*Proc. Natl. Acad. Sci. USA*
Vol. 92, pp. 11965–11969, December 1995
Biochemistry

# The putative actin-binding role of hydrophobic residues Trp$^{546}$ and Phe$^{547}$ in chicken gizzard heavy meromyosin

HIROFUMI ONISHI*†, MANUEL F. MORALES‡, KAZUO KATOH*, AND KEIGI FUJIWARA*

*Department of Structural Analysis, National Cardiovascular Center Research Institute, Fujishiro-dai, Suita, Osaka 565, Japan; and ‡University of the Pacific, San Francisco, CA 94115

*Contributed by Manuel F. Morales, September 1, 1995*

**ABSTRACT** **In the course of myosin-catalyzed ATP hydrolysis, certain amino acid residues in myosin interact with counterparts in actin to produce the relational changes that underlie muscle contraction; some of these interactions are ionic, but the stronger interactions are hydrophobic. In an effort to identify myosin residues participating in hydrophobic interactions, myosin (from smooth muscle) fragments with mutations at suspected sites were engineered and compared with wild-type fragments. It was found that the ATPase of doubly mutated (Trp546Ser and Phe547His) fragments was minimally activated by actin and did not decorate actin well to form the regular arrowhead pattern characteristic of myosin binding to actin filaments. Thus, we suggest that Trp$^{546}$ and Phe$^{547}$ are important participants in the hydrophobic actin–myosin interaction.**

It is now generally believed that the changing relationship between actin and myosin during the course of myosin-catalyzed ATP hydrolysis underlies the overall contractile process, and so we are attempting to identify the residues in myosin that interact with the residues in actin. These interactions appear to be primarily ionic or hydrophobic. If the former are defined as ionic strength-dependent, then it appears that in the absence of nucleotide (the so-called "rigor" condition), the forces that stabilize the actomyosin complex are overwhelmingly hydrophobic (1), whereas in the presence of nucleotide the forces seem to be ionic (2). How this interplay of forces enters into the contractile process has been ably discussed by Zhao and Kawai (3) and by Geeves and Conibear (4). At the protein level, identification of residues participating in ionic interactions has been easier because of the availability of bifunctional amino–carboxyl group crosslinkers (5, 6). Identification of "hydrophobic" sites has been circumstantial. For example, it has been observed that a nonpolar triplet of myosin residues (residues 541–543 in the skeletal myosin sequence) was adjacent to Cys$^{540}$, which was thought to move upon ATP binding (7), and might for this reason be a participant in the hydrophobic interaction (8). More solid progress began with the availability of crystallographic structures (9, 10), which suggested that in complexation certain nonpolar residues in one protein might come to lie near nonpolar residues in the other (11). Such inspection indicated to Rayment *et al.* (11) that residues 541–543, 535, 529, and 530 might be "hydrophobic" sites of myosin (Fig. 1). To test the significance of these sites by independent means, we examined the complexation behavior of myosins whose presumably critical sites have been mutated. For this purpose, we have employed an expression system recently devised by Trybus (12) and also by Onishi *et al.* (13). This system expresses a competent (14, 15) heavy meromyosin (HMM) fragment of myosin. The myosin fragment expressed is that from chicken smooth muscle (gizzard) myosin, whose sequence is slightly different from chicken skeletal myosin (for which crystallography has been done), but the functional homologies between them are clear (16). Met$^{541}$-Phe$^{542}$-Pro$^{543}$ of the skeletal muscle sequence corresponds to Trp$^{546}$-Phe$^{547}$-Pro$^{548}$ of the smooth muscle sequence.§ Initially we have limited ourselves to double mutants at residues 546 and 547 because mutation of Pro may more strongly distort local structure.

## MATERIALS AND METHODS

**Protein Preparations.** Chicken gizzard myosin light chain kinase and bovine testis calmodulin were purified as described by Adelstein and Klee (18) and Yazawa *et al.* (19), respectively. Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (20).

**Expression of Mutant HMM.** A transfer vector (pAcC/GHMW546SF547H) containing the full-length coding region of the mutant HMM heavy chain was prepared (13) with slight modification. Briefly, the GMH-6 cDNA clone, containing the 5′-terminal half of the HMM heavy chain coding region (17), was mutagenized with an oligonucleotide (5′-GCTGGATG-AAGAGTGCTCGCACCCCAAAGCTACTGACAC-3′; the underlined bases indicate the mutations imposed) to replace Trp$^{546}$ with Ser and Phe$^{547}$ with His. The mutagenized GMH-6 was digested with *Nco* I and *Eco*RI to obtain a 2.2-kb fragment. To obtain the vector containing the full-length coding region, the 2.2-kb fragment was then ligated into the *Nco* I/*Eco*RI site of pAcC4 containing the 3′-terminal half of the HMM heavy chain coding region. *Spodoptera frugiperda* cells (Sf9) were transfected with a mixture of linearized wild-type *Autographa Californica* nuclear polyhedrosis virus DNA and the transfer vector pAcC/GHMW546SF547H by the technique of cationic liposome-mediated transfection (Invitrogen). Recombinant viruses were obtained as described by Summers and Smith (21). Expression and purification of wild-type and mutant (Trp546Ser and Phe547His) HMMs were carried out as described by Onishi *et al.* (13).

**Gel Electrophoresis, Immunoblots, and Autoradiography.** SDS/PAGE was carried out as described by Laemmli (22). For immunoblots, peptide bands were electrophoretically transferred to a Durapore membrane (Millipore) (23). Polyclonal antibodies against the regulatory or the essential light chain of chicken gizzard myosin were used as probes and the reacting bands were detected by peroxidase-conjugated goat anti-rabbit IgG (Cooper Biomedical). Myosin light chain phosphorylation was performed at 25°C for 30 min by adding 1.8 μg of either wild-type HMM or the mutant to an assay medium (50 μl) containing 40 mM KCl, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.5), 0.3 mM dithiothreitol, and 50 μM [$\gamma$-$^{32}$P]ATP (0.9 TBq/mmol) with 0.6 mM EGTA or with 8 μg of chicken gizzard myosin light chain kinase per ml, 2 μg of bovine testis calmodulin per ml, and 0.05 mM $CaCl_2$. Assay samples con-

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Abbreviation: HMM, heavy meromyosin.

†To whom reprint requests should be addressed.

§The heavy chain numbering system is based on the deduced amino acid sequence from Yanagisawa *et al.* (17).

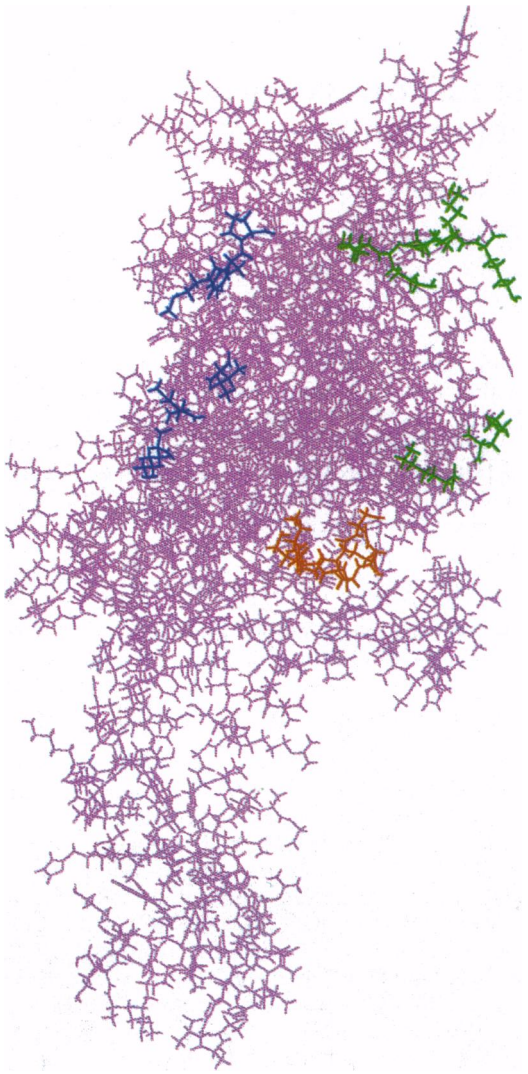

FIG. 1. Residues in the chicken skeletal myosin that may have interaction with actin. The hydrophobic residues are labeled in blue and are, from top to bottom, residues 541–543 (546–548), 535 (540), and 529–530 (534–535). The numbers in parentheses correspond to amino acid positions in the chicken smooth myosin sequence. The positively charged Lys residues in the two loops are labeled in green and are, from top to bottom, residues 626–647 (628–657) and 571–575 (575–579). This figure also shows the ATP-binding consensus sequence GXXXXGKT of residues 179–186 (177–184) in brown. Backbone atoms, side chains, and loops were reconstituted from $\alpha$-carbon coordinates kindly supplied by I. Rayment (University of Wisconsin). Reconstructions (courtesy of H. M. Martinez, University of California San Francisco) employed the DISCOVER module of Biosym software and were implemented on an Indigo "Extreme" Silicon Graphics computer. There are several "low energy" conformations of the loop containing residues 626–647, because it contains many Gly residues. These conformations are energetically equivalent and so the loop jumps around under thermal agitation.

taining equal amounts of HMM (0.6 $\mu$g) were subjected to SDS/PAGE and [$^{32}$P]phosphate incorporation was detected autoradiographically by exposing the gel to Kodak XAR-5 x-ray film (13).

**ATPase Assays.** The high salt HMM ATPase activity was measured at 25°C by adding 16 $\mu$g of either wild-type or mutant HMM to an assay medium (80 $\mu$l) consisting of 0.4 M KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 20 mM Tris·HCl (pH 7.5), and 1 mM ATP. The low salt HMM ATPase activity was also measured at 25°C by adding either 1.6–3.2 $\mu$g of wild-type HMM or 4 $\mu$g of the mutant to an assay medium (80 $\mu$l) consisting of 40 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 20 mM Tris·HCl (pH 7.5), and 1 mM ATP with 0.6 mM EGTA or with 8 $\mu$g of chicken gizzard myosin light chain kinase per ml, 2 $\mu$g of bovine testis calmodulin per ml, and 0.05 mM $CaCl_2$. Actin-activated ATPase activity was measured by adding various concentrations of rabbit skeletal muscle actin to the assay medium. Reactions were stopped by adding aliquots (20 $\mu$l) to 180 $\mu$l of a stop solution consisting of 0.2 M perchloric acid. Amount of inorganic phosphate was measured colorimetrically (24). Three time points were taken at 15- to 35-min intervals to determine whether the release of inorganic phosphate was linear with time.

**Negative Staining.** Rabbit skeletal muscle actin filaments (2 $\mu$g/ml) were decorated by adding 16 $\mu$g of either wild-type or the mutant HMM per ml. The decorated actin filaments were negatively stained with 4% (wt/vol) uranyl acetate in distilled water and observed with a JOEL 2000FX electron microscope operated at 80 kV.

## RESULTS

**Isolation of Mutant HMM.** Coinfection of Sf9 cells with two recombinant viruses, encoding the mutant gizzard HMM heavy chain (Trp546Ser and Phe547His) and the regulatory and the essential light chains, resulted in the expression of a mutant protein. We purified the mutant HMM by the same protocol used for isolating the wild-type HMM (13). A total of about 32 $\mu$g of protein was obtained from 0.4 g (wet weight) of virus-infected cells. This amount of isolatable mutant HMM was only about one-sixth of the amount of the wild-type HMM isolated by the same procedure. The remaining mutant HMM heavy chain either precipitated with cell debris (Fig. 2*A*) or remained in the supernatant after centrifugation of the actin–HMM complex in the absence of ATP (Fig. 2*B*). Analysis of the purified mutant protein by SDS/PAGE (Fig. 2*C*) revealed the presence of the three expected peptides, the 140-kDa HMM heavy chain, the 17-kDa essential light chain, and the 20-kDa regulatory light chain. Both the wild-type and the mutant HMM heavy chains had stoichiometric amounts of the regulatory and the essential light chains (Fig. 2*C*). The presence of the two types of light chains in the mutant HMM was also confirmed by immunoblot analysis with polyclonal antibodies against each of them (Fig. 2 *D* and *E*).

**Light Chain Phosphorylation.** For smooth muscle myosin ATPase, light chain phosphorylation is important. We there-

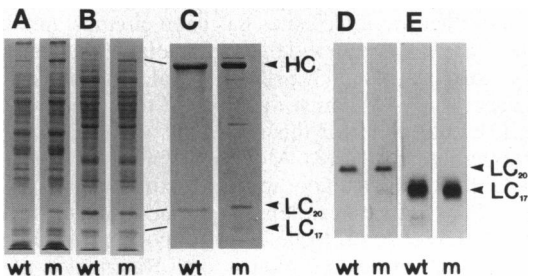


FIG. 2. SDS/PAGE gels and immunoblots of wild-type (wt) and mutant (m) HMMs. Samples subjected to SDS/PAGE on 13% gels were the pellet of the cell lysate after centrifugation at 15,000 × *g* (*A*), the material that did not coprecipitate with filamentous actin after centrifugation of the actin–HMM complex at 100,000 × *g* (*B*), and pooled HMM-containing fractions after Mono Q column chromatography (*C*). Due to higher amounts of the starting material, the mutant HMM fractions contain some contaminating proteins. Gels are stained with Coomassie brilliant blue. For immunoblots, HMM samples were subjected to SDS/PAGE on 15% gels. Peptide bands were electroblotted to Durapore membranes and then probed with polyclonal antibodies directed against chicken gizzard regulatory (*D*) or essential (*E*) light chains, respectively. Note that both wild-type and mutant HMMs contain the two types of light chains. HC, HMM heavy chain; $LC_{20}$, regulatory light chain; $LC_{17}$, essential light chain.

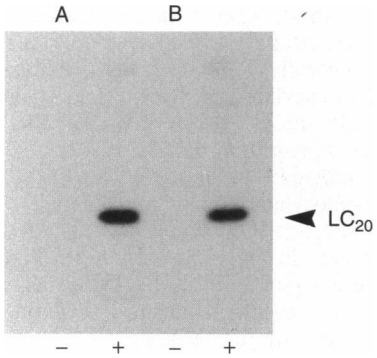


FIG. 3. Autoradiograms showing regulatory light chain phosphorylation by myosin light chain kinase. Wild-type (*A*) and mutant (*B*) HMMs were phosphorylated with [$\gamma$-$^{32}$P]ATP in the absence (−) or presence (+) of myosin light chain kinase, calmodulin, and $Ca^{2+}$ for 30 min. Samples were analyzed by SDS/PAGE on 15% gels and then subjected to autoradiography. The regulatory light chain ($LC_{20}$) of both wild-type and mutant proteins is phosphorylated in a kinase-dependent manner.

fore tested whether the mutant HMM light chain can be phosphorylated. Fig. 3 *A* and *B*, respectively, shows autoradiograms of SDS/polyacrylamide gels of purified wild-type and mutant HMMs that were either untreated or treated with myosin light chain kinase, calmodulin, and $Ca^{2+}$. In both wild-type and mutant HMMs, kinase addition converted the regulatory light chain ($LC_{20}$) to the phosphorylated form.

**Enzymatic Properties.** ATPase activities of unphosphorylated wild-type and unphosphorylated mutant HMMs were measured in a solution of 0.4 M KCl, 5 mM $MgCl_2$, and 20 mM Tris·HCl (pH 7.5). In such a high salt medium, the conformation of smooth muscle HMM is in the 7.5S state and independent of both ATP and light chain phosphorylation (25, 26). The activity of unphosphorylated mutant HMM (4.4 nmol of $P_i$ per min per mg of HMM) was similar to that of unphosphorylated wild-type HMM (3.7 nmol of $P_i$ per min per mg of HMM) (Table 1). This result suggests that the mutation scarcely affects the intrinsic ATPase activity of HMM.

In a low salt medium (40 mM KCl/2 mM $MgCl_2$/20 mM Tris·HCl, pH 7.5), the ATPase activity of phosphorylated wild-type HMM (3.2 nmol of $P_i$ per min per mg of HMM) was ≈2-fold higher than that of unphosphorylated wild-type HMM (1.3 nmol of $P_i$ per min per mg of HMM) (Table 1). This level of increase in activity is comparable to that obtained with HMM prepared from chicken gizzard myosin by proteolytic digestion (27). Previous studies indicated that the 2-fold

Table 1. ATPase activities of wild-type and mutant HMMs

| HMM | MgATPase, nmol of $P_i$ per min per mg of protein | | Actin-activated MgATPase | |
|---|---|---|---|---|
| | High salt | Low salt | $V_{max}$, nmol of $P_i$ per min per mg | $K_a$, $\mu M^{-1}$ |
| Wild-type | | | | |
| − kinase | 3.7 | 1.3 | 76 | 22 |
| + kinase | — | 3.2 | 637 | 104 |
| Mutant | | | | |
| − kinase | 4.4 | 2.7 | 49 | 27 |
| + kinase | — | 5.8 | 65 | 19 |

$V_{max}$ is the maximum actin-activated ATPase activity of HMM and $K_a$ is the apparent binding constant for HMM to actin, which is defined to be the reciprocal of the apparent $K_m$ from the double reciprocal plots (Fig. 4). To phosphorylate the regulatory light chain of HMM, myosin light chain kinase, calmodulin, and $Ca^{2+}$ were added to the ATPase assay medium. —, Not measured.

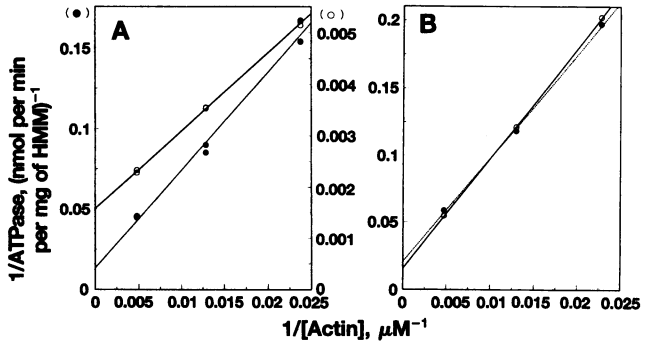


FIG. 4. Double reciprocal plots of MgATPase activities of wild-type (*A*) and mutant (*B*) HMMs vs. actin concentration. Solid circles, unphosphorylated HMM; open circles, myosin light chain kinase, calmodulin, and $Ca^{2+}$ were added to the assay medium to phosphorylate the regulatory light chain of HMM. The ATPase activity of each HMM alone (see Table 1) was subtracted from each measured value to estimate actin-activated ATPase activity.

increase in activity is associated with a conformational transition of HMM from the 9S to the 7.5S state, because the 7.5S state has higher specific ATPase activity and also because it is the preferred form of phosphorylated HMM (25, 26). Although the activities of the phosphorylated (5.8 nmol of $P_i$ per min per mg of HMM) and unphosphorylated (2.7 nmol of $P_i$ per min per mg of HMM) mutant HMMs were slightly higher than those of the wild type, a 2-fold increase in activity after

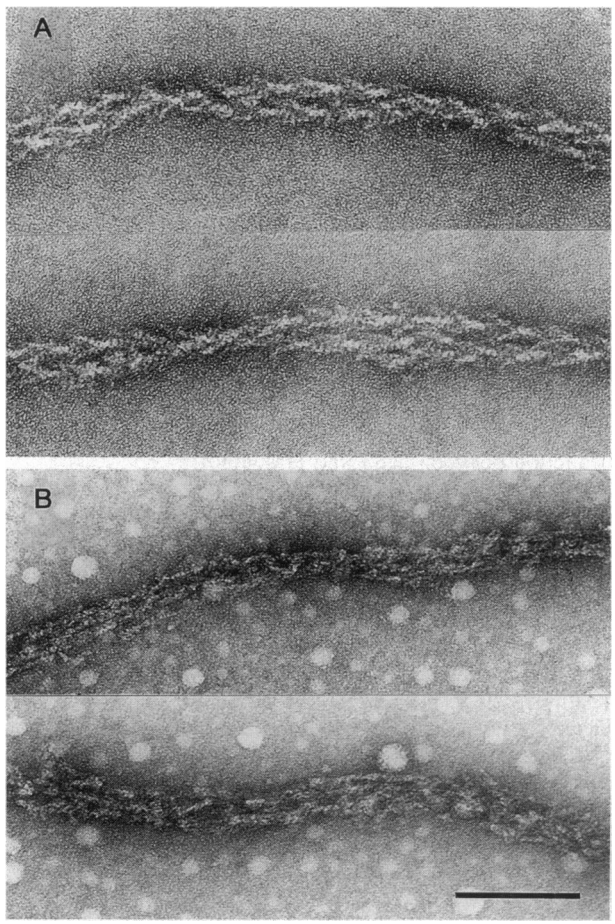


FIG. 5. Electron micrographs of actin filaments decorated by wild-type (*A*) or mutant (*B*) HMM. Rigor complexes were formed by mixing HMM and actin at a molar ratio of 1:1. Actin filaments in *A* show a typical arrowhead structure but those in *B* do not. (Bar = 100 nm.)

light chain phosphorylation was also observed with this mutant (Table 1). The result suggests that mutant HMM can change its conformation similarly to wild-type HMM.

Since the two residues we mutagenized are in a putative actin-associating segment of the HMM heavy chain, the mutations may have effects on the actin-activated ATPase activity. To test whether this is indeed the case, we measured the dependence of ATPase activity on actin concentration for both the wild-type and the mutant HMMs. The double reciprocal plots of these data gave straight lines, but their intercepts with the *y* axis and slopes were very different (Fig. 4). The maximum actin-activated ATPase activities ($V_{max}$) for the phosphorylated wild-type and mutant HMMs were 637 and 65 nmol of $P_i$ per min per mg of HMM, respectively, and the apparent binding constants for these HMMs to actin ($K_a$) were 104 and 19 $\mu M^{-1}$, respectively (Table 1). These data indicate that the mutations have caused a 10-fold decrease in $V_{max}$ and a 4-fold decrease in $K_a$. As a result of both changes in $V_{max}$ and $K_a$, the mutant activity at a given concentration of actin was much less than the wild-type activity.

Neither $V_{max}$ nor $K_a$ of the mutant was strongly dependent on light chain phosphorylation. We have also expressed a triple mutant of HMM, with the following replacements: $Trp^{546}$ to Ser, $Phe^{547}$ to His, and $Pro^{548}$ to Gly. The actin-activated ATPase activity of the phosphorylated triple mutant was affected to the same extent as that of the double mutant (data not shown), indicating that the third mutation (Pro to Gly) did not make much additional difference.

**Actin Filaments Decorated with the Mutant HMM.** As shown in Fig. 5*A*, wild-type HMM formed an arrowhead structure along actin filaments, when the HMM was mixed with actin in a molar ratio of 1:1 in the absence of ATP. Actin filaments decorated with the mutant HMM appeared thicker than undecorated actin filaments and the decorated filaments also tended to associate side-by-side (Fig. 5*B*). These results clearly indicate that the mutant HMM interacts in some way with actin filaments. However, the mutant HMM-decorated actin filaments did not show a typical arrowhead structure (Fig. 5*B*). Several explanations are possible. For example, decoration of actin filaments with the mutant heads may not be complete. It is also possible that the bound heads of the mutant protein make diverse angles with the axis of actin filaments. Another possibility is that the weaker mutant HMM binding to actin is insufficient to withstand the negative staining procedure. Any combination of these possibilities is also plausible. Although we are unable to say for sure which interpretation is correct, our result indicates that the mutations affect the interaction between actin and HMM.

## DISCUSSION

In mutating proteins one must consider that tertiary structure may be severely altered by the imposed mutations. In the present study, only amino acid residues known to be at the HMM surface were mutated by site-directed mutagenesis. Therefore, it is likely that mutant HMM has the same tertiary structure as the wild-type HMM. There are three additional pieces of evidence that wild-type structure is preserved in our purified mutant HMM. (*i*) In our purification protocol, the protein was precipitated with rabbit skeletal muscle actin, thus allowing only functional HMM to be purified. (*ii*) Analysis by SDS/PAGE revealed that each mutant HMM heavy chain has stoichiometric amounts of two types of light chains (Fig. 2), suggesting that in solution these polypeptide chains form a 1:1:1 complex. (*iii*) The mutant HMM had an intrinsic ATPase activity that was similar to the level obtained for the wild-type HMM (Table 1).

In spite of having an intrinsic activity similar to that of wild-type HMM, phosphorylated mutant HMM showed a dramatically different actin-activated ATPase activity from the wild-type counterpart. The mutations resulted in a 10-fold decrease in $V_{max}$ and a 4-fold decrease in $K_a$ for actin (Fig. 4 and Table 1). Since the regulatory light chain of the mutant HMM was phosphorylated by myosin light chain kinase (Fig. 3), defective light chain is not responsible for the decreased actin activation of the mutant HMM. $V_{max}$ and $K_a$ values of the mutant HMM showed little increase when its regulatory light chain was phosphorylated (Fig. 4 and Table 1). These results suggest that intact $Trp^{546}$ or/and $Phe^{547}$ residues are required for a large increase in the actin-activated ATPase activity of myosin. The yield of purified mutant HMM was insufficient for conventionally measuring its affinity to actin. Instead, we tested whether the mutant HMM forms arrowhead structure with actin filaments. Some sort of binding of mutant HMM to actin filaments was suggested by electron microscopy but the mutant HMM-decorated filaments did not show the typical arrowhead structure observed with wild-type HMM (13). These results suggest that HMM binds to actin filaments weakly and/or abnormally; for example, it could be that the smooth muscle homologs of the other sites (residues 529, 530, and 535; see Fig. 1) continue to function normally but that impairment of the homologs at residues 541 and 542 weakens the interaction and destroys its stereospecificity. A simple interpretation for all of these observations is that actin activation of the phosphorylated mutant HMM decreases, because the actin-binding affinity decreases, and that this is caused by the disabled (or weakened) hydrophobic interactions. Our results demonstrate the importance of hydrophobic triplet for the normal interaction between actin and HMM. Another interesting implication of this work is that the rate-limiting step in actin-activated myosin ATPase seems to depend on the integrity of the hydrophobic interaction between the two proteins.

We are grateful to Prof. Ivan Rayment for the $\alpha$-carbon structure of myosin subfragment 1 and to Prof. Hugo M. Martinez for the filling-in of the backbone and side chains. We also thank Mrs. Tokuko Asada for her technical assistance. This work was supported by Research Grants for Cardiovascular Diseases from the Ministry of Health and Welfare of Japan, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and by Special Coordination Funds for Promoting Science and Technology (Encouragement System of Center of Excellence) from the Science and Technology Agency of Japan.

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