SH1 (Cysteine 717) of Smooth Muscle Myosin: Its Role in Motor Function†

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ABSTRACT: To determine if a thiol group called SH1 has an important role in myosin's motor function, we made a mutant heavy meromyosin (HMM) without the thiol group and analyzed its properties. In chicken gizzard myosin, SH1 is located on the cysteine residue at position 717. By using genetic engineering techniques, this cysteine was substituted with threonine in chicken gizzard HMM, and that mutant HMM and unmutated HMM were expressed in biochemical quantities using a baculovirus system. The basal EDTA−, Ca^{2+}−, and Mg^{2+}−ATPase activities of the mutant were similar to those of HMM whose SH1 was modified by *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS). However, while the chemically modified HMM lost the function of the light chain phosphorylation-dependent regulation of the actin-activated ATPase activity, the mutant HMM exhibited the normal light chain-regulated actin-activated ATPase activity. Using an in vitro motility assay system, we found that the IAEDANS-modified HMM was unable to propel actin filaments but that the mutant HMM was able to move actin filaments in a manner indistinguishable from filament sliding generated by unmutated HMM. These results indicate that SH1 itself is not essential for the motor function of myosin and suggest that various effects observed with HMM modified by thiol reagents such as IAEDANS are caused by the bulkiness of the attached probes, which interferes with the swinging motion generated during ATP hydrolysis.

Cys707 (the so-called SH1) of the vertebrate skeletal muscle myosin heavy chain is known to be selectively labeled with various thiol reactive reagents (*1*−*3*). The SH1 modification causes a decrease in the EDTA−ATPase activity and an increase in the Ca^{2+}−ATPase activity of myosin. The crystal structure of rabbit skeletal muscle myosin S1[1] (*4*) revealed that the region containing this Cys residue consisted of two short α-helices linked with a turn (hereafter, we call this region the thiol region). Several lines of evidence suggest that upon ATP hydrolysis the thiol region changes its conformation, producing the power stroke (*5*−*11*).

SH1 is also conserved at Cys717 in vertebrate smooth muscle myosin (*12*, *13*). In the previous study, we modified chicken gizzard full-length myosin with *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS) under various conditions (*14*). Although there are 15 cysteine residues in the chicken gizzard myosin heavy chain, substantial modification occurs only at SH1 in the absence of ATP at low ionic strengths, which was determined by the partial amino acid sequence of the labeled myosin (*12*). In contrast, IAEDANS labeling of SH1 was nearly completely inhibited in the presence of ATP (*14*). By comparing the effects of modifications under different conditions, we could demonstrate that myosin biochemistry was strongly affected by SH1 modification and exclude the possibility that such effects were not derived from labeling of other sites. After SH1 modification, smooth muscle myosin exhibited changes in the EDTA− and Ca^{2+}−ATPase activities similar to those observed with skeletal muscle myosin (*14*, *15*). An actin-activated ATPase activity of vertebrate smooth muscle myosin is regulated by phosphorylation of its regulatory light chain by myosin light chain kinase (*16*−*18*). When SH1 of smooth muscle myosin was modified with IAEDANS, the actin-activated ATPase activity of phosphorylated myosin decreased, whereas that of dephosphorylated myosin increased (*14*). As a result, the phosphorylation-dependent regulation nearly disappeared. These observations were also reported for the modification with *N*-ethylmaleimide (*19*). A possible idea has been proposed that in smooth muscle myosin SH1 modification mimics light chain phosphorylation (*20*). Such an idea is consistent with the fact that myosin filaments are stabilized in the presence of ATP either by light chain phosphorylation or by SH1 modification (*14*, *19*, *21*). If this idea is correct, SH1-modified smooth muscle myosin is expected to have an ability to translocate actin filaments. However, recent in vitro studies demonstrated that in skeletal muscle myosin, SH1 modification with thiol reagents eliminated myosin's ability to support actin filament sliding (*22*−*24*). To examine whether SH1-modified smooth muscle myosin retains the motor activity, we made chicken gizzard heavy meromyosin (HMM) by using a baculovirus expression system and carried out in vitro motility assays with or without SH1 modification.

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[1] Abbreviations: HMM, heavy meromyosin; S1, subfragment 1; RLC, myosin regulatory light chain; IAEDANS, *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; CBB, Coomassie Brilliant Blue.

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A disadvantage of thiol modification experiments is the difficulty in distinguishing if the observed effects are due to blocking of the thiol group itself or to the secondary conformational distortion caused by a bulky labeling group. Indeed, some effects of thiol modification on skeletal muscle myosin were reported to depend on the size of the attached probes (*25*, *26*), suggesting that some changes introduced by SH1 modification are caused by the secondary perturbation, not by the elimination of the thiol group. To address this problem more closely, we have expressed a mutant smooth muscle HMM, Cys717 (SH1) of which is replaced with Thr. With such a mutation, the problem of the probe's bulkiness can be avoided because the side chains of Thr and Cys are roughly the same size. We found that, in contrast to the chemical modification, the C717T mutation has no significant effects on either the phosphorylation-dependent regulation of actin activation or the ability to move actin filaments. These results indicate that the intact thiol group is not necessary for the myosin motor function. Our interpretation of the results of the chemical modification is that a secondary conformational distortion by the labeling probe causes steric hindrance around the thiol region and interferes with the progression of the actin-activated ATPase pathway.

MATERIALS AND METHODS

Protein Preparations. Myosin light chain kinase was purified from chicken gizzards by the method of Adelstein and Klee (*27*). Calmodulin was prepared from bovine testes as described previously (*28*). Actin was purified from rabbit skeletal muscle according to the method of Spudich and Watt (*29*).

Construction of Recombinant Baculoviruses. All molecular biological procedures were essentially like those of Sambrook et al. (*30*). Transfer vectors for chicken gizzard HMM heavy chains linked to His and myc tags were newly constructed in this study.

A myc tag was attached to the C-terminus of the HMM heavy chain as follows. A modified GMH-5 cDNA fragment, encoding the C-terminal half (Phe730−Thr1318) of the HMM heavy chain (*13*, *31*), was subcloned in pBluescriptII SK(−) and named pBS-C-HMM. This plasmid was mutagenized by the method of Kunkel (*32*) to create a *Kpn*I site with a primer, 5′-AGAGGATCCTTAGGTACCA-CATCTTTAGTT-3′. A DNA fragment containing a coding sequence for the myc tag was amplified by a polymerase chain reaction using a plasmid, pKT10-mycC (kindly supplied by T. Yokoo), as a template. The amplified fragment was inserted into the *Kpn*I−*Bam*HI site of pBS-C-HMM. An *Eco*RI−*Xba*I fragment containing the coding region is recovered from the resultant plasmid and subcloned in pFastBacHTa (Life Technologies) to obtain pFastBacHT-C-HMM-myc.

A modified GMH-6, a cDNA encoding the N-terminal half of the HMM heavy chain, was subcloned in pBluescriptII SK(−) and named pBS-N. To substitute Cys717 with Thr, pBS-N was mutagenized with a primer, 5′-GGAAGGTAT-TCGTATCACCCGGCAAGGGTTC-3′, by Kunkel's method (*32*). An *Nco*I−*Eco*RI fragment containing the 5′-coding region of unmutagenized or mutagenized pBS-N was inserted into pFastBacHT-C-HMM-myc to obtain a transfer vector encoding the full-length sequence of the wild-type (WT) or C717T mutant HMM heavy chain. The recombinant HMM heavy chains had the myc-tag sequence V-P-R-D-L-S-S-A-E-Q-K-L-I-S-E-E-D-L, instead of Ala1317 and Thr1318, at its C-terminal end and the His-tag sequence M-S-Y-Y-H-H-H-H-H-D-Y-D-I-P-T-T-E-N-L-Y-F-Q-G-A at its N-terminal end. The coding sequence of the His tag was obtained from pFastBacHTa.

Recombinant viruses (AcNPV/HC[His-WT-myc] and AcNPV/HC[His-C717T-myc]) for expressing the tagged HMM heavy chains were produced via the manufacturer's instructions (Life Technologies). Two other viruses, AcNPV/ELC/RLC (for essential and regulatory light chains) and AcNPV/HC[WT] (for the wild-type HMM heavy chain without tags), were prepared as described previously (*31*).

Purification of Expressed Heavy Meromyosin. Sf9 cells were coinfected with AcNPV/ELC/RLC and either AcNPV/HC[His-WT-myc] or AcNPV/HC[His-C717T-myc] (for each virus, the virus-to-cell ratio was 6). The expressed HMMs (His-WT-myc and His-C717T-myc) were mixed with F-actin, and the actin−HMM precipitates were collected as described previously (*31*). By suspension and homogenization in 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 100 μM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, and 1 mM ATP, HMM was dissociated from F-actin. After centrifugation at 100000*g* for 90 min, the supernatant was incubated on a roller shaker at 4 °C for 2 h with Ni−NTA resin (Qiagen) which was placed into a column. The column was washed with C-buffer [20 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM 2-mercaptoethanol, and 1 μg/mL leupeptin] and then C-buffer containing 20 mM imidazole. HMM was eluted with C-buffer containing 100 mM imidazole. The eluted sample was concentrated by using a Centriprep-30 concentrator (Amicon) and dialyzed once against 20 mM Tris-HCl (pH 7.5), 40 mM KCl, 2 mM $MgCl_2$, 0.3 mM dithiothreitol, 20 μM phenylmethanesulfonyl fluoride, and 0.6 μg/mL leupeptin. Finally, purified HMM was dialyzed against a suitable buffered solution. All the steps were carried out below 4 °C. Protein concentrations were determined with a Coomassie Protein Assay Reagent (Pierce).

Wild-type HMM without tags (WT) was expressed by coinfection of Sf9 cells with AcNPV/HC(WT) and AcNPV/ELC/RLC (for each virus, the virus-to-cell ratio was 6). WT HMM was purified on a mono-Q column (Pharmacia) as described previously (*31*).

SDS−PAGE, Western Blot, and Phosphorylation Assay. SDS−PAGE was carried out according to the method of Laemmli (*33*) usually with a 13% polyacrylamide gel. A discontinuous gel, in which the polyacrylamide concentrations were 15 and 9% for the lower two-thirds and the upper one-third of the gel, respectively, was used to show the polypeptide composition of each HMM preparation (Figure 1A). In this experiment, 5 μg of HMM was loaded per lane. For Western blot analyses (Figure 1B), SDS−PAGE (0.5 μg of HMM per lane) was performed with an 8 (for heavy chain) or 15% (for essential and regulatory light chains) polyacrylamide gel. These peptides were electrophoretically transferred to Immobilon transfer membranes (Millipore) by the method of Towbin et al. (*34*). As primary antibodies, anti-chicken gizzard essential light chain [rabbit polyclonal (*31*)], anti-chicken gizzard regulatory light chain [rabbit

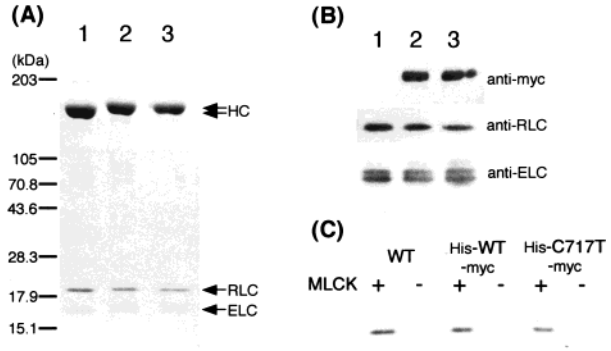

FIGURE 1: Polypeptide analyses of purified HMMs. (A) CBB staining of an SDS−PAGE gel showing the presence of the heavy chain (HC), the essential light chain (ELC), and the regulatory light chain (RLC). (B) Western blot analyses with anti-c-myc monoclonal (clone 9E10), anti-chicken gizzard ELC polyclonal, and anti-chicken gizzard RLC polyclonal antibodies. (C) An RLC phosphorylation assay. HMM was incubated with [γ-^{33}P]ATP in the presence (+) or absence (−) of MLCK. The samples were subjected to SDS−PAGE, and the gels were autoradiographed.

polyclonal (*31*)], and anti-c-myc (mouse monoclonal from clone 9E10; Genosys Biotechnologies) were used. Biotinylated secondary antibodies were purchased from Zymed Laboratories. Antibody−horseradish peroxidase complexes were made by using a VECTASTATIN ABC-HRP kit (Vector Laboratories). Konica Immunostain HRP-1000 (Konica) was used as the horseradish peroxidase substrate. An RLC phosphorylation assay (Figure 1C) was performed as described previously (*35*) except for using 50 μM [γ-^{33}P]-ATP (18.5 TBq/mmol). Incorporated phosphate was autoradiographically detected using XAR5 X-ray film (Kodak).

ATPase Assay. An actin-activated ATPase activity was assayed in 20 mM Tris-HCl (pH 7.5), 40 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM $CaCl_2$, 4 μg/mL chicken gizzard MLCK, 1 μg/mL bovine testis calmodulin, and 1 mM ATP for the activity of phosphorylated HMM. For the analysis of the actin-activated ATPase activity of unphosphorylated HMM, EGTA was added to a final concentration of 2 mM, instead of $CaCl_2$/MLCK/calmodulin. The HMM concentration was 50 μg/mL. The actin concentration was varied from 1.0 to 7.0 mg/mL.

The basal Mg^{2+}−ATPase activity was measured in 20 mM Tris-HCl (pH 7.5), 0.04 or 0.45 M KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM ATP containing 180−300 μg/mL HMM. The basal Ca^{2+}−ATPase activity assay was performed in 20 mM Tris-HCl (pH 7.5), 0.6 M KCl, 10 mM $CaCl_2$, 1 mM dithiothreitol, and 1 mM ATP containing 25 μg/mL HMM. The basal EDTA−ATPase activity measurement was carried out in 20 mM Tris-HCl (pH 8.0), 0.6 M KCl, 10 mM EDTA, 1 mM dithiothreitol, and 1 mM ATP with 25 μg/mL HMM.

Each reaction was started by adding ATP and stopped by adding $HClO_4$ at a final concentration of 0.18 M. All assays were performed at 25 °C. The amount of released phosphate was determined by the method of Kodama et al. (*36*).

IAEDANS Labeling. N-Iodoacetyl-*N*′-(5-sulfo-1-naphthyl)-ethylenediamine (IAEDANS) was purchased from Molecular Probes (Eugene, OR). Labeling of HMM was carried out at 20 °C for 1 h in 20 mM Tris-HCl (pH 7.5), 40 mM KCl, 2 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.8 mM IAEDANS in the presence or absence of 1.5 mM ATP. The concentration of HMM was 0.4 mg/mL. The reaction was stopped by adding 2-mercaptoethanol at a final concentration of 30 mM.

In Vitro Motility Assay. F-Actin was labeled by tetramethylrhodamine as described previously (*37*). Immediately prior to use, HMM preparations were centrifuged in the presence of F-actin and ATP at 100000*g* for 30 min to eliminate HMM heads that irreversibly bound to F-actin in the presence of ATP. The standard assay buffer consisted of 25 mM KCl, 3 mM $MgCl_2$, and 20 mM HEPES (pH 7.8). All procedures except for the motility assay were carried out at room temperature.

Anti-c-myc monoclonal antibodies (clone 9E10; Genosys Biotechnologies) at a concentration of 0.3 mg/mL in 150 mM NaCl and 10 mM sodium phosphate (pH 7.6) were adsorbed for 1 h to a nitrocellulose-coated glass surface of a flow cell, followed by blocking for 15 min with 25 mg/mL bovine serum albumin. HMM at a concentration of 0.35 mg/mL was infused into the flow cell and allowed to bind to the antibody-coated glass surface for 10 min. Bound HMM was phosphorylated by incubating for 20 min with 12 μg/mL chicken gizzard myosin light chain kinase and 3 μg/mL bovine testis calmodulin in the presence of 1 mM ATP and 0.15 mM $CaCl_2$. Then a solution of 1 μg/mL rhodamine-phalloidine-labeled F-actin in 100 mM KCl and 10 mM HEPES (pH 7.8) was infused into the flow cell. The movement of fluorescently labeled actin filaments was initiated by exchanging the solution with the standard assay buffer containing 2 mM ATP. This assay buffer also contained 0.5% methylcellulose (Sigma, 400 cP in a 2% solution) as a viscosity-enhancing reagent, and 4.5 mg/mL glucose, 216 μg/mL glucose oxidase, and 36 μg/mL catalase as an oxygen scavenger system.

The movement of fluorescently labeled actin filaments was observed with an Olympus IX70 inverted microscope equipped with epifluorescence optics and a rhodamine filter set (excitation at 546 nm and the emission cutoff at 580 nm). The objective lens was an Olympus UPlanApo oil immersion 100× (NA of 1.35). The flow cell was kept at 30 °C by using a heat stage. Fluorescent images were recorded via a Hamamatsu photonics SIT camera (C2400) by a Sony Hi8 videorecorder (CVD-1000). The video images were converted to a TIFF by an Apple Power Macintosh 9500/150 computer via a Scion LG-3/PCI video capture board. The velocity of each actin filament was analyzed with NIH Image software.

Trypsin Digestion. IAEDANS-labeled HMM (0.35 mg/mL) was digested with 20 μg/mL trypsin at 20 °C. Digestion was carried out either for 12.5 min in the absence of ATP or for 30 min in the presence of ATP and stopped by the addition of a trypsin inhibitor at a final concentration of 40 μg/mL. After trichloroacetic acid precipitation, samples were subjected to SDS−PAGE. Gels were fixed with 50% (v/v) methanol, and IAEDANS-labeled bands were visualized under UV light (365 nm). After photographs had been taken, gels were stained with Coomassie Brilliant Blue (CBB).

RESULTS

Preparation of Tagged HMM. Both the wild-type and the C717T mutant HMMs were expressed as N-terminally His-tagged and C-terminally myc-tagged forms. The His tag allowed us to use Ni^{2+} resin affinity column chromatography

Table 1: EDTA− and Ca^{2+}−ATPase Activities (s^{-1}) of His-WT-myc and His-C717T-myc HMMs

	His-WT-myc	His-C717T-myc
EDTA−ATPase	4.27	1.74
Ca^{2+}−ATPase	1.09	2.48

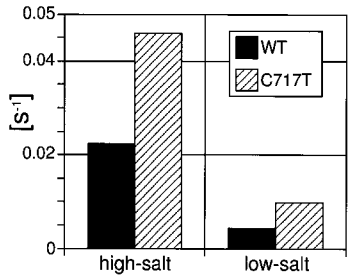

FIGURE 2: Basal Mg^{2+}−ATPase activities under high-salt (0.45 M KCl) or low-salt (0.04 M KCl) conditions: (black) His-WT-myc HMM and (hatched) His-C717T-myc HMM.

for purification procedures. SDS−PAGE patterns of affinity-purified tagged HMMs were essentially identical to that of the nontagged HMM purified by mono-Q column chromatography (Figure 1A). This result suggests that the two tagged HMMs have the heavy chain and the regulatory and essential light chains at a molar ratio of approximately 1:1:1. The presence of the two light chains was also confirmed by Western blotting analyses (Figure 1B). The regulatory light chains of His-WT-myc and His-C717T-myc HMMs, as well as nontagged HMM, were phosphorylated by myosin light chain kinase (Figure 1C).

Basal and Actin-Activated ATPase Activities. Both basal and actin-activated ATPase activities of HMM were not affected by the addition of both His and myc tags (data not shown). Therefore, His-WT-myc HMM was used as control wild-type HMM in the following enzymatic and motility assays.

The C717T mutation produced some effects on the basal EDTA−, Ca^{2+}−, and Mg^{2+}−ATPase activities. As shown in Table 1, His-C717T-myc HMM had $^1/_{2.5}$-fold reduced EDTA−ATPase and 2.3-fold enhanced Ca^{2+}−ATPase activities compared with those of His-WT-myc HMM. Under both low-salt and high-salt conditions, the Mg^{2+}−ATPase activity of C717T mutant HMM was 2-fold higher than that for His-WT-myc HMM (Figure 2). These changes in activities agreed with the previous reports that selective modifications of SH1 on various types of myosin increased Ca^{2+}− and Mg^{2+}−ATPase activities and decreased an EDTA−ATPase activity (*2*, *14*, *38*). All of the available data, including our results presented here, suggest that the changes in the basal ATPase activities are primarily due to some perturbation around SH1 either by the chemical modification or by the mutation.

Then we measured the ATPase activities of His-WT-myc and His-C717T-myc HMMs in the presence of 2 mg/mL F-actin. In both unphosphorylated and phosphorylated states of HMM, the C717T mutation had no significant effects on the ATPase activities (the first and third panels in Figure 3A). To compare their kinetic parameters more closely, we measured ATPase activities of the phosphorylated His-WT-myc and His-C717T-myc HMMs at various F-actin concentrations. The double-reciprocal plots of these data are shown in Figure 4. The maximum activities (V_{max}) were 3.7 and

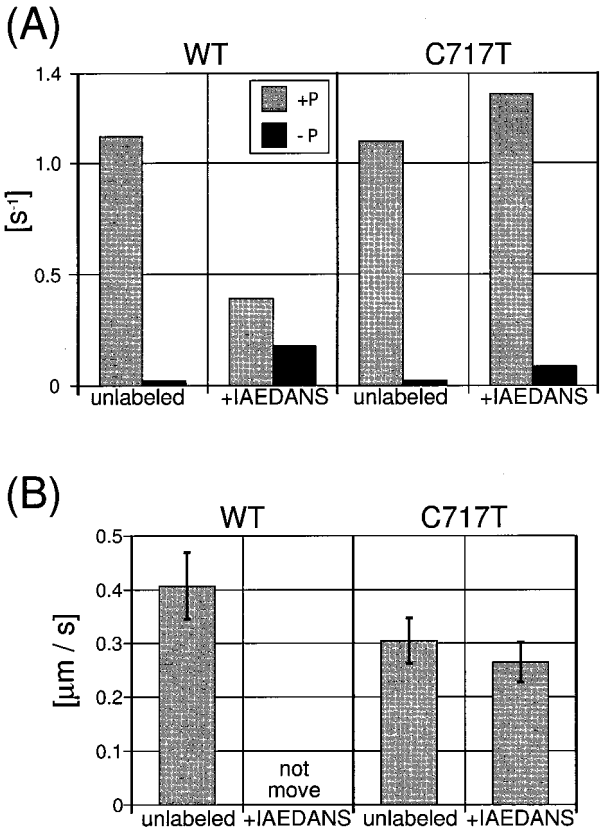

FIGURE 3: Effects of SH1 substitution and modification on acto−myosin interactions. (A) Actin-activated ATPase activities (s^{-1}) of IAEDANS-labeled and unlabeled HMMs in the presence of 2.0 mg/mL F-actin: (gray) RLC-phosphorylated HMM and (black) RLC-unphosphorylated HMM. (B) F-Actin sliding velocities (micrometers per second) measured by in vitro motility assays.

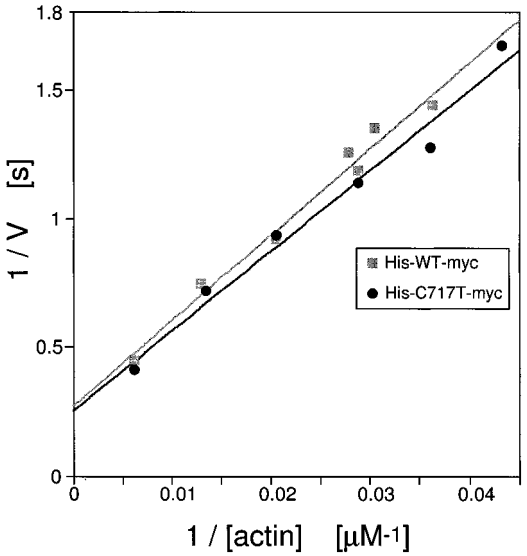

FIGURE 4: Actin-activated ATPase activities of RLC-phosphorylated His-WT-myc and His-C717T-myc HMM. A double-reciprocal plot of Mg^{2+}−ATPase activity vs actin concentration is shown: (gray squares) His-WT-myc and (black circles) His-C717T-myc.

3.5 s^{-1} for the wild-type and mutant HMMs, respectively, and the apparent affinity constants for actin (K_a) were 120 μM for both HMMs. No significant difference in these kinetic parameters suggests that the change in the local environment by the C717T mutation is not critical for the ATPase reaction in the presence of F-actin.

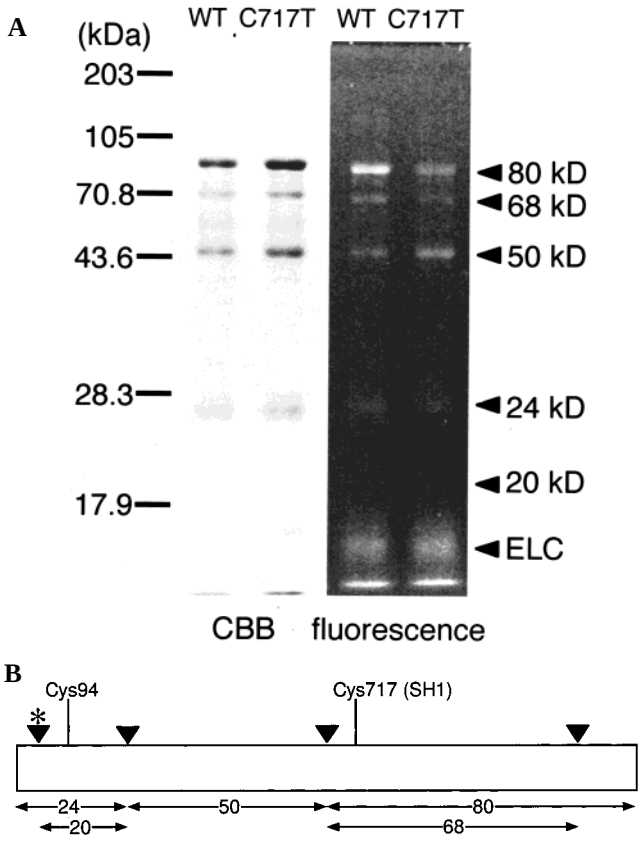

FIGURE 5: SH1 modification of His-WT-myc and His-C717T-myc with IAEDANS. (A) SDS−PAGE. HMMs were labeled with IAEDANS in the absence of ATP. After trypsin digestion, the samples (5 μg per lane) were loaded: (left) CBB staining and (right) fluorescence viewing of the same gel. (B) Schematic drawing of trypsin digestion sites (▼). The site near the N-terminus (asterisk) was digested only in the presence of ATP.

We also measured the actin-activated ATPase activities of His-WT-myc and His-C717T-myc HMMs which were reacted with IAEDANS in the absence of ATP at low ionic strengths. Under this condition, SH1 of the full-length myosin could be efficiently labeled with IAEDANS (*14*). To confirm SH1 labeling of the expressed HMMs, IAEDANS-treated His-WT-myc and His-C717T-myc HMMs were digested with trypsin, subjected to SDS−PAGE, and compared. The CBB staining patterns exhibited major bands at 24, 50, and 80 kDa, and a minor one at 68 kDa (the left panel of Figure 5A). On the basis of our previous proteolysis studies with smooth muscle myosin (*39*), we deduced digestion sites of the expressed HMM as shown in Figure 5B. The 80 kDa band which includes SH1 was strongly labeled with IAEDANS in His-WT-myc HMM (the right panel in Figure 5A). As expected, the IAEDANS fluorescence of the 80 and 68 kDa bands was reduced in His-C717T-myc HMM, corresponding to the loss of the thiol group of Cys717. The 24 kDa, 50 kDa, and essential light chain bands were weakly labeled with IAEDANS. However, fluorescence intensities of these bands were not significantly different between His-WT-myc and His-C717T-myc HMMs.

Actin-activated ATPase activities of wild-type and C717T mutant HMMs after labeling with IAEDANS were measured in the presence of 2 mg/mL F-actin (the second and fourth panels in Figure 3A). The effects of modification of Cys residues other than SH1 were studied by analyzing the ATPase activities of His-C717T-myc HMM before and after IAEDANS treatment. Although the actin-activated ATPase activity in the unphosphorylated state was increased by the modification, the activity was kept high in the phosphorylated state, indicating that the regulation by light chain phosphorylation remained essentially intact. When His-WT-myc HMM was labeled with IAEDANS, additional modification occurred at Cys717, causing a $^1/_3$-fold decrease in the activity in the phosphorylated state and an 8-fold increase in the unphosphorylated one. As a result, the phosphorylation-dependent regulation was lost. On the basis of the fact that the 80 and 68 kDa bands were the only bands in which IAEDANS labeling was different between His-WT-myc and His-C717T-myc HMMs, we conclude that the loss of regulation is due to labeling of Cys717, that is, SH1. Our results indicate that the mutation and the chemical modification of SH1 produced clearly different effects on the ATPase cycle in the presence of F-actin.

In Vitro Motility Assay of SH1-Modified and C717T Mutant HMMs. The effects of the SH1 modification and the C717T mutation on motor function were examined by using an in vitro motility assay. Each HMM was tethered onto the glass surface via anti c-myc monoclonal antibodies to improve the quality of the assay. In the unphosphorylated state, neither wild-type nor mutant HMM could slide actin filaments (data not shown). After phosphorylation of the regulatory light chain, both His-WT-myc and His-C717T-myc HMMs supported smooth movement of actin filaments at roughly the same velocity (the first and the third lanes in Figure 3B). In contrast to the unlabeled one, His-WT-myc HMM after labeling with IAEDANS did not support actin filament movement (the second lane in Figure 3B). Since labeled His-C717T-myc HMM did support sliding movements (the fourth lane in Figure 4B), it is clear that the disruption of the motor function observed in IAEDANS-labeled His-WT-myc is actually due to modification of Cys717 but not to other Cys residues. When a mixture of unlabeled and labeled HMMs at a molar ratio of 1:4 was used, actin filaments slid at almost the same velocity as unlabeled HMM alone, although actin sliding in the mixture stagnated occasionally (data not shown). Thus, the binding of labeled HMM to actin is likely to be too weak to slow actin sliding that is driven by the unlabeled HMM.

In Vitro Motility Assay of HMM Labeled at Cys94 with IAEDANS. When chicken gizzard myosin was treated with IAEDANS in the presence of ATP, the N-terminal 20 kDa segment was labeled to a great extent (*14*). We identified Cys94 as the modified residue on this fragment by amino acid sequencing (*40*). This thiol group is located near the thiol region and exposed to the protein surface in an ATP-dependent manner (*14*). Despite these suggestive data, the role of Cys94 in the myosin motor function has not been investigated. Thus, we treated His-C717T-myc HMM with IAEDANS in the presence of ATP. The mutant HMM was chosen because the effect of labeling of SH1, which occurred to a slight extent in the wild-type HMM even in the presence of ATP, could be excluded. The SDS−PAGE pattern of the trypsin digests (Figure 6A) exhibited a 24 kDa band with strong fluorescence, suggesting that Cys94 is efficiently labeled with IAEDANS. An in vitro motility assay indicated that this labeled His-C717T-myc HMM could support

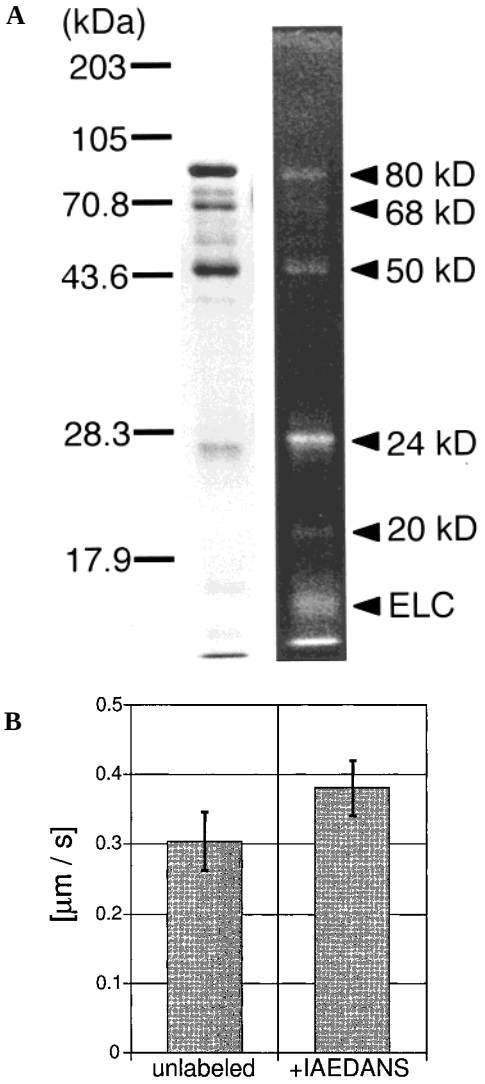

FIGURE 6: Effect of Cys94 modification with IAEDANS. (A) SDS−PAGE of IAEDANS-labeled His-C717T-myc HMM. HMM was labeled with IAEDANS in the presence of ATP. After trypsin digestion, the sample (5 μg per lane) was loaded: (left) CBB staining and (right) the fluorescence pattern of the same gel. (B) Actin sliding velocities measured by in vitro motility assays.

movement of actin filaments roughly at the same velocity as that for the unlabeled one (Figure 6B). The actin-activated ATPase activity did not significantly change with this chemical modification in either the phosphorylated or unphosphorylated state (data not shown). These results suggest that Cys94 has no significant effect on motor function.

DISCUSSION

Seidel first reported that the modification of thiol groups in smooth muscle myosin enhanced the actin-activated ATPase activity in a manner similar to that caused by light chain phosphorylation (*20*). Thus, he has proposed that thiol modification mimics light chain phosphorylation. Later, it was shown that, under physiological conditions where unphosphorylated myosin filaments were readily disassembled by adding ATP, formation of the ATP-resistant myosin filaments was also commonly observed after light chain phosphorylation and SH1 modification (*14*, *19*, *21*). These results are consistent with Seidel's proposal. However, the study presented here shows that light chain phosphorylation and SH1 modification are rather different with respect to their effects on motor function. SH1-modified HMM did not support sliding movement of actin filaments. This property of SH1-modified HMM is similar to that of unphosphorylated HMM rather than the phosphorylated form (*41*). Thus, our result does not support the simple view proposed by Seidel (*20*).

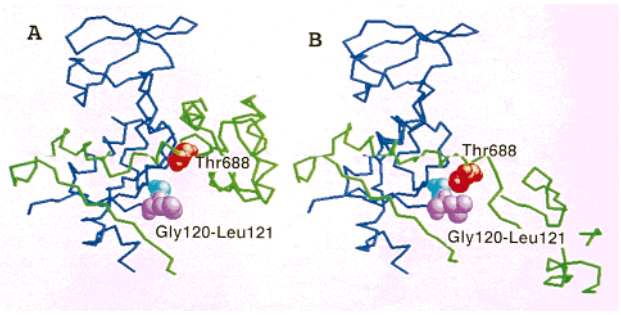

FIGURE 7: Crystal structures of the *Dictyostelium* myosin head. (A) The complex with ADP and beryllium fluoride (BeF_x). (B) The complex with ADP and vanadate. Backbone atoms of residues 2−130 of the heavy chain are blue, and residues 647−759 are green. Gly120 (cyan), Leu121 (purple), and Gly688 (red) are shown as balls and sticks. Note that the C-terminal part of the heavy chain containing Thr688 rotates as HMM undergoes transition from the myosin−ATP state (BeF_x structure) to the transient myosin−ADP−P_i state (vanadate structure).

Several lines of evidence have shown that the thiol region is important for producing the power stroke of myosin (*5*, *6*, *9*−*11*). Recent crystallographic studies with the myosin head have provided evidence supporting the swinging lever arm model, in which myosin heads are thought to generate the power stroke via conformational rearrangement within the thiol region (*4*, *7*, *8*). This study has demonstrated that, even after substituting SH1 (Cys717) with Thr, smooth muscle myosin retains the motor function. This result clearly indicates that the intact SH1 group is not essential for the motor function of myosin. In contrast, chemical modification of SH1 leads to disruption of the myosin motor. What mechanism can explain the difference between the chemical modification and the mutation? On the basis of the swinging lever arm hypothesis, the following interpretation may be possible. Figure 7 shows the crystal structures of the *Dictyostelium* myosin motor domain with MgADP·beryllium fluoride (*7*) and MgADP·vanadate (*8*) which are thought to correspond to myosin−ATP and transient myosin−ADP−P_i states, respectively. These head structures suggest that the C-terminal part starting from Gly680 moves like a lever arm during the ATP hydrolysis cycle. The Thr688 residue, equivalent to SH1 in *Dictyostelium* myosin, is located near the putative pivot (Gly680) of the lever arm. As shown in Figure 7, Thr688 changes its position relative to the nearest pair of Gly120 and Leu121 residues; the distance between the two segments is about 6.5−8.5 Å in the BeF_x structure, whereas it is about 3.5−4.5 Å in the vanadate structure. Therefore, it is plausible that bulky probes attached to SH1 could come close to the Gly120 and Leu121 residues particularly in the myosin−ADP−P_i state. Such steric hindrance may interfere with the swing motion of the lever arm. In the case of the C717T mutation, the change in the size of the side chain is presumed to be too small to lead to such steric hindrance.

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