the region of the tail that folds, also exists in two conformations, an extended form sedimenting at 7.5 S and a folded form sedimenting at 9 S (7, 10), the extended form of HMM having a 4–5-fold greater ATPase activity than the flexed form in the absence of actin (10). HMM's sediments as a single peak, reflecting an equilibrium between the folded and extended forms, with the observed ATPase activity and sedimentation velocity being determined by the fraction of HMM in each form. The sedimentation coefficient decreases on phosphorylation or on raising the ionic strength and increases on addition of MgATP. Electron micrographs of samples of HMM prepared from solutions of low ionic strength indicate that about half of the molecules are flexed at the neck, the heads projecting towards the tail and making about a 45° angle with it, while in samples prepared at high ionic strength, only about 10% of the molecules are flexed and the rest are extended (10).

The rate of proteolytic cleavage of gizzard myosin with papain depends on the state of phosphorylation of the light chain and on the presence or absence of MgATP (11–13), suggesting that these variables affect the conformation of the molecule at the S1-S2 junction. Changes in digestibility of myosin induced by MgATP and by phosphorylation have been attributed to shifts in the distribution of myosin between the folded and extended forms (11, 13) or between folded and filamentous forms (12). MgATP decreases digestibility under conditions where it induces the folded form (11, 12) and phosphorylation increases digestibility when it converts myosin to the extended form (11, 13). Phosphorylation also

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increases the digestion rate of HMM (13), but the effects of ionic strength and MgATP on digestibility of HMM have not been reported, nor has the relationship between the digestibility and the sedimentation velocity of HMM been addressed.

The present studies were carried out to define the relationships between the two parameters and to establish a method of phosphorylation, MgATP, and increasing ionic strength. The results provide evidence that phosphorylation changes the papain degradation rates of the HMM heavy chain by decreasing the fraction of HMM in the flexed form. On the other hand, MgATP decreases the degradation rate not by changing the fraction of flexed HMM but by decreasing the degradation rate of the flexed form.

MATERIALS AND METHODS

Myosin from chicken gizzard was prepared by the method of Ebashi (15), and phosphorylated according to Aiba et al. (16). Phosphorylated gizzard HMM was obtained by digesting phosphorylated myosin, 8-10 mg/ml, with a-chymotrypsin, 0.04 mg/ml, in a solution containing 0.5 M NaCl, 1 mM EDTA, 1 mM diithiothreitol, and 20 mM Tris-HCl, pH 7.5, at 37°C (17). The digestion was terminated by adding phenylmethanesulfonyl fluoride to a final concentration of 0.3 mM, the digested protein was dialyzed against a solution containing 0.025 M NaCl, 0.1 mM EDTA, 0.5 mM diithiothreitol, and 2 mM MOPS, pH 7.0, and the aggregated light meromyosin and undigested myosin were removed by centrifugation at 20,000 x g for 30 min. At least 90% of the 20-kDa light chain of HMM remained phosphorylated as indicated by gel electrophoresis in 6 M urea (18). Dephosphorylated HMM was obtained by incubation with partially purified light chain light phosphatase, 0.05 mg/ml in the presence of 10 mM MgCl2 (19). Phosphorylated and dephosphorylated HMM were purified by gel filtration on Sephacryl S-300 in a solution containing in 50 mM NaCl, 1 mM EDTA, 0.5 mM diithiothreitol, and 20 mM Tris-HCl, pH 7.5. At least 90% of the 20-kDa light chain of both phosphorylated and dephosphorylated HMM remained intact as judged by gel electrophoresis in sodium dodecyl sulfate (20).

Subfragment-1 was prepared by digesting phosphorylated myosin, 8-10 mg/ml, in a solution containing 0.5 M NaCl, 1 mM EDTA, 0.1 mM diithiothreitol, and 20 mM Tris-HCl, pH 7.5, and papain, 5 μg/ml, for 6 min at 25°C. Papain was activated for 1 h at 37°C at a concentration of 1 mg/ml in a solution containing 50 mM freshly dissolved cysteine, 10 mM EDTA, and 5 mM Tris-HCl, pH 7.5. The digestion was stopped by addition of iodoacetic acid to a final concentration of 2 mM, and the digested myosin was dialyzed against a solution containing 0.025 M NaCl, 0.1 mM EDTA, 0.1 mM diithiothreitol, and 5 mM MOPS, pH 7.0, and centrifuged at 20,000 x g for 30 min to remove insoluble myosin rod and undigested myosin. This procedure yielded S1 containing at least 90% of the 20-kDa light chain intact, as indicated by gel electrophoresis in sodium dodecyl sulfate, and at least 90% of the light chain was phosphorylated as indicated by gel electrophoresis in 6 M urea. Dephosphorylated S1 was prepared by incubation with light chain phosphatase as described above for HMM.

Determination of the degradation rates of HMM or S1 was carried out by digestion of HMM or S1, at a concentration of 0.8 mg/ml, at 25°C in a solution containing 0.02-0.5 M NaCl, 2 mM MgCl2, 20 mM Tris-HCl, pH 7.5, or 5 μg/ml papain, and when added, 1 mM ATP. The esterase activity of papain was determined spectrophotometrically at 255 nm with benzoyl-L-arginine ethyl ester, the reaction being carried out in a solution containing 20 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 0.3 mM diithiothreitol, 0.016 mg/ml papain, and 0.2 mM benzoyl-L-arginine ethyl ester at 20°C. Papain was found to have the same esterase activity at 0.025 and 0.4 mM NaCl. At the concentrations of HMM and ATP used in these experiments, and at steady-state rates of ATP hydrolysis of 4 and 1 nmol/min for the extended and flexed forms of HMM, respectively (10), the steady state of ATP hydrolysis would persist for 50 min at 300 mM NaCl and for 200 min at 25 mM NaCl. Digestion was started by adding activated papain, aliquots of the digest were added to a solution containing iodoacetic acid at a final concentration of 2 mM to stop the digestion, and an equal volume of a solution containing 8% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 0.125 mM Tris-HCl, pH 6.8, was added. The samples were immediately heated at 95°C for 1 min, and polyacrylamide gel electrophoresis was carried out in solutions containing 0.1% sodium dodecyl sulfate using 14% polyacrylamide gels (20). The gels were stained with 0.12% Coomassie Brilliant Blue R250 and scanned with a Zeineh soft laser scanning densitometer SL-504, emitting at 630 nm.

The rates of degradation of the heavy and light chains were estimated from plots of log c/co where c is the area under the scanning peak at time t and c0 is the area under the peak at time 0. Semilogarithmic plots of the decay under the scanning peaks against the time of incubation with papain were linear (Fig. 3) and pseudo first order rate constants were calculated from the slopes (21). The sum of the areas under the 134- and 90-kDa peaks was used to determine the degradation rate of the HMM heavy chain, while the sum of the areas under the 95- and 90-kDa peaks was used to determine the degradation rate of the heavy chain of S1. In the absence of ATP, papain cleaves the heavy chain at two sites, 72 and 95 kDa from the NH2 terminus, while in the presence of ATP, it also cleaves at a site 5 kDa from the NH2 terminus (Fig. 1). Ratios determined in this way include contributions from cleavage at sites 72 and 95 kDa from the NH2 terminus, but little or no contribution from cleavage at the 5-kDa site, since the sum of the concentrations of the 134- and 129-kDa peptides is taken as the concentration of the intact heavy chain of HMM and the sum of the concentrations of the 95- and 90-kDa peptides are used for S1. The area under the 20-kDa band was used to determine the degradation rate of the 20-kDa light chain. The degradation rates were expressed per μg of papain used in the digestion and normalized to the rate of degradation of the heavy chain of dephosphorylated HMM determined in the presence of 0.5 mM NaCl at the same concentration of papain.

Protein concentrations were determined by the biuret method. Sedimentation velocity was measured in a Beckman Model-E analytical ultracentrifuge at 20°C as described previously (10).

RESULTS

Effects of Nucleotides on the Degradation of the Heavy Chain—Papain cleaves the heavy chain at two sites, 72 and 95 kDa from the NH2 terminus in the absence of ATP, while cleavage at a third site 5 kDa from the NH2 terminus occurs only in the presence of ATP (Fig. 1). Cleavage of the heavy chain occurs more rapidly at the 95-kDa site, the S1-S2 junction, than at the 72-kDa site, as indicated by the rapid formation of the 95-kDa fragment during the first 3-10 min of digestion (Fig. 2). Most of the digestion at the 72-kDa site occurs after the 95-kDa site has been cleaved.

Quantitative estimates of the degradation rates were obtained from semilogarithmic plots of the concentration of the undigested heavy or light chains against time of incubation with papain. All these plots are linear for digestion of the heavy or light chains of phosphorylated or dephosphorylated HMM. A typical plot for digestion of the heavy chains of dephosphorylated HMM is shown (Fig. 3). The degradation rate of the heavy chain (kH) reflects cleavage occurring at the 72- and 95-kDa sites but not at the 5-kDa site (see "Materials and Methods").

![Schematic diagram of cleavage sites of the heavy chain of gizzard heavy meromyosin with papain (P) and chymotrypsin (CT). The 72- and 23-kDa fragments constitute the heavy chain of S1 (40), the 72- and 62-kDa fragments make up the heavy chain of HMM (41, 42), and the 39-kDa peptide constitutes S2 (43). Both papain and chymotrypsin cleave at the site 5 kDa from the NH2 terminus in the absence but not in the presence of ATP (11, 42).](image-url)
to addition of papain does not change the rate of proteolytic degradation (not shown). The decrease in the degradation rate produced by MgADP was measured in the presence of the myokinase inhibitor, 1,5-diadenosyl tetraphosphate, which had no effect on the degradation rate when added in the absence of ADP. Each of these nucleotides also decreases the susceptibility of myosin to digestion with papain (12). The effects of AMP-PNP and ADP indicate that the decrease in digestion rates of both HMM and myosin can be attributed largely to nucleotide binding.

Effects of Phosphorylation and Ionic Strength on the Degradation of the Heavy Chain—In the presence of MgATP, phosphorylation increases the degradation rate of the heavy chain of HMM by a factor of two at 0.025 mM NaCl (Fig. 4A). This increase is accompanied by a more rapid formation of the 95- and 72-kDa fragments (Fig. 2), indicating that phosphorylation accelerates the degradation of HMM at the S1-S2 junction in agreement with an earlier finding (13). However, in the absence of nucleotides, phosphorylation decreases the degradation rate by about 30%, an effect not previously found in digestion rates of both HMM and myosin.

MgATP decreases the rate of papain digestion of the heavy chain of phosphorylated or dephosphorylated HMM by as much as 90% at 0.025 mM NaCl (Fig. 4); the decreased rate involves cleavage at the S1-S2 junction (the 95 kDa site) as shown by a decrease in the rate of formation of the 39- and 52-kDa fragments (Fig. 2). The difference between the degradation rates of phosphorylated and dephosphorylated HMM becomes smaller with increasing concentrations of NaCl and disappears at 0.5 mM, indicating that MgATP does not affect the degradation of the extended form of HMM.

MgAMP-PNP, a nonhydrolyzable analog of MgATP, decreases kₜₙ by 80% as does MgADP (Table I). Similar decreases in digestion rates are observed for phosphorylated HMM (not shown). The effect of MgAMP-PNP cannot be attributed to ATP present as a contaminant; as little as 25 μM AMP-PNP in the presence of 8 μM HMM heads produces a nearly maximal decrease in degradation rate (Table I), and preincubation of AMP-PNP with HMM for as long as 80 min prior to addition of papain does not change the rate of proteolytic degradation (not shown). The decrease in the degradation rate produced by MgADP was measured in the presence of the myokinase inhibitor, 1,5-diadenosyl tetraphosphate, which had no effect on the degradation rate when added in the absence of ADP. Each of these nucleotides also decreases the susceptibility of myosin to digestion with papain (12). The effects of AMP-PNP and ADP indicate that the decrease in digestion rates of both HMM and myosin can be attributed largely to nucleotide binding.
Digestion of smooth muscle heavy meromyosin with papain

Effect of ATP and ATP analogues on the degradation rates of the heavy chain of dephosphorylated HMM

Digestion with papain was carried out in a solution containing 25 mM NaCl, 20 mM Tris, pH 7.5, 2 mM MgCl₂, 0.3 mM dithiothreitol, and 1.4 mg/ml dephosphorylated HMM as described under "Materials and Methods." When digesting in the presence of ADP, 20 μM diadenosyl tetraphosphate was included to prevent formation of ATP by myokinase present in the HMM preparation. Samples containing ADP or AMP-PNP were preincubated with HMM for 20 min before adding papain in order to hydrolyze any contaminating ATP. Degradation rates are normalized to those in solutions containing 25 mM NaCl, 20 mM Tris, pH 7.5, 2 mM MgCl₂, 0.3 mM dithiothreitol, and 1.4 mg/ml dephosphorylated HMM.

| Addition | Concentration (mM) | Relative digestion rate |
|----------|--------------------|------------------------|
| None     | 1.0                | 1.0                    |
| ATP      | 1.0                | 0.107                  |
| AMP-PNP  | 1.0                | 0.194                  |
| AMP-PNP  | 0.025              | 0.234                  |
| ADP      | 1.0                | 0.212                  |

Degradation rates of the heavy chain and the 20-kDa light chain of subfragment-1

Digestion of subfragment-1 was carried out at 20 °C in a solution containing NaCl as indicated, 2 mM MgCl₂, 20 mM Tris-Cl, pH 7.5, 0.7 mg/ml phosphorylated or dephosphorylated S₁, and 5 μg/ml papain, in the presence or absence of 1 mM ATP. The proteolytic fragments were separated by gel electrophoresis in sodium dodecyl sulfate, and the degradation rates were determined by densitometry as described under "Materials and Methods." The rates have been normalized to the degradation rate of the heavy chain of dephosphorylated HMM measured in a solution containing 0.5 M NaCl, 2 mM MgCl₂, 20 mM Tris-Cl, pH 7.5, 0.8 mg/ml HMM, and 5 μg/ml papain.

| Subfragment-1 | ATP | Degradation rate of heavy chain, kₜ₁ |
|---------------|-----|-------------------------------------|
| Phosphorylated| None | 0.11                               |
| Phosphorylated| 1 mM | 0.09                               |
| Phosphorylated| 1 mM | 0.32                               |

| Subfragment-1 | ATP | Degradation rate of light chain, kₜ₂ |
|---------------|-----|-------------------------------------|
| Phosphorylated| None | 1.28                               |
| Phosphorylated| 1 mM | 1.36                               |
| Phosphorylated| 1 mM | 0.19                               |

Fig. 5. Dependence of the degradation rates of HMM on S₂₀,ₙₐ. Degradation rates from Fig. 4 have been plotted against sedimentation velocity determined under the same conditions except for the absence of papain as described under "Materials and Methods." A, heavy chain; B, 20-kDa light chain; triangles, dephosphorylated HMM; circles, phosphorylated HMM; open symbols, digested without ATP; closed symbols, digested in the presence of 1 mM ATP.

Fig. 6. The effect of MgATP on the products of papain digestion of dephosphorylated subfragment-1. Digestion of subfragment-1 was carried out at 20 °C in a solution containing 30 mM NaCl, 2 mM MgCl₂, 20 mM Tris-Cl, pH 7.5, 0.7 mg/ml dephosphorylated S₁, and 5 μg/ml papain, A, no ATP; B, 1 mM ATP.

The values of kₜ₁ obtained with increasing ionic strength are plotted against S₂₀,ₙₐ, essentially linear relationships are observed (Fig. 5A). The observation that data for phosphorylated and dephosphorylated HMM fall on the same straight line suggests that phosphorylation does not affect the degradation rate of extended form.

The magnitude of the changes in kₜ₁ produced by phosphorylation are about one-third of those produced by raising the ionic strength (Fig. 4). Since the corresponding changes in S₂₀,ₙₐ and ATPase activity follow the same pattern (10), raising the ionic strength appears to convert all of HMM to the extended form, while phosphorylation converts only about one-third of it to this form. When the values of kₜ₁ obtained with increasing ionic strength are plotted against S₂₀,ₙₐ, essentially linear relationships are observed (Fig. 5A). The observation that data for phosphorylated and dephosphorylated HMM fall on the same straight line suggests that phosphorylation changes kₜ₁ by increasing the fraction of HMM in the extended form.

Degradation of Subfragment-1—Subfragment-1, whose sedimentation coefficient shows no response to MgATP, ionic strength, or phosphorylation (10), also shows little change in kₜ₁ in response to any of these variables (Table II). The values of kₜ₁ obtained on digestion of S₁ are in most cases lower than those obtained with HMM, which may reflect the absence of reported. This decrease also appears to involve cleavage at the S₁-S₂ junction, as indicated by the slower disappearance of the 62-kDa segment, the COOH-terminal half of the heavy chain (Fig. 2). To relate the degradation rates to the sedimentation coefficient, the concentration of NaCl was varied over a range in which S₂₀,ₙₐ changes from 9 to 7.5 S (10). The effects of raising the ionic strength on kₜ₁ are similar to those of phosphorylation; kₜ₁ decreases in the absence and increases in the presence of MgATP (Fig. 4A). At an ionic strength of 0.5, all rates converge reaching an intermediate value that is independent of phosphorylation, indicating that phosphorylation does not effect the degradation rate of extended form.
the 95-kDa cleavage site in S1. MgATP accelerates cleavage of the heavy chain of S1 at the 5-kDa site (Fig. 6), showing that the increased rate of cleavage at the 5-kDa site does not depend on conformational changes associated with changes in $s_{20,w}$.

**Degradation of the 20-kDa Light Chain**—The degradation rate of the 20-kDa light chain ($k_l$) of dephosphorylated HMM depends on MgATP and ionic strength in the same manner as does the degradation rate of the heavy chain (Fig. 4). On raising the ionic strength, $k_l$ varies linearly with sedimentation velocity (Fig. 5), suggesting that the degradation rates of both the heavy and light chains depend on the distribution of HMM between the extended and flexed forms. However, when HMM is phosphorylated, the dependence of $k_l$ on ionic strength is substantially diminished in the presence of MgATP and $k_l$ undergoes little or no change as the ionic strength is raised.

**DISCUSSION**

The dependence of the sedimentation velocity of HMM on ionic strength has been interpreted in terms of shifts in an equilibrium between a flexed form sedimenting at 9 S and an extended form of HMM sedimenting at 7.5 S (7, 10). The ATPase activity in the absence of actin and the sedimentation velocity of mixtures of the two forms are determined largely by the fraction of HMM in each form (10). This provides a molecular basis on which to relate the changes in proteolytic degradation rates to the reorientation of HMM heads that is reflected in changes in the sedimentation velocity. On this view, the changes in degradation rates with increasing ionic strength reported in this paper can be attributed to an increase in the fraction of HMM in the extended form.

**Effects of Nucleotides on Papain Digestion of Myosin and HMM**—Earlier studies indicate that under conditions where myosin is in the folded form, e.g., in the presence of MgATP, the susceptibility to papain is low (11, 12), while under conditions where it is in the extend form, the susceptibility to papain is much greater (11–13). These observations led to the suggestion that the effects of phosphorylation and MgATP on digestibility of myosin could be attributed to their ability to induce a papain-susceptible extended form or a papain-resistant folded form, respectively (11, 13).

However, our present results are not consistent with the view that MgATP decreases the papain degradation rate of HMM by converting it from the extended form to a papain-resistant flexed form. MgATP exerts its maximal effect on $k_H$ at low ionic strengths, where two-thirds of HMM is already flexed in the presence or absence of nucleotides (10), and decreasing the fraction of flexed HMM by raising the ionic strength does not increase the response. Furthermore, in the absence of nucleotides, the flexed form is more susceptible to papain than is the extended form.

These observations imply the presence of two flexed forms of HMM differing in their sensitivity to papain. A papain-susceptible flexed form exists in the absence of nucleotides and a papain-resistant flexed form in the presence of ATP, AMP-PNP, or ADP. The decrease in digestibility induced by nucleotides can be explained most simply by a local conformational change that affects only the flexed form, because MgATP has no effect on the digestibility of the extended form at high ionic strengths (Fig. 5). This conclusion is consistent with the absence of MgATP-induced changes in the degradation rates of S1, a fragment that does not undergo the conformational transition characteristic of HMM (10).

The decrease in $k_H$ induced by MgATP cannot be attributed to changes in enzymatic activity of papain, because the degradation of subfragment-1 is not influenced by MgATP. Location of the ATP-binding site of skeletal muscle myosin approximately 140 A from the S1-S2 junction (26) appears to rule out a steric effect of ATP on access of papain to the cleavage sites of HMM. These considerations suggest that the decrease in $k_H$ reflects a conformational change at the S1-S2 junction that may involve long range interactions (27).

**Effects of Phosphorylation on Papain Digestion of Myosin and HMM**—Phosphorylation has been shown to increase the rate of papain cleavage of myosin at the S1-S2 junction in the presence of MgATP, as indicated by the increased rate of formation of subfragment-1 (13). Based on a similar MgATP-induced increase in digestibility of HMM, it was suggested that phosphorylation induces a local conformational change at the S1-S2 junction (13). Our present results confirm this increased digestibility induced by phosphorylation in the presence of MgATP and show in addition that the changes in $k_H$ induced by phosphorylation are associated with a decrease in the sedimentation coefficient, reflecting a conformational change in the neck region. This correlation provides strong evidence that the increase in digestibility of the heavy chain induced by phosphorylation can be attributed to an increase in the fraction of HMM in the extended form. These results further suggest that the changes in the degradation rate of the heavy chain produced by phosphorylation are closely associated with the reorientation of HMM heads that appears to be the molecular change responsible for the changes in $s_{20,w}$.

The effects of phosphorylation on $k_l$ are in agreement with previous results showing that phosphorylation increases the proteolytic digestibility of myosin and HMM in the presence of MgATP and shifts the cleavage site from a point 4 kDa from the NH$_2$ terminus to one 2 kDa closer to the NH$_2$ terminus (11–13, 17). The dependence of $k_l$ on ionic strength demonstrated in the present study provides evidence that the conformation of the light chain depends on the spatial distribution of the HMM heads relative to the tail (10) and that phosphorylation substantially diminishes this dependence.

During ATP hydrolysis, phosphorylation appears to increase $k_H$ and decrease $s_{20,w}$ by increasing the fraction of HMM in the extended form, which during ATP hydrolysis is predominantly an HMM-ADP-P$_i$ complex (22–25). Slow dissociation of P$_i$ from this complex appears to limit the steady-state rate of ATP hydrolysis by dephosphorylated HMM (25) under conditions of low ionic strength in which essentially all HMM is in the flexed form (10). This suggests that the slow product dissociation from dephosphorylated HMM represents dissociation from the flexed HMM-ADP-P$_i$ complex. Phosphorylation increases the rate of P$_i$ dissociation from the HMM-ADP-P$_i$ complex at low ionic strengths by a factor of three (28) and increases the steady-state rate of ATP hydrolysis by a factor of two (10). The parallel changes in $R_{A10}$, $s_{20,w}$, and ATPase activity suggest that in the absence of actin, phosphorylation changes all three of these parameters by increasing the fraction of HMM in the extended form.

The regulatory light chains of scallop or vertebrate striated muscle myosins overlap the S1-S2 junction or neck of the molecule as indicated by cross-linking studies (29, 30) and by electron microscopic observations (31–35). Photocross-linking of the two regulatory light chains of molluscan myosin at a cysteine residue near position 50 suggests that the NH$_2$ terminal region of the light chain is close to the S1-S2 junction (36, 37). A monoclonal antibody directed against the divalent metal binding site of the 19-kDa light chain of skeletal muscle myosin, which contains amino acid residues between 35 and 50, has been shown to bind to the neck of the myosin molecule (34). On this basis, the authors suggested that Ser-19 of the...
20-kDa light chain of vertebrate smooth muscle myosin, the primary site of phosphorylation by myosin light chain kinase (38, 39), may be close to the S1-S2 junction.

The present studies are consistent with this view. The nearly identical dependence of $k_3$ and $k_4$ of dephosphorylated HMM on ionic strength and the location of the phosphorylation-dependent cleavage site of the heavy chain at the S1-S2 junction (13, 14) indicate that the 95-kDa cleavage site of the heavy chain and 4-kDa site of the 20-kDa light chain are in a region of HMM that includes the S1-S2 junction. The proximity of the 95- and 4-kDa cleavage sites would in turn suggest that Ser-19, which lies between the 2- and 4-kDa cleavage sites of the light chain (17), is close to the Sl-S2 junction.

REFERENCES

1. Suzuki, H., Onishi, H., Takahashi, K., and Watanabe, S. (1978) J. Biochem. (Tokyo) 84, 1529-1542
2. Suzuki, H., Takahashi, K., Onishi, H., and Watanabe, S. (1982) J. Biochem. (Tokyo) 91, 1587-1998
3. Trybus, K. M., Huiatt, T. W., and Lowey, S. (1982) J. Biol. Chem. 257, 18564-18571
4. Onishi, H., and Wakabayashi, T. (1982) J. Biochem. (Tokyo) 92, 871-879
5. Onishi, H., and Watanabe, S. (1984) J. Biochem. (Tokyo) 95, 899-902
6. Kumon, A., Yasuda, S., Murakami, N., and Matsumura, S. (1984) Eur. J. Biochem. 140, 265-271
7. Ikebe, M., and Hartshorne, D. J. (1985) Biochemistry 24, 2380-2386
8. Elbash, S. (1976) J. Biochem. (Tokyo) 79, 229-231
9. Alba, T., Ohtsuka, I., Onishi, H., and Watanabe, S. (1979) J. Biochem. (Tokyo) 86, 1275-1282
10. Suzuki, H., Stafford, W. F., and Watanabe, S. (1982) Proc. Natl. Acad. Sci. U. S. A. 30, 436-438
11. Onishi, H., and Watanabe, S. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2060-2064
12. Kumon, A., Yasuda, S., Murakami, N., and Matsumura, S. (1984) Eur. J. Biochem. 140, 265-271
13. Ikebe, M., and Hartshorne, D. J. (1985) Biochimica et Biophysica Acta 80, 1163-1164
14. Ikebe, M., and Hartshorne, D. J. (1985) Biochemistry 24, 2380-2386
15. Elbash, S. (1976) J. Biochem. (Tokyo) 79, 229-231
16. Alba, T., Ohtsuka, I., Onishi, H., and Watanabe, S. (1979) J. Biochem. (Tokyo) 86, 1275-1282
17. Sellers, J. R., Pato, M. D., and Adelstein, R. S. (1981) J. Biol. Chem. 256, 13137-13142
18. Perrie, W. T., Smillie, L. B., and Perry, S. V. (1973) Biochem. J. 135, 151-164
19. Onishi, H., Iijima, S., Anzai, H., and Watanabe, S. (1979) J. Biochem. (Tokyo) 86, 1283-1290
20. Laemmli, U. K. (1970) Nature 227, 680-685
21. Ueno, H., and Harrington, W. F. (1984) J. Mol. Biol. 173, 35-61
22. Marston, S. B., and Taylor, E. W. (1980) J. Mol. Biol. 139, 737-600
23. Onishi, H. (1982) J. Biochem. (Tokyo) 91, 157-166
24. Rosenfeld, S. S., and Taylor, E. W. (1984) J. Biol. Chem. 259, 11908-11919
25. Sellers, J. R. (1985) J. Biol. Chem. 260, 15815-15819
26. Sutoh, K., Yamamoto, K., and Wakabayashi, T. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 212-216
27. Botts, J., Takashi, P., Torgerson, T., Hozumi, T., Muhrlad, A., Hornet, D., and Morales, M. F. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2060-2064
28. Greene, L., and Sellers, J. R. (1985) Biochem. J. 228, 87-95
29. Wallimann, T., Hardwicke, P. M. D., and Szent-Györgyi, A. G. (1982) J. Mol. Biol. 156, 153-173
30. Hardwicke, P. M. D., Wallimann, T., and Szent-Györgyi, A. G. (1983) Nature 301, 478-481
31. Flicker, P. F., Wallimann, T., and Vibert, P. (1983) J. Mol. Biol. 169, 723-742
32. Winkelmans D. A., Lowey, S., and Press, J. L. (1983) Cell 34, 295-306
33. Margossian, S. S., Bhan, A. K., and Slayter, H. S. (1983) J. Biol. Chem. 258, 13359-13369
34. Shimizu, T., Reina, F. C., Masaki, T., and Fischman, D. A. (1985) J. Mol. Biol. 183, 271-282
35. Winkelmans D. A., and Lowey, S. (1986) J. Mol. Biol. 188, 595-612
36. Hardwicke, P. M. D., and Szent-Györgyi, A. G. (1985) J. Mol. Biol. 183, 203-211
37. Vibert, P., Cohen, C., Hardwicke, P. M. D., and Szent-Györgyi, A. G. (1985) J. Mol. Biol. 183, 283-286
38. Pearson, R. B., Jakes, R., John, M., Kendrick-Jones, J., and Kemp, B. E. (1984) FEBS Lett. 168, 108-112
39. Ikebe, M., Hartshorne, D. J., and Elzinga, M. (1986) J. Biol. Chem. 261, 36-39
40. Sobieszek, A., and Small, J. V. (1976) J. Mol. Biol. 102, 75-92
41. Seidel, J. C. (1980) J. Mol. Biol. 135, 153-164
42. Okamoto, Y., Okamoto, M., and Sekine, T. (1980) J. Biochem. (Tokyo) 88, 361-371
43. Cross, R. A., Bardsley, R. G., Ledward, D. A., Small, J. V., and Sobieszek, A. (1984) Eur. J. Biochem. 145, 305-310
44. Suzuki, H., Kondo, Y., Carlos, A. D., and Seidel, J. C. (1986) Biophys. J. 49, 1984a