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Functional transitions in myosin: Formation of a critical salt-bridge and transmission of effect to the sensitive tryptophan

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ABSTRACT **For analyzing the mechanism of energy transduction in the "motor" protein, myosin, it is opportune both to model the structural change in the hydrolytic transition, ATP (myosin-bound) + $H_2O \rightarrow ADP{\cdot}P_i$ (myosin-bound) and to check the plausibility of the model by appropriate site-directed mutations in the functional system. Here, we made a series of mutations to investigate the role of the salt-bridge between Glu-470 and Arg-247 (of chicken smooth muscle myosin) that has been inferred from crystallography to be a central feature of the transition [Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., & Rayment, I. (1995) *Biochemistry* 34, 8960–8972]. Our results suggest that whether in the normal, or in the inverted, direction an intact salt-bridge is necessary for ATP hydrolysis, but when the salt-bridge is in the inverted direction it does not support actin activation. Normally, fluorescence changes result from adding nucleotides to myosin; these signals are reported by Trp-512 (of chicken smooth muscle myosin). Our results also suggest that structural impairments in the 470–247 region interfere with the transmission of these signals to the responsive Trp.**

The "head" (subfragment 1; S1) or the joined "heads" (heavy meromyosin; HMM) of myosin is now considered the seat of energy transduction, in the sense that a sequence of interactions between the head(s) and bound ATP-derivatives cause the protein to deform sequentially, transmitting a pattern of mechanical effects to actin and the rest of the contractile apparatus. The precise analysis of how the chemical free energy of the interacting system gets converted into mechanical work has been strongly advanced by Rayment's initiation of myosin crystallography (1, 2). By comparing appropriate stable analog states that could otherwise only be observed transiently, Rayment and his colleagues (see refs. 3 and 4) have provided reasonable and detailed guesses about important transitions thought to occur in the real, transient case. Sometimes a useful way to check inferences from crystallography is to make strategic mutations (impairments) in a functional system and to see if its differences with the normal system are comprehensible. It is in this context that we have examined previously a crystallographically based idea that the transition in which myosin-bound ATP hydrolyzes to myosin-bound $ADP{\cdot}P_i$ is accompanied by a rotation around Ile-466–Ala-467–Gly-468.[‖] The behavior of the G468A mutant suggested that hindering the rotation does block hydrolysis (5). We examine here another idea that formation of a salt-bridge between Glu-470 and Arg-247 stabilizes a "rotated" state in the $ADP{\cdot}P_i$-bound myosin head.

Two established techniques were used here for detecting myosin-bound hydrolysis. One is the measurement of an acid-labile phosphate liberation in the pre-steady state (the so-called initial burst) that is formed as a result of the rapid cleavage of bound ATP (6, 7). Another technique is enhancement of protein fluorescence (8), but until recently this method has been empirical; now it appears to originate at Trp-512 (4, 9–12). As it happens, the fluorescence changes used to detect hydrolysis are affected by the mutations used in analyzing salt-bridge formation; this circumstance provided us with some new information on how Trp-512 is perturbed.

MATERIALS AND METHODS

Protein Preparations. F-actin was prepared from rabbit skeletal muscle as in (13). Myosin light chain kinase was prepared from chicken gizzard according to Adelstein and Klee (14) and calmodulin from bovine testis according to Yazawa *et al.* (15).

Preparation of Recombinant HMMs. Wild-type chicken gizzard HMM and its mutant E470A were prepared as described (5, 16). Construction of four other mutant HMM heavy chain cDNAs, viz. R247A, E470R, R247E, and E470R/R247E, was carried out by the same method as described for E470A. Briefly, GMH-6, a cDNA encoding the N-terminal half (Met-1–Glu-729) of chicken gizzard HMM heavy chain (17), was used as a template. A GMH-6 derivative, within which a unique *Nco*I site was introduced around the initiation codon, was mutagenized by the method of Kunkel *et al.* (18) with three oligonucleotides (the underlined bases indicate mutations imposed): 5′- GAATGACAACTCCTCCGCCTTTGGCAAATTTATC-3′ to replace Arg-247 with Ala, 5′-GATATTGCTGGATTTCGCATTTTTGAGATCAATT-3′ to replace Glu-470 with Arg, and 5′-GAATGACAACTCCTCCGAATTTGGCAAATTTATC-3′ to replace Arg-247 with Glu. A cDNA construct with both E470R and R247E was obtained by ligation of two cDNA fragments with mutations E470R and R247E at a unique *Bgl*II site. cDNAs with four different sequences, viz. R247A, E470R, R247E, and E470R/R247E, were then digested with *Nco*I and *Eco*RI to obtain

Abbreviations: HMM, heavy meromyosin; S1, subfragment 1; AMP-PNP, adenosine 5′-[γ-imido]triphosphate.

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[‖]Because our experiments were largely performed with smooth muscle myosin, its sequence numeration is used throughout this paper, but, to facilitate citations to homologous residues used by others, we note that Arg-247, Ile-466, Gly-468, Glu-470, and Trp-512 corresponds in chicken skeletal muscle to Arg-245, Ile-464, Gly-466, Glu-468, and Trp-510, and in *Dictyostelium* myosin to Arg-238, Ile-455, Gly-457, Glu-459, and Trp-501.

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2.2-kb fragments. Each of these fragments was ligated into the *Nco*I/*Eco*RI site of a derivative of a baculovirus transfer vector (pAcC4) containing the coding region of the C-terminal half (Phe-730–Thr-1318) of the HMM heavy chain. Recombinant baculovirus was isolated for each cDNA construct as described by Summers and Smith (19). Expression and purification of recombinant HMMs were carried out as described (5, 16).

Fluorescence Measurements. Fluorescence assays were performed at 25°C on a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo) in a medium containing 0.12 mg/ml of HMM, 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.5), and 0.5 mM DTT. Excitation wavelength was 293 nm. Emission spectra were recorded from 315 to 375 nm.

Gel Electrophoresis and Autoradiography. SDS/PAGE was carried out as described by Laemmli (20). Light chain phosphorylation was performed as described (5). Samples were subjected to SDS/PAGE and ^{32}P incorporation into regulatory light chains was detected by exposing the gel to an imaging plate BAS-SR 2025 and analyzing the plate with a bioimaging analyzer BAS 5000 (Fuji, Kanagawa, Japan).

Actin Filament Decoration. F-Actin was decorated with expressed HMMs at a molar ratio of actin/HMM = 2, negatively stained with a 4% uranyl acetate solution, and observed with a 2000FX electron microscope (JEOL, Tokyo) operated at 80 kV.

Actin Cosedimentation Assays. Binding of expressed HMMs for F-actin was measured by using cosedimentation assays as described (5). Phalloidin-stabilized F-actin (0.017 mg/ml) and each of the HMMs (0.065 mg/ml) were mixed in a solvent containing 0.04 M KCl, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.5), and 0.5 mM DTT in the presence or absence of 1 mM ATP. The assay samples were centrifuged at 108,000 × *g* for 20 min in a Himac CS 120 ultracentrifuge (Hitachi Koki, Hitachi-Naka, Japan). The proteins in the supernatant were estimated by scanning SDS/PAGE gels with an Ultroscan LX laser densitometer (LKB Prodakter, Bromma, Sweden).

ATPase Assays. The steady-state ATPase activity was measured at 25°C in an assay medium containing 0.24 mg/ml of HMM, 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.5), 0.5 mM DTT, 0.5 mM ATP, and 0.8 mM EGTA. An actin-activated ATPase activity was measured as a function of actin concentration in an assay medium containing 0.054 mg/ml (wild type) or 0.24 mg/ml (mutants) of HMM, 0.04 M KCl, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.5), 0.5 mM DTT, 1 mM ATP, 4 μg/ml of chicken gizzard myosin light chain kinase, 1 μg/ml of bovine testis calmodulin, and 0.05 mM $CaCl_2$. Inorganic phosphate was estimated colorimetrically by using the malachite green reagent (21). Rates were calculated from three time points.

The initial phosphate burst was measured at 25°C in an assay medium containing 0.24 mg/ml of HMM, 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.5), 0.5 mM DTT, 4 μM [γ-^{32}P]ATP (0.4 TBq/mmol). The reaction was stopped at 15, 30, 45, and 60 sec by adding trichloroacetic acid to a final concentration of 5%. Released inorganic phosphate was extracted into an organic solvent as phosphomolybdate by the method of Martin and Doty (22), and its radioactivity was counted in Tri-Carb 2700TR liquid scintillation analyzer (Packard). The size of the initial burst was estimated by extrapolating the steady-state phosphate liberation to zero time.

RESULTS

Construction of Mutant HMMs. To disable the salt-bridge between Glu-470 and Arg-247, four single mutants, viz. E470A, R247A, E470R, and R247E, of the chicken gizzard HMM heavy chain were constructed. We also constructed a heavy chain double mutant in which Glu-470 and Arg-247 were replaced with Arg and Glu, respectively (E470R/R247E). It was thought that this mutant might restore the salt-bridge. The five different mutants of the HMM heavy chain were expressed with both regulatory and essential wild-type light chains in cultured Sf9 cells and purified. On SDS/PAGE gel patterns, each of the purified mutant HMMs appeared as three bands indistinguishable from those of the wild-type HMM (Fig. 1*A*). Relative mobilities of the three bands corresponded to those of the 140-kDa HMM heavy chain, the 17-kDa essential light chain, and the 20-kDa regulatory light chain. In addition to these main bands, several faint bands were present in the molecular mass range between 70 and 200 kDa. Because these peptides eluted from the Superose column at the same time as HMM did, they may be proteolytic digests from HMM that remain associated under nondenaturing conditions. Incubation of both wild-type and E470R/R247E HMMs with myosin light chain kinase, calmodulin, and Ca^{2+} resulted in phosphorylation of their regulatory light chains (Fig. 1*B*).

Steady-State ATPase Activity, Initial Phosphate Burst, and Tryptophan Fluorescence Enhancement of Mutant HMMs. The steady-state ATPase activities of the five mutant HMMs were measured at 0.45 M KCl and compared with the activities observed with the wild-type HMM (Fig. 2). The wild-type and the E470R/R247E HMMs exhibited similar ATPase activities. The activity of the R247A HMM was 1/2.7 that of wild-type HMM. Activities of the three other mutant HMMs, viz., E470A, E470R, and R247E, were substantially lower (less than 1/10 wild type).

To test if mutations at Glu-470 and/or Arg-247 also affect the formation of M·ADP·P_i (Taking "M" to mean HMM), we measured the initial phosphate bursts for the wild-type HMM and the five mutant HMMs. The burst sizes of wild-type HMM and of E470R/R247E HMM were quite similar (0.70 and 0.68 mol per mol of HMM head, respectively), whereas the other four HMMs exhibited no phosphate burst (Fig. 3).

The Trp fluorescence of myosin is enhanced by ATP binding and hydrolysis. These enhancements, along with chemical measures, have been accepted as signatures of the chemical transitions of myosin ATPase (23, 24). So, we studied Trp responses of the wild type, and five mutant HMMs upon adding various nucleotides. The fluorescence levels observed with the wild-type HMM and the E470R/R247E HMM were similar to each other in the presence of ATP, adenosine

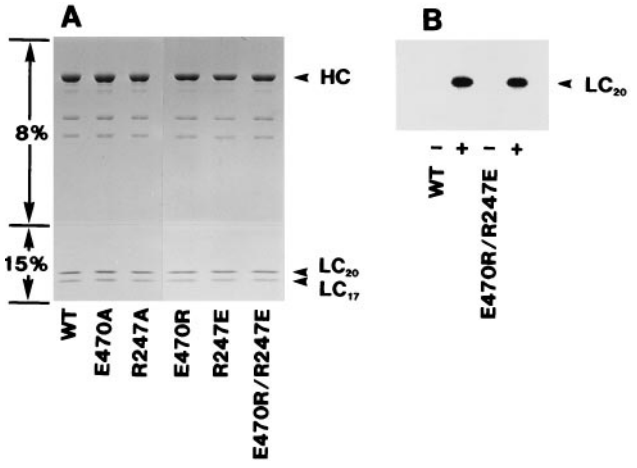

FIG. 1. SDS/PAGE gels and autoradiograms of wild-type (WT) and its mutant HMMs. Samples of the purified HMMs were subjected to SDS/PAGE on 8 and 15% discontinuous acrylamide gels. Gels were stained with Coomassie brilliant blue (*A*). Light chain phosphorylation was performed in the presence (+) or absence (−) 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 (*B*). HC, HMM heavy chain; LC_{20}, regulatory light chain; and LC_{17}, essential light chain.

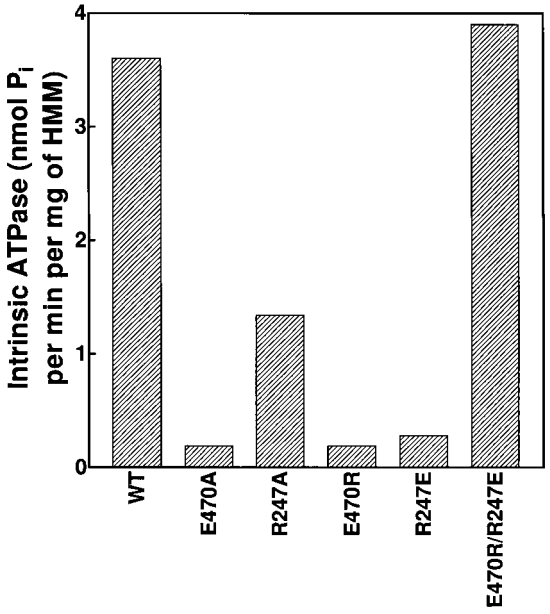

FIG. 2. Intrinsic ATPase activities of wild-type (WT) and five mutant HMMs. Assay conditions were 0.24 mg/ml of wild-type HMM or each of five mutant HMMs in 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.5), 0.5 mM DTT, 0.8 mM EGTA, and 0.5 mM ATP at 25°C.

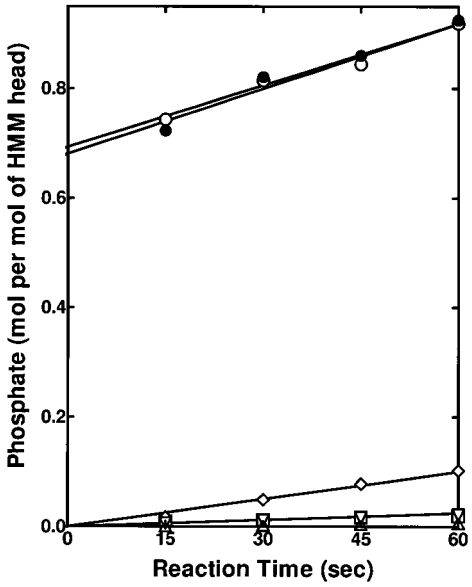

FIG. 3. Initial phosphate bursts in the hydrolysis of ATP by wild-type and five mutant HMMs. Assay conditions were 0.20 mg/ml of each of wild-type (○), E470A (□), R247A (◇), E470R (△), R247E (▽), and E470R/R247E (●) HMMs, 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.5), 0.5 mM DTT, and 4 μM [γ-^{32}P]ATP. Burst sizes for wild-type, E470A, R247, E470R, R247E, and E470R/R247E HMMs were 0.68, 0.00, 0.00, 0.00, 0.00, and 0.70 mol of P_i/mol of HMM head, respectively.

5′-[γ-imido]triphosphate (AMPPNP), or ADP (Fig. 4 *A* and *F*). The binding of ADP or AMPPNP to the two HMMs caused some fluorescence enhancement (11% for ADP and 18% for AMPPNP). The binding of ATP, followed by its hydrolysis to ADP·P_i, resulted in an even greater enhancement (24%). Thus we conclude that during ATP hydrolysis, both the wild-type and E470R/R247E HMMs are in the conformational state conventionally designated M·ADP·P_i** (see Fig. 5).

Upon adding ATP or AMPPNP, the fluorescence of E470A HMM was enhanced to the same level (18%) (Fig. 4*B*). As described previously (5), this enhancement was also similar to that of wild-type HMM upon adding a nonhydrolyzable ATP analogue. Because data (Figs. 2 and 3) suggest that this mutant cannot hydrolyze ATP, we conclude that its conformational state in the presence of ATP is M·ATP* (see Fig. 5). The fluorescence of this mutant was also enhanced by adding ADP, but its enhancement (5%) was somewhat lower than that (11%) obtained with wild-type HMM. On the other hand, the fluorescence intensities of the R247A (Fig. 4*C*), the E470R (Fig. 4*D*), and the R247E (Fig. 4*E*) HMMs were not enhanced at all by ATP. This finding suggests that these enzymatically disabled HMMs cannot isomerize into a state with enhanced fluorescence (see Fig. 5).

Actin-Activated ATPase Activity. Actin-activated ATPase activities at 2.2 mg/ml actin were measured for the wild-type HMM and the five mutant HMMs (data not shown). No actin activation was observed in the ATPase activities of the four single-mutant HMMs, viz. E470A, R247A, E470R, and R247E. The activity for the E470R/R247E HMM was detectably activated by actin, but at a level only 1/100 that of wild-type HMM. To test if the reduced actin activation is caused by a weak affinity of the double-mutant HMM for actin, we also measured the dependence of ATPase activity on actin concentration for both the wild-type and the double-mutant HMMs. Double reciprocal plots (Fig. 6) show that there is no significant difference in the apparent binding constant to actin (K_a), whereas the maximal actin-activated ATPase activity (V_{max}) of the double-mutant HMM is 100 times smaller than that of wild-type HMM. The decrease in the actin-activated V_{max} of the double-mutant HMM is not caused by a defect in phosphorylation capability; its regulatory light chain could be phosphorylated by myosin light chain kinase as well as could that of the wild-type HMM (Fig. 1*B*).

Rigor Complex Formation of Mutant HMMs with Actin and its Dissociation by ATP. Electron microscope observations indicate that the R247E (Fig. 7*A*), the E470R (Fig. 7*B*), the R247E (Fig. 7*C*), and the E470R/R247E (Fig. 7*D*) HMMs decorate actin filaments with the similar arrowhead appearance to that observed previously with wild-type HMM (16). To investigate if the R247A, the E470R, the R247E, and the E470R/R247E HMMs can interact with ATP, we studied the binding of the single mutants with F-actin in the presence or absence of ATP by using cosedimentation assays and compared it with actin binding of wild-type or E470R/R247E HMM. No significant difference between the HMMs examined were observed either in the presence or absence of ATP. When centrifuged in the absence of actin, little of these HMMs was pelleted; about 90% of the proteins remained in the supernatant (first column in Table 1). When the centrifugation was carried out in the presence of actin, almost 100% of HMMs were pelleted (second and third columns in Table 1). When ATP was added, most of the HMMs were in the supernatant (fourth and fifth columns in Table 1). This result suggests that, like the wild-type and the double-mutant HMMs, the three single-mutant HMMs can dissociate from F-actin upon ATP addition. In other words, the three single-mutant HMMs can interact with ATP and change their conformation so that they now bind weakly to actin, although they show no Trp fluorescence enhancement (Fig. 4 *C–E*).

DISCUSSION

An important finding here is that E470R/R247E HMM, like wild-type HMM, has a steady-state ATPase activity, an initial phosphate burst, and a high-level tryptophan fluorescence enhancement corresponding to M·ADP·P_i**, whereas the constituent single-mutant HMMs, E470R and R247E, have no steady-state ATPase activity, no phosphate burst, and no

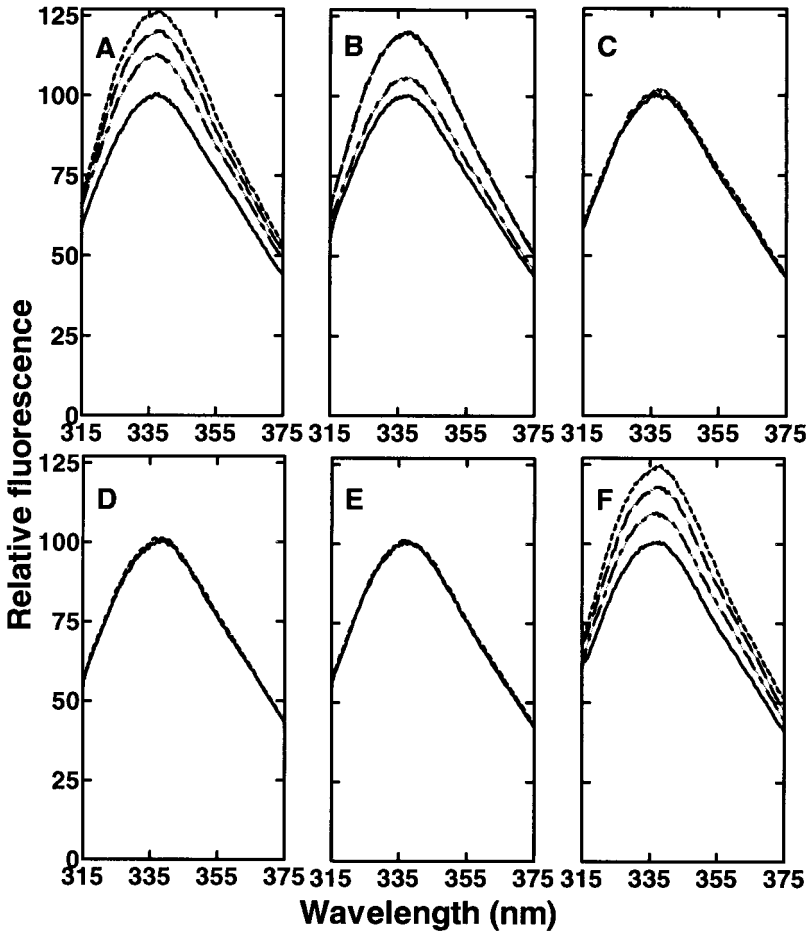

FIG. 4. Tryptophan fluorescence spectra from wild-type HMM and five mutant HMMs. Conditions were 0.12 mg/ml of each of wild-type (*A*), E470A (*B*), R247A (*C*), E470R (*D*), R247E (*E*), and E470R/R247E (*F*) HMMs, 0.45 M KCl, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.5), and 0.5 mM DTT at 25°C (——). A total of 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 nucleotides. In *B*, fluorescence spectra obtained in the presence of ATP and AMPPNP were overlapped mostly.

fluorescence enhancement. In wild-type and the double-mutant HMMs, the salt-bridge is constituted from the same residues but in opposite order. This finding has dual significance. First, it means that mechanical integrity of the salt-bridge is important for intrinsic functions, because these functions are preserved whatever the order. Second, it means that differences inverted by changing the order, such as strains or altered interactions with neighboring structures, have only second-order effects on these functions.

In ref. 5, we noted that with the knowledge of that time, it was ambiguous whether mutation E470A failed to mediate hydrolysis because it disabled half of an essential salt-bridge (3) or because it was the elusive proton acceptor. Our present result favors the interpretation of Fisher *et al.* (3), because our double-mutant HMM participates in intrinsic hydrolysis just as

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

R247A, E470R, R247E ? E470A wild-type E470R/R247E

FIG. 5. Myosin mutants and their major intermediates in the ATPase reaction. The asterisks (* and **) denote two different states with enhanced Trp fluorescence. Three single mutants, R247A, E470R, and R247E, can interact with ATP to form a weakly bound state for actin, although no ATP-induced fluorescence enhancement is associated.

well as wild-type HMM, even though the putative "acceptor" has been relocated to another environment.

It is well known that specific occupants of the ATP binding site generate specific enhancements of the fluorescence from an S1 tryptophan (8). Recently, several findings (9–12) have suggested a homologous location; in chicken skeletal muscle myosin, this "ATP-responsive Trp" seems to be 510, and in smooth muscle myosin, 512. This Trp is connected by a stiff strand to the flexible triplet Ile-466–Ala-467–Gly-468 (Fig. 8). It is plausible that the aforementioned rotating lower piece of 50 kDa, which bears Trp-512 at its tip, can move and perturbs this fluorophore. Smith and Rayment (4) have reported that the environment of this Trp is different in the MgADP-beryllium fluoride (M·ATP type) and in the MgADP-vanadate ($M{\cdot}ADP{\cdot}P_i$ type) complexes of the truncated *Dictyostelium* myosin head. Such a difference would be consistent with assuming that this Trp (or its homologs in other myosins) is perturbed in the course of hydrolysis. We note, however, that the binding of ADP or of AMPPNP (neither of which hydrolyzes) also enhances fluorescence, but does so in the absence of the rotation originally described by Fisher *et al.* (3). Therefore, it should be assumed that with both wild-type and double-mutant HMMs, the observed enhancements upon adding ATP are the sum of two distinct enhancements. The hydrolysis- and rotation-associated enhancement would be the difference, 24–18%. Of course this difference appears to be much reduced because it is measured against the unchanging emissions from other Trps.

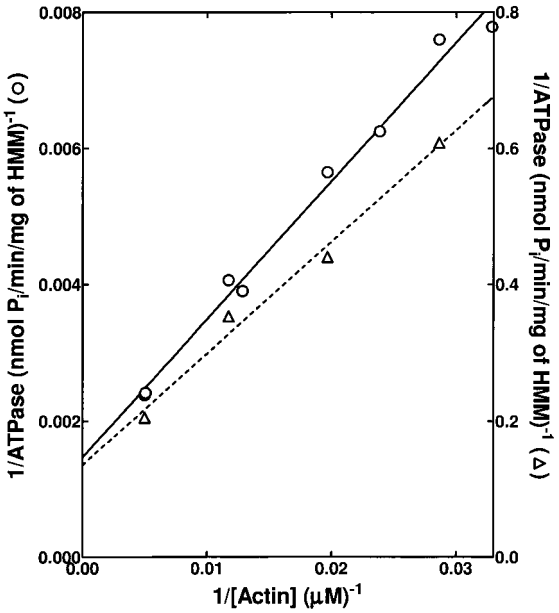

FIG. 6. Double reciprocal plots of ATPase activities of wild-type (circles) and E470R/R247E (triangles) HMMs versus actin concentration. Myosin light chain kinase, calmodulin, and Ca^{2+} were added to the assay medium to phosphorylate regulatory light chains of HMM. Actin-activated ATPase activity was obtained by subtracting the activity of HMM itself from each measured value. The maximal actin-activated ATPase activity and the apparent binding constant for actin were 740 nmol P_i/min/mg and 0.64×10^4 M^{-1}, respectively, for wild-type HMM, and 8 nmol P_i/min/mg and 0.74×10^4 M^{-1}, respectively, for E470R/R247E HMM.

Although the intrinsic ATPase of E470R/R247E appears normal, some phase of its interaction with actin is defective. This defect is not in association/dissociation from actin but is in a process that occurs within the complex of the two proteins. In normal activation, actin is thought to intervene to shorten a rate-limiting substrate-leaving process, but in this newly found situation it is unknown which reaction establishes itself as rate-limiting (whereas steady-state intrinsic ATPase, phosphate burst, and tryptophan enhancement are unaffected). We will elaborate on this matter elsewhere, but we will say here only that although the double-mutant HMM preserves the mechanical integrity of the bridge, it may be in other regards very dissimilar to wild-type HMM, for example, in its interactions with its immediate neighbors or by virtue of being a reversed dipole.

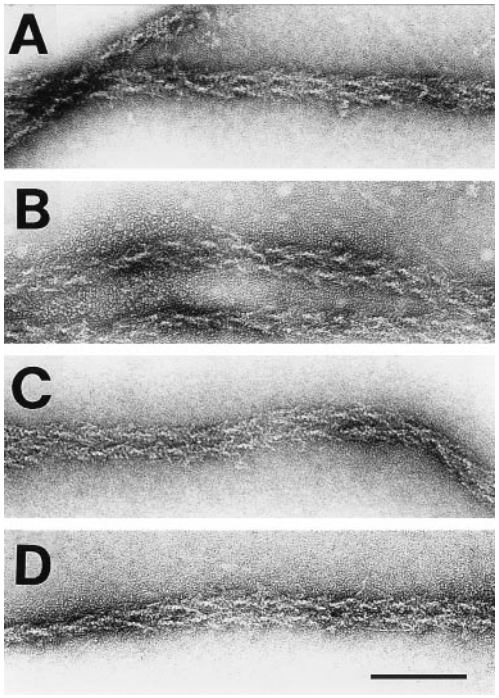

FIG. 7. Electron micrographs of actin filaments decorated by R247A (*A*), E470R (*B*), R247E (*C*), and E470R/R247E (*D*) HMMs. Rigor complexes were formed by mixing HMM and actin at a molar ratio of 1:2. (Bar = 100 nm.)

Table 1. Binding of actin with wild-type (WT) HMM and four mutant HMMs in the presence or the absence of ATP

	HMM alone, HC	HMM + actin		HMM + actin + ATP	
		HC	Actin	HC	Actin
WT	93	1	2	85	4
R247A	91	0	5	81	4
E470R	87	5	0	71	6
R247E	89	0	0	83	6
E470R/R247E	92	0	3	88	4

Percentages of proteins remaining in the supernatant were quantified by laser densitometry of gels. HC, heavy chain.

We examined several single mutations. Before discussing these mutants, we note that their effects are neither global nor gross. Specifically, these mutants bind to actin filaments and dissociate with ATP. In the electron microscope, they are seen to decorate actin filaments in characteristic "arrowhead" manner. Also, as

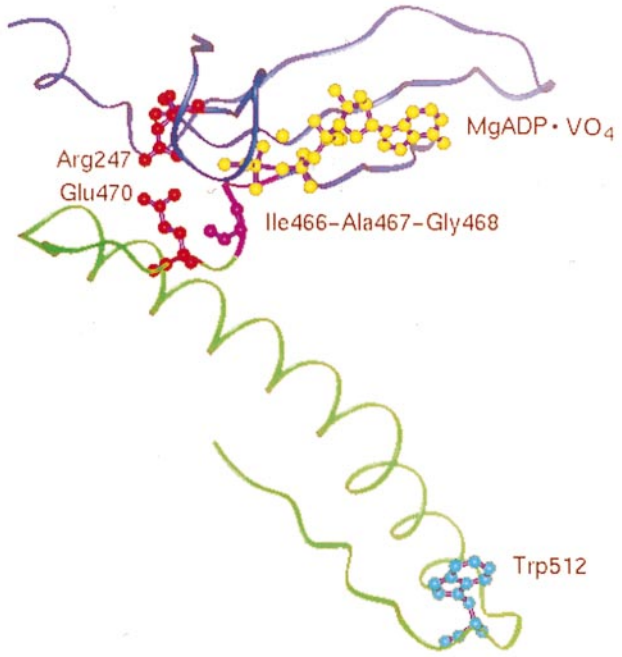

FIG. 8. The neighborhood of the Glu–Arg salt-bridge, and the "sensitive" tryptophan. The entire structure of the truncated myosin head from *Dictyostelium* ligated at the active site with MgADP·VO_4 was solved by Smith and Rayment (4). To get an idea of how this neighborhood might look in the smooth muscle heavy meromyosin used in this work, the residues located by Smith and Rayment have been labeled as though they were the conserved homologs of the smooth muscle sequence (17). Backbone atoms of the 235–278 and 458–465 sequences of the heavy chain are colored blue, and the 469–521 sequence is colored green. Residues Ile-466–Ala-467–Gly-468 are colored magenta. Residues 247 (in red), 470 (in red), and 512 (in cyan) are shown as balls and sticks, as is the analog MgADP·VO_4 (in yellow), simulating MgADP·P_i.

noted above, their defects cannot be attributed to imperfect phosphorylation (data not shown).

Because the E470R/R247E HMM is functional, it seems reasonable to attribute defects in the single-mutant HMMs to lack of an integral bridging member. Mutations E470R and R247E both lack such a member; both replace the integrity of either wild-type or reverse bridges by repulsive disruptions certain to preclude such a member. This repulsive strain could interfere with effects transmitted to a responsive Trp. In fact, we found that these mutants give no hydrolysis and also null fluorescence enhancements (just "background" fluorescence). The mutation E470A removes a negative charge from one of two constituents of the bridge, so that the bridge formation is impaired but no repulsive strain is generated. Therefore, the mutation totally blocks hydrolysis,but does not impair communication with a responsive Trp (which then produces fluorescence enhancement corresponding to M·ATP*). On the other hand, interpretation of R247A may be rather complicated. This mutant shows some detectable ATPase activity (≈40% wild type), but no phosphate burst. This result suggests that the reaction mechanism of this ATPase is greatly different from the wild-type mechanism. In contrast to E470A, this mutant give null fluorescence enhancement. Simple ideas cannot explain the effect of losing Arg, because the mutation R247A should not generate the repulsive strain described above. Although Arg-247 is not directly connected to a responsive Trp (see Fig. 8), we tentatively assume that the local field of Arg-247 may have some significance for the communication with a responsive Trp. This speculation has to be followed by a more serious structural examination, by energy minimization, and by electrostatic consideration. We note in passing the careful report of Shimada *et al.* (25) using *Dictyostelium* myosin, who studied R238A (corresponding to chicken smooth muscle R247A) and neighboring single mutations. Their results differ from ours in detail, though not in general thrust; however, by single mutation studies it was not possible to recognize the essentiality of the bridging member for function.

In summary, two important inferences from crystallography concerning the functional significance of certain structures have been borne out by observing systems that were mutated and then compared with the fully functional normal system. In the hydrolytic transition $M{\cdot}ATP + H_2O \rightarrow M{\cdot}ADP{\cdot}P_i$, it was inferred that Gly-468 participated in a crucial rotation (3); it was found that in G468A neither hydrolysis nor phosphate burst could occur (5). It was inferred that formation of a salt-bridge between Arg-247 and Glu-470 was required for hydrolysis (and its accompaniments) (3). It was found here that single mutations impaired the hydrolysis function, but that salt-bridge formation (even in reverse order) restored (intrinsic) function. It is reasonable to suppose that both the rotation and the bridge formation have important functions in energy transduction.

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