Effects of Mutations in the γ-Phosphate Binding Site of Myosin on Its Motor Function* 

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The role of the highly conserved residues in the γ-phosphate binding site of myosin upon myosin motor function was studied. Each of five residues (Ser181, Lys185, Asn235, Ser236, and Arg238) in smooth muscle myosin was mutated. K185Q has neither a steady state ATPase nor an initial P_i burst. Although ATP and actin bind to K185Q, it is not dissociated from actin by ATP. These results indicate that the hydrolysis of bound ATP by K185Q is inhibited. S236T has nearly normal basal Mg^2+-ATPase activity, initial P_i burst, ATP-induced enhancement of intrinsic tryptophan fluorescence, and ATP-induced dissociation from actin. However, the actin activation of the Mg^2+-ATPase activity and actin translocation of S236T were blocked. In contrast S236A has nearly normal enzymatic properties and actin-translocating activity. These results indicate that 1) the hydroxyl group of Ser236 is not critical as an intermediate of proton transfer during the ATP hydrolysis step, and 2) the bulk of the extra methyl group of the threonine residue in S236T blocks the acceleration of product release from the active site by actin. Arg238, which interacts with Glu459 at the Switch II region, was mutated to Lys and Ile, respectively. R238K has essentially normal enzymatic activity and motility. In contrast, R238I does not hydrolyze ATP or support motility, although it still binds ATP. These results indicate that the charge interaction between Glu459 and Arg238 is critical for ATP hydrolysis by myosin. Other mutants, S181A, S181T, and N235I, showed nearly normal enzymatic and motile activity.

Myosins are molecular motors that translocate actin filaments using energy from ATP hydrolysis and participate in diverse biological contractile events, such as muscle contraction, cytokinesis, cell locomotion, and organelle movements. During the last few years, a number of myosin-like proteins have been identified, and it is now clear that myosin consists of a large family of actin-based motors (1–4). While the C-terminal tail portion of these myosins is quite diverse, the N-terminal 80-kDa motor domain, which contains the ATP binding site and actin binding sites, is highly conserved among various myosins (4). Therefore, it is thought that the mechanism by which the chemical energy of ATP is converted to mechanical energy is universal in all myosin family members. Myosin-based motility occurs as the result of cyclic association and dissociation of myosin molecules and F-actin molecules with concomitant hydrolysis of ATP. This cycle can lead to relative sliding of the actin and myosin and the production of work. Equation 1 contains the generally accepted mechanism of actomyosin ATP hydrolysis, where AM and M represent actomyosin and myosin, respectively.

ATP binding to actomyosin is rapid and produces a rapid dissociation of myosin from actin (>1000 s⁻¹). The rate of M-ATP hydrolysis to M-ADP-P_i is also much faster than the steady state rate of hydrolysis by myosin, which in the absence of actin, is limited by slow release of inorganic phosphate, P_i (5). Actin increases the steady state rate of ATP hydrolysis by increasing the rates of product release >100-fold (6, 7). The transitions between weakly bound states, AM-ATP and AM-ADP, and the strongly bound states, AM-ADP and AM are thought to be associated with movement and production of force (8–10). While the molecular details of the mechanism in Equation 1 are not fully understood, recent determination of the three-dimensional structure of the myosin head determined by x-ray crystallography (11-13) significantly increased the understanding of the molecular mechanism of myosin-based enzymatic activity and contractility. The myosin head contains several clefts, which divide the protein into distinct domains. A cleft splitting the 50-kDa central segment of myosin S1 extends from the nucleotide binding pocket to the actin-binding interface. It is proposed that this cleft opens upon ATP binding and closes after hydrolysis (11, 14). These are coupled with weak and strong binding states, respectively. Furthermore, it is suggested that the closure of the nucleotide binding pocket triggers a conformational change to generate a bent configuration (15), which is coupled with cross-bridge movement. The triphosphate moiety of ATP is accommodated in a narrow tunnel formed by the two loops, i.e. Gly179-Glu187 and Asn233-Gly240, which are highly conserved among various myosin subfamilies (4). A number of interactions between the triphosphate and these amino acid residues of myosin occur during substrate binding and hydrolysis. The phosphate binding loop contributes six hydrogen bonds from its main chain amide nitrogen and additional interactions from the side chains of Ser181, Lys185, and Thr186. The other loop (233–240) also provides six hydrogen bonds, including the ones from the side chain of Ser236, Ser237, and Arg238 (12,13). Among the residues contributing to form the phosphate binding site, Ser181 and Ser234

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1 We have used sequence numbers for Dictyostelium myosin for ease in comparison with the structural studies of the active site. The corresponding gizzard smooth muscle myosin sequence numbers (in parentheses) are Ser181 (179), Lys185 (183), Asn233 (244), Ser236 (245), and Arg238 (247).
(skeletal) are known to be photomodified by vanadate upon irradiation of the Si1MgADP-vanadate complex (16). This is consistent with the notion that these two residues are involved in γ-phosphate binding. The purine binding site is formed by the Gly-rich loop (181–184) on one side and the Asn237-Tyr35 strand on the other side. Trp131 (skeletal) folds over the purine ring, shielding it from external solvent and is known to be photolabeled by the ATP analogue, 2-azoido ATP (17).

A major goal of this work was to identify the residues that are necessary for ATP hydrolysis and motor activities of myosin. While recent structural analysis of the myosin head provides important information for understanding the structure of the active site of myosin, the functional roles of the amino acid residues that form the active site and how they couple the catalytic activity to movement and work production is not well understood. In the present study, we have produced a series of mutant myosins in which the residues involved in the formation of the phosphate binding site are altered in order to address these questions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Smooth muscle myosin was prepared from frozen turkey gizzards as described previously (18). Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (19). Smooth muscle myosin light chain kinase was prepared from frozen turkey gizzards (20). Recombinant calmodulin of Xenopus oocyte (21) was expressed in *Escherichia coli* as described elsewhere (22).

**Expression of the Recombinant Smooth Muscle Myosin Mutants—** Site-directed mutagenesis was performed using the construct (6D3) as described previously (23). The baculovirus transfer vectors of smooth muscle myosin light chains were produced as described (25). Recombinant baculoviruses for the heavy chain and the light chains were produced according to the protocols described by O'Rielly et al. (26). To express smooth muscle myosin mutants, Sf9 insect cells were infected with three separate viruses expressing the heavy chain and two light chains. The recombinant smooth muscle myosin was purified as described previously (25) with slight modification. After extraction as described elsewhere (25), the extract was submitted to ammonium sulfate fractionation (20–25%) to remove endogenous myosin. After dialysis against 0.3 M KCl, 20 mM Tris-HCl (pH 7.5) for 2 h, the fraction was incubated with 10% glucose and 20 units/ml hexokinase at 4 °C for 1 h to eliminate residual ATP. F-actin (0.2 mg/ml) was added to the supernatant and incubated at 4 °C for 30 min, and the expressed myosin fragment was co-purified with F-actin. The pellet was resuspended with 5 mM ATP to release the expressed myosin-HMM from F-actin as described previously (25). For mutant HMM preparations, in which no phosphate burst or tryptophan fluorescence enhancement was observed consistent with the notion that these two residues are involved in the binding of the ATP to the active site, utilizing the relatively low protein concentrations of expressed proteins that were available. However, the size of the burst is unchanged as long as kburst >> ksteady state.

**Stopped-flow Measurements of ATP Hydrolysis—** Chemical quench measurements were done using a computer-controlled stepper motor-driven quench-flow apparatus used in a pulse-quench mode. Syringes of the quench-flow sample handling unit (model 27001, Kintek Corp., State College, PA) were driven by a 34A109E stepper motor (Anahiem Automation, Anaheim CA), which was powered by an IMS Panther HI2 microstepper motor controller (Servo-Systems, Montville, NJ). A program written in turbo basic and running on a Zenith 140 PC provided the timing. Solutions of HMM (20 μl) and ATP (15 μl) containing 10,000–20,000 dpm [γ-32P]ATP (DuPont, BLU-002) were loaded into the sample loops of the 27001 and driven into a delay line by buffer from the drive syringes. After incubation for the desired amount of time, a second drive was used to expel the reaction mixture and quench it with acid (2 N HCl and 0.35 μl KH2PO4) to give a final sample volume of 1.0 ml. Mixing times down to 100 ms could be obtained using this configuration. A 0.4-ml portion of the quenched sample was mixed with an equal volume of a 10% slurry of charcoal (Sigma, C-8368) in 2 N HCl and centrifuged to remove the unhydrolyzed ATP. The total radioactivity of ATP in each sample was determined by counting 0.2 ml directly. The percent hydrolysis was obtained from the ratio of the radioactivity in charcoal-treated to directly counted samples after subtracting background from each. Control experiments indicate that more than 99% of the unhydrolyzed ATP is bound to charcoal. The experiments were done under “single turnover” conditions in which [HMM] sites > [ATP]. The data were then fit to a two-exponential equation using a simplex fitting routine, to obtain amplitude and rate information. The rate of the burst in these experiments is limited by the rate of binding of the ATP to the active site, utilizing the relatively low protein concentrations of expressed proteins that were available. However, the size of the burst is unchanged as long as kburst >> ksteady state.

**Stopped-flow Measurements of Tryptophan Fluorescence Enhancement by ATP—** Stopped-flow measurements of tryptophan fluorescence enhancement were done as described previously (30). WT and mutant HMM (1–2 μm) were mixed with 200 μM MgATP. The rate of the hydrolysis step is estimated by the rate of tryptophan fluorescence increase at saturating ATP concentration (32). The observed increases in tryptophan fluorescence were fit to one or two exponential terms by the method of moments (31).

**Emission Spectroscopy Labeling of Myosin Mutants with ATP—** Phoaffinity labeling was performed as described by Maruta and Korn (34) with some modification. Myosin mutants (0.1 mg/ml) were mixed with 4 or 40 μM ATP (0.5 ± 1 Tq/mm mol) in 100 μl of 30 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 1 mM DTT, 5% sucrose. The solution was irradiated at a distance of approximately 4 cm for 15 min with ultraviolet light (18.4 watts) at 254 nm. The protein was precipitated by the addition of cold 5% trichloroacetic acid containing 1% sodium pyrophosphate and collected by
TABLE I

ATPase activities of myosin mutants

| Mutantsa | Phosphorylated | Dephosphorylated | Degree of regulation | Ca2+-ATPase | K+(EDTA)-ATPased |
|----------|----------------|------------------|----------------------|-------------|-----------------|
|          | $V_m$ a | $K_m$ $\mu M$ | $V_m$ $s^{-1}$ | $K_m$ $\mu M$ | $s^{-1}$ | $s^{-1}$ |
| WT       | 0.525 ± 0.069 | 59.0 ± 14.3 | 0.017 ± 0.005 | 54.8 ± 13.3 | 30.9 | 0.795 ± 0.060 | 1.001 ± 0.148 |
| S181A    | 0.363 ± 0.063 | 54.8 ± 2.8  | 0.023 ± 0.007 | 52.1 ± 12.4 | 15.8 | 1.576 ± 0.146 | 0.160 ± 0.015 |
| S181T    | 0.240 ± 0.002 | 70.7 ± 9.5  | 0.032 ± 0.011 | 160.5 ± 40.5 | 7.5  | 1.260 ± 0.146 | 0.267 ± 0.066 |
| K185Q    | 0           | 0               | 0                    | 0            | 0    | 0             |
| N235I    | 0.300 ± 0.025 | 41.4 ± 18.6 | 0.032 ± 0.009 | 44.0 ± 10.0 | 9.4  | 1.672 ± 0.214 | 0.201 ± 0.112 |
| S236A    | 0.292 ± 0.050 | 31.9 ± 3.1   | 0.015 ± 0.004 | 105.2 ± 7.4 | 19.5 | 0.556 ± 0.050 | 0.194 ± 0.003 |
| S236T    | 0           | 0               | 0                    | 0            | 0    | 0             |
| R238I    | 0           | 0               | 0                    | 0            | 0    | 0             |
| R238K    | 0.822 ± 0.052 | 48.1 ± 7.1   | 0.030 ± 0.006 | 78.3 ± 9.8  | 27.4 | 0.426 ± 0.019 | 0.654 ± 0.220 |

a Wild type and mutants, except K185Q and R238I, were purified by actin coprecipitation. K185Q and R238I were purified by immunoprecipitation.

b Assay conditions were 0.1 mg/ml myosin mutant, 0.3 mM ATP, 32 mM KCl, 30 mM Tris-HCl, pH 7.5, 1 mM MgCl2, and various concentration of actin. 0.2 mM CaCl2, 15 $\mu$g/ml MLCK, and 10 $\mu$g/ml calmodulin were added to measure the activity of phosphorylated HHMMs, whereas 1 mM EGTA was added to do the dephosphorylated one. Mg2+-ATPase activity in the absence of actin is subtracted. Results are means ± S.D. A computed nonlinear least squares curve-fitting program was used to estimate the maximum actin-activated ATPase activity ($V_{max}$), and the apparent dissociation constant for actin ($K_a$) based on the equation $V = V_{max}K_a/(1 + K_a[actin])$. Degree of regulation is the ratio of the phosphorylated to dephosphorylated actin-activated Mg2+-ATPase. The activity is per S1-head.

c Ca2+-ATPase activity was measured at 25 °C in 10 mM CaCl2, 0.5 M KCl, 30 mM Tris-HCl, pH 8.5. The activity is per S1-head.

d K+ (EDTA) ATPase activity was measured at 25 °C in 10 mM EDTA, 0.5 M KCl, 30 mM Tris-HCl, pH 8.5. The activity is per S1-head.

RESULTS

In the present study, several highly conserved residues in the γ-phosphate binding loops of smooth muscle myosin were mutated, expressed in Sf9 cells, and biochemically characterized. A high percentage of the expressed myosin-HMM heavy chains were recovered in the supernatant of the extraction buffer. However, a fraction of myosin heavy chain was insoluble, which suggested that some of the heavy chain was improperly folded. Sf9 cells were coinfected with a heavy chain expressing virus alone does not produce soluble myosin (33), it is therefore likely that some of the heavy chain was not coinfected with all three viruses. Previously, we found that infection of Sf9 cells with a heavy chain expressing virus alone does not produce soluble myosin (33), it is therefore likely that the insoluble myosin is due to the lack of coexpression with the light chains in some of the cells.

ATPase Activities of Mutant Myosins—Table I summarizes ATPase activity of mutations to a series of conserved amino acids in the γ-phosphate binding site of smooth muscle myosin. The substitution of the conserved Ser181 to Ala or Thr decreased K+ (EDTA) ATPase activity and increased Mg2+-ATPase activity. The mutation of Ser181 also resulted in near normal actin-activated ATPase activity. These results show that, although this residue is completely conserved among myosins including unconventional myosins (4), relatively conservative substitutions do not severely affect the ATPase activities. In contrast, K185Q showed no detectable Ca2+-, K+(EDTA), Mg2+-, or actin-activated ATPase activity, indicating that Lys185 is critical for ATP hydrolysis. The mutation of Asp236 to Ile moderately decreased the K+ (EDTA) and actin-activated ATPase and increased the Ca2+ ATPase activity. Of consider-
labeled by both \([\alpha-\text{32P}]\text{ATP}\) and \([\gamma-\text{32P}]\text{ATP}\) (Fig. 2). The extent of labeling was similar, although the extent of labeling of these mutants was significantly lower than that of the WT. Labeling by \([\gamma-\text{32P}]\text{ATP}\) indicates that K185Q and R238I can bind ATP but cannot hydrolyze the phosphoester bond of ATP to produce ADP and Pi. The low labeling efficiency for the mutants might have been due to the decrease in affinity for ATP. To test this possibility, the ATP concentration was increased 10 times (40 mM). However, the relative labeling efficacy for these two mutants against the WT was unchanged, suggesting that the low labeling efficiency is not due to the decrease in the affinity for ATP for these mutants (not shown). The addition of F-actin to these mutant myosins did not alter the photo-induced labeling of ATP, indicating that the actin binding did not affect ATP binding or hydrolysis.

**P, Burst of the Mutant Myosins**—Myosin rapidly binds and hydrolyzes ATP to form a metastable M-ADP-P ternary complex, which is followed by rate-limiting release of products. Acid quenching of the reaction of myosin with ATP reveals a rapid burst of hydrolysis corresponding to the formation of M-ADP-P followed by a second slow phase from the steady state breakdown of M-ADP to products. Formation of M-ADP-P, as the predominant steady state intermediate is necessary for normal myosin motor function (35). It is therefore a critical test to determine whether S236T, which has near normal basal steady state Mg\(^{2+}\)-ATPase activity but no actin-activation of ATPase activity, exhibits a P burst. As shown in Fig. 3, S236A and S236T have an initial P burst, followed by slow steady state P$_r$, release similar to WT. The results of fitting the data to two exponential rate processes are summarized in Table II. The ratio of the amplitudes of the fast \(k_{\text{burst}}\) and slow \(k_{\text{steady state}}\) phases of hydrolysis give an accurate measure of the equilibrium constant, \(K_H\), of the enzyme bound hydrolysis (M-ATP \(\leftrightarrow\) M-ADP-P$_r$). \(K_H \approx k_{\text{burst}}/k_{\text{steady state}}\). The observed rate of the phosphate burst can be used to estimate the second order rate constant of ATP binding \(k_{\text{burst}}/(\text{sites})\). The values for ATP binding and hydrolysis by S236A and S236T are within a factor of two of WT and indicate that these steps are not significantly impaired in S236A or S236T.

**Stopped-flow Measurements of ATP Binding to WT, S236A, and S236T**—ATP binding to the active site of myosin increases the intrinsic fluorescence of tryptophan residues. At low con-
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FIG. 3. Stopped-flow fluorescence (left) and single turnover quench flow measurements (right) of ATP binding and hydrolysis by WT (A and B), S236A (C and D), S236T (E and F). WT (1.1 μM), S236A (1.5 μM), or S236T (2.2 μM) HMM were mixed with half the respective concentrations of 32P[γ-ATP] and quenched at the indicated times. The solid lines represent the best fit of the data to the equation \( e^{-kt} \), where \( k \) is the rate constant of the burst and \( t \) is the time elapsed. The values used to calculate the theoretical curves are listed in Table II. Stopped-flow fluorescence measurements were observed upon mixing equal volumes of WT (A), S236A (C), and S236T (E) HMM with 200 μM ATP. Solid lines through the data for WT and S236A are for a single exponential equation (\( I = I_0 - A e^{-kt} + C \)) fits of 28 s \(^{-1} \) (A), 54 s \(^{-1} \) (C), S236T is fit to a double exponential equation (\( E \)): \( 0.7e^{-70t} + 0.3e^{-t} + C \).

Many chemical modifications of myosin (e.g. GTP, ADP-Pi) result in partial or complete loss of the tryptophan fluorescence enhancement normally observed in the presence of ATP. A second characteristic associated with such modifications is that the hydrolysis step (rather than product release) is rate-limiting and M-ATP (or M-GTP) rather than M-ADP-Pi (or M-GDP-Pi) is the predominant steady state intermediate. Such modifications also result in inhibition of in vitro motility in solution (39) and force production in fibers (40). In Fig. 3 an increase in tryptophan fluorescence was observed upon mixing 200 μM ATP with WT, S236A, and S236T, which is characteristic of myosin in which there is rapid ATP hydrolysis followed by slow product release. The kinetics of the fluorescence enhancement for S236A and WT are fit reasonably well by a single exponential equation (\( k_{SS} = 54 \text{ s}^{-1}, k_{WT} = 28 \text{ s}^{-1} \)) for WT and S236A are for a single exponential equation (\( I = I_0 - A e^{-kt} + C \)) fits of 28 s \(^{-1} \) (A), 54 s \(^{-1} \) (C), S236T is fit to a double exponential equation (\( E \)): \( 0.7e^{-70t} + 0.3e^{-t} + C \).

**TABLE II**

| HMM  | [ATP] | \( f_{SS}^{max} \) | \( f_{max}^{SS} \) | \( k_{burst} \) | \( k_{SS} \) | \( k_{burst} \) | \( k_{SS} \) |
|------|-------|---------------------|------------------|----------------|--------|----------------|--------|
| WT   | 1.0   | 0.50                | 0.61             | 0.11           | 0.007  | 1.5            | 28     | 0.11           |
| S236A| 0.74  | 0.37                | 0.69             | 0.14           | 0.003  | 2.3            | 54     | 0.19           |
| S236T| 1.0   | 0.25                | 0.80             | 0.14           | 0.017  | 4.0            | 8      | 0.14           |

\( a \) Parameters from quench flow data in Fig. 3.
\( b \) Parameters from fluorescence data in Fig. 3.
\( c \) Parameters from quench flow data in Fig. 3.
\( d \) Parameters from quench flow data in Fig. 3.

**Actin-translocating Activity of the Mutant Myosins**—The motor activity of the various myosin mutants was directly determined by measuring the actin-translocating velocity using an in vitro motility assay. Both S181T and S181A mutants have actin-translocating activity indistinguishable from that of WT (Fig. 4). K185Q has no actin-translocating activity. This was consistent with the observation that 1) K185Q did not hydroly-
lyze ATP, and 2) K185Q did not dissociate from actin upon addition of ATP. N235I, which has normal actin-activated ATPase activity, translocated actin filaments in the in vitro motility assay well, although the velocity was slightly lower than that of WT. The mutation of Ser236 to Thr decreased the actin-translocating velocity to approximately 5–10% of the rate in WT, although there was no significant actin-activated ATPase activity. On the other hand, S236A translocated actin filaments at half the rate of WT, which is consistent with the fact that this mutant has good actin-activated ATPase activity. The mutation of Arg238 yielded completely opposite results for two different substituted residues. R238I, in which the positive charge of Arg was abolished, did not support actin sliding at all. On the other hand R238K, which retained the positive charge of the side chain, had normal actin-translocating activity.

**DISCUSSION**

Recent three-dimensional structural analysis of the myosin motor domain revealed that the triphosphate moiety of bound ATP lies in the pocket formed by the two loops, i.e. Gly179–Asn188 and Asn235–Gly240, composed of highly conserved amino acid residues among various myosins (Fig. 5). In the present study, five conserved residues in those loops which form hydrogen bonds with the triphosphate moiety of ATP were mutated in order to understand the importance of the interaction between each conserved residue and the triphosphate moiety of ATP, in myosin mechano-enzymatic function.

The side chain of Ser181 interacts with γ-phosphate group of ATP (12, 13). It has also been shown that this residue is photo-oxidized with the vanadate moiety of the myosin-ADP-Vi complex, which resembles the metastable reaction intermediate of myosin ATPase (41). The substitution of Ser181 for either Ala or Thr did not significantly alter the myosin motor function (Table III). This suggests that, although Ser181 is completely conserved among conventional and unconventional myosins (4), the H-bonding between the side chain of Ser181 and the γ-phosphate of ATP is not required for myosin motor function. Lys235 forms H-bonds with the β- and γ-phosphate moieties of ATP, with its ε-amino group. The substitution of Lys235 for Gln is expected to alter this interaction. The results shown in Fig. 2 and Table I indicate that K185Q could still bind to ATP but hydrolysis was abolished. This indicates that these H-bonds are necessary for normal hydrolysis. The side chain of Asn235 is H-bonded to a water molecule, which in turn forms an H-bond with the Mg2+ coordinating with the γ-phosphate moiety of ATP. This residue is completely conserved in all myosins sequenced so far. N235I eliminates this interaction but has actin-activated ATPase and actin-translocating activity that is 50–60% of that of WT (Table III). These results suggest that Asn235 is not essential for myosin ATPase and motor function.

Although K185Q and R238I showed no ATP hydrolysis activity, photoaffinity labeling of ATP to these mutants indicated that ATP binds to the active site. ATP concentration required for the labeling was similar to that of wild type, suggesting that the affinity for ATP is not markedly decreased by these mutations. However, the affinity labeling efficiency was significantly lower than that of WT. It is likely that the relative configuration between the adenine ring of ATP and the residues involved in forming an adenine binding site is slightly altered in these mutants, which would reduce both the UV cross-linking efficiency with ATP and the catalytic activity.

While the mutation of Arg238 to Ile abolished the hydrolysis of ATP, the substitution by Lys did not disrupt normal myosin functions, i.e. the actin-activated ATPase activity and ATP induced dissociation from actin and the actin-translocating activity (Table III). The side chain of Arg238 forms a salt bridge with the side chain of Glu459 in the Switch II region (13). Recently, it was shown that mutation of Glu459 to Ala abolished the actin-activated ATPase activity and eliminated the initial Pi burst (45). Together with the present results, this indicates that the salt bridge between Arg238 and Glu459 plays a critical role in myosin motor function. The side chain of Glu459 forms an ion bond with Arg238 and a water molecule in a position to attack the γ-phosphate of the ATP (13). It is plausible that the ion bond is necessary to position Glu459 and water since the abolition of the ionic interaction by substituting Arg238 with Ile but not Lys completely abolished the hydrolysis of the β-γ phosphoester bond, although the ATP molecule still binds to the active site of the myosin molecule.

The OH group of Ser236 forms a hydrogen bond with the γ-phosphate of ATP, and it was proposed that the OH of Ser236 acts as an intermediary in the proton transfer from the hydrolytic water molecule to the γ-phosphate (12). However, the ATPase activities of S236A myosin (Ca2+-ATPase, K+(EDTA)-ATPase, Mg2+-ATPase, and the actin-activated ATPase) (Table I) are all at least 50% that of WT. Furthermore, S236A showed an initial Pi burst and intrinsic tryptophan-fluorescence enhancement upon ATP binding (Table III), indicating that the ATP hydrolysis step is essentially normal, and S236A undergoes the conformation change upon ATP binding and hydrolysis which is critical for myosin motor function. Consistent with these results, S236A showed good actin-translocating activity. These results indicate that the OH of Ser236 is not required for the hydrolysis of ATP. Similar results have recently been reported for S236A in Dictyostelium myosin (42) in which the actin-activated ATPase activity was decreased 3-fold and the actin-sliding velocity 7-fold. We report here that in vertebrate smooth muscle myosin S236A has smaller changes in activity, i.e. a 1.8-fold decrease in the actin-activated ATPase activity and 1.9-fold decrease in the actin-sliding velocity.

A second mutation in which Ser236 was replaced by Thr to preserve the H-bonding properties of the side chain produced much more severe changes in the enzymatic and motor functions of myosin (Table III). Actin binding of S236T is ATP-dependent, and S236T showed an initial Pi burst, with a size within a factor of two of WT, indicating that the equilibrium constant of the ATP hydrolysis step is not significantly altered. The kinetics of the tryptophan fluorescence enhancement of S236T observed upon ATP binding are biphasic with observed rates slower and faster than those of WT. The simplest interpretation of the fluorescence data is that the fast component is
associated with binding and the slow component is associated with hydrolysis. The two rates are within a factor of two for WT and S236A but hydrolysis is slower for S236T. However, the rate of the hydrolysis for S236T is still 100 times faster than the steady state rate myosin ATP hydrolysis and 6 times greater than the steady state rate of WT actomyosin ATP hydrolysis. The somewhat slower rate of hydrolysis is therefore unlikely to limit the steady state rate or significantly change the distribution of steady state intermediates. Moreover, there is no correlation between the rate of the hydrolysis step (S236A > WT > S236T) and either the rates of actin-activated ATP hydrolysis or motility. Thus ATP binding to myosin, ATP-dependent dissociation from actin, and hydrolysis to form M-ADP-P, which are necessary for motor function are essentially normal for S236A and S236T. Although the steady state Mg\textsuperscript{2+}-ATPase activity of S236T is the same as that of WT, it is not accelerated by actin. This indicates that normal actin acceleration of product release from myosin is inhibited by \textgrec{100}-fold in S236T. Although the reason why this substitution abolished the actin induced acceleration of the product release is unclear, it is less likely that the hydrogen bonding between the OH group of the Thr and the \gamma-phosphate plays a role since S236A has only a slight reduction of the actin activation of myosin ATPase activity. It is more likely that the extra CH\textsubscript{2} group

![FIG. 5. Three dimensional structure of the \gamma-phosphate binding site of myosin-MgADPV\textsubscript{0} complex. Shown is a ribbon representation of the \gamma-phosphate binding site of myosin along with the bound ADP/V. Also shown is the interactions of the conserved amino acid residues in the loops with the triphosphate moiety of ATP. A and B, the whole myosin head domain; C and D, the expanded view of the active site of myosin. A-D show the three dimensional structure from different angles. The number of amino acid residues are based on the Dictyostelium myosin II heavy chain sequence. The number of the corresponding gizzard smooth myosin heavy chain residues is shown in parentheses. The N-terminal, central, and C-terminal segments of the heavy chain are colored in green, red, and blue, respectively. ADP, V, Mg\textsuperscript{2+}, and water are colored in magenta, gray, yellow, and red, respectively.](#)

**TABLE III**

*Motor and enzymatic properties of myosin mutants*

The ATPase activities are calculated per S1-head. Conditions are described in Table 1.

| Mutants | WT | S181A | S181T | K185Q | N235I | S236A | S236T | R238I | R238K |
|---------|----|-------|-------|-------|-------|-------|-------|-------|-------|
| Basal Mg\textsuperscript{2+} ATPase (s\textsuperscript{-1}) | 0.059 ± 0.010 | 0.028 ± 0.009 | 0.017 ± 0.007 | 0 | 0.092 ± 0.007 | 0.015 ± 0.003 | 0.048 ± 0.010 | 0 | 0.060 ± 0.008 |
| AM ATPase (s\textsuperscript{-1}) | 0.325 ± 0.069 | 0.363 ± 0.063 | 0.240 ± 0.002 | 0 | 0.300 ± 0.025 | 0.292 ± 0.050 | 0 | 0 | 0.822 ± 0.052 |
| Dissociation by ATP | + | + | + | - | + | + | + | + | + |
| P\textsubscript{i} burst (K\textsubscript{burst}/sites) (\mu M\textsuperscript{-1} s\textsuperscript{-1}) | 0.11 | ND\textsuperscript{b} | ND | ND | ND | ND | 0.19 | 0.14 | ND |
| P\textsubscript{i} burst (fraction) | 0.61 | ND | ND | ND | ND | ND | 0.69 | 0.80 | ND |
| Fluorescence enhancement\textsuperscript{c} | + | ND | ND | ND | ND | ND | + | + | ND |
| Motility activity (\mu m/s) | 0.27 ± 0.04 | 0.22 ± 0.05 | 0.28 ± 0.04 | 0 | 0.16 ± 0.02 | 0.14 ± 0.02 | 0.014 ± 0.006 | 0 | 0.32 ± 0.09 |

\textsuperscript{a} The actin-activated ATPase activity. Mg\textsuperscript{2+}-ATPase activity in the absence of actin is substracted.

\textsuperscript{b} Not determined.

\textsuperscript{c} ATP induced intrinsic tryptophan fluorescence enhancement.
prevents the release of the hydrolyzed γ-phosphate by blocking the release of phosphate from the myosin-active site. Myosin forms a metastable state after hydrolysis (myosin-ADP-P) and the kinetic studies indicate that the release of P_i is prior to the release of ADP (43). The analysis of the 3D crystal structure of myosin-ADP-vanadate suggests that the phosphate cannot leave from the myosin-active site via the same route as ATP enters. It was therefore proposed that the phosphate leaves the active site through the bottom of the active site pocket (44). It is plausible that S236T prevents the P_i release through the “back door” which is opened by actin binding, presumably due to the relative movement between the γ-phosphate and Ser236, and that the extra methyl group of Thr prevents the P_i release through the back door even after actin binds.

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