Corrections

CHEMISTRY, GENETICS. For the article “Genomewide studies of histone deacetylase function in yeast” by Bradley E. Bernstein, Jeffrey K. Tong, and Stuart L. Schreiber, which appeared in number 25, December 5, 2000, of Proc. Natl. Acad. Sci. USA (97, 13708–13713; First Published November 28, 2000; 10.1073/pnas.250477697), the authors note the following corrections. As a result of an error at the proof stage, there is a shift in the references. Ref. nos. 9–16 and 31–34 in the text should be 10–17 and 30–33, respectively.

BIOCHEMISTRY. For the article “Mapping the intrinsic curvature and flexibility along the DNA chain” by Giampaolo Zaccheri, Anita Scipioni, Valeria Calviere, Giuseppe Gargiulo, Pasquale De Santis, and Bruno Samori, which appeared in number 6, March 13, 2001, of Proc. Natl. Acad. Sci. USA (98, 3074–3079; First Published February 27, 2001; 10.1073/pnas.051631198), the authors note the following correction. In the last part of the Discussion, the following DNA base steps were termed incorrectly: AT→TA should be AT→TA; TA→AT should be TA→TA; CG→GC should be CG→CG; and GC→CG should be GC→CG. The other sections and the figure legends are not affected.

ANTHROPOLOGY. For the article “A highly variable segment of human subterminal 16p reveals a history of population growth for modern humans outside Africa” by Santos Alonso and John A. L. Armour, which appeared in number 3, January 30, 2001, of Proc. Natl. Acad. Sci. USA (98, 864–869; First Published December 19, 2000; 10.1073/pnas.011244998), the authors note the following corrections. Table 1 on page 865 was misaligned; therefore, a corrected table is printed below. In addition, the circle representing lineage e in Fig. 1b should be split into two sections to indicate equal representation of this haplotype in Pygmies and Kenyans.

Table 1. Polymorphic positions

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Dots represent the same state as in the ancestor sequence. + and – in polymorphism number 5 represent presence or absence of a 5-bp motif, respectively. Abbreviations: B, Basques; J, Japanese; K, Kenyans; P, Pygmies; and U, U.K.
BIOCHEMISTRY. For the article “Functional transitions in myosin: Formation of a critical salt-bridge and transmission of effect to the sensitive tryptophan” by Hirofumi Onishi, Shin-ichiro Kojima, Kazuo Katoh, Keigi Fujiwara, Hugo M. Martinez, and Manuel F. Morales, which appeared in number 12, June 9, 1998, of Proc. Natl. Acad. Sci. USA (95, 6653–6658), the authors note the following correction. Recently, it has been discovered that the heavy meromyosin (HMM) mutant described as E470R/R247E HMM was actually P548G HMM. Examination and subsequent experiments with authentic E470R/R247E HMM revealed that although its tryptophan fluorescence is increased upon addition of ADP or ATP, its intrinsic ATPase at all ATP concentrations examined, 0.5–4 mM, was far less than that of wild type. As before, it was not actin-activated. Therefore, our revised conclusions are: (i) our observations do not conflict with Raymont’s suggestion that at some stage preceding hydrolysis, bridge formation occurs; (ii) for fluorescence enhancement, the reversed dipole of the mutant is at least partly effective; (iii) although ATP binds as suggested by the partial tryptophan enhancement, the salt bridge does not form properly, so hydrolysis is therefore precluded; and (iv) we cannot deduce anything about actin activation because intrinsic ATPase is absent. It seems that in E470R and R247E HMMs, electrical repulsion precludes bridge formation, and therefore, hydrolysis.

www.pnas.org/cgi/doi/10.1073/pnas.1111491998

CELL BIOLOGY. For the article “Integrin-mediated mechanotransduction requires its dynamic interaction with specific extracellular matrix (ECM) ligands” by Shila Jalali, Miguel A. del Pozo, Kuang-Den Chen, Hui Miao, Yi-Shuan Li, Martin A. Schwartz, John Y.-J. Shyy, and Shu Chien, which appeared in number 3, January 30, 2001, of Proc. Natl. Acad. Sci. USA (98, 1042–1046; First Published January 23, 2001; 10.1073/pnas.031562998), the authors note the following correction. The first sentence on page 1045, right column, fourth paragraph, lines 1–3, reads, “Recent study (23) has indicated that vascular endothelial growth factor receptor (VEGF-R) may be involved in integrin/Shc association.” This sentence should be changed to read, “Recent study (23) indicated that vascular endothelial growth factor receptor (VEGF-R) may interact with integrin for VEGF signaling.”

www.pnas.org/cgi/doi/10.1073/pnas.111142899

GENETICS. For the article “Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10” by Hyong Doo Shin, Cheryl Winkler, J. Claiborne Stephens, Jay Bream, Howard Young, James J. Goedert, Thomas R. O’Brien, David Vlahov, Susan Buchbinder, Janis Giorgi, Charles Rinaldo, Sharyne Donfield, Anne Willoughby, Stephen J. O’Brien, and Michael W. Smith, which appeared in number 26, December 19, 2000, of Proc. Natl. Acad. Sci. USA (97, 14467–14472), the authors note the following: “The discovery described by Shin et al. in this paper was the subject of U.S. patent application no. PCT/US00/09355 filed on behalf of the U.S. Department of Health and Human Services on April 9, 1999, and internationally on April 6, 2000. M. W. Smith, H. D. Shin, and S. J. O’Brien are listed as inventors on the patent.”

www.pnas.org/cgi/doi/10.1073/pnas.101139899

MEDICAL SCIENCES. For the article “Heparin and cancer revisited: Mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis” by Lubor Borsig, Richard Wong, James Feramisco, David R. Nadeau, Nissi M. Varki, and Ajit Varki, which appeared in number 6, March 13, 2001, of Proc. Natl. Acad. Sci. USA (98, 3352–3357), the authors note the following correction. On page 3355, the URL for nearcount software was incorrectly listed as http://vis.sdsc.edu. The correct URL is http://vis.sdsc.edu.

www.pnas.org/cgi/doi/10.1073/pnas.111148898

MICROBIOLOGY. For the article “Efficient use of a small genome to generate antigenic diversity in tick-borne ehrlichial pathogens” by Kelly A. Brayton, Donald P. Knowles, Travis C. McGuire, and Guy H. Palmer, which appeared in number 7, March 27, 2001, of Proc. Natl. Acad. Sci. USA (98, 4130–4135), the authors note the following correction. The correct address for Donald P. Knowles is Animal Disease Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Pullman, WA 99164-6630.

www.pnas.org/cgi/doi/10.1073/pnas.111153498

SPECIAL FEATURE, MICROBIOLOGY. For the article “Chains of magnetite crystals in the meteorite ALH84001: Evidence of biological origin” by E. Imre Friedman, Jacek Wierzchos, Carmen Ascaso, and Michael Winklhofer, which appeared in number 5, February 27, 2001, of Proc. Natl. Acad. Sci. USA (98, 2176–2181), the authors note the following correction. On page 2178, left column, 2nd line from the bottom, insert after “fractured”, “along existing microscopic cracks to expose carbonate globules.”

www.pnas.org/cgi/doi/10.1073/pnas.0911151998

NEUROBIOLOGY. For the article “Dopamine D1/D5 receptor modulation of excitatory synaptic inputs to layer V prefrontal cortex neurons” by Jeremy K. Seamans, Daniel Durstewitz, Brian R. Christie, Charles F. Stevens, and Terrence J. Sejnowski, which appeared in number 1, January 2, 2001, of Proc. Natl. Acad. Sci. USA (98, 301–306; First Published December 26, 2000; 10.1073/pnas.011518798), the authors note the following correction. Line 10 of the abstract should read: “With 20Hz synaptic trains we found that the D1/D5 agonists increased the depolarization produced by summatng NMDA excitatory postsynaptic potentials (EPSPs).” In addition, on line 3 of page 305, “signal” should be “single.”

www.pnas.org/cgi/doi/10.1073/pnas.0911152998

PHYSIOLOGY. For the article “Choline acetyltransferase mutations cause myasthenic syndrome associated with episodic apnea in humans” by Kinji Ohno, Akira Tsujino, Joan M. Brengman, C. Michel Harper, Zeljko Bajzer, Bjarne Udd, Roger Beyring, Stephanie Robb, Fenella J. Kirkham, and Andrew G. Engel, which appeared in number 6, March 13, 2001, of Proc. Natl. Acad. Sci. USA (98, 2017–2022), the authors note the following correction. Dr. Xin-Ming Shen’s name and affiliation were inadvertently omitted from the list of authors. Dr. Shen’s affiliation is Department of Neurology and Neuromuscular Research Laboratory, Mayo Clinic, Rochester, MN 55905. The corrected list of authors is: Kinji Ohno, Akira Tsujino, Xin-Ming Shen, Joan M. Brengman, C. Michel Harper, Zeljko Bajzer, Bjarne Udd, Roger Beyring, Stephanie Robb, Fenella J. Kirkham, and Andrew G. Engel.

www.pnas.org/cgi/doi/10.1073/pnas.101139998
Functional transitions in myosin: Formation of a critical salt-bridge and transmission of effect to the sensitive tryptophan

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Contributed by Manuel F. Morales, April 6, 1998

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) + H₂O → ADP-Pi (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.

Glu-470 and Arg-247 stabilizes a “rotated” state in the ADP-Pi-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 (Met1–Glu–729) of chicken gizzard HMM heavy chain (17), was used as a template. A GMH-6 derivative, within which a unique NcoI 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’-GAATGACAACCTCTCCCGTTT-3’ and 3’-GATATTGCTGAGATTTGACATT-5’.

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

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Abbreviations: HMM, heavy meromyosin; S1, subfragment 1; AMP-PNP, adenosine 5’-(γ-ribo)triphosphate.
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‡e-mail: hiro@sonic.net.
§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.
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 S9 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. L4). 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\textsuperscript{2+} resulted in phosphorylation of their regulatory light chains (Fig. 1B).

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, (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 5'-(γ-thio) triphosphate (ATPγS), ADP, or AMP-PNP.

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 4). Light chain phosphorylation was performed in the presence (+) or absence (−) of myosin light chain kinase, calmodulin, and Ca\textsuperscript{2+} for 30 min. Samples were analyzed by SDS/PAGE on 15% gels and then subjected to autoradiography (B). HC, HMM heavy chain; LC\textsubscript{α}, regulatory light chain; and LC\textsubscript{β}, essential light chain.
significant difference in the apparent binding constant to actin HMMs. Double reciprocal plots (Fig. 6) show that there is no concentration for both the wild-type and the double-mutant
we also measured the dependence of ATPase activity on actin caused by a weak affinity of the double-mutant HMM for actin, wild-type HMM. To test if the reduced actin activation is
ably activated by actin, but at a level only 1
single-mutant HMMs, viz. E470A, R247A, E470R, and
activation was observed in the ATPase activities of the four
HMM and the five mutant HMMs (data not shown). No actin
fluorescence (see Fig. 5).

Rigor Complex Formation of Mutant HMMs with Actin and its Dissociation by ATP. Electron microscope observations indicate that the R247E (Fig. 7A), the E470R (Fig. 7B), the R247E (Fig. 7C), and the E470R/R247E (Fig. 7D) 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 was 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·P₄, whereas the constituent single-mutant HMMs, E470R and R247E, have no steady-state ATPase activity, no phosphate burst, and no 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. 1B).

**Fig. 2.** Intrinsic ATPase activities of wild-type (WT) and five mutant HMMs. Assay conditions were 0.24 mg/ml of each of wild-type (○), E470A (□), R247A (△), E470R (▲), and E247E (▼) HMMs, 0.45 M KCl, 2 mM MgCl₂, 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 (▲), and E247E (▼) HMMs, 0.45 M KCl, 2 mM MgCl₂, 20 mM Tris·HCl (pH 7.5), 0.5 mM DTT, and 4 μM [γ⁻³²P]ATP. Burst sizes for wild-type, E470A, R247, E470R, and E247E, and E470R/R247E HMMs were 0.68, 0.00, 0.00, 0.00, and 0.70 mol of Pᵢ/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·Pᵢ, 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·P₄ (see Fig. 5).

Upon adding ATP or AMPPNP, the fluorescence of E470A HMM was enhanced to the same level (18%) (Fig. 4B). 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. 4C), the E470R (Fig. 4D), and the R247E (Fig. 4E) 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 (Kₒ), 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
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 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\text{z}\text{ATP type}) and in the MgADP-vanadate (M\text{z}\text{ADP}_{2}\text{Pi 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. 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, interact with ATP to form a weakly bound state for actin, although no ATP-induced fluorescence enhancement is associated.
Although the intrinsic ATPase of E470R\(^{y}\)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.

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

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

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.

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. 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.)

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 \(\times\) 10\(^4\) M\(^{-1}\), respectively, for wild-type HMM, and 8 nmol P\(_i\)/min/mg and 0.74 \(\times\) 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.

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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-ATP + H₂O → M-ADP-P₇, 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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