Blebbistatin Stabilizes the Helical Order of Myosin Filaments by Promoting the Switch 2 Closed State

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ABSTRACT Blebbistatin is a small-molecule, high-affinity, noncompetitive inhibitor of myosin II. We have used negative staining electron microscopy to study the effects of blebbistatin on the organization of the myosin heads on muscle thick filaments. Loss of ADP and Pi from the heads causes thick filaments to lose their helical ordering. In the presence of 100 μM blebbistatin, disordering was at least 10 times slower. In the M·ADP state, myosin heads are also disordered. When blebbistatin was added to M·ADP thick filaments, helical ordering was restored. However, blebbistatin did not improve the order of thick filaments lacking bound nucleotide. Addition of calcium to relaxed muscle homogenates induced thick-thin filament interaction and filament sliding. In the presence of blebbistatin, filament interaction was inhibited. These structural observations support the conclusion, based on biochemical studies, that blebbistatin inhibits myosin ATPase and actin interaction by stabilizing the closed switch 2 structure of the myosin head. These properties make blebbistatin a useful tool in structural and functional studies of cell motility and muscle contraction.

INTRODUCTION

Blebbistatin is a small-molecule, high-affinity, noncompetitive inhibitor specific for most forms of myosin II. It derives its name from its ability to inhibit cell blebbing by interfering with nonmuscle myosin II function (1). In addition to its effect on nonmuscle myosin, blebbistatin has also been found to inhibit several striated muscle myosins (2,3) and some types of smooth muscle myosin (2,4), but to have no effect on unconventional myosins (2). The molecule binds at the apex of the 50 kDa cleft of the myosin head, close to the γ-phosphate pocket of the ATP binding site (5). In so doing, it appears to slow Pi release from the heads, prolonging the M·ADP·Pi state by stabilizing the switch 2 closed structure of the nucleotide binding pocket (5–7). Stabilization of the M·ADP·Pi state, by preventing complete closure of the 50 kDa cleft, is thought to keep the myosin head in a weak actin-binding state, preventing completion of the actomyosin crossbridge cycle and thus inhibiting motility. These properties have made blebbistatin a useful molecule for cellular and molecular studies involving myosin II function. For example, cell activities requiring myosin II can be detected by their inhibition by blebbistatin (1,8,9). In muscle studies, blebbistatin is valuable as an uncoupler of excitation-contraction coupling in electrophysiological studies (10). Here we complement previous biochemical observations of blebbistatin (2,6,7) by studying its structural effects on native myosin filaments from muscle and its consequent utility as a tool in structural studies of myosin.

In muscle, myosin II molecules are assembled into filaments (the ''thick'' filaments) (11,12) that interact with actin (''thin'') filaments to generate contraction (13). In nonmuscle cells, myosin II transiently forms filaments that interact with actin to generate many forms of cell motility (14–16). The thick filaments of diverse relaxed muscles are all characterized by helical ordering of their surface array of myosin heads (12,17–29). This ordered structure is lost on muscle activation or when myosin heads lose their ATP (17,30–32). Helical ordering appears to require the closing of switch 2 of the nucleotide binding site on the myosin head (33,34), which prevents Pi release. In this state myosin heads are sharply bent (35) and undergo intramolecular head-head interactions (36–38). In intrinsically regulated myosins (those regulated by phosphorylation of the regulatory light chain or Ca^{2+} binding to the essential light chain), these interactions appear to switch off myosin activity, leading to relaxation (36,37,39–41). Similar interactions are also present in unregulated filaments (38).

Our greatest insights into thick filament structure and function have come from studies of tarantula filaments, whose stable helical ordering has made them an excellent model system for structural studies (20,37,42). In this study we used tarantula filaments to study blebbistatin's effects on filament structure. Our observations support previous biochemical data suggesting that blebbistatin stabilizes the closed switch 2 state of the myosin head, inhibiting Pi release, ATPase activity, and actin binding. The results show that it is a useful tool for stabilizing the helical ordering of myosin heads in labile filaments, improving the detail visible in structural studies (38), and for stabilizing head-head interaction in weakly interacting structures (41). Our observations also show directly that blebbistatin inhibits actin-myosin binding, which has proved to be important for separating thick and thin filaments in filament studies (38).

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MATERIALS AND METHODS

Solutions

Rigor solution consisted of 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 5 mM PIPES, and 1 mM NaN_3, pH 7.0. Relaxing solution was rigor solution containing 1 mM MgATP. Rigor rinse contained 100 mM NaAc, 3 mM $MgCl_2$, 0.2 mM EGTA, 2 mM imidazole, and 1 mM NaN_3, pH 7.0. Relaxing rinse was rigor rinse containing 1 mM MgATP, and ADP rinse was rigor rinse containing 1 mM MgADP, 100 μM AP_5A, 0.1 mg/mL hexokinase, and 1 mM glucose (see below).

Preparation of thick filaments

Relaxed, native thick filaments were purified from saponin-skinned tarantula leg muscle according to Hidalgo et al. (43), except that the Ca^{2+}-insensitive gelsolin fragment used to fragment the thin filaments was obtained by bacterial expression (44).

Effect of different nucleotide states on thick filament structure

ATP removal

Purified thick filaments in relaxing solution were centrifuged at 18,000 × *g* for 20 min, resuspended in rigor solution containing 100 μM AP_5A, 0.1 mg/mL hexokinase, and 1 mM glucose, and then incubated at room temperature for 30 min to remove any residual ATP. This process was repeated twice more in rigor solution (without added AP_5A, hexokinase, and glucose). The final suspension contained thick filaments in rigor solution. The heads in these filaments would be in the apo state.

Time course of structural change as ADP and Pi dissociate from the myosin heads

Purified thick filaments were resuspended in relaxing solution, and apyrase (Sigma A6535) was added to a final concentration of 16 units/mL. This would hydrolyze all ATP and ADP in solution in less than 1 min.

Presence of ADP

Rigor thick filaments were prepared as described under ''ATP removal'' and resuspended in rigor solution containing 1 mM MgADP. The solution also contained AP_5A and hexokinase (as above) to remove contaminating ATP from the ADP, and AP_5A (100 μM) to inhibit conversion of ADP to ATP by contaminating adenylate kinase. This reduces ATP levels to <1 μM in 20 min (33).

The effects of blebbistatin on the structures observed in these conditions were studied by adding blebbistatin (EMD, Darmstadt, Germany) to the filament suspensions at a final concentration of 100 μM. Experiments were carried out in covered tubes to avoid light inactivation of blebbistatin (45).

Electron microscopy and image analysis

A 6-μL drop of filament suspension was applied to a 400-mesh grid coated with a thin carbon film supported by a holey carbon film. The grid was then rinsed with three to four drops of the appropriate acetate-based rinsing solution (20), and negatively stained with 1% (w/v) uranyl acetate. Drying was performed at ~80% relative humidity (46). Conventional dose images of negatively stained specimens were recorded at 15,500× magnification on Kodak 4489 film using a Philips (FEI, Hillsboro, OR) CM10 electron microscope operated at 80 kV. For image processing, films were scanned on a FlexTight scanner at a pixel size of 1.1 nm in the original specimen. Fourier transforms of boxed areas of selected filaments were computed and averaged using ImageJ (v1.34s, NIH, Bethesda, MD). Averaged Fourier transforms in Figs. 1–5 (from 40, 20, 20, 20, and 10 filament segments, all of the same length, respectively) were brightness- and

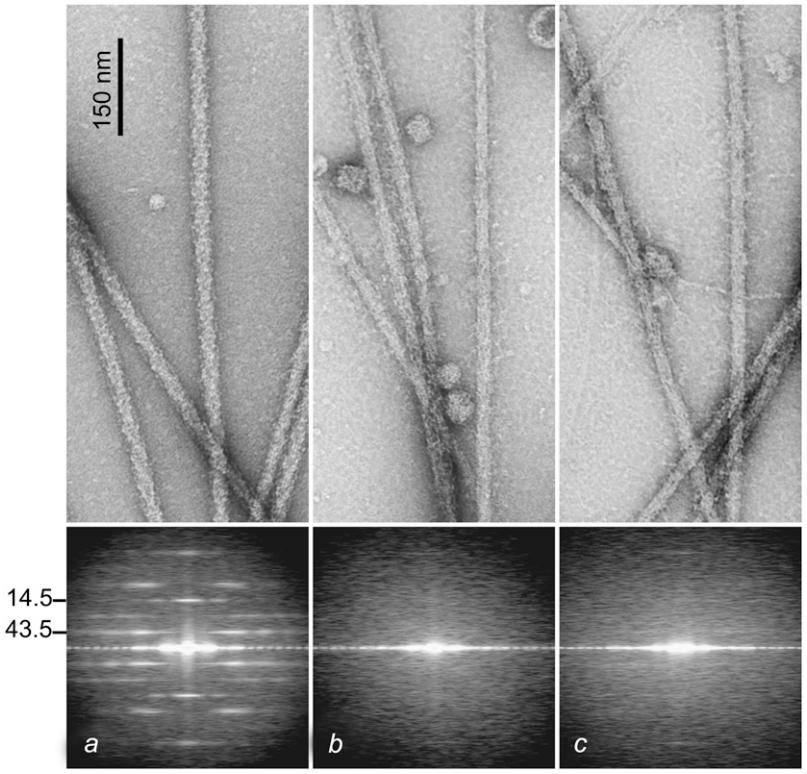

FIGURE 1 Effects of different nucleotides on helical ordering of myosin heads. (*a, Top*) tarantula thick filament negatively stained in relaxing conditions (M·ADP·Pi state), showing helical ordering of myosin heads. Ordering along helical tracks is most apparent when viewing along the filament axis at a glancing angle, and is confirmed by the appearance of layer lines at orders of 43.5 nm in averaged Fourier transform (*bottom*). (*b*) Filament negatively stained after removal of solution ATP and allowing time for ADP and Pi to dissociate (apo state). Heads are disordered and project away from the filament backbone (compare with the compact structure of *a*); the averaged Fourier transform lacks significant layer lines, confirming disorder. (*c*) Filament negatively stained in the presence of MgADP (M·ADP state). The filament image and averaged Fourier transform show disorder. Weak meridional reflections at the 6th order (7.2 nm) of the 43.5 nm repeat in (*b*) and (*c*) probably originate from the filament backbone, which is more exposed in the disordered filaments. Averaged transforms in *a–c* are from 40 filament segments, all of the same length.

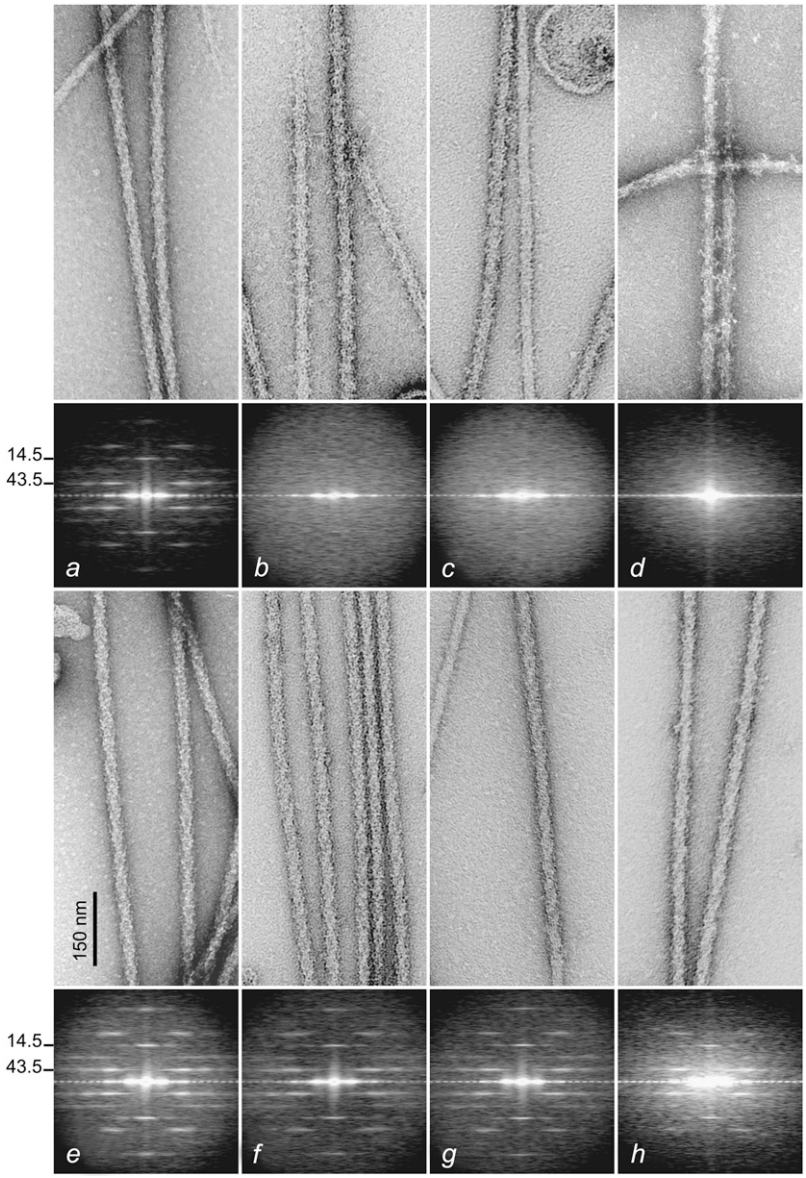

FIGURE 2 Blebbistatin maintains head ordering in the absence of free nucleotide. (*a–h*) Time points at 0, 5, 60, and 90 min after treatment of thick filament suspension with apyrase (to remove ATP and ADP from solution) in the absence (*a–d*) and presence (*e–h*) of 100 μM blebbistatin. Average Fourier transforms of filaments confirm visual impression of order or disorder. Helical order is retained for at least 1 h in the presence of blebbistatin, but is lost in its absence. At 90 min (*d* and *h*) and 120 min (not shown), only the 43.5 and 14.5 nm reflections are prominent, showing that over long time periods helical order weakens even in the presence of blebbistatin.

contrast-adjusted to show a clear signal; adjustments for each condition were identical so that the transforms could be objectively compared.

RESULTS

Myosin heads lose their relaxed helical order in M·ADP and apo states

Relaxed thick filaments (M·ADP·Pi state) showed clear helical ordering of the myosin heads when visualized by negative staining (Fig. 1 *a*), as found in previous studies (20,42). This was confirmed by the presence of strong layer lines in their Fourier transforms, which extended to at least the sixth order of the basic 43.5 nm helical repeat. The first and fourth off-meridional layer lines and the third and sixth meridional layer lines were all strong, with weaker reflections on the second and fifth layer lines (Fig. 1 *a*) (20,42). Filaments in which all nucleotide had dissociated from the heads (apo state) appeared completely disordered, both by eye and from the absence of layer lines in their Fourier transforms (Fig. 1 *b*) (30–32). Filaments in which MgADP, but not Pi, was present on the heads (M·ADP state) were also disordered (Fig. 1 *c*). These controls confirm earlier studies (33).

Blebbistatin inhibits Pi release from myosin heads

We determined the effect of blebbistatin binding to the myosin heads on the maintenance of the ordered state after removal of ATP and ADP from a relaxed thick filament suspension. Purified thick filaments in relaxing solution (containing 1 mM MgATP) were divided into three aliquots: 1), an untreated control; 2), the same as the control but con-

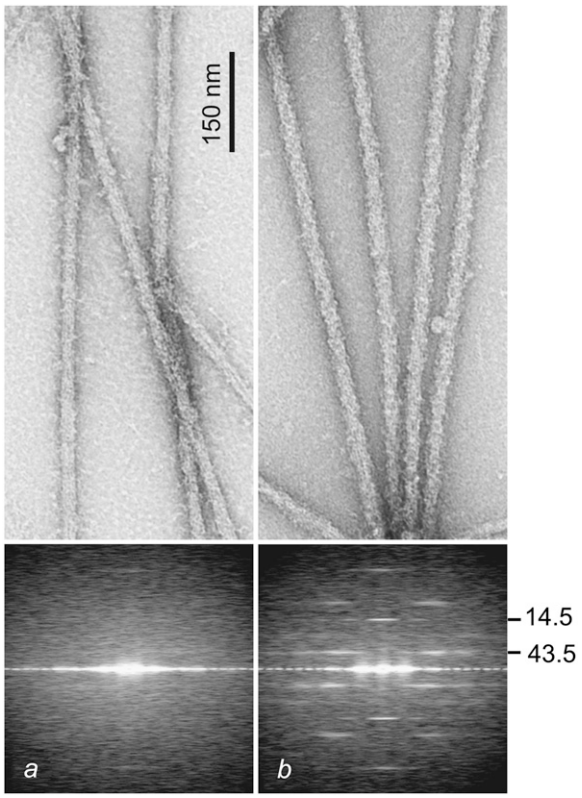

FIGURE 3 Blebbistatin restores helical order to disordered M·ADP thick filaments. (*a*) In rigor solution containing 1 mM MgADP, heads are disordered and project away from the filament backbone. (*b*) After treatment with blebbistatin, helical ordering is restored.

taining apyrase (16 units/mL) to hydrolyze ATP and ADP in solution; and 3), the same as aliquot 2 but also containing blebbistatin (100 μM). Filaments from each aliquot were negatively stained at different times during a 2-h room-temperature incubation.

Relaxing solution control

The control in relaxing solution retained the helical order of the myosin heads throughout the 2-h period, as judged by both the visual appearance of the filaments and the layer lines in their Fourier transforms (cf., Fig. 1 *a*). Dissociation of ADP and Pi during this time would have been followed immediately by rebinding of ATP from solution, followed by hydrolysis to ADP and Pi, thus maintaining the relaxed helical structure (20,33).

Treatment with apyrase

When filaments were incubated with apyrase at room temperature, they appeared fully ordered at 0 min (Fig. 2 *a*), but had lost their order within 5 min (Fig. 2 *b*), an appearance that continued through 60 min (Fig. 2 *c*) and beyond (Fig. 2 *d*). Fourier transforms showed little or no sign of myosin layer lines. Here, ATP and ADP present in solution are rapidly hydrolyzed ($<$1 min) by apyrase. Thus, when bound Pi and

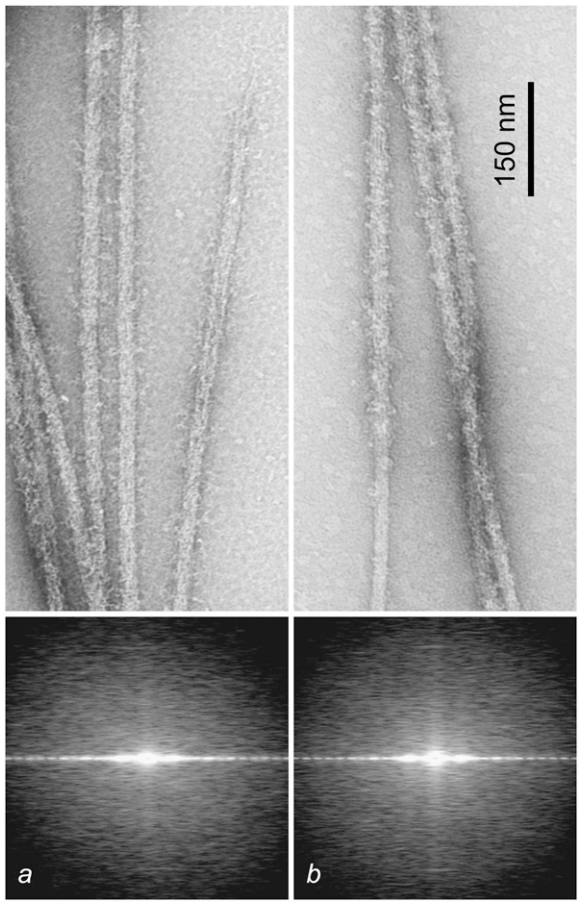

FIGURE 4 Blebbistatin does not restore order to apo thick filaments. (*a*) Under rigor conditions, in which all ATP and ADP have been removed from solution, and all bound nucleotide has had time to dissociate, myosin heads are disordered and project away from the filament backbone. (*b*) Treatment with blebbistatin fails to restore helical ordering to such rigor filaments.

ADP are released from the myosin heads, they would not be replaced, and the heads would go into the apo state, causing disorder (cf., Fig. 1 *b*). Our observations suggest that this happens within 5 min (Fig. 2 *b*).

Treatment with apyrase in the presence of blebbistatin

When the same experiment was performed in the presence of blebbistatin, the helical order appeared excellent in most filaments, even at 60 min, and this was confirmed by the persistence of layer lines similar to those in relaxing conditions (Fig. 2 *e–g*). With longer times (up to 2 h), filaments started to lose their order and their helical layer lines became weaker, even in the presence of blebbistatin (Fig. 2 *h*), but this effect was small and more than an order of magnitude slower than in its absence.

Our results thus show that in the presence of 100 μM blebbistatin, disordering of thick filaments is slowed at least 10-fold, consistent with biochemical studies (6). Because the M·ADP·Pi state promotes helical ordering (33,47), this is consistent with blebbistatin acting as an inhibitor of Pi release from the nucleotide pocket.

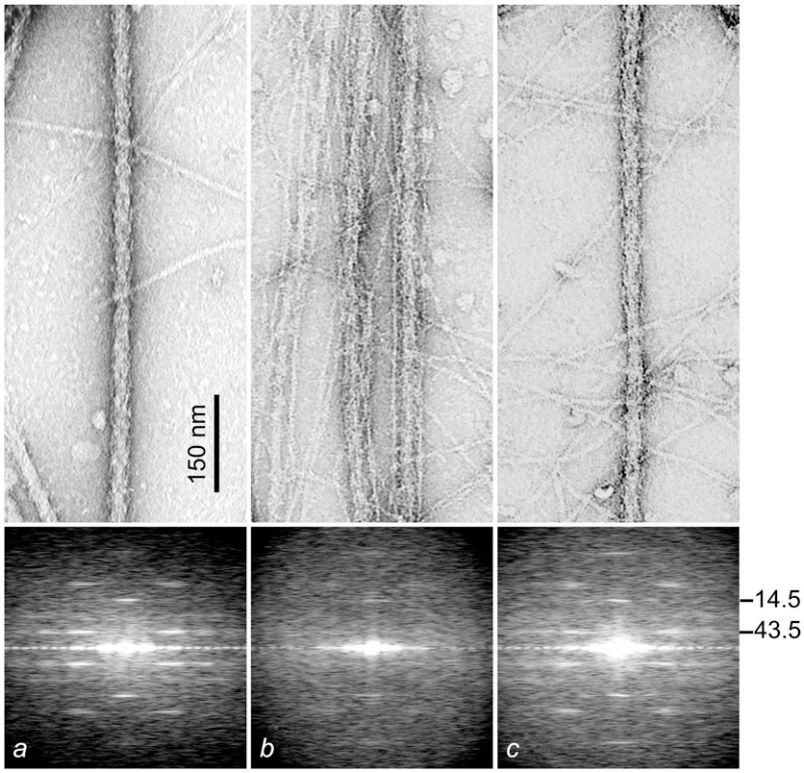

FIGURE 5 Inhibition of thick-thin filament interaction by blebbistatin. (*a*) Relaxed muscle homogenate containing thick and thin filaments showing very few interactions. Thick filaments reveal helical order by eye and in the averaged Fourier transform. (*b*) When a relaxed muscle homogenate containing thick and thin filaments is activated by Ca^{2+}, numerous densely stained filament aggregates are formed, suggesting thick-thin filament interaction. These aggregates are difficult to image due to the density of staining. Smaller aggregates, like the one shown, reveal many filament interactions and a generally disordered array of heads. (*c*) When treated with Ca^{2+} in the presence of blebbistatin, thick and thin filaments show minimal interaction with each other, aggregates are absent, and the heads have a degree of helical ordering similar to that of relaxed filaments (compare the transform with that in *a*).

Blebbistatin reorders M·ADP thick filaments

Having shown that blebbistatin prolongs the ordered state of myosin filaments after removal of nucleotide, we wanted to determine whether it could also restore order to disordered filaments. Apo filaments were suspended in rigor solution containing 1 mM MgADP (see Materials and Methods). Under these conditions (M·ADP state), the myosin heads appeared fully disordered, and this was confirmed by the absence of layer lines in their Fourier transforms (Fig. 3 *a*). Thus MgADP alone fails to restore order to apo filaments, confirming earlier findings (33). However, when 100 μM blebbistatin was included with the MgADP solution, the disordered M·ADP filaments became ordered again (Fig. 3 *b*). The same result was obtained whether blebbistatin was added before, after, or together with the MgADP. The ordering and the layer line pattern to which it gave rise were indistinguishable from those of relaxed filaments (compare Figs. 1 *a* and 3 *b*). Based on earlier studies (33,34), we conclude that blebbistatin promotes the closing of switch 2 of the nucleotide binding site when ADP is present.

Blebbistatin does not reorder rigor thick filaments

Since blebbistatin can reorder disordered filaments in the M·ADP state, we next tested its ability to restore order to apo-state filaments. Thick filaments in rigor conditions consistently showed disorder of the myosin heads (Figs. 1 *b* and 4 *a*). There was no return of order after incubation with 100 μM blebbistatin (Fig. 4 *b*). Thus blebbistatin does not reorder nucleotide-free heads on thick filaments.

Blebbistatin inhibits thick-thin filament interaction

We also determined whether blebbistatin has an inhibitory effect on actin-myosin interaction. At high concentration, unpurified homogenates of tarantula muscle (containing native thick and thin filaments) have a turbid appearance in relaxing conditions. When viewed by electron microscopy, thick and thin filaments are seen to be separate from each other, with only occasional interactions, and the thick filaments are ordered (Fig. 5 *a*) (20). On addition of ~0.4 mM free Ca^{2+} to the homogenate there was an immediate visual ''granulation'' of the suspension and adherence of granular material to the wall of the tube, as reported previously (48). Negative stain images revealed numerous thick-thin filament interactions (Fig. 5 *b*) and often large filament bundles, consistent with this macroscopic appearance. The thick filaments were generally disordered (30). In the presence of 100 μM blebbistatin, these Ca^{2+}-induced changes were absent macroscopically, and negative stain images showed separate thick and thin filaments, with the thick filaments retaining their helical order (Fig. 5 *c*). These results show that blebbistatin inhibits actin-myosin interaction.

DISCUSSION

Our observations on native myosin filaments demonstrate that blebbistatin slows the rate of disordering of myosin heads

after removal of ATP, reorders the disordered array of heads occurring in the M·ADP state (but not the disorder of the apo state), and inhibits actin-myosin interaction occurring in an activated filament preparation. These structural observations, together with previous findings on the requirements for thick filament order (33,34), directly support earlier studies that found that blebbistatin stabilized the closed state of the switch 2 element of the nucleotide binding site (5,6). In this state, in which both ADP and Pi are bound in the active site, myosin binds only weakly to actin. These earlier studies were based on enzyme kinetics, x-ray crystallography, and observations of actin motility in the presence of blebbistatin (2,5–7). Our results reveal the structural manifestations of blebbistatin inhibition that would occur in the thick filaments in intact muscle.

Our observations are consistent with previous findings on the selectivity of blebbistatin, showing that it inhibits a variety of myosin II species (but not unconventional myosins) from nonmuscle, striated muscle, and some smooth muscle sources (2,4). The finding that blebbistatin inhibits tarantula (this study) as well as scallop (2) striated myosins, regulated by phosphorylation of the regulatory light chain (30) and Ca^{2+} binding to the essential light chain (49,50), respectively, suggests that it is likely to inhibit most types of invertebrate striated myosin IIs.

Recent studies of tarantula and rabbit striated muscle thick filaments have revealed that helical ordering of the heads requires the closed switch 2 state (33,34). Our observation that blebbistatin stabilizes helical ordering therefore suggests that it promotes the closing of switch 2. Helical ordering has been shown to involve intramolecular interaction between myosin heads (37), the actin-binding region of one head binding to the converter and essential light chain of the other (36,37,39). Stabilization of helical ordering thus suggests that head-head interaction is likewise favored by blebbistatin. This has been shown to be the case in single-molecule studies of myosin (41). When switch 2 is closed, myosin binds only weakly to actin (51), which would explain the inhibition of thick-thin filament interaction that we observe in activated filament preparations. We conclude that when muscles are treated with blebbistatin, their thick filaments become stabilized in an ''enhanced'' relaxed state, with their heads in stable helical arrays, interacting with each other but not with actin, even under activating conditions.

Our conclusions are consistent with x-ray diffraction data from rabbit psoas muscle treated with blebbistatin (52,53). Blebbistatin enhanced the strength of layer lines already present in x-ray patterns of relaxed muscle, and induced layer lines in conditions in which they were otherwise absent (MgADP or absence of nucleotide). It was concluded that blebbistatin shifts the equilibrium of the myosin head toward the switch 2 closed state. The detection of weak reordering in rigor filaments, which was not observed in our studies, may reflect the averaging of large numbers of filaments that occurs with x-ray diffraction, making possible the detection of small changes.

These properties of blebbistatin make it an excellent tool in studies of myosin II. Functional studies, in which inhibition by blebbistatin has been used to determine the role of myosin II in different cell processes, were referred to earlier (1,8,9). Blebbistatin has also been used, for example, as an uncoupler of excitation-contraction coupling, making it possible to carry out fluorescence imaging of electrical activity in muscle while avoiding motion artifacts (10). Blebbistatin has also proved to be an invaluable tool for muscle structural studies. Its ability to promote the relaxed helical ordering of the myosin heads on the thick filament backbone helped in the isolation of vertebrate cardiac thick filaments (38) by minimizing thick-thin filament interactions, which are common in cardiac filament preparations, even under relaxing conditions (28). In the same work (38), blebbistatin also played a crucial role in stabilizing the helical array of myosin heads on the filament surface, which is labile and easily disrupted in mammals. This made possible the most detailed three-dimensional reconstruction of vertebrate thick filaments yet available, revealing new features of myosin head organization (38). The use of blebbistatin in structural studies of the more stable vertebrate skeletal thick filament may also be of great value.

Invertebrate thick filaments, regulated by regulatory light chain phosphorylation or Ca^{2+} binding to the essential light chains, are intrinsically well ordered (19,20,23,24) and have been the source of our most detailed insights so far into myosin II filament structure (37). The use of blebbistatin to further enhance invertebrate filament order may improve the resolution even more. In studies of single myosin molecules, it has been shown that myosin heads interact with each other intramolecularly in the inactive (switched-off) state (36,39,40). As is the case with filaments, this interaction is relatively stable in regulated myosins, but much weaker in unregulated (vertebrate striated) myosin (41). Thus only a small number of vertebrate molecules show a head-head interaction, making image averaging difficult. By stabilizing the switch 2 closed structure with blebbistatin, the number of molecules that show interacting heads increases dramatically, providing structural insights that are otherwise inaccessible (41).

Blebbistatin might also prove valuable in x-ray crystallographic studies of myosin. Although myosin heads have been crystallized in numerous studies, leading to many new insights into their function (35,54,55), attempts to crystallize myosin in its functional, two-headed form have not been successful. A likely difficulty is motion of the heads about their junction with the myosin tail. Blebbistatin should help to minimize motion by favoring the closing of switch 2 and thus formation of the more rigid structure in which the heads interact with each other.

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