THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 22, Issue of June 1, pp. 19491–19494, 2001
Printed in U.S.A.

The Myosin Relay Helix to Converter Interface Remains Intact throughout the Actomyosin ATPase Cycle*

Received for publication, December 4, 2000, and in revised form, February 20, 2001
Published, JBC Papers in Press, February 21, 2001, DOI 10.1074/jbc.M010887200

William M. Shih‡ and James A. Spudich§

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Crystal structures of the myosin motor domain in the presence of different nucleotides show the lever arm domain in two basic angular states, postulated to represent prestroke and poststroke states, respectively (Rayment, I. (1996) *J. Biol. Chem.* 271, 15850–15853; Dominguez, R., Freyzon, Y., Trybus, K. M., and Cohen, C. (1998) *Cell* 94, 559–571). Contact is maintained between two domains, the relay and the converter, in both of these angular states. Therefore it has been proposed by Dominguez *et al.* (cited above) that this contact is critical for mechanically driving the angular change of the lever arm domain. However, structural information is lacking on whether this contact is maintained throughout the actin-activated myosin ATPase cycle. To test the functional importance of this interdomain contact, we introduced cysteines into the sequence of a "cysteine-light" myosin motor at position 499 on the lower cleft and position 738 on the converter domain (Shih, W. M., Gryczynski, Z., Lakowicz, J. L., and Spudich, J. A. (2000) *Cell* 102, 683–694). Disulfide cross-linking could be induced. The cross-link had minimal effects on actin binding, ATP-induced actin release, and actin-activated ATPase. These results demonstrate that the relay/converter interface remains intact in the actin strongly bound state of myosin and throughout the entire actin-activated myosin ATPase cycle.

Myosins are molecular motors that transduce the chemical energy of ATP hydrolysis to mechanical work in the form of the vectorial translocation of substrate actin filaments. In the swinging lever arm model of actin-myosin motor action, myosin binds to the actin with its globular catalytic domain and then rotates its carboxyl-terminal lever arm domain (reviewed in Ref. 5). The anchoring of the end of the lever arm domain results in the translocation of the catalytic domain and the attached actin filament.

Crystal structures of the motor domain of myosin show the lever arm domain in two basic angular classes, which have been postulated to represent prestroke and poststroke states (2, 3, 6). Scallop myosin complexed with ADP has been crystallized in a third angular state, proposed as a myosin·ATP actin-detached state (7). Recent dynamic studies of the lever arm position using steady state and time-resolved fluorescence energy transfer measurements support a swing of the lever arm from a prestroke state to a poststroke state through an angle of more than 70 degrees (4). The lever arm domain consists of a disc-shaped "converter domain" from which a long α-helix, bound by two calmodulin-like light chains, emerges. In both crystal states, the converter domain maintains a contact on a face of its radial edge to a rigid helix extending from the lower domain of the large cleft, referred to as the relay helix (3). This helix has to undergo a small conformational change, primarily a rigid body translation and rotation, to accommodate the angular rotation of the converter domain. Because this domain-domain interface is maintained between the two angular states, it has been proposed that this interface is important for mechanically driving the angular change. However, structural information is lacking as to whether this contact is maintained throughout the actin-activated myosin ATPase cycle, including in an actin strong binding state.

We have used a cysteine engineering approach to address the question as to whether the relay/converter contact is maintained in the actin strongly bound state. We constructed myosinII alleles containing cysteine-light mutations (C49S, C312Y, C442S, C470I, C599L, and C678Y) (4) and substituted cysteine codons into positions corresponding to either residue 499 or 738 or both (Fig. 1). We show that the mutant myosins are functional *in vivo* and *in vitro*. The cross-linked myosin (containing cysteines at both positions 499 and 738) retained the ability to bind to actin in the absence of ATP as well as the ability to be released from actin in the presence of ATP. The cross-linked myosin also retained actin-activated ATPase activity.

MATERIALS AND METHODS

Nomenclature—The changes made in a given mutant myosin are described within parentheses. For example, myosin(CL, I499C) refers to a full-length myosin gene with the cysteine-light (CL)[1] mutations (C49S, C312Y, C442S, C470I, C599L, and C678Y) (4) and the mutation I499C.

Mutagenesis and Subcloning—Subcloning procedures were carried out using standard protocols (8). myosin(CL, I499C), myosin(CL, R738C), and myosin(CL, I499C, R738C) were generated by splice overlap extension mutagenesis (9) using myosin(CL) as a template. Myosin genes were subcloned into the expression vector pTIKL-Myo. The introduced mutations were verified by dideoxy-DNA sequencing. The S1 gene fragments were then subcloned into pTIKLOES1, an expression vector for producing S1 with a carboxyl-terminal His_6 tag on the heavy chain.

The following oligonucleotides were used for mutagenesis: I499C-F, 5′-TATCTTAAAGAGAAATGTAATTGGACTTTCATC-3′; I499C-R, 5′-GATGAAAGTCCAATTACATTTCTCTTTAAGATA-3′; R738C-F, 5′-GATCCAGAACAATATTGTTTCGGTATCACCAAG-3′; and R738C-R, 5′-CTTGGTGATACCGAAACAATATTGTTCTGGATC-3′.

Transformation into Dictyostelium Cells—*Dictyostelium* cells were grown in HL-5 medium as described previously (10). Cells were grown at 22 °C in HL-5 supplemented with 17% FM medium (Life Technologies, Inc.), 100 units/ml penicillin, and 100 units/ml streptomycin.

* This work was supported by Grant AR42895 from the National Institutes of Health (to J. A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a Howard Hughes Predoctoral Fellowship.

§ To whom correspondence should be addressed: Dept. of Biochemistry, Beckman Center B400, Stanford University School of Medicine, Stanford, CA 94305-5307. Tel.: 650-723-7634; Fax: 650-723-6783; E-mail: jspudich@cmgm.stanford.edu.

[1] The abbreviations used are: CL, cysteine-light; PAGE, polyacrylamide gel electrophoresis.

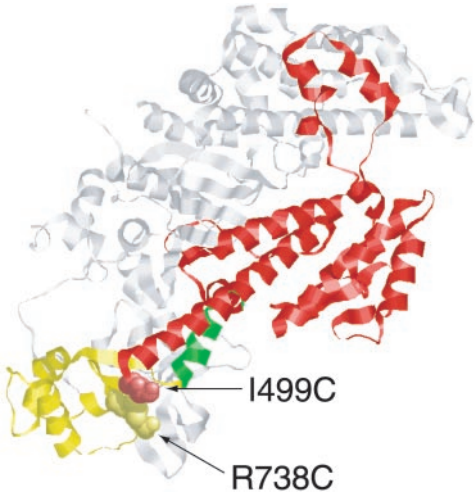

FIG. 1. **Introduction of two cysteines into the structure of *Dictyostelium* myosinII.** The structure of the *Dictyostelium* myosinII catalytic domain complexed with ADP·BeF_x is depicted (1mmd in the PDB data base). The lower jaw of the large cleft is colored *red*, and the converter domain is colored *yellow*. The SH1-SH2 helix is colored *green*. The actin binding face is on the *right*, and the lever arm would extend from the structure on the *lower left*. Ile^{499} from the relay helix and Arg^{738} from the converter domain are rendered in the Corey-Pauling-Koltun space filling model. These two positions were mutagenized to cysteines for this study.

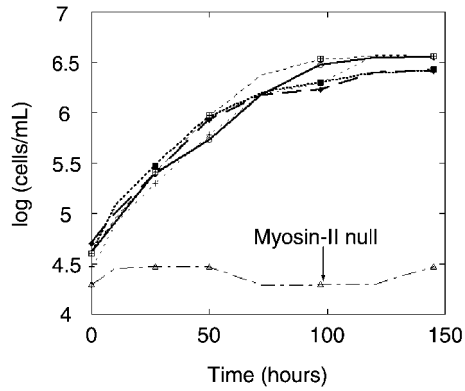

FIG. 2. ***Dictyostelium* growth in suspension.** *Plus signs*, wild type myosin; *open circles*, myosin(CL); *dark diamonds*, myosin(CL, I499C); *dark boxes*, myosin(CL, R738C); *tetrasected boxes*, myosin(CL, I499C, R738C); and *open triangles*, myosinII null. All myosinII alleles tested in this study rescued the growth-in-suspension defect exhibited by the myosinII null *Dictyostelium* cells.

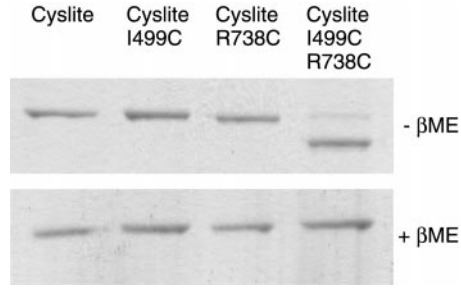

FIG. 3. **Effect of disulfide cross-linking on the electrophoretic mobility of cysteine-engineered myosin motor domains.** Purified S1 proteins were treated with 25 μM 5,5′-dithiobis(nitrobenzoic acid) and then analyzed by SDS-PAGE. Only the S1 with cysteines at both positions 499 and 738 exhibited increased mobility upon treatment with the cross-linking reagent. This mobility shift was reversed when the S1 was electrophoresed in the presence of the reducing agent β-mercaptoethanol. *Cyslite*, cysteine light.

Transformations were performed as described previously (11). The *mhcA* null cell line HS1 was transformed with 10 μg of each of the pTIKL-Myo plasmids bearing wild type or mutant versions of the full-length myosin, whereas the AX3-ORF+ cell line was transformed with 10 μg of each of the pTIKLOES1 plasmids bearing wild type or mutant versions of the S1 fragment of myosin. Clonal cell lines that grew in the presence of HL-5 supplemented with penicillin, streptomycin, and 8 μg/ml G418 were isolated, and these cell lines were further characterized.

Growth in Suspension Assay—Cells were grown on plates to near confluence before they were transferred to shaking flasks. Cells were diluted to 4×10^4 cells/ml in 25 ml of total volume HL-5 in 125-ml Erlenmeyer flasks and shaken at 200 rpm at 22 °C for 6 days. A small aliquot was removed at regular intervals, and the number of cells was counted using a hemocytometer.

Protein Purification—Dictyostelium S1 His_6 was expressed in *Dictyostelium* AX3-ORF+ cells (grown in suspension) and purified as described (12).

Actin-activated ATPase Assay—S1 ATPase activities were measured as the release of labeled P_i using γ-^{32}P, as reported previously (12). The *plotted points* and *error bars* of Fig. 5 represent measurements from three independent trials for each of two different protein preparations.

Cross-linking Assay—Cross-linking was induced by the addition of 25 μM 5,5′-dithiobis(nitrobenzoic acid) to S1 at a concentration of 1–2 μM. The buffer conditions were 25 mM HEPES, pH 7.0, 25 mM NaCl, and 10 mM $MgCl_2$. The cross-linking reaction was quenched by the addition of 1 mM dithiothreitol. (The Cys^{499}-Cys^{738} disulfide cross-link is not reduced by 1 mM dithiothreitol under nondenaturing conditions.) Disulfide cross-linking was assayed by mobility shift in SDS-PAGE behavior in the absence of reducing agent. Confirmation of disulfide bond formation was made by SDS-PAGE analysis in the presence of reducing agent reversing the mobility shift.

Actin Cosedimentation Assay—The buffer conditions used were 25 mM HEPES, pH 7.0, 25 mM NaCl, and 10 mM $MgCl_2$. For the noncompetitive assay, S1 (0.8 μM final concentration) was mixed with F-actin (3.0 μM final concentration) for 10 min, and then the mixture was centrifuged at 100,000 $\times$ *g* for 10 min. The supernatant and the resuspended pellet were examined by SDS-PAGE to determine whether the S1 cosedimented with the F-actin. The same assay was repeated in the presence of 2 mM Mg-ATP. For the competitive assay, the same procedure was used but with S1 at a final concentration of 1.3 μM and F-actin at a concentration of 0.5 μM.

RESULTS

The Cysteine Mutant MyosinII Is Functional in Vivo—Dictyostelium cells that lack the myosinII gene are unable to divide in suspension, instead becoming large and multinucleate before eventually lysing and dying (13, 14). Thus the transformation of the mutant myosinII gene into these myosinII null cells can lead to a simple assay for *in vivo* function (assaying for the rescue of the growth in suspension defect). The ability to rescue an *in vivo* defect serves as a useful benchmark to demonstrate that mutant myosins behave in a functional manner. The design of this experiment relies on the un-cross-linked double-cysteine mutant myosin to behave in a functional manner and then to assay for biochemical differences upon specific cross-linking.

Arg^{738} (with its β and γ carbons) and Ile^{499} form part of the hydrophobic interface between the relay helix and the converter domain, respectively (Fig. 1). Disruption of this hydrophobic interface thus could potentially be destabilizing for the myosin. Cysteines are relatively hydrophobic, however, and should be good candidates for replacement side chains for packing in the interface.

Fig. 2 shows a growth curve examining the growth of *Dictyostelium* cells that were missing the genomic copy of myosinII but were supplied with another copy on an extrachromosomal plasmid. All of the mutant myosinII genes introduced (myosin(CL), myosin(CL, I499C), myosin(CL, R738C), and myosin(CL, I499C, R738C)) rescued growth in suspension to a rate comparable with that of the wild type. The parent strain lacking a copy of myosinII, however, failed to grow in suspension. Therefore it appears that the introduction of either cysteine,

both individually or in tandem, is well tolerated by the structure of myosin.

A Specific Cross-link Is Inducible between Introduced Cysteines at Positions 499 and 738—According to the crystal structures available, the side chains of residues at positions 499 and 738 are in close proximity (2, 15). Previous studies have shown that cysteines placed at nearby positions in a structure usually can be induced to form a disulfide cross-link, which is catalyzed either by ambient oxygen or by a disulfide exchange reagent (16). The cross-linking of two residues in the structure of a protein that are separated by a large number of residues results in a covalently closed large loop within the primary sequence. Denatured proteins containing such a loop might be expected to exhibit a different mobility during gel electrophoresis; examples have been found where cross-linking induces either a gel mobility increase (17) or a gel mobility decrease (18). Fig. 3 shows that treatment with the disulfide exchange reagent dithionitrobenzoate induces an SDS-PAGE mobility increase in over 85% of a cysteine-light myosin S1 only when cysteines are introduced at both positions 499 and 738 but not when the cysteines are introduced individually. This gel mobility shift is reversible if the protein is loaded onto the gel in the presence of a reducing agent such as β-mercaptoethanol. Thus a specific cross-link is formed between introduced cysteines at positions 499 and 738.

Curiously, the disulfide cross-link that is formed is quite robust against reduction under native conditions. Overnight treatment in the presence of 1 mM dithiothreitol, 1 mM β-mercaptoethanol, or 1 mM tricarboxyethylphosphine followed by passage through a Sephadex spin column (to remove the dithiothreitol) fails to significantly reduce the disulfide, as analyzed by SDS-PAGE (data not shown). Only after SDS denaturation does the disulfide become accessible to reduction. In the absence of the disulfide exchange reagent and reducing agents, the un-cross-linked protein is converted slowly to the cross-linked form over a period of weeks when stored at 4 °C, presumably catalyzed by ambient dissolved oxygen (data not shown).

Strong Actin Binding (in the Absence of ATP) and ATP Disruption of This Strong Binding State Are Not Perturbed by Cross-linking the Introduced Cysteines—Wild type S1 precipitates with F-actin in the absence of ATP during centrifugation at sufficient speeds. Small, unattached proteins sediment much more slowly and therefore remain in the supernatant. This assay can be used to investigate the binding of S1 to actin. The property of the cross-linked form of S1 (that it migrates more rapidly during SDS-PAGE, relative to the un-cross-linked form) lends itself as a special advantage in the F-actin cosedimentation assay because this assay allows for the analysis of the supernatant (actin-detached) and pellet (actin-attached) fractions using SDS-PAGE. Thus the behavior of the two forms can be analyzed simultaneously in the same sample volume.

Fig. 4*A* shows S1 binding to F-actin in the presence of excess F-actin and in the absence of ATP. Both the cross-linked and un-cross-linked forms of S1 cosediment with the F-actin. Fig.

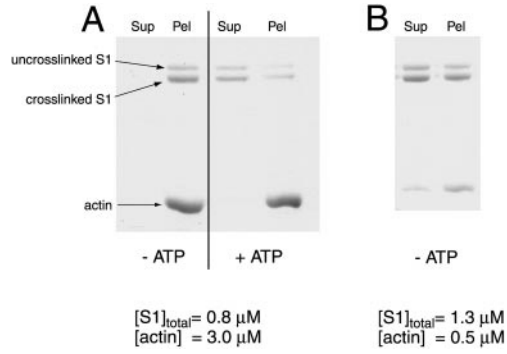

FIG. 4. **Actin cosedimentation assays.** The SDS-PAGE lanes containing supernatant samples are labeled *Sup*, whereas the gel lanes containing pellet samples are labeled *Pel*. *A*, both the cross-linked and un-cross-linked forms of S1(CL, I499C, R738C) bind to F-actin and are released by ATP, in the presence of excess F-actin. *B*, the cross-linked and un-cross-linked forms of the S1(CL, I499C, R738C) have a similar affinity to F-actin, as analyzed by actin cosedimentation using substoichiometric amounts of F-actin.

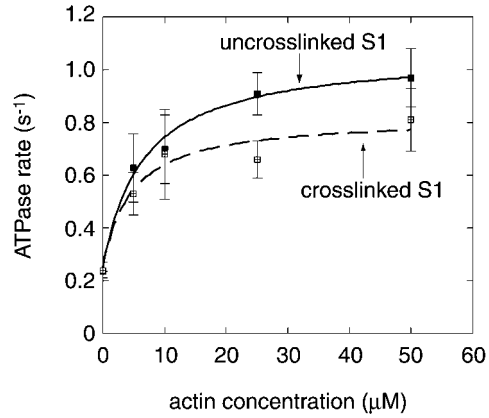

FIG. 5. **Actin-activated ATPase assays.** The cross-linked and un-cross-linked forms of the S1(CL, I499C, R738C) exhibit similar basal ATPase rates (0.24 s^{-1}). The cross-linked S1 exhibits an actin-activated ATPase (V_{max} = 0.82 s^{-1}) that is 30% less than the activity of the un-cross-linked S1 (V_{max} = 1.06 s^{-1}). The cross-linked S1 exhibits a K_m for the actin activation (K_m = 4.6 μM) about 30% lower than that shown by the un-cross-linked S1 (K_m = 6.5 μM).

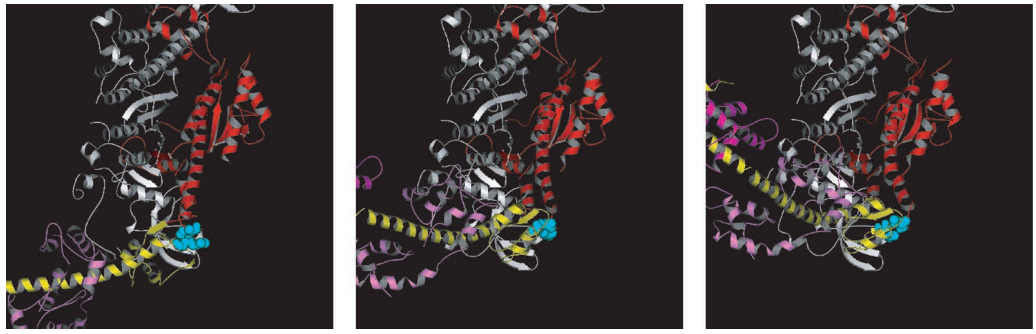

FIG. 6. **Conformational changes in the relay helix to converter domain interface.** Shown here are three successive *frames*, going from *left* to *right*, depicting the myosin S1 conformational change in transforming from the putative poststroke angle state (based on 1mmd in the PDB data base) to the putative prestroke angle state (based on 1vom in the PDB data base). The *second frame* was generated as described (4). The relay helix (colored *red*), which forms an interface with the converter domain (colored *yellow*), undergoes a rotation and translation. This change drives the attached converter domain to rotate and translate. This results in a large movement upward of the lever arm α-helix (attached to the converter domain and also colored *yellow*). The action is reminiscent of a hand turning a steering wheel. Residues 499 and 738 are colored in *cyan*.

4*A* also shows S1 binding to F-actin in the presence of excess F-actin and ATP. Both the cross-linked and un-cross-linked forms of S1 are similarly released from the actin by the ATP. Fig. 4*B* shows S1 binding to F-actin in the presence of limiting F-actin. If the cross-linked form of S1 had a significantly different binding affinity to F-actin as compared with the un-cross-linked form of S1, then the ratio of the un-cross-linked to cross-linked bands should be significantly different in the supernatant and pellet lanes. However, Fig. 4*B* shows that no significant difference is evident. Thus the cross-link does not perturb the binding of myosin to F-actin in its strong affinity mode.

Cross-linking the Introduced Cysteines Results in a Modest Decrease in the Actin Activation of the ATPase of Myosin—The rate-limiting step in the ATPase cycle of myosin in the absence of F-actin is the product release (19). The presence of F-actin stimulates the myosin to release its products more rapidly (20). We examined the actin-activated ATPase activity of the cross-linked form *versus* the un-cross-linked form to see if the actin stimulation of product release might be impaired. Fig. 5 shows that the actin activation of the cross-linked species is decreased by 29% and that the K_m for the actin activation is decreased by 29%. The basal rate of ATP hydrolysis (*i.e.* in the absence of F-actin) is the same in the cross-linked and un-cross-linked forms. Thus phosphate release in the cross-linked myosin is still stimulated by F-actin, although the amount of the stimulation is slightly decreased.

DISCUSSION

The crystal structures of the *Dictyostelium* myosinII catalytic domain have been solved in complex with a number of nucleotide analogues, including ADP beryllium fluoride (BeF_x), ADP aluminum fluoride (AlF_4^-), and ADP vanadate (V_i), all thought to mimic myosin states with a weak affinity for actin. It is only upon phosphate release that the myosin can adopt a conformation that allows it to bind to actin with a strong affinity. The crystal structures fall into two basic conformational classes: one that is thought to represent the myosin with its lever arm in a putative poststroke angle and one that is thought to represent the myosin with its lever arm in a putative prestroke angle.

The lever arm extends off from a 60-residue globular domain, often referred to as the converter domain. This converter domain shares in both structures a hydrophobic interface with the back end of the lower jaw of the large cleft of the catalytic domain, referred to as the relay helix, despite a 70° rotation of the converter domain between the two structures. Fig. 6 shows this interface in the two structures, as well as in an intermediate structure that was generated as an interpolation between the crystal structure models (4). Inspection of the crystal structures suggests a mechanical picture describing the sequence of forces that are behind the power stroke. It has been proposed that changes in the nucleotide binding site, triggered by the formation of a bond between the backbone amide of switch II residue Gly^{457} with the γ-phosphate in the prestroke state, are amplified into a larger change in the position of the relay helix. This change drives a rotation and translation of the lever arm through its converter domain (3, 15).

These structures, however, are thought to represent myosin in conformations that have a weak affinity to actin (21). Myosin undergoes a conformational change that allows it to bind to actin with a much higher affinity; this conformational change in myosin can be monitored by the pyrene labeling of Cys^{374} on actin (22) or with mant-ADP in the active site of the myosin (23). These spectroscopic probes, however, give no structural details of the nature of this conformational change. Therefore currently there is no experimental evidence that allows one to build a reliable model of myosin in its strong affinity to actin state.

One proposal has been that the large cleft completely closes to achieve this strong affinity to actin state (1, 6). One possible extension of this model is that the complete closure of the large cleft causes the relay helix to slip away from the converter domain. The converter domain, after its release from the relay helix, snaps back to its poststroke conformation, thus completing the power stroke. In this model, the lever arm behaves as a torque spring that is wound by 70° by the back end of the lower jaw and then suddenly released once actin strong binding has been achieved.

Another model is that the interface between the converter domain and the relay helix is maintained in the strong actin affinity state of myosin as well. In this case, the relay helix may mechanically transmit the changes to the lever arm both in the actin-detached stages (the recovery stroke) and in the actin-attached stages (the power stroke).

If the first model were correct (that the interface must slip), then the cross-linking of the interface would prevent that slipping and should prevent the myosin from achieving its actin strong binding affinity state. On the other hand, if the second model were correct (that the interface is maintained during the whole cycle), then the cross-linking of the interface should have little or no effect on the transition to the strong affinity to actin state.

Our experiments demonstrate that cross-linking these two domains together through a cysteine at position 499 on the relay helix and a cysteine at position 738 on the converter domain does not inhibit myosin from achieving a strong affinity to actin state nor does it inhibit the effect of ATP in shifting the myosin back to the weak affinity to actin state. Thus our results are consistent with the model that the interface is maintained both in the actin-detached and actin-attached stages of the actomyosin ATPase cycle.

Acknowledgments—We thank András Málnási-Csizmadia and Clive Bagshaw for an advance copy of their manuscript and discussions. We thank Wen Liang for assistance with generating the growth curves, and we thank Doug Robinson for helpful discussions.

REFERENCES

1. Rayment, I. (1996) *J. Biol. Chem.* **271,** 15850–15853
2. Smith, C. A., and Rayment, I. (1996) *Biochemistry* **35,** 5404–5417
3. Dominguez, R., Freyzon, Y., Trybus, K. M., and Cohen, C. (1998) *Cell* **94,** 559–571
4. Shih, W. M., Gryczynski, Z., Lakowicz, J. L., and Spudich, J. A. (2000) *Cell* **102,** 683–694
5. Goldman, Y. E. (1998) *Cell* **93,** 1–4
6. Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G., and Holden, H. M. (1993) *Science* **261,** 50–58
7. Houdusse, A., Kalabokis, V. N., Himmel, D., Szent-Gyorgyi, A. G., and Cohen, C. (1999) *Cell* **97,** 459–470
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed, Cold Spring Harbor Laboratory Press, New York
9. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* (*Amst.*) **77,** 51–59
10. Murphy, C. T., and Spudich, J. A. (1998) *Biochemistry* **37,** 6738–6744
11. Egelhoff, T. T., Titus, M. A., Manstein, D. J., Ruppel, K. M., and Spudich, J. A. (1991) *Methods Enzymol.* **196,** 319–334
12. Giese, K. C., and Spudich, J. A. (1997) *Biochemistry* **36,** 8465–8473
13. De Lozanne, A., and Spudich, J. A. (1987) *Science* **236,** 1086–1091
14. Knecht, D. A., and Loomis, W. F. (1987) *Science* **236,** 1081–1086
15. Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., and Rayment, I. (1995) *Biochemistry* **34,** 8960–8972
16. Falke, J. J., and Koshland, D. E. J. (1987) *Science* **237,** 1596–1600
17. Careaga, C. L., and Falke, J. F. (1992) *J. Mol. Biol.* **226,** 1219–1235
18. Murai, N., Makino, Y., and Yoshida, M. (1996) *J. Biol. Chem.* **271,** 28829–28234
19. Lymn, R. W., and Taylor, E. W. (1970) *Biochemistry* **9,** 2975–2983
20. Lymn, R. W., and Taylor, E. W. (1971) *Biochemistry* **10,** 4617–4624
21. Werber, M. M., Peyser, Y. M., and Muhlrad, A. (1992) *Biochemistry* **31,** 7190–7197
22. Criddle, A. H., Geeves, M. A., and Jeffries, T. (1985) *Biochem. J.* **232,** 343–349
23. Woodward, S. K., Eccleston, J. F., and Geeves, M. A. (1991) *Biochemistry* **30,** 422–430

ENZYME CATALYSIS AND REGULATION:

The Myosin Relay Helix to Converter Interface Remains Intact throughout the Actomyosin ATPase Cycle

William M. Shih and James A. Spudich

J. Biol. Chem. 2001, 276:19491-19494.
doi: 10.1074/jbc.M010887200 originally published online February 21, 2001