Functional Transitions in Myosin: Role of Highly Conserved Gly and Glu Residues in the Active Site†

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ABSTRACT: It has been proposed from crystallographic comparisons [Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., & Rayment, I. (1995) *Biochemistry 34*, 8960−8972] that in one of the important transitions of myosin head (M), M·ATP → M·ADP·P_i, a rotation occurs in Gly^{468} (of chicken smooth muscle myosin). We find that mutation of this Gly to Ala does block the transition. Searching for proton acceptors in ATPase catalysis, we also find that mutation of a candidate (Glu^{470} of chicken smooth muscle myosin) blocks the transition. Interpretations of both findings are examined.

The advent of highly-resolved crystal structures of actin (Kabsch et al., 1990), and of the myosin "head" (Rayment et al., 1993a,b) has reduced the general problem of energy transduction in muscle (Morales & Botts, 1979) to one of figuring out how—under the influence of their various ligands—these molecules move relative to one another. Because the molecules are large and the movements are multiple, the task has to be undertaken in tractable steps. Since the functional contractile system is surrounded by water, reasoning from crystallography has to be supplemented by other methods.

From comparing the crystal structures of myosin head ligated with different nucleotide analogs, Fisher et al. (1995) suggested that as ligated head (M) passes from a "ground" to a "transition" state (M·ATP to M·ADP·P_i), a major, effectively interdomain rotation occurs by virtue of changes in the backbone angles of two residues (Ile^{464} and Gly^{466}).[1] One of our objectives is to check this idea by examining the status of this transition in the functional system when Gly^{466} is mutated to Ala, a substitution expected (Richardson & Richardson, 1989) to hinder backbone angle change.

As a second objective we search for the residue(s) of myosin head that participate in the proton acceptance (for example, a residue which, acting as a base, may accept the ejected proton, thus lowering the activation free energy and speeding up the process) phase of catalysis. Although sensible arguments against its participation have been advanced (Fisher et al., 1995), we test—because of its appropriate character and location—whether function is affected if Glu^{468} is mutated to Ala.

MATERIALS AND METHODS

Preparation of Proteins. Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt (1971). Myosin light chain kinase from chicken gizzard and calmodulin from bovine testis were prepared according to Adelstein and Klee (1981) and Yazawa et al. (1980), respectively.

Expression of Mutant HMMs. GMH-6 is a plasmid encoding the N-terminal half (Met^1−Glu^{729}) of the chicken smooth muscle HMM[2] heavy chain (Yanagisawa et al., 1987). The GMH-6 derivative, whose initiation codon was changed to an *Nco*I site (Onishi et al., 1995), was mutagenized with two oligonucleotides: 5′-CCTTGATATT-5′-CCTTGATATTGCTGCCTTTGAGATTTTTGAG-3′ (the underlined bases indicate the mutations imposed) to replace Gly^{468} with Ala; and 5′-GATATTGCTGGATTTGCCATTTTTGAGATCAAT-3′ to replace Glu^{470} with Ala. Mutagenesis was performed by the method of Kunkel et al. (1987) and mutations were confirmed by dideoxy sequencing. Both G468A and E470A mutant sequences were digested with *Nco*I and *Eco*RI to obtain 2.2-kb fragments. To obtain transfer vectors encoding the full-length HMM heavy chains with mutations at 468 and 470 (pAcC/GHMG468A and pAcC/GHME470A, respectively), the 2.2-kb fragments were then ligated into the *Nco*I/*Eco*RI site of a derivative of pAcC4 containing the coding sequence of the C-terminal half (Phe^{730}−Thr^{1318}) of the HMM heavy chain (Onishi et al., 1995). *Spodoptera frugiperda* (Sf9) cells were co-transfected with linearized *Autographa californica* nuclear polyhedrosis virus DNA and the transfer plasmid for either G468A or E470A mutant by cationic liposome, according to the manufacture's protocol (Invitrogen Co., San Diego, CA). Recombinant viruses were purified and amplified by the method of Summers and Smith (1987). The virus for

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[1] Because different sequences but homologous residues are considered here, we note for convenience that Arg^{245}, Ile^{464}, Gly^{466}, and Glu^{468} in the chicken skeletal muscle myosin sequence (taken as reference) have residue numbers 238, 455, 457, and 459 in *Dictyostelium* II myosin and 247, 466, 468, and 470 in chicken smooth muscle myosin.

[2] Abbreviations: HMM, heavy meromyosin; S1, subfragment 1; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; AMPPNP, 5′-adenylylimido diphosphate; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

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expression of both the regulatory and the essential light chains was obtained as in Onishi et al. (1995). Sf9 cells were co-infected with the HMM heavy chain-containing virus, and the virus containing both light chains (for each virus, the virus-to-cell ratio was 8). Purification of wild-type HMM and G468A and E470A mutants was carried out as in Onishi et al. (1995). After HMM-enriched fractions eluted from the MonoQ column were pooled, HMM was further purified by gel filtration using a Superose 6HR column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.1 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, and 0.5 mM dithiothreitol. This column was very useful for removing trace impurities with ATPase activity. About 0.18 mg of the G468A and the E470A HMMs was purified from 1.5 g (wet weight) of virus-infected cells by this purification procedure.

Gel Electrophoresis, Immunoblots, and Autoradiography. SDS/PAGE was carried out according to Laemmli (1970), using 8−25% gradient polyacrylamide gels (Pharmacia Biotech, Uppsala, Sweden). For immunoblots, 15% SDS−polyacrylamide gels were used and peptide bands were electrophoretically transferred to an Immobilon transfer membrane (Millipore Co., Bedford, MA) (Towbin et al., 1979). Polyclonal antibodies against the regulatory or the essential light chain of chicken gizzard myosin (Onishi et al., 1995) were used as probes, and the reacting bands were detected by peroxidase-conjugated goat anti-rabbit IgG (Cooper Biomedical Inc., West Chester, PA). Light chain phosphorylation was performed by incubating HMM (0.04 mg/mL) at 25 °C for 30 min in an assay medium containing 0.04 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, 0.5 mM DTT, and 50 μM [γ-^{32}P]ATP (0.9 TBq/mmol) either with 0.8 mM EGTA or with 4 μg/mL of chicken gizzard myosin light chain kinase, 1 μg/mL of bovine testis calmodulin, and 0.05 mM $CaCl_2$. Samples containing the same amount (0.6 μg) of HMM were subjected to SDS/PAGE, and phosphate incorporation was detected autoradiographically by exposing the gel to XAR-5 X-ray film (Kodak, Rochester, NY).

Cosedimentation Assays. Cosedimentation assays between HMM and phalloidin-stabilized F-actin were carried out at 20 °C in the presence or absence of 1 mM ATP in a solvent containing 0.04 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, and 0.5 mM dithiothreitol. HMM samples (0.065 mg/mL) were mixed with 0.017 mg/mL of F-actin. After incubation for 20 min, the assay samples were centrifuged in a himac CS 120 ultracentrifuge (Hitachi Koki, Hitachi-Naka, Japan) at 108000*g* for 20 min. Pellets and supernatants of each sample were examined by SDS/PAGE. Gels were stained with Coomassie Brilliant Blue, and the bands were quantified using an ultroscan II laser densitometer (LKB Produkter, Bromma, Sweden). A given amount of bovine serum albumin was added to each sample to normalize peak areas of HMM heavy chain and actin.

Actin Filament Decoration. Rabbit skeletal muscle actin filaments (2 μg/mL) were mixed with 16 μg/mL of HMM. The decorated actin filaments were negatively stained with 4% uranyl acetate in distilled water and observed with a 2000FX electron microscope (JOEL, Tokyo, Japan) operated at 80 kV.

ATPase Assays. The steady-state HMM ATPase activity was measured at 25 °C in an assay medium containing 0.24 mg/mL of HMM, 0.04 or 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, 0.5 mM dithiothreitol, 0.5 mM ATP, and 0.8 mM EGTA. Actin-activated ATPase activity was measured in an assay medium containing 0.054 mg/mL (wild-type) or 0.17 mg/mL (mutants) of HMM, 0.04 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, 0.5 mM dithiothreitol, 1 mM ATP, 4 μg/mL of chicken gizzard myosin light chain kinase, 1 μg/mL of bovine testis calmodulin, and 0.05 mM $CaCl_2$. Actin concentrations were 2.2 and 6.9 mg/mL. ATPase reactions were stopped by adding aliquots to "stop solution" containing perchloric acid at a final concentration of 0.3 M. The mixture was centrifuged at 4200*g* for 5 min to remove precipitated proteins. Amounts of released phosphate were quantified using the malachite green reagent (Ohno & Kodama, 1989). Unreacted molybdate was quenched with sodium citrate according to Lanzetta et al. (1979). Three time points for each assay were taken at 5.5−35-min intervals to determine whether the release of inorganic phosphate was linear with time.

The initial burst of ^{32}P-labeled phosphate liberation from [γ-^{32}P]ATP was measured at 25 °C in an assay medium containing 0.21 mg/mL HMM, 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, 0.5 mM dithiothreitol, and 4 μM [γ-^{32}P]ATP (0.2 TBq/mmol). After 15, 30, 45, or 60 s of incubation, ATPase reaction was stopped by adding trichloroacetic acid (with unlabeled inorganic phosphate carrier) to a final concentration of 5%. The protein precipitate was removed by centrifugation. Inorganic phosphate was separated from organic phosphate as phosphomolybdate according to the method of Martin and Doty (1949). Radioactive phosphate was counted in an LS 5801 scintillation counter (Beckman Instruments, Fullerton, CA). The size of the initial phosphate burst was determined by extrapolating the steady state phosphate liberation to zero time.

Fluorescence Spectra. Tryptophan fluorescence spectra of HMM were recorded at 25 °C using an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan; excitation, 293 nm) in a medium containing 0.12 mg/mL of HMM, 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, and 0.5 mM dithiothreitol. The emission wavelength is as indicated on Figure 5. The slit widths for excitation and emission were adjusted to be 2.5 and 5.0 nm, respectively. A square quartz cuvette with a light path length of 0.5 cm was used. To increase reproducibility, ATP, AMPPNP, or ADP was added directly into the cell without removing it from the holder.

RESULTS

Preparation of Expressed Mutant HMMs. Two different mutants of chicken gizzard HMM heavy chain were co-expressed with the regulatory and the essential light chains in Sf9 insect cells, viz., G468A, and E470A. SDS/PAGE patterns of the two purified mutant HMMs were indistinguishable from those of the wild-type HMM (Figure 1A). Scanning of the peptide bands on SDS/PAGE gels gave a molar ratio of 1:1:1 for the 140-kDa heavy chain and the 17-kDa essential and the 20-kDa regulatory light chains. Furthermore, the presence of the two types of light chains in the mutant HMMs was confirmed by immunoblot analyses with polyclonal antibodies against each of them (Figure 1B and C). Phosphorylation of the regulatory light chain of the wild-type HMM was critical for actin activation of its

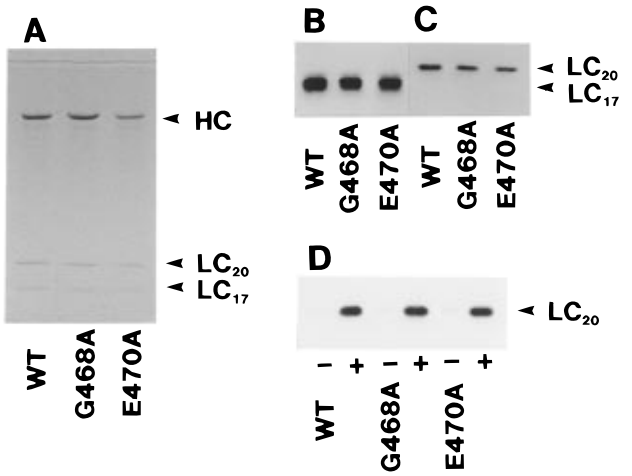

FIGURE 1: SDS/PAGE gels, immunoblots, and autoradiograms of the wild-type (WT) and the G468A and the E470A HMMs. Samples subjected to SDS/PAGE on 8−25% gradient gels were pooled HMM-containing fractions after Superose 6HR column chromatography. Gels were stained with Coomassie Brilliant Blue (A). For immunoblots, samples were subjected to SDS/PAGE on 15% gels. Peptide bands were electroblotted to Immobilon transfer membranes and then stained with polyclonal antibodies directed against chicken gizzard regulatory (B) or essential (C) light chains. Light-chain phosphorylation with [γ-^{32}P]ATP was performed in the presence (+) or absence (−) of myosin light chain kinase, calmodulin, and Ca^{2+} for 30 min (D). Samples were analyzed by SDS/PAGE on 15% gels and then subjected to autoradiography. The regulatory light chain of the wild-type and the two mutant HMMs was phosphorylated in a kinase dependent manner. HC, HMM heavy chain; LC_{20}, regulatory light chain; and LC_{17}, essential light chain.

ATPase activity (Onishi et al., 1995). Like the wild-type HMM, incubation of the two mutant HMMs with myosin light chain kinase, calmodulin, and Ca^{2+} resulted in phosphorylation of their regulatory light chains (Figure 1D).

Binding of Mutant HMMs to Actin. Both Gly468Ala and Glu470Ala HMMs (Figure 2B and C, respectively) decorated actin filaments with an arrowhead appearance indistinguishable from that of the wild-type HMM (Figure 2A). The binding of the two mutant HMMs with actin filaments was also tested by cosedimentation in the presence or absence of ATP. When the wild-type and the two mutant HMMs were centrifuged in the absence of actin, more than 90% of the HMMs remained in the supernatant (lanes a and b in Figure 3; first column in Table 1). When the centrifugation was performed in the presence of actin, 99% of the wild-type HMM, 90% of the G468A HMM, and 100% of the E470A HMM were pelleted with actin (lanes c and d in Figure 3; second and third columns in Table 1). In the presence of ATP, the amount of pelleted HMMs significantly decreased (lanes e and f in Figure 3; fourth and fifth columns in Table 1). These results indicate that, in the absence of ATP, both mutant HMMs produce tight-binding "rigor" complexes with actin and that, upon the addition of ATP, the complexes dissociate into actin and HMM.

ATPase Activity of Mutant HMMs. Actin-activated ATPase activities at two different concentrations of actin of the phosphorylated wild-type HMM, the phosphorylated G468A HMM, and the phosphorylated E470A HMM are shown in Table 2. The actin-activated ATPase activity for the phosphorylated wild-type HMM was increased with increasing the concentration of actin. On the other hand, the two

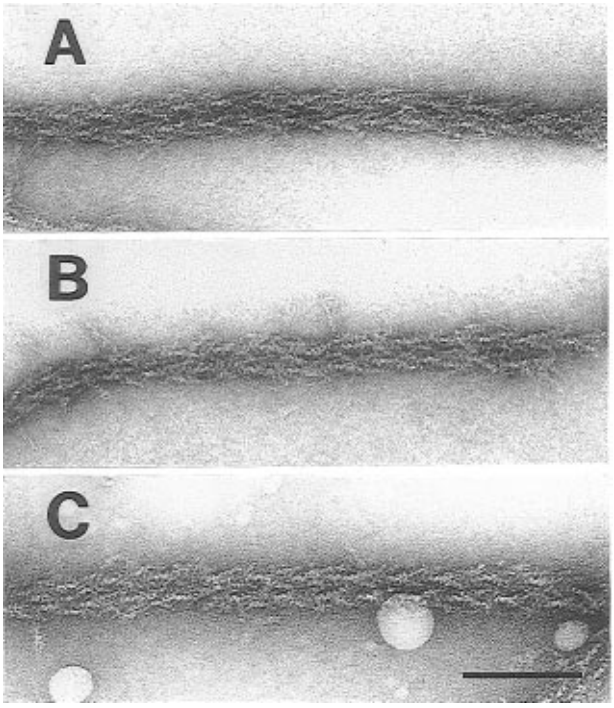

FIGURE 2: Electron micrographs of actin filaments decorated by the wild-type (A), the G468A (B) and the E470A (C) HMMs. Rigor complexes were formed by mixing HMM and actin at a molar ratio of 1:2. Actin filaments in A−C show a typical arrowhead structure (bar = 100 nm).

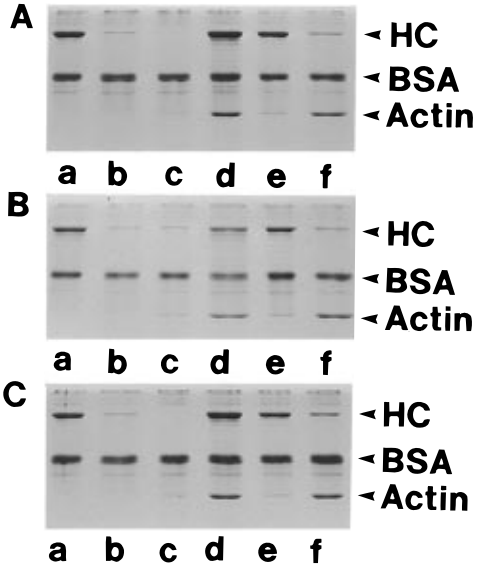

FIGURE 3: SDS/PAGE patterns of the supernatant and the pellet obtained by centrifugation of actin and HMM in the presence or absence of ATP. (A) Wild-type HMM; (B) G468A HMM; (C) E470A HMM. F-actin (0.017 mg/mL) was incubated with 0.065 mg/mL HMM at 20 °C in a solvent containing 0.04 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, and 0.5 mM dithiothreitol (c and d). The same incubation was done in the presence of 1 mM ATP (e and f). Mixtures of F-actin and HMM were pelleted by centrifugation at 108000*g* for 20 min. HMM alone was also centrifuged under the same condition (a and b). Supernatant (a, c, and e) and pellet (b, d, and f) samples were run on SDS/PAGE and quantified by densitometry (summarized in Table 1).

mutant HMMs showed practically no actin-activated ATPase activity.

Since the amino acid residues we mutated are in or near the γ-phosphate pocket as described in the introduction, alterations of these residues may produce serious damage

Table 1: Binding of Actin with the Wild-Type (WT) and Two Mutant HMMs in the Presence or Absence of ATP[a]

	HMM alone	HMM + actin		HMM + actin + ATP	
	HC	HC	actin	HC	actin
WT	94	1	7	89	14
G468A	90	10	11	81	13
E470A	91	0	11	91	11

[a] Percentages of proteins remaining in the supernatants were quantified by laser densitometry of SDS/PAGE gels (Figure 3). HC, heavy chain.

Table 2: ATPase Activities [in nmol of P_i/min/(mg of HMM)] of the Wild-Type (WT) and Two Mutant HMMs in the Presence of Actin[a]

actin (mg/mL)	WT	G468A	E470A
2.2	183	0.1	0.0
6.9	378	0.4	0.1

[a] Assay conditions were 0.054 mg/mL of the wild-type HMM, 0.17 mg/mL of the G468A HMM, or 0.17 mg/mL of the E470A HMM in 2.2 or 6.9 mg/mL of rabbit skeletal muscle actin, 0.04 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, 1 mM ATP, and 0.5 mM dithiothreitol at 25 °C. Chicken gizzard myosin light chain kinase (4 μg/mL), bovine testis calmodulin (1 μg/mL), and $CaCl_2$ (0.05 mM) were also added to the assay medium to phosphorylate the regulatory light chain of HMM. The ATPase activity of each HMM alone was subtracted from each measured value to estimate actin-activated ATPase activity.

Table 3: Intrinsic ATPase Activities [in nmol of P_i/min/(mg of HMM)] of the Wild-Type (WT) and Two Mutant HMMs[a]

KCl (M)	WT	G468A	E470A
0.04	0.91	0.04	0.04
0.45	3.92	0.19	0.19

[a] Assay conditions were 0.24 mg/mL of the wild-type HMM, the G468A HMM, or the E470A HMM in 0.04 or 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, 0.5 mM ATP, 0.5 mM dithiothreitol, and 0.8 mM EGTA at 25 °C.

not only to the actin-activated ATPase activity but also to the intrinsic ATPase activity of the mutant HMMs. To test whether or not this is so, we measured the steady-state ATPase activities of the two mutant HMMs and compared them with the activity of the wild-type HMM (Table 3). In a solution (0.45 M KCl) in which the HMM conformation is in the 7.5S state (Suzuki et al., 1982, 1985), the activity of the wild-type HMM was 3.92 nmol of P_i/min/(mg of HMM). On the other hand, the activity obtained in a solution containing 0.04 M KCl was only $^1/_4$ as high [0.91 nmol of P_i/min/(mg of HMM)]. Previous studies have indicated that the decrease in activity in a low-salt medium is due to a decrease in the rate of M·ADP·P_i degradation (Onishi, 1982; Cross et al., 1986). In both high- and low-salt media, the steady-state activities of both mutant HMMs were much lower (less than 1/20) than that of the wild-type HMM. These results suggest that these mutants hardly go through certain step(s) in ATP hydrolysis by HMM.

Even if the rate-limiting step of the overall ATPase reaction is blocked in the mutants, it is possible that the formation of M·ADP·P_i occurs normally. If this were the case, we should be able to detect an initial phosphate burst of the same level as that of the wild-type HMM. However, the data in Figure 4 indicate that the initial phosphate bursts for the G468A and the E470A HMMs are only 1/300 and 1/100, respectively, of the burst levels of the wild-type HMM

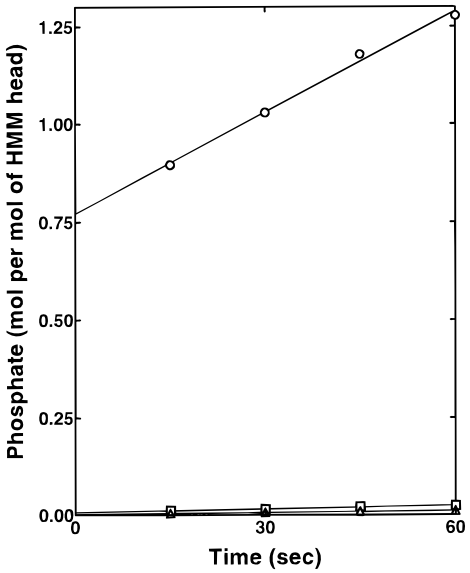

FIGURE 4: Initial phosphate burst in the hydrolysis of ATP by the wild-type HMM and the two mutant HMMs. Assay conditions were 0.21 mg/mL of the wild-type HMM (○) or the G468A HMM (△) or the E470A HMM (□), 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, 0.5 mM dithiothreitol, and 4 μM [γ-^{32}P]ATP. Burst sizes, determined by extrapolating the steady-state phosphate liberation to zero time, of the wild-type, the G468Ala, and the E470A HMMs were 0.78, 0.00, and 0.01 mol of P_i/(mol of HMM head), respectively.

(0.78 mol/mol of HMM head). It is thus clear that the formation of M·ADP·P_i is also blocked by these mutations, although our "burst" test cannot completely exclude the possibility that the formation of this intermediate occurs very slowly.

Earlier kinetic studies on the ATP-induced enhancement of S1 tryptophan fluorescence (Bagshaw & Trentham, 1974; Johnson & Taylor, 1978) have led to the following scheme for ATP binding and hydrolysis by myosin:

$$M + ATP \rightleftarrows M{\cdot}ATP \rightleftarrows M{\cdot}ATP^* \rightleftarrows M{\cdot}ADP{\cdot}P_i{}^{**}$$

where the asterisks, * and **, denote different states of enhanced fluorescence. To test whether G468A and E470A HMMs can form M·ATP*, we measured their tryptophan fluorescence enhancements. Comparison between the left (A) and the right (C) panels in Figure 5 indicates that upon ATP addition, the tryptophan fluorescence of the E470A HMM is increased to a level (119%) between that (125%) achieved by the wild-type HMM with ATP and that (112%) achieved by the wild-type HMM with ADP. This increase is comparable to the level (119%) achieved by the wild-type HMM upon adding a nonhydrolyzable ATP analogue, AMPPNP (Yount et al., 1971), as shown in the left panel (A) in Figure 5. Since it is thought that the intermediate observed in the presence of AMPPNP is mostly M·ATP* (Bagshaw et al., 1974), these results suggest that the formation of M·ATP* occurs when this E470A binds ATP. The middle panel (B) in Figure 5, in contrast, shows that upon ATP addition, the fluorescence level is not enhanced. This result suggests that the G468A mutation causes HMM to lose its ability to isomerize into a state of enhanced fluorescence. However, this does not mean that there is no interaction between this mutant HMM and ATP. As described above, the rigor complex of G468A HMM is

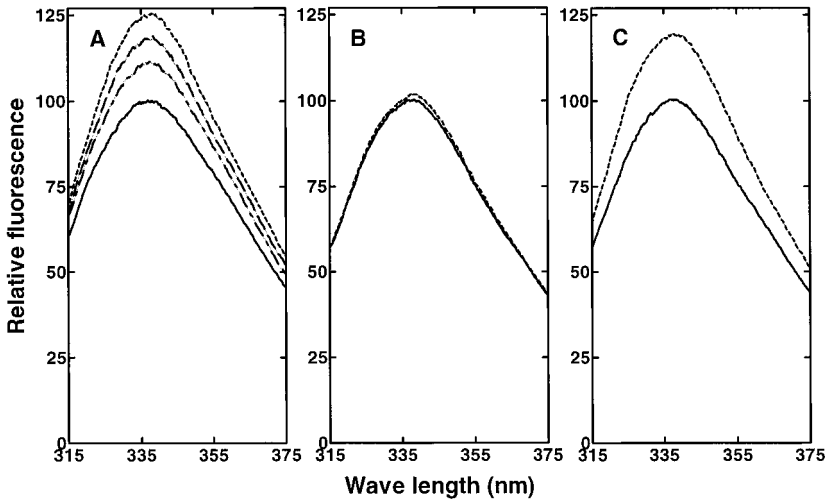

FIGURE 5: Tryptophan fluorescence spectra from the wild-type HMM and the two mutant HMMs. Conditions were 0.12 mg/mL of the wild-type HMM (A), the G468A HMM (B), or the E470A HMM (C) with 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.5, and 0.5 mM dithiothreitol at 25 °C (—). 0.1 mM ATP (- - -), AMPPNP (— — —), or ADP (— · —) was added to the reaction mixture. Fluorescence spectra were recorded at 1−3 min following the addition of nucleotide. The fluorescence level was enhanced by adding nucleotide in A and C but not B.

dissociated upon the addition of ATP. This result suggests that even G468A interacts with ATP in some manner.

DISCUSSION

If it is true—as Fisher et al. (1995) surmised from comparing crystal structures—that the M·ATP to M·ADP·P_i transition includes a significant rotation in Gly^{466}, then one would expect that seriously hindering this rotation in the functional system would impair the transition and so block hydrolysis. When we imposed this hindrance (by G468A in chicken smooth muscle HMM), we did, in fact, block hydrolysis. Our finding is therefore consistent with the ideas of Fisher et al. (1995). Our additional observation is also pertinent. In the wild-type functional system, M·ATP is a state with enhanced fluorescence and M·ADP·P_i is a state with even more enhanced fluorescence, but our mutated HMM·ATP had no enhanced fluorescence. In the mutant, the pyrophosphate moiety of ATP seems properly bound (it caused a seemingly normal dissociation of HMM from actin), but its adenosine moiety may be differently directed and unable to perturb the fluorophore. [Such a "dark" state is not implausible because bound inorganic pyrophosphate (which also dissociates HMM from actin) also fails to enhance fluorescence (Werber et al., 1972).] The block, therefore, may have a complicated origin.

How various residues of the active site participate in catalysis is largely unexplored. Fisher et al. (1995) pointed out that among potentially catalytic residues none is within 5 Å of the point of hydrolytic attack. Glu^{468}, however, is just outside of this radius, has the right attitude, and could be reached after a small conformational change. A preliminary examination of its role was therefore of interest. As reported above, its substitution (by E470A in chicken smooth muscle HMM) abolishes hydrolysis, even though nucleotide appears to bind properly, adequately dissociating actin from the myosin heads and producing the fluorescence enhancement expected from binding unsplit ATP (compare the result of G468A above). This is just the behavior expected if Glu^{468} is an essential base catalyst in the hydrolytic process. To make this conclusion firm, however, requires eliminating an alternative interpretation, viz., that hydrolysis stops because a salt bridge, Arg^{245}—Glu^{468}, that stabilizes the rotated state (Fisher et al., 1995) is disabled in the mutation.

ACKNOWLEDGMENT

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NOTE ADDED IN PROOF

While our paper was under review, we learned that the Glu459Val mutation of *Dictyostelium* II myosin resulted in the loss of both actin-activated and intrinsic activities [Ruppel, K. M., & Spudich, J. A. (1996) *Mol. Cell. Biol. 7*, 1123−1136].

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