Two Conserved Lysines at the 50/20-kDa Junction of Myosin Are Necessary for Triggering Actin Activation*

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Actin stimulates myosin’s activity by inducing structural alterations that correlate with the transition from a weakly to a strongly bound state, during which time inorganic phosphate (P,) is released from myosin’s active site. The surface loop at the 50/20-kDa junction of myosin (loop 2) is part of the actin interface. Here we demonstrate that elimination of two highly conserved lysines at the C-terminal end of loop 2 specifically blocks the ability of heavy meromyosin to undergo a weak to strong binding transition with actin in the presence of ATP. Removal of these lysines has no effect on strong binding in the absence of nucleotide, on the rate of ADP binding or release, or on the basal ATPase activity. We further show that the 16 amino acids of loop 2 preceding the lysine-rich region are not essential for actin activation, although they do modulate myosin’s affinity for actin in the presence of ATP. We conclude that interaction of the conserved lysines with acidic residues in subdomain 1 of actin either triggers a structural change or stabilizes a conformation that is necessary for actin-activated release of P, and completion of the ATPase cycle.

The generation of force and movement by the myosin-actin interaction results from an ATP-driven cycle that alternates between dissociated states, weakly bound actomyosin states, and strongly bound actomyosin complexes. This cyclic interaction between actin and myosin is thought to involve the steps illustrated in Scheme 1, where A is actin and M is myosin.

| Reaction | Constant |
|----------|----------|
| A + M ⇄ [AM] | $K_c$ |
| [AM] + ATP ⇄ [AM(ADP)] | $K_{c1}$ |
| [AM(ADP)] + P, ⇄ [AM(ADP)P,] | $K_{c2}$ |
| [AM(ADP)P,] + M ⇄ [M(ADP)P,] | $K_{c3}$ |

Scheme 1

Hydrolysis of ATP occurs principally with the myosin dissociated from actin. Following hydrolysis, the formation of the weakly bound, A-M-ADP-P, state (K,) is governed by electrostatic interactions between myosin and actin. Progression to the strongly bound AM-ADP state (K,) requires major conformational changes that result in force generation and P, release and involves structural elements in both the lower and upper 50-kDa domains that lie on either side of a deep cleft within the motor domain (reviewed in Ref. 1). Because actin catalyzes the release of P,, it must stabilize a myosin conformational state from which P, can be released and strong binding can then follow. Although a number of the residues on both actin and myosin that are involved in formation of the weak and strong interactions have been delineated (reviewed in Ref. 2), the interactions that trigger the transition from weak to strong binding and concurrent P, release are unknown.

One of the components of the actomyosin interface that is believed to be involved in the initial interaction between actin and myosin is a surface loop at the 50/20-kDa proteolytic junction (loop 2). Although the loop residues are not well resolved in most crystal structures of the myosin head (3–5), there is a large body of biochemical evidence that implicates loop 2 in the interaction between actin and myosin. Proteolytic cleavage within the C-terminal cluster of lysine residues weakened binding of actin to myosin and markedly reduced actin-activated ATPase activity (6), and this cleavage was prevented by binding to actin (7). Furthermore, actin can be cross-linked to this lysine-rich region (8), and a peptide corresponding to loop 2 bound to the N-terminal region of actin (9). Cross-linking of a negatively charged “antipeptide” to the lysine-rich region on myosin significantly reduced actin binding (10). The sites on actin that presumably interact with positively charged residues on myosin include the acidic cluster of residues at positions 1–4, 24/25, and 99/100, all in subdomain 1 (11–16).

Mutational studies that added additional positive charge to loop 2 (17), removed large segments of loop 2 (18, 19), or replaced loop 2 of one species with that from another (20–22) all concluded that loop 2 affects the affinity for actin in the presence of ATP. In some studies the source of loop 2 also appeared to affect $V_{max}$ of the actin-activated ATPase in a way that reflected the ATPase rate of the donor myosin (21, 22). Such data led Spudich (23) to propose the provocative hypothesis that the variable portion of loop 2 determines or tunes the ATPase properties of the myosin motor.

Unlike any of the previous mutational studies, we focused on the conserved rather than the variable residues of loop 2. We noted that two positively charged residues (generally two lysines) at the C-terminal end of loop 2 are invariant in all myosins and thus postulated that the positioning and conservation of these residues is a critical feature for actomyosin function. We demonstrate here that these two lysine residues are absolutely required for actin activation of myosin activity. In contrast, 16 residues from the remainder of the loop can be deleted with minimal impact on actin activation of myosin.

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MATERIALS AND METHODS

Plasmid Construction—The cDNA for chicken gizzard smooth muscle myosin was truncated after the nucleotides coding for amino acid 1112 to create an HMM1-like fragment. Nucleotides coding for a Myc epitope (24) followed by the FLAG epitope (DYKDDDDK) and a stop signal were appended. The HMM-like construct was subcloned into the baculovirus transfer vector pVL1393 (Invitrogen). Site-directed mutagenesis was used to create three mutant constructs. For “AA” mutant HMM, the nucleotides coding for lysines at amino acids 652 and 653 were replaced with nucleotides coding for two alanines. For “d16” mutant HMM, the nucleotides coding for amino acids 627 through 651 were deleted.

Extraction and Purification of Proteins—Recombinant baculovirus encoding the HMM-like constructs were prepared by conventional protocols (25). SF9 insect cells in suspension culture were coinfected with virus coding for either a WT HMM or mutant HMM construct and for virus coding for both smooth muscle regulatory light chains (RLC) and essential light chains (in p2Bac vector; Invitrogen). Three days after infection, SF9 cells were lysed in 10 mM sodium phosphate, pH 7.2, 0.1 M NaCl, 5 mM MgCl2, 3 mM NaN3, 7% sucrose, 2 mM EDTA, 1% Nonidet P-40, 2 mM MgATP, 4 mM DTT, and protease inhibitors. Protein that fractionated between 40 and 70% ammonium sulfate was dialedyzed in the presence of RNAse A (pretreated with protease inhibitors; Sigma). The HMM was isolated on an anti-FLAG affinity column, eluted with FLAG peptide, and dialyzed against 10 mM imidazole, pH 7.0, 40 mM NaCl, 1 mM MgCl2, 1 mM DTT, 1 mg/ml leupeptin, and 50% glycerol.

The RLC on HMM was phosphorylated by addition of ATP, Ca2+, calmodulin, and myosin light chain kinase at 0 °C. The purity of the preparations was determined with SDS polyacrylamide gels (26). Phosphorylation of the RLC was confirmed using 40% glycerol gels with samples dissolved in 7.5 M urea (27). HMM concentration was determined by Bradford reagent with bovine serum albumin as a standard.

In Vitro Motility Assay—Phosphorylated HMM at 0.1 mg/ml was mixed with 0.04 mg/ml actin and 1 mg/ml MgATP and centrifuged for 20 min at 350,000 × g to remove HMM that was unable to dissociate from actin in the presence of ATP. The motility assay was performed at 30 °C in 25 mM imidazole, pH 7.5, 25 mM KC1, 4 mM MgCl2, 1 mM EGTA, 0.5% methylcellulose, 1 mM MgATP, 10 mM DTT, 5 mM glucose, 0.1 M Mg2+ chloride, and 0.018 mg/ml catalase essentially as described (28). Assays were also conducted with 60 mM KC1 and 0.7% methylcellulose. An anti-rod monoclonal antibody (Ab S2.1) (29) was used to bind the HMM to the nitrocellulose-covered coverslip.

ATPase Assays—Actin-activated ATPase activity was measured at 37 °C in 8 mM KC1, 10 mM imidazole, pH 7.0, 1 mM DTT, 1 mM NaN3, 1 mM MgCl2, 1 mM EGTA, and 10 μM HMM except for HMM in samples for and for the AA mutant HMM, which were 20 μM. The reactions were initiated by the addition of 2 mM MgATP. The reactions were stopped with SDS at six time points per actin concentration, and inorganic phosphate was determined colorimetrically (30). NH4+-ATPase activity was measured at 37 °C in 0.4 M NH4Cl, 2 mM EDTA, 25 mM Tris base, 0.2 M sucrose, and 1.5 μM Mg2+ phosphorylated HMM. The reactions were initiated by addition of 4 mM NH4ATP. The reactions were stopped at six time points with SDS, and inorganic phosphate was determined colorimetrically.

Cosedimentation Assay—Varying concentrations of actin were mixed with 0.2 mg/ml HMM and 0.1 mg/ml bovine serum albumin in 5 mM imidazole, pH 7.0, 5 mM KC1, 2 mM DTT, and 1 mM MgCl2 and allowed to sit for 10 min at room temperature. 3 mg MgATP was added, and the samples were centrifuged in the Beckman TL-100 at 350,000 × g for 20 min. Equal proportions of supernatant and dissolved pellet were run on 12% SDS polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R. Gel images were captured in digital format using a Kodak Digital Science DC120 zoom digital camera. The band intensities were quantified using the Kodak Digital Science ID image analysis software package to determine the percentage of HMM bound to pelleted actin. A plot of band intensity versus micrograms of HMM per lane gave a linear relationship over the range of band intensities found in the experimental samples.

Fluorimetry Assay for HMM-Actin Binding—Actin was labeled with pyrene-iodoacetamide as described (31). The protein concentration of pyrene-labeled actin was determined by Bradford reagent with unlabeled actin as standard. Fluorescence titrations were carried out on an ISS PC1 photon counting spectrophuorometer at 20 °C using excitation and emission wavelengths of 365 and 407 nm, respectively, and excitation/emission bandwidths of 1 nm. 1 μM pyrene-actin in 10 mM imidazole, pH 7.5, 0.1 mM KC1, 0.1 mM MgCl2, and 1 mM NaCl2 was titrated with increments of 0.25 μM dephosphorylated HMM until the fluorescence was maximally quenched. 1 mM MgATP was added to reverse the actin-HMM binding, and fluorescence was remeasured.

Transient Kinetic Experiments—All kinetic experiments were done in 10 mM HEPES, pH 7.0, 0.1 M NaCl, 5 mM MgCl2, 1 mM EGTA, 1 mM NaN3, 1 mM DTT using a Kin-Tek stopped flow spectrophotometer and a 100-watt mercury lamp. For tryptophan fluorescence (ATP binding measurements), the exciting beam was passed through a 294-nm interference filter and the emission was detected after passing through a 340-nm interference filter. For 90° light scattering (ADP release measurements), the exciting beam was passed through a 294-nm interference filter, and the emission was detected with a 294-nm interference filter. For pyrene-actin fluorescence (ADP release measurements), pyrene-actin was excited using a 360-nm interference filter, and emission was detected with a 400-nm cut-off filter. ATP and ADP stocks were prepared with an equimolar amount of mannitol. The transients shown are the average of three or four independent mixings. The signal averaging and fitting were done using Kin-Tek software. Single exponential data were fit to the equation y = a × exp (−λt) + c, where c is a constant, and λ is the amplitude of the signal.

RESULTS

Description of the Constructs—WT HMM and three mutant HMM constructs derived from chicken gizzard smooth muscle myosin were coexpressed with light chains in a baculovirus/SF9 cell system. The AA mutant HMM has two highly conserved lysines (amino acids 652 and 653) at the C-terminal end of loop 2 (25 and 60 μM) to determine whether the mutations made in these amino acids affected movement in vitro (motility assays). The yields of the constructs were indistinguishable from each other in their heavy and light chain components. The RLCs of the four HMM constructs were completely phosphorylated by myosin light chain kinase as judged by electrophoresis of HMM samples in 7.5% urea on 40% glycerol AA gels.

In Vitro Motility of Mutant HMMs Differ from WT HMM—In vitro motility assays were performed at two KCl concentrations (25 and 60 mM) to determine whether the mutations made in loop 2 of HMM affect the ability of the HMM to move actin (Fig. 2). Essentially all actin filaments moved in motility assays performed with WT HMM. However, in motility assays performed with two different preparations of phosphorylated AA mutant HMM, less than 1% of actin filaments moved, and with a third preparation no movement of actin filaments was detected.

FIG. 1. Schematic illustrating the location and sequence of loop 2 in wild type HMM (WT), AA mutant HMM (AA), and d16 mutant HMM (d16). 25, 50, and 20 kDa define the tryptically derived fragments. The loop 2 amino acids shown for WT HMM are amino acids 627 to 656. AA mutant HMM has the lysines at positions 652 and 653 mutated to alanines (indicated in boldface). d16 mutant HMM has the 16 amino acids indicated deleted. A d16 mutant HMM (used only in an in vitro motility assay) extends the deletion in the d16 mutant HMM by two amino acids toward the C-terminal end of loop 2 (K and T).
sharp contrast, the extrapolated HMM had essentially no actin-activated ATPase activity. In as a function of actin concentration (Fig. 3). The AA mutant HMM, and d16 mutant HMM was then measured actin-activated ATPase activity of phosphorylated WT HMM, catalytic ATPase function in the absence of actin (Table I). The indications that the mutations do not interfere with the intrinsic.

ments moved in assays with WT HMM and d16 mutant HMM, whereas less than 1% of actin filaments moved in the three assays with AA mutant HMM. The motility of this very minor population of filaments that moved in the AA mutant was 90% slower than the values obtained with WT HMM.

tected. The velocity of the few filaments of actin moved by phosphorylated AA mutant HMM was ~90% slower at both salt concentrations compared with WT HMM. In contrast, essentially all actin filaments moved in motility assays with phosphorylated d16 mutant HMM, with a velocity that was reduced 29% at 25 mM KCl and 16% at 60 mM KCl compared with WT HMM. Thus, the loss of two lysines from loop 2 had a drastic effect on *in vitro* motility, whereas deletion of 16 other amino acids from loop 2 had a minimal effect.

The amino acids deleted in the d16 mutant HMM included one of the two lysines in the variable portion of loop 2. Because deletion of this one lysine did not have a major effect on *in vitro* motility, we performed motility assays with a phosphorylated d18 mutant HMM in which both of the lysines in the variable portion of loop 2 have been removed. Essentially all actin filaments moved in the assays. The velocity was reduced 41% at 25 mM KCL and 29% at 60 mM KCL compared with WT HMM (average of three separate preparations). Thus, the loss of two lysines in the variable portion of loop 2 had much less effect on motility than mutation of the two conserved lysines.

**ATPase Activities**—We first established that the WT and mutant HMM constructs had the same *NH₄⁺*-ATPase activity, indicating that the mutations do not interfere with the intrinsic catalytic ATPase function in the absence of actin (Table 1). The actin-activated ATPase activity of phosphorylated WT HMM, AA mutant HMM, and d16 mutant HMM was then measured as a function of actin concentration (Fig. 3). The AA mutant HMM had essentially no actin-activated ATPase activity. In sharp contrast, the extrapolated *V*ₘₐₓ values obtained from the

![Fig. 2. Velocity of actin filaments sliding in the *in vitro* motility assay over phosphorylated WT HMM, AA mutant HMM, and d16 mutant HMM. The assays were performed both in 25 mM KCl (with 0.5% methylcellulose) and 60 mM KCl (with 0.7% methylcellulose). Values are mean velocities ± S.D. for eight assays for WT HMM (on three independent preparations), three assays of AA mutant HMM (on three independent preparations), and five assays of d16 mutant HMM (on three independent preparations). The assays were performed both in 25 mM KCl (23 s⁻¹) and 60 mM KCl (17 s⁻¹).](http://www.jbc.org/)

**Table I.** *NH₄⁺*-ATPase activity of WT and mutant HMMs

| HMM construct | Rate (s⁻¹) |
|---------------|------------|
| WT            | 36 ± 2     |
| d16           | 36 ± 2     |
| AA            | 34 ± 2     |

![Fig. 3. Actin-activated ATPase activity of phosphorylated HMM. Shown are WT HMM (○), AA mutant HMM (●), and d16 mutant HMM (▲). Values are averages of four assays with three independent preparations of each HMM. Data were fit to the Michaelis-Menten kinetic model. *V*ₘₐₓ was 4.4 ± 0.3 s⁻¹ for WT HMM and 4.4 ± 0.2 s⁻¹ for d16 mutant HMM. *K*ₘₐₜ was 41 ± 6 μM for WT HMM and 11 ± 2 μM for d16 mutant HMM.](http://www.jbc.org/)

illustrated best fit curves were essentially identical for WT HMM and d16 mutant HMM (4.4 ± 0.3 and 4.4 ± 0.2 s⁻¹, respectively). Interestingly, the *K*ₘₜ for the d16 mutant HMM was much lower than the *K*ₘₜ for WT HMM (11 ± 2 versus 41 ± 6 μM) indicating that the deletion of 16 amino acids in loop 2 increased the affinity for actin. Note that the deletion of the 16 amino acids increases the net positive charge of loop 2 by one (Fig. 1). Thus, loss of two lysines from loop 2 essentially abolished actin-activated ATPase activity, but deletion of 16 other amino acids from loop 2 had no effect on *V*ₘₐₓ.

**Mutant HMMs Bind to Actin in the Presence of ATP**—To determine whether the loss of motility and actin-activated ATPase activity for AA mutant HMM could be due to changes in the weak binding affinity for actin, cosedimentation assays were performed. Phosphorylated WT HMM and mutant HMMs were cosedimented with increasing concentrations of actin in the presence of 3 mM ATP (Fig. 4). The points in the graph are fitted to curves that assume 100% binding at infinite actin concentration, because all three constructs show complete binding to actin in the absence of nucleotide. The *K*ₜ values from these curves are 14 ± 1 μM for WT HMM, 89 ± 15 μM for AA mutant HMM, and 5.4 ± 0.4 μM for d16 mutant HMM.

Although the AA mutant HMM in the presence of ATP has a weaker affinity for actin than WT HMM, the reduction in affinity is not sufficient to explain the drastic effect that the loss of two lysines has on motility and actin-activated ATPase activity. Under conditions where about 40% of the AA mutant...
HMM is bound to actin, there is still no detectable actin-activated ATPase activity. The d16 mutant HMM's stronger affinity for actin in the presence of ATP than that of WT HMM corroborates the decrease in $K_m$ determined in the actin-activated ATPase assay.

Rigor Binding of AA HMM to Actin Is Unimpaired—Because the degree of reduction in weak binding affinity for actin shown by AA mutant HMM was inadequate to explain the drastic reduction in motility and actin-activated ATPase activity, the ability of AA mutant HMM to form a strongly bound rigor state with actin was investigated. When HMM binds to pyrene-labeled actin, the fluorescence of pyrene-actin undergoes quenching as the weakly bound state converts to a strongly bound rigor state. The fluorescence of pyrene-actin was measured in the presence of increasing concentrations of WT HMM or AA mutant HMM (Fig. 5). The degree of quenching of pyrene-actin fluorescence by AA mutant HMM and WT HMM was identical in two independent experiments using different preparations of both HMMs. At the end of the experiment, addition of ATP dissociated the actin-HMM complex and restored 80–90% of the pyrene-actin fluorescence for both the WT HMM and AA mutant HMM samples, confirming that the quenching was because of binding of actin to HMM. Hence AA mutant HMM is capable of forming a strongly bound rigor state with actin.

Pyrene-actin was also titrated with d16 mutant HMM. The degree of fluorescence quenching by d16 mutant HMM was identical to that of WT HMM, and pyrene-actin fluorescence was restored by addition of ATP (data not shown).

Tryptophan Fluorescence Shows WT and AA Mutants Bind and Hydrolyze ATP at Similar Rates—To determine whether the loss of motility and actin-activated ATPase activity seen...
with AA mutant HMM could be because of impaired binding of ATP, the rate of ATP binding was determined from the increase in intrinsic tryptophan fluorescence (Fig. 6). At low ATP concentrations, the rate of the transient increased linearly with ATP, yielding a second order rate constant for ATP binding of $8 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$ for WT HMM and $1 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$ for the AA mutant HMM. Thus ATP binding was not reduced for AA mutant HMM. Fitting the data at all ATP concentrations to a hyperbola yielded a maximum rate of of $33 \text{ s}^{-1}$ for WT HMM and of $25 \text{ s}^{-1}$ for the AA mutant HMM. The maximum rate of the fluorescence change has been equated with the rate of ATP cleavage determined by quench flow experiments (32). Hence, the rate of ATP hydrolysis may be slightly reduced for the AA mutant HMM.

The Rates of ADP Release from WT and Mutant HMMS Are Equal.—The rate of ADP release was also examined as a possible explanation for the decrease in motility and actin-activated ATPase seen with AA mutant HMM. The rate of ADP release was measured at three temperatures (Table II). An actoHMM-ADP complex (actin or pyrene-labeled actin) was rapidly mixed with ATP in a stopped flow apparatus. The rate of dissociation of the actoHMM complex by ATP is rate-limited by the rate of ADP dissociation. Thus the rate of the decrease in light scattering or the rate of increase in pyrene fluorescence measures the rate of ADP release. Both methods showed very similar values for WT HMM, AA mutant HMM, and d16 mutant HMM. This step cannot account for the abnormal motility and actin-activated ATPase seen with AA mutant HMM.

DISCUSSION

Here we show that the highly conserved C-terminal lysines of loop 2 are essential residues for triggering actin-induced phosphate release and the concurrent strong binding to actin ($K_s$ in Scheme 1) that is necessary for actin-activated ATPase activity and actin movement. In previous mutational studies, these two lysines have never been altered, because other investigators focused on the variable portion of loop 2. Mutation of the conserved two lysines to alanines essentially obliterates both actin activation of ATPase activity and the ability of myosin to move actin filaments in a motility assay.

A number of assays verified that the major functional defect caused by mutation of the two conserved lysines occurs only as actoHMM progresses through the cycle from weak to strong binding and that other steps in the cycle are not compromised. The intrinsic myosin ATPase activity and the rate of ATP binding and hydrolysis of the AA mutant HMM are similar to WT. The rate of ADP release from actoHMM and the ability to strongly bind actin in the absence of ATP are also native. The reduction in affinity for actin in the presence of ATP is not sufficient to account for the complete loss of actin-activated ATPase activity and motility. This reduction in affinity implies that either these two lysines are also involved in the weak binding to actin or that the loss of strongly bound intermediates (because the mutant cannot proceed through the cycle) results in an apparent decrease in actin affinity. Because substantial binding of the mutant myosin to actin did remain, other residues must contribute to weak binding.

In striking contrast to the effect of removing the conserved lysines, the deletion of 16 residues from loop 2 that precede the lysine-rich region had little negative impact on myosin function. Extending the deletion to 18 residues (thereby removing both of the lysines in the variable portion of loop 2) did reduce motility. However, the reduction was small compared with the complete lack of motility observed upon deletion of the conserved lysines. This highlights the greater importance of the conserved lysines. Consistent with these observations, earlier biochemical studies had shown that cleavage at residues in loop 2 N-terminal to the conserved lysines had little effect on actin-activated ATPase activity (6, 33). Here we find that the affinity for actin in the presence of ATP was actually significantly increased by the deletion of 16 residues, as evidenced by a 3.7-fold decrease in $K_m$ in an actin-activated ATPase assay and by an increase in affinity for actin in a cosedimentation assay. This increase in weak binding affinity may relate to the fact that the deletion of 16 residues increased the net positive charge of the loop by one. Notably, this increase in net positive charge had no effect on the $V_{max}$ of the actin-activated ATPase activity. Consistent with this observation, Furch et al. (17) reported that adding 4 to 12 positive charges to loop 2 of Dictyostelium myosin motor domain significantly decreased the apparent $K_m$ for actin by 10-fold.

The only functional deficit resulting from the deletion of the 16 amino acids was a modest reduction in the rate at which the d16 mutant HMM moved actin in a motility assay. The slower in vitro motility may result from the fact that increasing the strength of the weak pre-powerstroke states creates a larger internal drag on the actin filament. Increasing the ionic strength would reduce this effect by decreasing the weak interactions, consistent with less inhibition at higher salt levels (16% decrease at 60 mM KCl versus 29% at 25 mM KCl). Our results with the 16-residue deletion in loop 2 differ markedly from the results of Knetsch et al. (18) who shortened the Dictyostelium myosin loop 2, which is already much shorter than the smooth muscle myosin loop, by 8 residues. Their mutation reduced actin activation of ATPase activity 5-fold, lowered the affinity for actin 100-fold, reduced the rate of ADP dissociation 2.5-fold, and abolished the ability of the mutant to enter the strong binding state as judged by pyrene-labeled actin. The mutation in Dictyostelium myosin reduced the loop size to about six flexible residues, whereas our d16 mutant myosin retained 14 flexible residues in loop 2. We interpret these differences between our results and those of Knetsch et al. (18) to mean that there is a minimal size of loop 2 that is required to allow opening of the cleft in the 50-kDa fragment and release of phosphate.

Two studies have reported an effect of the variable portion of loop 2 on the $V_{max}$ of actin-activated ATPase activity in a way that reflects the ATPase activity of the donor myosin (21, 22). Furch et al. (17) reported an increase in $V_{max}$ of 2–3-fold upon addition of more positive charge to loop 2. The rate-limiting step for actin-activated ATPase activity is thought to be $K_p$ (Scheme 1), which involves a conformational change in myosin, and the release of P, as the weakly bound state isomerizes to the strongly bound state (34, 35). Accurate determination of $V_{max}$, which is necessary to prove an increase in the rate-

### Table II

| Temp  | WT     | d16   | AA    |
|-------|--------|-------|-------|
| 15 °C | 28.8 ± 0.1 | 52.9 ± 0.1 | 53.3 ± 0.5 |
| 20 °C | 52.3 ± 0.3 | 53.3 ± 0.5 | 53.3 ± 0.5 |
| 25 °C | 52.3 ± 0.3 | ND    | 74.1 ± 1.0 |
The actin partners of the C-terminal lysine residues of loop 2 are likely in subdomain 1 of actin. The effects of removing negative charge from the N terminus, residues 24/25, or residues 99/100 of actin (15, 16, 36) are similar to the effects of removing two conserved positive charges from loop 2 of myosin. Mutations in either myosin or actin caused a large decrease in actin activation of ATPase activity, a more modest reduction in actin activation of ATPase activity, and impairments in motility. Our HMM mutation was more severe in that even at low ionic strength, motility was not observed. Based on mutations in actin, Reisler and colleagues (16) proposed that the interaction between acidic residues in subdomain 1 of actin and the residues in loop 2 facilitates the flux from weak to strong binding states. Here we come to similar conclusions but further show that the conserved positive residues in loop 2 of myosin are essential for triggering any flux from the weak to strong binding state. Because loop 2 straddles a cleft separating the upper and lower domains of the 50-kDa fragment from which phosphate is thought to be released, we propose that the interaction of the lysines of loop 2 with acidic residues in subdomain 1 of actin facilitates the opening of this cleft and thus the release of phosphate (1, 4–5).

In summary, the data suggest that more than half of loop 2 is not essential for in vitro function of the myosin, although it plays a modulatory role by altering the affinity of myosin for actin. In striking contrast, the two highly conserved lysines at the C-terminal end of loop 2 are essential for triggering Pi release concurrent with the transition between the weakly bound and strongly bound actomyosin states. It is interesting to note that the properties of the AA mutant are identical to the properties displayed by the inhibited, dephosphorylated state of WT smooth HMM. It is possible that in the inhibited state, the presentation of these essential lysine residues to actin is not allowed and that this is also a component of the mechanism that prevents dephosphorylated smooth muscle HMM from proceeding through the contractile cycle.

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