

On the tryptophan residue of smooth muscle myosin that responds to binding of nucleotide

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Initially, we asked which (of 10) smooth muscle myosin head residues responds to MgADP or MgATP binding with enhanced fluorescence emission (Trp-441 and Trp-512 were leading candidates)? To decide, we prepared sham-mutated smooth muscle heavy meromyosin (HMM), W441F HMM, and W512F HMM. On adding MgATP, emission of wild-type and W441F HMMs increased by 25–27%, but that of W512F HMM by 5%. So, in myosin, 512 is the “sensitive Trp.” Unexpectedly, properties of W512F HMM [elevated Ca²⁺-ATPase, depressed EDTA (K⁺)-ATPase, no regulation of its basal or actin-activated Mg²⁺-ATPase by phosphorylation of its “regulatory” light chain, limited actin activation, and inability to move actin filaments in a motility assay] are strikingly like those of smooth muscle myosin reacted at Cys-717 with thiol reagent. From crystallography-based [Houdusse, A., Kalabakis, V. N., Himmel, D., Szent-Györgyi, A. G. & Cohen, C. (1999) *Cell* 97, 459–470] simulations, we found that in wild-type HMM with MgADP added, Trp-512 is in a “hydrophobic pocket,” but that pocket becomes distorted in W512F HMM. We think that there is a “path of influence” from 512 to 717 to the active site. We suggest that the mutational changes at 512 are transmitted along this path to Cys-717, where they induce changes similar to those caused by reacting wild-type HMM with thiol reagent.

Discoveries that on binding MgATP (or MgADP) the intrinsic UV absorbance (1) and fluorescence intensity (2) of myosin changes have enabled the quantitative—albeit empirical—study of myosin ATPase kinetics (3, 4). Earlier studies have attempted to distinguish between various classes of Trp and to determine the nature and identity of the Trp residues, whose fluorescence is most influenced on addition of nucleotide (5, 6). However, it has not been known which tryptophan residue [there are seven for skeletal muscle (7) or 10 for smooth muscle (8) in the unitary myosin fragment, subfragment 1] responds on binding. During the last decade there have been reports (9, 10) that the ATP-sensitive tryptophan residue in rabbit skeletal myosin is Trp-512^{**}, but recently, Reshetnyak *et al.*^{††} claimed that it is Trp-441. By combining site-directed mutation of *Dictyostelium* with homology, Batra and Manstein (12) find that it is Trp-512. As our present paper was being readied for publication, Yengo *et al.*^{‡‡}, using a “one-Trp-only” strategy in mutating smooth muscle myosin, more directly confirm Trp-512. In this paper, using single Phe substitution of either Trp-441 or Trp-512, we, too, document that Trp-512 is the responsive one and are aware that, in a novel spectroscopic approach, Park and Burghardt^{§§} reached the same conclusion. Thus the controversy seems to us ended. Fortuitously, however, our “one substitution at a time” strategy has led us to find interesting additional findings. It turns out that the mutant W512F heavy meromyosin (HMM) (constructed originally to contrast with wild-type HMM) has ATPase properties strikingly like those of wild-type HMM that has been chemically reacted with a common thiol reagent. From this fact, we infer that changes at position 512 are transmitted to position 717, where they cause the same changes as are alternatively caused by reacting Cys-717 with thiol reagent. The changes at

Cys-717, caused either way, are, in turn, transmitted to the active site. We say that there is a “path of influence” (15) extending from 512, to 717, to the active site. Some details about this path are given below.

Materials and Methods

Protein Preparations. Rabbit skeletal muscle actin was purified by the method of Spudich and Watt (16). Chicken gizzard myosin light chain kinase and bovine testis calmodulin were prepared as described by Adelstein and Klee (17) and Yazawa *et al.* (18), respectively.

Construction of Recombinant Baculoviruses. cDNA constructs for wild-type and two mutant (W441F and W512F) HMM heavy chains linked to a His tag (at the N terminus) and a myc tag (at the C terminus) were prepared as described in Kojima *et al.* (19). Briefly, GMH-6, a cDNA encoding the N-terminal half (Met-1 to Glu-729) of the chicken gizzard HMM heavy chain (8), was mutagenized according to the method of Kunkel *et al.* (20) with two oligonucleotides: 5'-TTTGAACGTCTCTTCCGTTTCAT-TCTAACTCGTGTAAC-3' to replace Trp-441 with Phe and 5'-CAGCGTGAGGGCATTGAATTTAATTTTCATTGACTTTGGCC-3' to replace Trp-512 with Phe. The underlined nucleotides indicate the mutations imposed. Wild-type and the two mutagenized MGH-6 were digested with *Nco*I and *Eco*RI and then ligated into a plasmid [named pFastBacHT-C-HMM-myc (19)] containing the C-terminal (Phe-730 to Thr-1318) half of the HMM heavy chain. DH10Bac *Escherichia coli* cells were transformed by pFastBac plasmids including wild type and the two mutant HMM heavy chain coding regions according to the manufacturer's protocol (Life Technologies, Rockville, MD) to produce recombinant bacmid DNAs encoding wild type and two mutant full-length HMM heavy chains, respectively. Recombinant baculoviruses were produced by transfecting Sf9 cells with these bacmid DNAs. AcNPV/ELC/RLC viruses (for essential and regulatory light chains) were prepared as described (21).

Expression and Isolation of Recombinant HMMs. The wild-type and mutant HMMs were expressed in insect cells as described (21)

Abbreviation: HMM, heavy meromyosin.

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^{**}Because different sequences but homologous residues are considered here, we note for convenience that Trp-441, Trp-512, Cys-717, Pro-722, Asn-765, Arg-768, and Arg-777 in the chicken smooth muscle sequence (taken as reference) have residue numbers 440, 510, 707, 712, 756, 759, and 768 in chicken skeletal muscle myosin and 437, 507, 693, 698, 751, 754, and 763 in scallop myosin.

^{††}Reshetnyak, Y. K., Andreev, O. A., Borejdo, J. & Burstein, E. A. (1999) *Biophys. J.* 76, 50A.

^{‡‡}Yengo, C. M., Chrin, L., Rovner, A. S. & Berger, C. L. (2000) *Biophys. J.* 78, 243A.

^{§§}Park, S. & Burghardt, T. P. (2000) *Biophys. J.* 78, 115A.

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but with a slight modification (22). Briefly, the HMM heavy chain-containing virus and the virus containing both light chains were simultaneously infected into Sf9 cells to produce HMM proteins. After extraction from the infected cells as described (21), extracts were fractionated between 40% and 62% saturated ammonium sulfate. To reduce the ammonium sulfate and ATP concentrations, this fraction was dialyzed for 4 h against 0.2 M KCl, 2 mM MgCl₂, 20 mM Tris·HCl (pH 7.5), and 0.3 mM DTT with a medium exchange. The dialyzed fraction was incubated with 10% glucose and 20 units/ml hexokinase at 4°C for 30 min to consume residual ATP and then mixed with F-actin (0.25 mg/ml). After incubation at 4°C for 2 h, the actin-HMM complex was collected by centrifugation. Pellets were resuspended in 2 mM ATP, 0.2 M KCl, 2 mM MgCl₂, 20 mM Tris·HCl (pH 7.5), and 7 mM 2-mercaptoethanol to release HMM from F-actin. His-tagged HMMs were further purified by affinity chromatography using Ni²⁺-NTA agarose (Qiagen, Valencia, CA) as described in Kojima *et al.* (19).

Sequencing of Viral DNA. According to the manufacturer's instruction (Invitrogen), viral DNAs containing W441F and W512F HMM heavy chain coding regions were isolated from the medium, in which virus-infected Sf9 cells were cultured for 3 days. Briefly, recombinant viruses were collected by centrifugation in 10% polyethylene glycol and 0.5 M NaCl and then suspended in distilled water. Viral coat proteins were digested by incubating with 1 mg/ml protease K. After the protease was denatured by phenol and chloroform (1:1), viral DNAs were collected by centrifugation in 70% ethanol. Three PCR products (nucleotide residues 1–874, 543–1826, and 1609–2376 of the HMM heavy chain) were produced by using purified viral DNAs as a template and sequenced by using a 373 DNA sequencer and a *Taq* dye terminator cycle sequence kit (Applied Biosystems). A possible interpretation for the functional changes in the W512F mutation (see *Results*) is that an unexpected DNA misreading occurs during *in vitro* mutagenesis, but this interpretation was not supported by DNA sequencing of both recombinant viruses.

SDS/PAGE and Phosphorylation Assay. SDS/PAGE was carried out according to the method of Laemmli (23). Phosphorylation assays for the regulatory light chain were performed as described (21).

ATPase Assays. The Ca²⁺-ATPase assay of HMMs (0.1 mg/ml) was carried out in 0.45 M KCl, 10 mM CaCl₂, 20 mM Tris·HCl (pH 7.5), 0.5 mM DTT, and 1 mM ATP. The EDTA (K⁺)-ATPase assay was performed by using 0.04 or 0.15 mg/ml HMM in 10 mM EDTA, 20 mM Tris·HCl (pH 8.0), 0.5 mM DTT, and 1 mM ATP. The Mg²⁺-ATPase assay of HMMs (0.24 mg/ml) was carried out in a medium containing various KCl concentrations from 0.04 to 0.45 M, 2 mM MgCl₂, 20 mM Tris·HCl (pH 7.5), 0.5 mM DTT, and 0.5 mM ATP with 0.8 mM EGTA (for unphosphorylated HMMs) or with 4 μg/ml of chicken gizzard myosin light chain kinase, 1 μg/ml of bovine testis calmodulin, and 0.05 mM CaCl₂ (for phosphorylated HMMs). A total of 0.1 mg/ml BSA also was added to the assay medium to stabilize the enzyme activity. All of the assays were carried out at 25°C. The ATPase reaction was stopped by adding 0.3 M perchloric acid. The amount of released phosphate was determined by the malachite green method (24, 25).

The actin-activated Mg²⁺-ATPase activity was measured as a function of actin concentration (0–200 μM) in an assay medium containing 0.03–0.24 mg/ml HMM, 40 mM KCl, 2 mM MgCl₂, 20 mM Tris·HCl (pH 7.5), 0.5 mM DTT, and 1 mM ATP with 0.8 mM EGTA (for unphosphorylated HMMs) or with 4 μg/ml of chicken gizzard myosin light chain kinase, 1 μg/ml of bovine testis calmodulin, and 0.05 mM CaCl₂ (for phosphorylated HMMs).

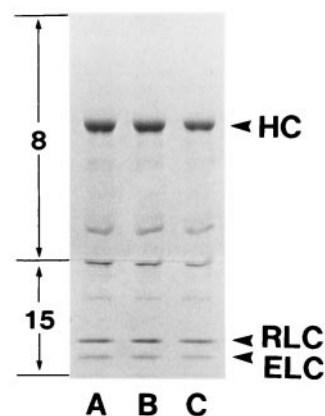


Fig. 1. SDS/PAGE gels of wild-type and mutant HMMs. Purified HMM samples (A: wild type; B: W441F; and C: W512F) were subjected to SDS/PAGE on 8% and 15% discontinuous acrylamide gels. Gels were stained with Coomassie brilliant blue. HC, HMM heavy chain; ELC, essential light chain; RLC, regulatory light chain.

Fluorescence Spectra. Tryptophan fluorescence spectra were recorded at room temperature by using a Hitachi F-4500 fluorescence spectrophotometer in a medium containing 0.12 mg/ml HMM, 0.45 M KCl, 2 mM MgCl₂, 20 mM Tris·HCl (pH 7.5), and 0.3 mM DTT. Excitation wavelength was 293 nm, and emission spectra were recorded from 315 to 375 nm. Excitation and emission slit widths were adjusted to 2.5 and 5.0 nm, respectively. A square quartz cuvette with a light path length of 5 mm was used. To secure reproducibility, the cuvette was not removed from the holder during a series of measurements.

In Vitro Motility Assay. The motility assay was performed as described (19). Briefly, c-myc-tagged HMMs were adsorbed via a mAb against c-myc (clone 9E10; Genosys, The Woodlands, TX) to a nitrocellulose-coated glass surface of a flow cell that was made by two parallel coverslips. Just before use, the regulatory light chain of HMMs was phosphorylated by myosin light chain kinase. Rhodamine phalloidine-labeled actin filaments were infused into the flow cell. Actin filament movement was initiated by perfusing a solution containing 2 mM ATP, 0.5% methylcellulose (Sigma), 25 mM KCl, 3 mM MgCl₂, 20 mM Hepes (pH 7.8), and 7 mM 2-mercaptoethanol. Fluorescent-labeled actin filaments were observed by an IX70 inverted microscope (Olympus) equipped with epifluorescence optics and rhodamine filters. Images were detected by using a C2400 SIT camera (Hamamatsu Photonics, Hamamatsu City, Japan).

Results

Preparation of Mutant HMMs. Wild-type and two mutant (W441F and W512F) HMMs were expressed in Sf9 insect cells as N-terminally His-tagged and C-terminally myc-tagged forms. The His tag allowed us to purify these HMMs by Ni²⁺-NTA resin affinity chromatography. SDS/PAGE patterns of the two affinity-purified mutant HMMs were essentially identical to that of wild-type HMM (Fig. 1). This result suggests that in the native form the two (17 and 20 kDa) light chains are correctly associated with the mutant HMM heavy chains.

Intrinsic Tryptophan Fluorescence. To determine which Trp (441 or 512) residue is responsible for the myosin fluorescence enhancement on binding ATP, we compared the fluorescence spectra of these HMMs with that of wild-type HMM. When excited at 293 nm, emission spectra of wild-type and mutant HMMs showed the highest emission at 337 nm. On addition of 100 μM ATP, the fluorescence intensity of W441F HMM increased by 27% (Fig.

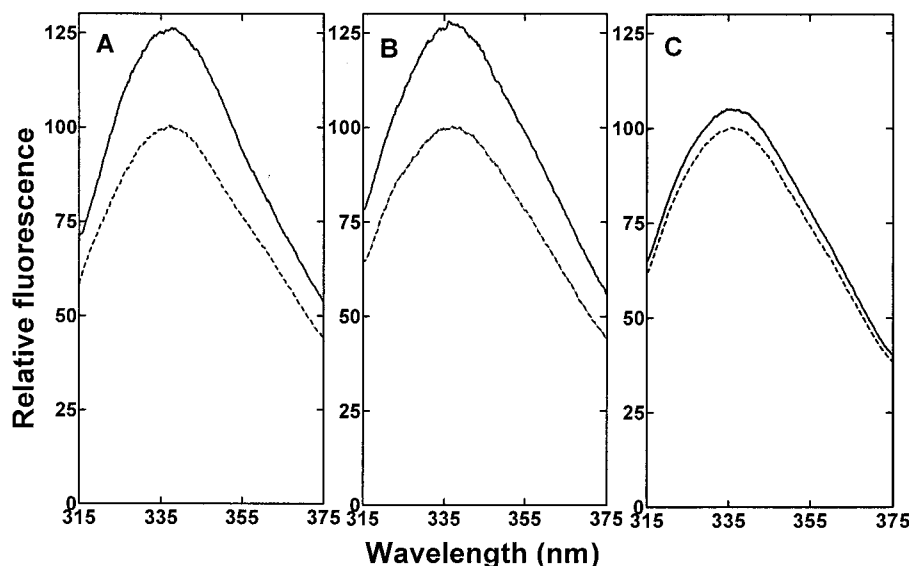


Fig. 2. Tryptophan fluorescence spectra of wild-type (A), W441F (B), and W512F (C) HMMs. Conditions were 0.12 mg/ml HMM, 0.45 M KCl, 2 mM MgCl₂, 20 mM Tris-HCl (pH 7.5), and 0.5 mM DTT at 25°C with (solid lines) or without (dotted lines) 0.1 mM ATP. Fluorescence spectra in the presence of ATP were recorded 1–3 min after the addition of ATP. Excitation wavelength was 293 nm.

2B), which was similar to the increase (25%) observed for wild-type HMM (Fig. 2A). On the other hand, the ATP-induced enhancement was only 5% for W512F HMM (Fig. 2C). As this mutant HMM hydrolyzed ATP more rapidly than wild-type HMM (see Figs. 3 and 4), it was possible that the low-level fluorescence enhancement was caused by a rapid ATP consumption. Therefore, we studied the time course of the fluorescence amplitude change after ATP addition. The enhanced level of fluorescence signal continued for 5 min after the addition of 100

μM ATP. Moreover, the signal amplitude of W512F HMM did not change by increasing the ATP concentration to 200 μM. These results exclude the possibility that the low level of fluorescence enhancement is the result of ATP consumption. We thus conclude that the ATP-induced fluorescence enhancement was greatly decreased by the W512F mutation, because the primary ATP-sensitive tryptophan residue had been replaced by the mutation.

Basal ATPase Activities. Basal Ca²⁺-ATPase and EDTA (K⁺)-ATPase activities often are used to characterize myosin. Table 1 shows that Ca²⁺-ATPase and EDTA (K⁺)-ATPase activities are similar in wild-type and W441F HMMs, whereas they are drastically changed by the W512F mutation. W512F HMM had about a 7-fold enhanced Ca²⁺-ATPase (3.9 s⁻¹) and about a

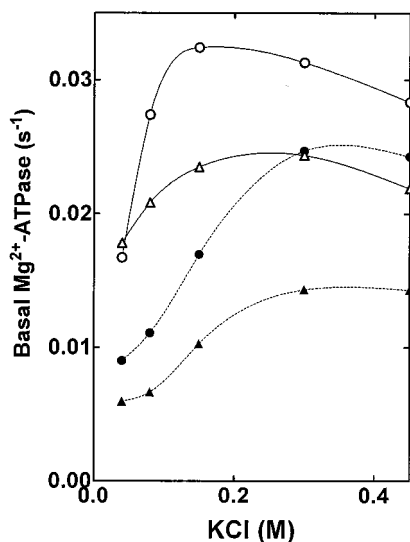


Fig. 3. Mg²⁺-ATPase activities of wild-type (○, ●) and W441F (△, ▲) HMMs as a function of KCl concentration. Assay conditions were 0.24 mg/ml HMM, 2 mM MgCl₂, 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT, and 0.5 mM ATP with 4 μg/ml myosin light chain kinase, 1 μg/ml calmodulin, and 0.05 mM CaCl₂, or with 0.8 mM EGTA. Before the assay, HMM was phosphorylated by incubating for 15 min at 25°C in a low-salt medium (40 mM KCl) with myosin light chain kinase, calmodulin, and CaCl₂ (○, △). Each assay was started by adjusting the KCl concentration to an appropriate value. For unphosphorylated HMM, EGTA was added to the medium instead of myosin light chain kinase/calmodulin/CaCl₂ (●, ▲).

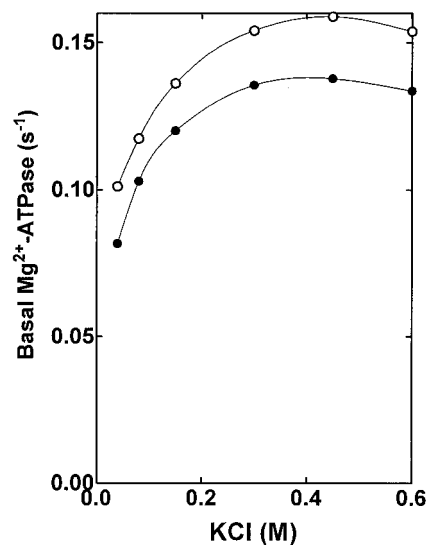


Fig. 4. Mg²⁺-ATPase activities of W512F HMM as a function of KCl concentration. Assays were the same as in Fig. 3, except that 0.06 mg/ml W512F phosphorylated (○) and unphosphorylated (●) HMMs were used.

Table 1. Basal and actin-activated ATPase activities of wild-type and two mutant HMMs

	Ca ²⁺ -ATPase*, s ⁻¹	EDTA(K ⁺)-ATPase†, s ⁻¹	Actin-activated Mg ²⁺ -ATPase‡			
			V _{max} , s ⁻¹		K _d , mM	
			P	UP	P	UP
Wild type	0.53	1.7	1.8	ND	0.1	ND
W441F	0.55	1.76	1.8	ND	0.09	ND
W512F	3.9	0.024	0.08	0.09	0.05	0.05

The ATPase activities are per HMM head.

*Assay conditions were HMM (0.1 mg/ml for wild type and W441F, and 0.02 mg/ml for W512F), 0.1 mg/ml BSA, 10 mM CaCl₂, 0.45 M KCl, and 20 mM Tris-HCl (pH 7.5).

†Assay conditions were HMM (0.04 mg/ml for wild type and W441F, and 0.15 mg/ml for W512F), 0.1 mg/ml BSA, 10 mM EDTA, 0.45 M KCl, and 20 mM Tris-HCl (pH 8.0).

‡The maximum actin-activated Mg²⁺-ATPase activity of HMM, V_{max}, and the apparent dissociation constant for actin, K_d, were calculated from the data sets in Fig. 5 for wild-type and W441F HMMs and in Fig. 6 for W512F HMM. P, Phosphorylated HMM; UP, unphosphorylated HMM; ND, not determined.

1/80-fold reduced EDTA (K⁺)-ATPase activity (0.024 s⁻¹) compared with that of wild-type HMM (0.53 s⁻¹ for Ca²⁺-ATPase and 1.7 s⁻¹ for EDTA-ATPase).

Previous ultracentrifugation studies have shown that the decreased Mg²⁺-ATPase activity of smooth muscle HMM is closely correlated with its conformational transition from the 7.5 S to 9 S state (11, 26). As shown in Fig. 3, the Mg²⁺-ATPase activity of unphosphorylated wild-type HMM was decreased by about 3-fold by lowering the KCl concentration from 0.3 to 0.04 M. Although in these experiments, we did not measure the sedimentation coefficient of our HMM samples, we believe that the observed activity decrease is caused by the HMM conformational change from the 7.5 S to the 9 S state. When the regulatory light chain of wild-type HMM was phosphorylated, the KCl concentration, which was required for the activity decrease, was shifted to less than 0.08 M. As a similar phosphorylation-dependent KCl concentration shift also was observed in the activity of W441F HMM, we believe that the conformational change described above is not affected by the W441F mutation. On the other hand, although the regulatory light chain of W512F HMM was phosphorylated (data not shown), the KCl concentration dependency of its Mg²⁺-ATPase activity was not shifted by the phosphorylation (Fig. 4). These results strongly suggest that Trp-512 is important for the phosphorylation-dependent regulation of the HMM conformation.

Actin-Activated Mg²⁺-ATPase Activities. The actin-activated Mg²⁺-ATPase activities of wild-type and the two mutant (W441F and W512F) HMMs were measured as a function of actin concentrations. The actin activation of wild-type and W441F HMMs was regulated by the phosphorylation of its regulatory light chains (Fig. 5). In the phosphorylated state, this mutant HMM showed almost the same maximum activity (V_{max}) and dissociation constant for actin (K_d) as that of wild-type HMM, namely, 1.8 s⁻¹ and 0.1 mM, respectively (Table 1). As the data for the unphosphorylated state showed a linear relation, we were unable to estimate V_{max} and K_d separately. However, the apparent second-order rate constant (V_{max}/K_d) could be estimated from their slopes. The value (0.07 s⁻¹·mM⁻¹ for wild type and 0.1 s⁻¹·mM⁻¹ for W441F) was not significantly different between wild-type and W441F HMMs. Therefore, we conclude that regardless of the phosphorylation, the actin-activated ATPase activity is not affected by the W441F mutation. On the other hand, the actin-activated ATPase activity of W512F HMM was much smaller (about 1/20-fold) than that of phosphorylated wild-type HMM. Actin activation of the mutant HMM was almost independent of the phosphorylation (Fig. 6 and Table 1). These results clearly suggest that the integrity of the structure

around Trp-512 is important also for the phosphorylation-dependent regulation of actin activation.

Sliding of Actin Filaments. The effects of the two Trp (441 and 512) mutations on motor function were examined by an *in vitro* motility assay. In the unphosphorylated state, neither wild-type nor the two mutant HMMs could move actin filaments (data not shown). After the regulatory light chain was phosphorylated, W441F HMM could move actin filaments at almost the same velocity (about 0.4 μm/s) as wild-type HMM (Table 2). In contrast, W512F HMM could not support filament movement (Table 2).

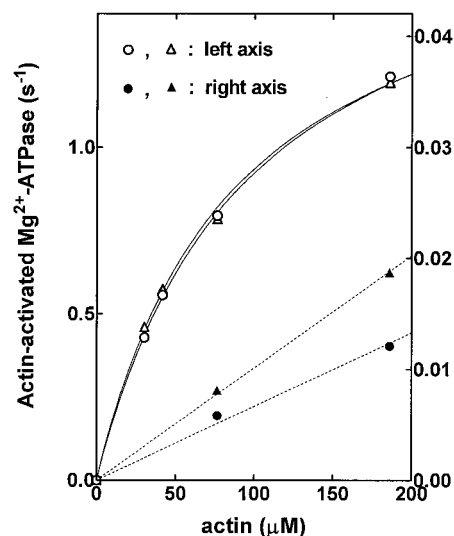


Fig. 5. Actin-activated Mg²⁺-ATPase activities of wild-type (○, ●) and W441F (△, ▲) HMMs as a function of actin concentration. Assay were carried out by incubating HMM at 25°C in the medium containing various concentrations (from 0 to 200 μM) of actin, 40 mM KCl, 2 mM MgCl₂, 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT, and 1 mM ATP. A total of 4 μg/ml myosin light chain kinase, 1 μg/ml calmodulin, and 0.05 mM CaCl₂ also were added to the assay medium to phosphorylate the regulatory light chain (○, △). For unphosphorylated HMMs, EGTA was added to the final concentration of 0.8 mM instead of myosin light chain kinase/calmodulin/CaCl₂ (●, ▲). The concentration of HMMs was 0.03–0.06 mg/ml in the former case and 0.24 mg/ml in the latter case. The ATPase activities of HMM alone and that of actin alone were subtracted from each measured value to estimate actin-activated ATPase activity. Straight (broken) lines were fitted to the initial slopes to define V_{max}/K_m for unphosphorylated HMMs. For phosphorylated HMMs, curves were fitted to the data sets by using the equation $V = V_{max}/(1 + K_d/[actin])$.

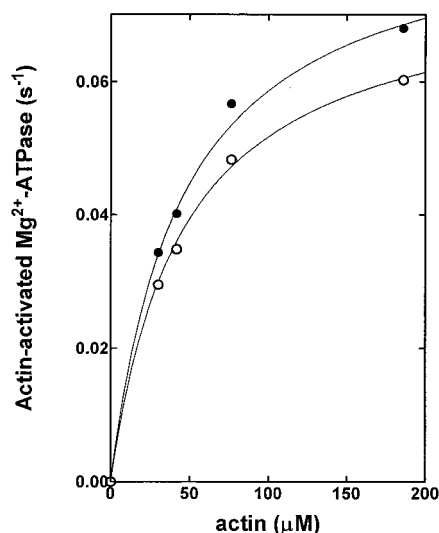


Fig. 6. Actin-activated Mg^{2+} -ATPase activities of W512F HMM as a function of actin concentration. Conditions were the same as in Fig. 5, except that W512F HMM was used. Concentrations of the phosphorylated (○) and unphosphorylated (●) forms of the mutant HMM were 0.14–0.24 mg/ml. For both phosphorylated and unphosphorylated HMMs, curves were fitted to the data sets by using the equation $V = V_{max}/(1 + K_d/[actin])$.

Discussion

Our present study finds that the ATP-responsive tryptophan in smooth muscle myosin is Trp-512, not Trp-441, as recently claimed by Reshetnyak *et al.*^{††}. Thus we support early suggestions by Johnson *et al.* (9), and by Hiratsuka (10) and the more definitive recent work, namely, the mutational work of Batra and Manstein (12), using the homology of Dictyostelium, and that of Yengo *et al.*^{‡‡}, applying a “one Trp only” strategy to smooth muscle myosin, and the recent spectral analysis of Park and Burghardt (14). Besides confirming recent work, application of a one substitution at a time strategy leads us to the finding, namely, that the enzymatic properties of W512F HMM are strikingly like those of smooth muscle myosin (26) whose “SH1s” (Cys-717s) have been reacted with thiol reagent. The mutant has a decreased EDTA (K^+)-ATPase activity and an increased Ca^{2+} -ATPase activity (Table 1). It has an increased basal Mg^{2+} -ATPase activity (Fig. 4) and a decreased actin-activated activity (Fig. 6). Neither of the latter activities becomes regulated by phosphorylation of the “regulatory” light chain of the mutant (Figs. 4 and 6). Also, this mutant cannot move actin filaments (Table 2). The last observation is very like our previous observation that SH1 modification of wild-type HMM did not support actin filament movement (19).

To facilitate our discussion, we adopt the terminology of Botts *et al.* (15) and describe, in noncommitting fashion, related events occurring at different times and different points in space. We say that an “influence” capable of causing circumstance C_a at a

travels along a path from a to b , and at b , it generates a new circumstance, C_b . Taking positive direction as “out-bound” from the active site to Cys-717, and continuing to Trp-512, and taking “in-bound” as the reverse direction, we say that some out-bound influence travels along a path from the active site to 717, because it is known (13) that binding of MgADP to the active site unwinds the helix bearing Cys-717. A path of influence is similarly indicated because MgADP binding also affects the chemical reactivity of Cys-717 (11, 26). In the reverse direction, reacting Cys-717 with a thiol reagent facilitates the “leaving” of bound MgADP (11, 26). Also in the same direction, an NMR signal (the resonance of the ^{31}P of the β phosphate of bound MgADP) is changed on reacting Cys-717 with a thiol reagent (14). In this perspective, in the presence of MgADP, we begin investigating an in-bound path of influence between position 512, position 717, and the active site.

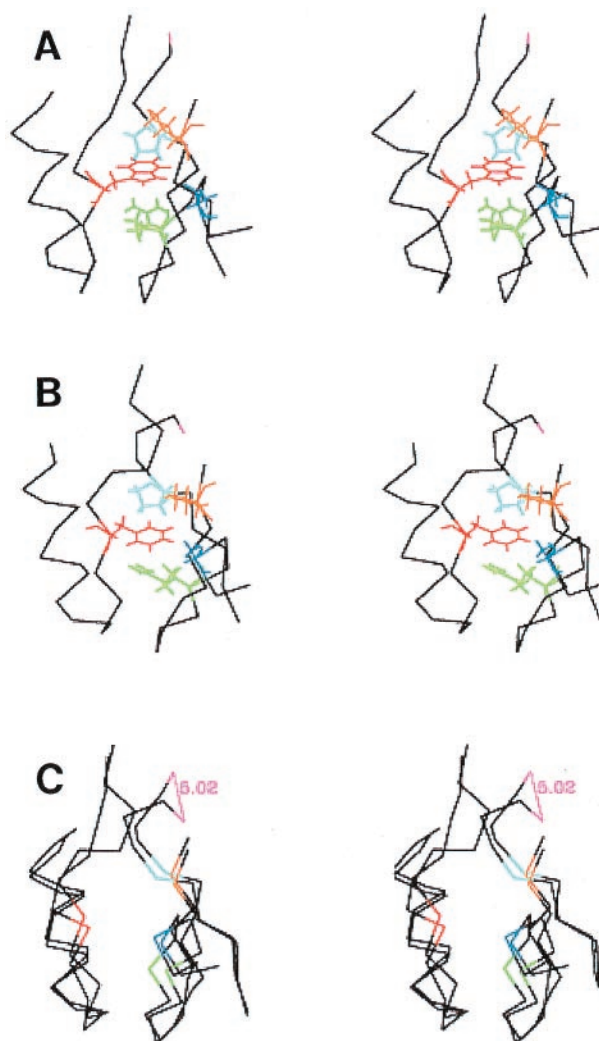


Fig. 7. Energy minimized neighborhood of the “responsive” tryptophan residue (Trp-512) and the “reactive” cysteine residue (Cys-717) of myosin in the presence of MgADP. This depiction is abstracted from Houdusse *et al.* (28), pdb1B7T, but known homology has been used to report sequence numeration as if it were that of the smooth muscle myosin used in this work. Trp-512 (red), Pro-722 (cyan), Asn-765 (blue), Arg-768 (orange), and Arg-777 (green) are identified by colors; only the α -carbon atoms of other residues are shown. (A) Wild type. (B) W512F mutant. (C) Regional structures of A and B arranged so that the foregoing five residues coincide. The rms separation distance for corresponding atoms is 1.42 Å. Note that the α -carbon of Arg-718 moves by 5 Å as a result of the mutation. The segment colored in pink shows this trajectory (beginning and end of the movement of the α -carbon).

Table 2. Actin sliding velocities of wild-type and two mutant HMMs measured by *in vitro* motility assays

	Velocity*, $\mu m/s$
Wild type	0.45 ± 0.05
W441F	0.41 ± 0.06
W512F	Not move

Motility assays were carried out at 30°C under the conditions described in *Materials and Methods*.

*The mean \pm the SD of velocities of moving actin filaments. $n = 50$.

Crystallography (13) reveals that if MgADP is introduced many significant structural changes occur, e.g., unwinding of the helix that bears Cys-717. We find in particular that Trp-512 becomes enclosed in a “hydrophobic pocket” formed by Pro-722 and Asn-765, and by the hydrophobic surfaces of Arg-768 and Arg-777. It appears that Trp-512 in this pocket emits at an intensity that is “over background.” As may be anticipated—and is actually found on modeling—the W512F mutation entails distortion of the pocket as the indole of Trp-512 is replaced by the phenyl ring of Phe-512. To model this distortion and estimate how it may propagate so as to involve Cys-717, we isolated a region (from ref. 28, pdb1B7T) consisting of three residue segments: 497–518, 718–727, and 768–777. This region contains Trp-512, the hydrophobic pocket, and enough residues to sense any effects of distorting the pocket. It does not contain Cys-717 (no coordinates for this residue are available), but does contain Arg-718, whose behavior suggests something about the behavior of Cys-717. This region (capped to neutralize the amino and carboxyl termini) was subjected to refinement by energy minimization and became the model of the wild-type structure. It is shown (Fig. 7A) in stereo fashion, featuring only Trp-512 and the residues of the hydrophobic pocket; only the α -carbons of other residues are shown. Fig. 7B is our model resulting from substituting Phe-512 and again energy-minimizing the structure of the

region. Fig. 7C compares the regional structures in Fig. 7A and B. For both wild-type and mutant HMMs, Fig. 7 depicts the different positions of all of the α -carbon atoms of the region when just the α -carbons of the 512 occupant and the pocket (10 atoms altogether) have been made to coincide. The comparison gives a rms separation between corresponding atoms of 1.42 Å. Of particular importance is that Arg-718 moves by 5 Å as a result of the model mutation. Alternatively expressed, a considerable force on Cys-717 may be applied if it maintains its position after mutation. It remains for future research to examine in more detail whether application of such a force on Cys-717 has the same effect as reaction with certain reagents. For now this seems plausible, so tentatively, we say that there may be in-bound path of influence connecting 512 with 717, then leading by a common path to the active site.

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