Cryo-atomic Force Microscopy of Unphosphorylated and Thiophosphorylated Single Smooth Muscle Myosin Molecules*

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The purpose of this study was to determine whether steric blockage of one head by the second head of native two-headed myosin was responsible for the inactivity of nonphosphorylated two-headed myosin compared with the high activity of single-headed myosin, as suggested on the basis of electron microscopy of two-dimensional crystals of heavy meromyosin (Wendt, T., Taylor, D., Messier, T., Trybus, K. M., and Taylor, K. A. (1999) J. Cell Biol. 147, 1385–1390; and Wendt, T., Taylor, D., Trybus, K. M., and Taylor, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4361–4366). Our earlier cryo-atomic force microscopy (cryo-AFM) (Zhang, Y., Shao, Z., Somlyo, A. P., and Somlyo, A. V. (1997) Biophys. J. 72, 1308–1318) indicates that thiophosphorylation of the regulatory light chain increases the separation of the two heads of a single myosin molecule, but the thermodynamic probability of steric hindrance by strong binding between the two heads was not determined. We now report this probability determined by cryo-AFM of single whole myosin molecules shown to have normal low ATPase activity (0.007 s⁻¹). We found that the thermodynamic probability of the relative head positions of nonphosphorylated myosin was approximately equal between separated heads as compared with closely apposed heads (energy difference of 0.24 kT (where k is a Boltzman constant and T is the absolute temperature)), and thiophosphorylation increased the number of molecules having separated heads (energy advantage of ~1.2 kT (where k is a Boltzman constant and T is the absolute temperature)). Our results do not support the suggestion that strong binding of one head to the other stabilizes the blocked conformation against thermal fluctuations resulting in steric blockage that can account for the low activity of nonphosphorylated two-headed myosin.

Received for publication, June 10, 2003, and in revised form, July 28, 2003
Published, JBC Papers in Press, August 6, 2003, DOI 10.1074/jbc.M306094200

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* This work was supported by National Institutes of Health Grants EB002017 and HL48807. 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.

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The abbreviations used are: RLC, regulatory light chain; EM, electron microscopy; AFM, atomic force microscopy; HMM, heavy meromyosin; kT, k is a Boltzman constant and T is the absolute temperature; ATPyS, adenosine 5’-O-(thiotriphosphate).

This paper is available on line at http://www.jbc.org
would have to be sufficiently stable in the absence of activation, and, consequently, the energy cost of the transition from the blocked to the open state should be significantly higher than the thermal energy. Otherwise, if regulation were solely or primarily dependent on the blocked-head model, thermal fluctuation would separate the two heads, leading to nearly continuous activity (12). Our results suggest only a minimal energy difference between the closed and the open forms of dephosphorylated myosin and do not support the mechanism of regulation based on intramolecular occlusion by strong forces binding one head to the other.

EXPERIMENTAL PROCEDURES

Protein Preparation—Turkey gizzard myosin was purified as described previously (13) and kept at 4 °C in buffer A (0.3 M KCl, 10 mM KH₂PO₄, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, pH 6.8) for less than 2 weeks or stored in buffer A with 50% glycerol at −20 °C without noticeable differences in both the function and the structure of the protein. Before use, myosin was extensively dialyzed at 4 °C against buffer A or low salt buffer (20 mM KH₂PO₄, 1 mM MgCl₂, 1 mM EGTA, 120 mM KCl, pH 7.2) to remove glycerol. To eliminate undesirable “unregulated” molecules, the unphosphorylated myosin was incubated with equimolar actin in buffer A containing 1 mM ATP and 4 mM MgCl₂, and subjected to centrifugation at 200,000 × g for 1 h (see “dead heads” removal protocol in Ref. 14). Myosin was thiophosphorylated by a standard procedure (15) as described previously (9); thiophosphorylation efficiency was determined for each preparation by one-dimensional urea-gel electrophoresis according to Ref. 16 and Fig. 1. More than 95% of myosin was thiophosphorylated, whereas only a single band of nonphosphorylated myosin was found in the absence of ATP and ATP-βS.

ATPase Activity Assay—The ATPase activity of the purified myosin (with or without the dead heads removed, as well as nonphosphorylated and thiophosphorylated) was measured under single turnover conditions (myosin = ATP) by monitoring the rate of inorganic phosphate release with the aid of linked phosphorylation reaction (Molecular Probes, EnzChek kit E-6645). Following the procedure described by Webb (17), solutions in two Hamilton syringes containing 50 μl of myosin (3–5 mg/ml) in 300 mM KCl and separately 50 μl of a stoichiometric amount of ATP together with the linked enzyme system (100 μM 2-amino-6-mercapto-7-methylurea riboside, 10 units/ml purine nucleoside phosphorylase) were injected simultaneously into a 100-μl spectrophotometric cuvette, and the time course of absorption at 360 nm was monitored. The ATPase rate was determined from single exponential fitting of the absorption increase at 360 nm over time. The “dead time” of the system was less than 1 s, giving adequate time resolution for the present study. The lower limit of rate measurements determined by the sensitivity to [Pi] changes (1 μM) was determined as 0.001 s⁻¹.

Unphosphorylated smooth muscle myosin hydrolyzed MgATP at a rate of 0.007 ± 0.001 s⁻¹ (n = 7) and at 0.04 ± 0.02 s⁻¹ (n = 5) after thiophosphorylation by myosin light chain kinase. The ATPase rate of unphosphorylated myosin without the dead heads was below the detection limit (<0.001 s⁻¹), in agreement with reports in the literature (6, 18, 19).

Specimen Preparation for Cryo-AFM—Mica was used as the substrate for preparing all AFM specimens. The freshly cleaved mica surface was negatively charged. Positively charged surfaces were prepared by treating mica with 1 mM spermidine followed by washing with deionized water. The treated mica was blown dry with nitrogen gas leaving sufficient spermidine retained on the surface to efficiently adsorb negatively charged molecules. After 20 μl of myosin was deposited on the substrate (1 μg/ml in buffer A or low salt buffer), excess protein was removed by washing with the same buffer. To avoid possible structural effects, including separation of the heads because of changes in ionic strength during evaporation of the volatile buffer, the surface-adsorbed molecules were cross-linked with the incubation buffer containing 0.5% glutaraldehyde for 2–3 min. The sample was then washed again with the incubation buffer before finally being flushed with 250 mM or 125 mM ammonium acetate, pH 7.2 (volatile buffer). The specimens were quickly transferred to the cryo-AFM for equilibration and imaging.

Cryo-AFM Imaging—All cryo-AFM images were collected at ~80 K with instrumentation described elsewhere (20). The scanner was calibrated with a standard grid, and the accuracy was verified by imaging various samples with known dimensions (21, 22). Full frame images were collected at a rate of 1 scan line/s. Distortion was minimal because of the high thermal stability of the system (23). No image processing was applied.

RESULTS
We first examined the distribution of different conformations of nonphosphorylated myosin on freshly cleaved mica. A typical image is shown in Fig. 2A. The relative arrangement of the two heads may be roughly divided into three different categories (see Fig. 2B): well separated by 180° (a); still resolvable, but at a smaller angle (b); no longer distinguishable even in stereo views and, therefore, may be considered closely associated (γ). Note that in well resolved molecules both the regulatory and catalytic domains of each of the heads can be resolved (e.g., see molecules in Fig. 2B). We also note that, because of the random
orientation of the molecules on the substrate, even the count of the γ conformation may yield an overestimate of closed states if, for example, two separated heads were viewed as one on top of the other. Counting the number of nonphosphorylated myosin molecules that were clearly resolved by cryo-AFM (n = 294), we found 25% in the α conformation, 20% in the β conformation and 55% in the γ conformation. In other words, the ratio between closely associated heads and open heads is almost 1:1.

However, as noted above, the two-dimensional crystals used for the cryo-EM study were formed on a positively charged lipid monolayer (2), whereas the results of Fig. 2 show molecules deposited on a negatively charged surface. Even though at such high ionic strength mica is only slightly negatively charged, we were concerned that the negative surface could still affect the relative arrangement of myosin heads during adsorption. Therefore, to more closely reconstitute the conditions used for the cryo-EM study, we also used a substrate with a positive surface charge. The adsorption efficiency did not change significantly when the surface charge was reversed, consistent with cryo-EM results. Moreover, the distribution of myosin in the three recognizable conformations remained essentially unchanged. A typical cryo-AFM image of nonphosphorylated smooth muscle myosin molecules on the positively charged substrate is shown in Fig. 3A. Simple counting (n = 349) indicates that in this case, 25% of myosin is in the α conformation, 19% in the β conformation, and 56% in the closed γ conformation (see Table I).

To determine whether phosphorylation affects the conformation of myosin heads, we examined samples containing more than 95% thiophosphorylated RLC. The distribution of molecular conformations of thiophosphorylated myosin on the positively charged surface was noticeably shifted between the three conformations, as made more obvious by color-coding the molecules in Fig. 3, B versus A. As summarized in Table I, thiophosphorylation reduced the fully closed γ conformation by about 50% (to 23%). To exclude the possibility that strong ionic shielding under high salt conditions (buffer A) could favor conformations otherwise disfavored at lower ionic strength, we further compared the distribution of nonphosphorylated and thiophosphorylated myosin in a buffer closer to physiological ionic strength and also similar to that used for the cryo-EM studies (20 mM K2PO4, pH 7.2, 1 mM EGTA, 1 mM MgCl2, 120 mM KCl, 1 mM ATP) (2). These results were essentially identical to those obtained under high salt conditions (Table I). As shown in Fig. 4, the head conformation of myosin molecules is essentially the same as observed at higher ionic strength (both nonphosphorylated and thiophosphorylated) except that the bends of the tail (characteristic of low salt conditions) are clearly seen. When observed in this buffer even in the absence of ATP, the structure of myosin molecules as well as the distribution of the relative head-to-head positions were not noticeably different (data not shown). However, most of the molecules were not in the completely folded 10 S conformation in this low, but close to physiological ionic strength buffer containing ATP, unlike those found in previous studies where myosin was imaged in 50 mM NaCl (24, 25). To further examine the possible sources of this discrepancy, we cross-linked myosin molecules in 60 mM KCl buffer containing 1 mM MgATP, similar to the ionic strength of the buffer used in a previous electron microscopic study (24). Only at this very low (unphysiological) ionic strength did we find a significant number of myosin molecules in the 10 S conformation (Fig. 5, 10 S gallery). Because the partition of molecules into different energy states is determined by the Boltzmann distribution, the statistics shown in Table I can be used to estimate the energy difference between the two states: "n_\gamma/n_\alpha = \exp(-\delta kT). We found that \delta is only 0.24 kT for dephosphorylated myosin, and when thiophosphorylated, the energy difference favoring the open state is significantly greater: \delta = -1.21 kT.

Table I

| Biochemical state | KCl | α State (open) | β State (open) | γ State (closed) | n |
|-------------------|-----|---------------|---------------|-----------------|---|
| Nonphosphorylated | 300 | 25            | 19            | 56              | 349 |
|                   | 120 | 23            | 25            | 52              | 291 |
| Thiophosphorylated| 300 | 64            | 13            | 23              | 336 |
|                   | 120 | 58            | 18            | 24              | 285 |

The summary of distribution of smooth muscle myosin (dead head removed) into three different conformational states as resolved under different ionic conditions by cryo-AFM on spermidine treated mica surface.
the two heads in a population sample of nonphosphorylated myosin molecules. Regardless of the preparatory method, the surface charge of the substrate, or the ionic strength, cryo-AFM of whole nonphosphorylated myosin molecules failed to show a predominant distribution of closely apposed heads. Based on the Boltzmann distribution, this suggests that the energy cost of separating the two heads is small, only about one-fourth of the thermal energy. Our results, obtained by direct imaging of single molecules, do not support the suggestion, based on cryo-EM image processing and modeling of two-dimensional crystals of HMM crystallized at low temperatures, that strong head-to-head interactions are responsible for the inhibited state of myosin in which RLC is not phosphorylated (2). The high proportion (~60%) of thiophosphorylated myosin heads in the completely open state (present study) confirms our original observation (3) about the effect of thiophosphorylation.

The implication that regulation of two-headed myosin is not because of strong head-to-head interactions in the unphosphorylated state is consistent with several relevant studies of myosin in solution. For example, chimeric myosins composed of the motor domain of skeletal muscle and regulatory domains and light chains of smooth muscle are regulated by RLC phosphorylation, arguing against the possibility that special properties of the smooth muscle heavy chain favor intramolecular binding between the two motor domains and are required for regulation (26). Furthermore, the myosin ATPase activity of HMM that contains only one motor domain is regulated by phosphorylation (Ref. 27, but see also Ref. 6). The two heads of dephosphorylated myosin have been shown to be photo-cross-linked through the RLCs (within 8.9 Å of each other) (28), and thiophosphorylation abolishes this cross-linking (29) consistent with the greater separation of the two thiophosphorylated heads in solution (present study and Ref. 3). In the present experiments, because myosin in buffer was deposited on the mica surface prior to cross-linking, only the conformation with closely apposed heads at the time of deposition would be cross-linked independent of the protein substrate interaction. A cryo-AFM image of myosin molecules represents a “snapshot” of all conformations in solution. Indeed, ~50% of the unphosphorylated heads were separated, indicating that the two states of different head-to-head interactions are equally present in solution. In other words, myosin molecules fluctuate between the “open” and “closed” states, because of their relatively small energy difference. Following completion of this manuscript, images of two-dimensional crystals of smooth muscle myosin interpreted as being consistent with 10 S conformation were published (30). The relatively high ionic strength of the crystallization buffer used in the study (20 mM phosphate, 90–120 mM NaCl) that would have further increased during crystallization is difficult to reconcile with conditions producing 10 S myosin in solution (24).

Considering the behavior of muscle itself, the blocked head model of Wendt et al. (2) would have to allow one head to bind to actin to be consistent with the ability of nonphosphorylated myosin in the permeabilized muscle strip to develop rigor stiffness (31), enabling dephosphorylated myosin filaments to hold actin in a rigor-like conformation preventing actin filaments from diffusing away into the solution in motility assays (32), and enabling filaments containing both phosphorylated and dephosphorylated heads to move actin at intermediate rates in comparison to fully phosphorylated heads (33). The blocked model requires the actin-binding domain of one head to be strongly bound to the converter domain of the second head to maintain myosin in the off state. It is also less likely to allow HMM to bind actin in the presence of MgATP, and would not allow the cross-bridge to mechanically cycle (34). The model is
also difficult to reconcile with the cooperative attachment and cycling of dephosphorylated cross-bridges induced by micromolar ATP or CTP in muscles in rigor, which results in force development of up to 40% of maximal active tension without phosphorylation (31); a recent report (35) showing that both heads of tissue-derived smooth muscle HMM bind to actin irrespective of RLC phosphorylation or the presence of ADP seems also incompatible with a model (2) that requires the actin-binding domain of one head to be strongly bound to the converter domain of the second head to maintain myosin in the off state.

Taking the appropriate Popperian view that hypotheses cannot be proven but only tested and destroyed, we conclude that the available data do not support the hypothesis that requires strong intramolecular head-to-head binding forces to maintain the inhibited state of nonphosphorylated myosin II. In contrast, several studies (13, 36–39) are consistent with an alternative mechanism in which the activating message from the regulatory to the catalytic domain is transferred through the long α helix of the heavy chain and the converter domain as the result of stiffening of the α helix induced by phosphorylation of RLC. It is plausible to suggest, given the structural homology between calmodulin and myosin light chains (10, 11), that phosphorylation of the myosin regulatory light chain causes a conformational change similar to that experienced upon calcium binding by calmodulin, causing it to collapse on the regulatory domain of the lever arm, resembling the interaction of calmodulin with myosin light chain kinase (40). What is not explained by such a mechanism is the original critical observation (4) that two-headed myosin II containing nonphosphorylated RLC is inhibited, but a single head is active. If there are no strong binding forces between the two catalytic domains (2), then what mechanism keeps the catalytic domain of one head informed about the proximity of the other? Is it the inhibitory effect of the rod portion of the molecule on the regulatory domain (41, 42), or is it the frequency of contacts (between regulatory and/or catalytic domains) driven by thermal fluctuations?

Acknowledgments—We thank Shizhen Luo for technical help on protein purifications and phosphorylation measurements, Dr. Dan Czajkowsky for helpful discussions, and Ann Folsom and Howard W. Phipps for preparation of the manuscript.

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