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Coupling between myosin head conformation and the thick filament backbone structure

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Abstract

The recent high-resolution structure of the thick filament from *Lethocerus* asynchronous flight muscle shows aspects of thick filament structure never before revealed that may shed some light on how striated muscles function. The phenomenon of stretch activation underlies the function of asynchronous flight muscle. It is most highly developed in flight muscle, but is also observed in other striated muscles such as cardiac muscle. Although stretch activation is likely to be complex, involving more than a single structural aspect of striated muscle, the thick filament itself, would be a prime site for regulatory function because it must bear all of the tension produced by both its associated myosin motors and any externally applied force. Here we show the first structural evidence that the arrangement of myosin heads within the interacting heads motif is coupled to the structure of the thick filament backbone. We find that a change in helical angle of 0.16° disorders the blocked head preferentially within the *Lethocerus* interacting heads motif. This observation suggests a mechanism for how tension affects the dynamics of the myosin heads leading to a detailed hypothesis for stretch activation and shortening deactivation, in which the blocked head preferentially binds the thin filament followed by the free head when force production occurs.

Keywords

Striated Muscle; cryoelectron microscopy; image reconstruction; muscle physiology

INTRODUCTION

Striated muscle fibrils comprise three ordered structures: (1) thin, actin-containing filaments, (2) thick, myosin-containing filaments, and (3) a Z-disk, which orders the thin filaments into a lattice and defines the ends of the sarcomeres. Each filament structure has accessory

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Author contributions

ZH collected the data, performed the data analysis & contributed to the writing

DWT prepared the thick filament specimens, contributed to the writing

RJE prepared myofibrils, & contributed to the writing

KAT supervised the experimental work and wrote the paper

proteins that perform key functions. Thin filaments from different muscle types and different species are quite similar in structure and composition; they all have in common a 2-stranded chain of actin subunits at their core and associated proteins. The actin filaments from all muscles host a long protein with an α -helical coiled coil structure called tropomyosin (Clark et al., 2002). In those muscles where tension production is regulated at the thin filament, changing concentrations of calcium ions alter myosin's access to actin via tropomyosin's interaction with the troponin (Tn) complex, consisting of three proteins, Tn-C, Tn-T and Tn-I (Lehman, 2016). Capping proteins occur at the ends to stabilize the length (Littlefield and Fowler, 1998). Although the thin filaments are polar structures, they are anchored into a bipolar arrangement at the Z-disk.

Striated muscle thick filaments also contain more than simply myosin, but they are quite variable in structure and composition between both species and muscle types. For example, the thick filaments of *Lethocerus* flight muscle are 2.4 μm long (Levine et al., 1976) whereas those from *Drosophila* flight muscle are 3.0 μm long (Reedy and Beall, 1993). The mechanism of thick filament length determination in invertebrate muscles is not clearly defined, but the presence of paramyosin in all invertebrate muscle thick filaments suggests a contributory role. Vertebrate striated muscles have a defined thick filament length of 1.6 μm , which is determined by the giant protein titin (Whiting et al., 1989). Although myosin filaments are variable in structure, they appear to have one feature in common; myosin heads arranged in so-called Interacting Heads Motifs or IHM characterize their relaxed filaments.

The IHM was first identified as the inhibited conformation of the heavy meromyosin fragment of smooth muscle myosin (Wendt et al., 2001), and later in a folded hair-pin structure that is soluble at physiological ionic strength, known as the 10S conformation because it sediments in solution at that apparent size (Liu et al., 2003). The IHM is characterized by an asymmetric interaction between otherwise identical heads (Fig. 1A,B). Thought originally to be specific for smooth muscle and vertebrate non-muscle myosin II, the IHM was later found in HMM and 10S related conformations in multiple myosin II species (Jung et al., 2008b). The most significant observation occurred when the IHM was found on relaxed thick filaments from Tarantula leg muscle, a striated muscle (Fig. 1C) (Woodhead et al., 2005). Since then, it has been found in all relaxed thick filament structures investigated (Al-Khayat et al., 2013; Pinto et al., 2012; Sulbaran et al., 2015; Woodhead et al., 2013; Zhao et al., 2009), usually in an orientation in which the IHM is approximately tangential to the surface of the filament backbone with the blocked head contacting the S2 fragment of the myosin rod. More recently and surprisingly, the IHM was found to characterize the relaxed thick filament structure from *Lethocerus* asynchronous flight muscle (Hu et al., 2016) but in what is so far a unique orientation; it is almost perpendicular to the filament backbone (Fig. 1D) with no S2-blocked head contact.

Thick filaments in muscle produce tension via their myosin motors contacting the thin filaments. Like the actin filament, they must also bear the tension produced by their myosin heads along the whole filament backbone in order that tension produced within each sarcomere be transferred to the ends of the muscle. The filament backbone was thought to be structured so as to transmit tension efficiently without having a specific role in how contraction might be regulated.

Thick filaments in relaxed vertebrate striated muscles have groups of myosin heads arranged around a 3-fold rotation axis, spaced 143 Å apart, giving rise to a strong 143-Å meridional reflection in the X-ray diffraction pattern (Huxley et al., 1994). These axial groupings of myosin heads are generally referred to as crowns (Reedy et al., 1973). The length of the myosin rod is 11 crowns or ~1600 Å. Upon activation, but before significant tension development, vertebrate thick filaments lengthen by about 1.5%, changing the crown spacing from 143 Å to 145.1 Å (Linari et al., 2015). In contrast, the crown spacing of *Lethocerus* flight muscle thick filaments is always ~145 Å, regardless of whether the muscle is active or relaxed (Perz-Edwards et al., 2011). *Lethocerus* thick filaments have a 4-fold rotation axis.

In either filament type, active tension development causes a further increase in axial spacing, by 0.2% in tetanized vertebrate striated muscle (Huxley et al., 1994), versus 0.05% in stretch-activated *Lethocerus* flight muscle (Perz-Edwards et al., 2011). Despite the 4-fold difference in spacing change, the elastic moduli of the filaments are similar in the two species, due to the larger diameter of the filament backbone in *Lethocerus*, and lower tension produced during stretch activation versus tetanus. Additionally, due to the high passive stiffness of *Lethocerus* flight muscle, changes in the axial spacing of 0.03% can also be observed by stretching a relaxed muscle (Perz-Edwards et al., 2011). In either stretch-activated or stretch-relaxed *Lethocerus* flight muscle, the magnitude and the timing of the thick filament stretch are proportional to and in phase with the total force on the muscle. In vertebrate striated muscle the 0.2% increase above the 145.1-Å active spacing is proportional to force, but the initial 1.5% change in vertebrate striated muscle spacing occurs before significant development of tension.

Lethocerus thick filaments also show signs of twisting when the muscles are stretched, amounting to -0.15° rotation/crown, representing an unwinding of right-handed helices (Perz-Edwards et al., 2011). The magnitude of the twist is similar regardless of whether the muscle is stretch-activated or stretch-relaxed, and the timing of the twist is in phase with the length change, not with the total force, which is delayed with respect to the length change during stretch activation. Therefore this twist was interpreted as a passive response of the thick filament (Perz-Edwards et al., 2011). To our knowledge, there are no published studies specifically assessing whether vertebrate thick filaments do or do not show a similar twist.

Recently, X-ray diffraction studies on vertebrate skeletal muscles have shown minimal change in thick filament structure during unloaded shortening, but large changes toward features characteristic of an isometric contraction when a load was imposed (Linari et al., 2015). This would suggest that most of the myosin heads on a thick filament remain in the IHM arrangement, but a few disordered heads can sense the activation state of the thin filament and shorten the muscle in the absence of a load. However, when a load is imposed, the remaining heads dissociate from the IHM in order to interact with actin. Thus, the thick filament is acting as a tension sensor, activating multiple heads to provide sufficient force to shorten the sarcomere. In stretch-activated or stretch-relaxed *Lethocerus* muscle, the X-ray diffraction pattern also shows changes in the intensity of some key reflections, notably the 14.5 nm meridional reflection that reports the orientation and order of the myosin heads. Thus, both the vertebrate and the flight muscle results suggest that applied tension, either

internal or external, alters the structure of the myosin filament backbone and that this alteration can be transmitted to the myosin heads thereby altering their arrangement. In other words, the filament backbone structure and the myosin head conformation appear to be coupled.

Reversing this logic, it follows that altering the conformation of the myosin heads, in the absence of any applied tension, can change the structure of the thick filament backbone. With this reasoning, images of filaments with apparently disordered heads might show a change in filament structure manifest as a change in helical pitch. Invertebrate thick filaments are preferable to vertebrate thick filaments for this purpose because the indications are that they change helical parameters, whereas vertebrate filaments, which have rotational symmetry rather than helical symmetry, change only the axial spacing. Magnification uncertainties may exceed the small amount of spacing change for invertebrate filaments. Here we use cryoEM and iterative helical real space reconstruction (IHRSR) to show that a population of filaments with apparently disordered heads has an altered helical symmetry and that the disorder in the heads is mostly confined to the blocked head of the IHM.

MATERIALS AND METHODS

Cryo-electron microscopy

The specimens and micrographs used in this study were the same ones used in our earlier report (Hu et al., 2016). In this case rather than selecting the apparently best-ordered filaments, we selected the least-ordered filaments. The total number of filament segments in this population was 100,000. The 3-D image classification and reconstruction were carried out the same way as the earlier work using the same methods and the same software. When 3D classification was initiated, the starting reference model was the 5.5-Å resolution map of the relaxed filament (Hu et al., 2016), which was low pass filtered to 60 Å. As before, only segments from classes showing a clear S2, ~50,000, were classified as best segments and the other ~50,000 that failed to show S2 clearly were classified as worst segments. Both groups of filament segments were carried individually through to a final 3D reconstruction. In addition, the ~50,000 segments rejected in our earlier report (Hu et al., 2016) were also carried through to a final 3D reconstruction. In all cases, the resolution was evaluated based on the golden FSC at 0.143 of the filament backbone.

Structure Comparison

Visual comparison of the relaxed thick filament structure with the “disordered” thick filament is sensitive to the contour threshold. To make comparisons between the four structures as valid as possible, we adopted the following convention. Maps being compared were low pass filtered to the same resolution for the different comparison, one for the rods and the other for the heads. For the rod comparison, the relaxed thick filament structure at 5.5 Å resolution was low pass filtered to 6.4 Å and the thresholds for the myosin rods selected to match as close as possible their outlines. Although the helical parameters of two data sets differed, we could segment the rods separately, align them and visually match their contours as closely as possible. This occurred for a threshold of 0.0776 for the relaxed thick

filament and 0.032 for the disordered thick filament. Here, both maps are non-normalized density maps, which directly result from Relion (Scheres, 2012).

The more difficult comparison was the head arrangement. Here the two reconstructions were low pass filtered to 20-Å resolution and the threshold selected that best matched the outline of the myosin rods and the S2 domain, which extends out from the filament backbone. This threshold also matches well the outlines of the free head. The contours were 0.2 for the relaxed filament structure and 0.25 for the disordered filament structure. Here, both maps are normalized density maps after reconstruction using Relion. In all other respects, image processing was the same as earlier (Hu et al., 2016).

RESULTS

In this report we compare IHRSR of four data sets, which are summarized in Table 1. All of the data come from the same set of electron micrographs from which the relaxed structure was previously reported (Hu et al., 2016). Although coming from the same set of images, the two data sets, “relaxed” and “disordered”, are completely independent in that no segments from the relaxed are contained in the disordered set and no segments from the disordered set are contained in the relaxed set. Since the images of the disordered thick filaments are intermingled among the better-ordered relaxed filaments, the two sets have been prepared equivalently and analyzed in the same way. The 3-D reconstruction process itself, which utilized 3-D classification within Relion, divided each of the two sets into a “best” set of filament segments and a “worst” set of filament segments, best and worst being relative terms.

Appearance of Disordered Filaments

Within the image set, the disordered filaments made up a larger population than the relaxed filaments. The disordered filaments generally show at least some features of the 145-Å axial periodicity, while at the same time showing features characteristic of detached myosin heads at higher radii (Fig. 2).

Previously, we obtained 74,000 segments of ordered, relaxed filaments from which the relaxed-best filament reconstruction utilized 24,000 segments (Hu et al., 2016). Less constrained by the requirement for order in the heads, we obtained 100,000 disordered segments from which 50,000 were utilized for the final disordered-best reconstruction. As previously, we selected segments only from classes that showed a clear S2 density to form the “best” disordered class. Despite having twice the number of segments, we were able to achieve only a resolution of 6.4 Å in the disordered-best filament reconstruction, compared to 5.5 Å in the relaxed-best reconstruction.

The reconstruction from the relaxed-best filament segments showed a rotation between crowns of 33.98°, which is slightly larger than the range observed by X-ray diffraction when relaxed *Lethocerus* fibers were cyclically stretched and released, ~33.75–33.9° (Perz-Edwards et al., 2011). Using 33.98° as the maximum angle when filaments are under no tension and 33.75° as the value of filaments under tension, the maximum angle difference is 0.23°. The disordered-best filaments show a rotation between crowns of 33.82°, a change of

0.16° from our relaxed thick filament reconstruction under no tension. The 33.82° rotation of the disordered-best reconstruction suggests that the disordered filaments may have a structure similar to native filaments under tension in a stretched muscle.

After obtaining the disordered-best reconstruction, we reconstructed by the same methods, the ~50,000 rejected segments of the relaxed filament data to compute the relaxed-worst reconstruction. We did not attempt to classify further the relaxed-worst filaments into different groups and obtained a reconstruction of 7.2 Å resolution and similar appearance in both the backbone and the IHM, except that the resolution of the heads was lower because the “hole” in the center was not visible (data not shown). The helical turn was 33.95°, only 0.03° less than the relaxed-best filament segments. The reconstruction obtained from the 50,000 disordered-worst filament segments was also similar to that obtained from the disordered-best segments and had a helical angle of 33.85°, a smaller change than that obtained for the disordered-best reconstruction.

The Disordered Filament Structure

When filtered to 6.4-Å resolution, both the relaxed-best thick filament and the disordered-best thick filament reconstructions look very similar (Fig. 3A). The myosin heads are mostly missing but the rods have excellent definition. When a myosin rod segmented from the disordered-best reconstruction is aligned to a similarly segmented rod from the relaxed-best thick filament at 6.4 Å resolution, the two maps superimpose over the entire length of the coiled-coil (Fig. 3B). When the relaxed-best and disordered-best filament maps are aligned at a crown corresponding to the myosin C-terminus, the main difference observed is a clockwise shift of the helices by an amount corresponding to the change in helical pitch. At the N-terminus, this shift corresponds to about half the diameter of an α -helix (Fig. 3C,D).

More dramatic changes are observed in the structure of the myosin heads (Fig. 4). In the relaxed-best reconstruction, the density corresponding to the IHM is reasonably high and well-defined, when contoured at a threshold that will encompass the blocked-head atomic model (Fig. 4A). Only the SH3 domain extends outside of the envelope, leaving some empty density positioned somewhat clockwise of the SH3 domain. Although it is tempting to assign the empty density to the SH3 domain, the large population of published crystal structures do not suggest any precedent for the large shift needed to make the SH3 domain fill the empty density, so this feature of the relaxed thick filament IHM remains to be explained (but see below).

The density of the IHM of the disordered-best thick filament is significantly different (Fig. 4B). When comparing maps from two independent reconstructions, the results are sensitive to the resolution and the choice of contour threshold. In order to show the heads, we low-pass filtered the two maps to the same resolution and then selected the thresholds that most closely matched the backbone features (because resolution was highest in the backbone). At the matching threshold for the backbone, the density corresponding to the blocked-head motor domain disappears from the disordered-best reconstruction leaving behind reduced density for the blocked-head ELC but strong density for the blocked-head RLC (Fig. 4B,C). The free-head density is strong for all features from the RLC to the motor domain. Moreover, the S2 domain is swung to the side just as it is in the relaxed-best thick filament

(Fig. 4C). The free-head motor domain may have enhanced mobility based on reduced density near the tip of the head (arrow in Figure 4) compared to the relaxed state, but mostly it remains unchanged.

DISCUSSION

Similarities among relaxed thick filaments

In muscles of all types, and to a certain extent non-muscle cells, thin filaments are quite similar in structure, having actin, tropomyosin, capping proteins and the troponin complex (if thin filaments are regulated by calcium). Although differing in sequence, the associated proteins are quite similar in their functions. The helical pitch of thin filaments may also vary but not by much (from 13/6 to 28/13 subunits/turn). On the other hand, thick filaments from different muscle types and species are quite variable in structure and composition seemingly having in common only the conformation of their heads in the relaxed state, which is highly conserved in muscle and non-muscle cells (Jung et al., 2008a). Thick filament structures range from short, bipolar types found in non-muscle cells (Scholey et al., 1980), side polar filaments of vertebrate smooth muscle cells (Cooke et al., 1989), bipolar filaments with helically arranged myosin in striated muscle of invertebrates (Pinto et al., 2012; Sulbaran et al., 2015; Woodhead et al., 2013; Woodhead et al., 2005; Zhao et al., 2009) and non-helical, or approximately helical filaments in vertebrates (Zoghbi et al., 2008).

Protein compositions of thick filaments are also quite variable, and mutually exclusive. Vertebrate thick filaments have myosin binding protein C (Bennett et al., 1999); invertebrates thick filaments have paramyosin (Levine et al., 1976). Vertebrate thick filaments have the giant protein titin (3 MDa), which spans the distance from the Z-disk to the center of the thick filament (the M-band); whereas invertebrate flight muscles have projectin, a shorter version of titin that only spans from the Z-disk the ends of the thick filaments. Thick filament structures vary widely among species and muscle types. However, they apparently share one thing in common in their relaxed state, the IHM.

In the majority of reported thick filament structures (Sulbaran et al., 2015; Woodhead et al., 2013; Woodhead et al., 2005; Zhao et al., 2009; Zoghbi et al., 2008), the IHM folds back onto the rod so that the blocked head contacts its own S2 domain (Fig. 1C). It has been suggested that this S2-blocked head interaction is essential to form a stable IHM (Jung et al., 2008b; Trybus et al., 1997), and that the amino acid residues potentially involved in the S2-blocked head interaction are conserved across myosin species that display the IHM (Alamo et al., 2016; Blankenfeldt et al., 2006). However, *Lethocerus* thick filaments display a perpendicular IHM (Fig. 1D), which does not fold back to contact its own S2 (Hu et al., 2016); and thus the S2-blocked head contact must not be strictly required to form a stable IHM. Moreover, the *Lethocerus* flight muscle myosin sequence lacks the net negative charge (Fee et al., 2017), which is thought to stabilize the folded-back S2-blocked head contact (Alamo et al., 2016; Blankenfeldt et al., 2006). We conclude that while the blocked head-S2 interaction provides stability in some myosin species, it is not essential to formation of the IHM in all cases.

The unique orientation of the *Lethocerus* IHM suggests some functional significance for the phenomenon of stretch activation, which we explore here. Stretch activation is defined as a delayed increase in active tension following a stretch (Pringle, 1978). The stretch itself causes an instantaneous increase in tension but this response in the muscle is transient; the subsequent active tension development occurs after a short delay and can be prolonged depending on the muscle type. Stretch activation has been observed in all striated muscles where it has been investigated, but it is not equally strong. The magnitude of the response correlates with the passive stiffness of the muscle itself, being strongest in the highly stiff asynchronous insect flight muscles and weakest in the more compliant vertebrate striated muscles; cardiac muscle stiffness is intermediate as is its stretch activation response (Moore, 2006). Various mechanisms for stretch activation have been described including stretch-induced thin filament activation (Perz-Edwards et al., 2011). What follows is not meant to preclude such mechanisms, which may operate concurrently, but to provide some novel ideas not previously proposed.

Differential mobility of the blocked and free heads

The high-resolution image of the relaxed *Lethocerus* flight muscle thick filament suggested higher mobility of the blocked head compared to the free head (Hu et al., 2016). The present reconstruction made from filament images that were rejected within the same population of micrographs because they appeared less ordered, has produced a 3-D image that is similar in nearly all features other than helical twist and blocked-head motor domain density, although at lower resolution. The criterion for selecting 50,000 segments for the disordered-best reconstruction from the 100,000 filament segments of disordered thick filaments, was visibility of the S2 domain in class averages. This criterion used to select segments for the disordered-best filament reconstruction, produces a biased subset of images. However, it's the same bias used for the better-ordered relaxed-best filament reconstruction. Despite having twice as many segments as the relaxed-best reconstruction, the disordered-best reconstruction could not be pushed to greater resolution. The reconstruction computed from ~50,000 relaxed-worst filament segments, which were rejected based on the lack of S2 visibility (Hu et al., 2016), was similar in most respects to the reconstruction of the relaxed-best filament segments except for the resolution within the IHM and a very slight change in the helical angle. The reconstruction computed from ~50,000 disordered-worst thick filament data set was similar to the disordered-best reconstruction. The only difference is the contour level selected to match the backbone features. Disordered heads can be produced in a variety of ways, some physiological, such as RLC phosphorylation (Levine et al., 1996), some in vitro, such as addition of EDTA, and some by experimental artifacts such as contact of the IHM with the air water interface. Nevertheless, the present result suggests the disorder in the blocked head is coupled to the change in helical angle. External tension applied to relaxed *Lethocerus* flight muscle also changes the helical angle and the diffraction pattern, particularly the intensity of the 145-Å reflection (Perz-Edwards et al., 2011). However, the interpretation of the intensity change must be modeled in order to deduce what happens in situ.

Although the blocked head in relaxed *Lethocerus* thick filaments, even the best ordered ones, appears more mobile than the free head, its mobility is highly restricted because the

free head remains bound to the filament backbone constraining mobility of the S2 tether. When the blocked head dissociates from the free head, it can only pivot around its junction with S2 because S2 motion is constrained by the free head-backbone association, which is very different from that seen in all other relaxed thick filaments to date. In relaxed *Lethocerus* thick filaments, the free-head contacts with the backbone involve the RLC, possibly the ELC, and the motor domain (Hu et al., 2016). The *Lethocerus* free-head RLC-rod contact has hydrophobic character giving it greater stability; it can be imaged at subnanometer resolution where an accurate atomic model can be built to show the residues involved.

In other thick filaments, of which those from Tarantula striated muscle are the most extensively described, there appears to be no contact between the IHM and the filament backbone other than through the blocked head, which contacts its own S2 as well as the S2 from a myosin molecule in the next pair of heads from the next Z-ward crown (Woodhead et al., 2005). Thus, the topology of IHM-backbone interaction in Tarantula is exactly the opposite of that of *Lethocerus*, where the blocked head makes no contact with anything other than the free head. A model has been proposed for activation in relaxed Tarantula thick filaments based on the blocked-head interactions with S2, in which the free head is dynamic and more easily phosphorylated than the blocked head, which is static until contact is made with actin (Alamo et al., 2016).

The S2 tether is a key parameter in modeling the possible interactions between myosin heads and actin subunits in muscle (Haselgrove and Reedy, 1978; Haselgrove and Reedy, 1984). The length of S2 is typically determined from proteolysis of monomeric myosin dissolved in high salt; the cleavage site is unavailable in filaments. The exact cleavage site is poorly defined but can be taken to be near R1175 in smooth muscle myosin, which corresponds to the site of the first coiled-coil bend in the 10S conformation (Burgess et al., 2007), resulting in an S2 length of ~ 480 Å. However, in *Lethocerus* flight muscle the effective length of the S2 tether has been measured experimentally by swelling rigor muscle and is only ~ 110 Å (Liu et al., 2006), far shorter than that presumed from proteolysis. We think that the effective length of the S2 in other striated muscle thick filaments will also prove to be shorter than the conventional length. In the *Lethocerus* case, the disordered thick filaments indicate that blocked-head dissociation can occur while free head remains docked onto the thick filament backbone. With the free head remaining docked, the blocked head will be constrained to pivoting movements about a stably positioned S2. Despite this constraint, the blocked-head motor domain is already very close to actin and does not require radial movements of its S2 tether to reach actin targets. Once detached from the free head, the blocked head need only straighten slightly for sufficient radial movement to contact actin (Fig. 5).

Consequences of differential head mobility

As illustrated in Figure 5, no radial movement of the S2 domain is needed for the dissociated blocked head to reach the thin filament; mere dissociation and straightening of the myosin head is sufficient. However, this would limit the possible actin azimuths that the blocked head could reach. We know from previous work that the force producing heads in *Lethocerus* flight muscle bind a very restricted region of the thin filament called target zones

(Reedy, 1968; Wu et al., 2010) that are positioned midway between Tn complexes. Myosin heads can also contact the Tn complex on the thin filament as well as contact TM on the M-ward side of the target zone (Wu et al., 2010; Wu et al., 2012). Tn contacts may contribute to stretch activation through some of the novel flight muscle extensions found in the TnI protein but how this works is not clear (Perz-Edwards et al., 2011). However, the involvement of the TnI extension in stretch activation seems unlikely since proteolytic cleavage seems to have little effect in honey-bee flight muscle (Iwamoto, 2013).

Blocked-head contacts near the target zone provide a useful focus. If they occur following the stretch, when the thin filament is fully activated, and the target zone is positioned at the correct azimuth, the blocked head could bind and generate tension. The S2 domain in *Lethocerus* is strategically angled azimuthally so that tension would straighten the S2 tether toward parallel with the filament axis, an orientation that would pull the free head loose from the filament backbone thereby activating it. Target zones consist of two actin subunits on each long pitch helical strand of the thin filament. Blocked-head attachment to one target zone actin would likely limit simultaneous target zone attachment of the newly freed free head to the second actin of the same target zone. Such two-headed target zone cross-bridges are indeed found in actively contracting flight muscle fibers (Wu et al., 2010; Wu et al., 2012). Conversely, after the free-heads complete their power stroke, and have bound and cleaved ATP, they may resume their relaxed configuration on the thick filament backbone, thus restricting the ability of blocked heads to locate another target zone, an effect that would contribute to shortening deactivation.

One key to this mechanism would be the rate of cleavage of ATP to yield transition state complexes and the refolding rate of the IHM. To our knowledge, there is no direct biochemical measurement of the kinetics of IHM formation. IHM formation appears to require cleavage of ATP to form transition state complexes because in all IHM structures observed in thick filaments to date, the lever arms are in the transition state position. At room temperature, the ATP hydrolysis rate of *Lethocerus* flight muscle myosin is ~30/sec (White et al., 1986) with a Q_{10} of 2.3 (Ruegg and Tregear, 1966). At the physiological muscle temperature of ~40 °C, the ATPase rate is 4× faster than the wing beat frequency of 20–40 Hz. Therefore, in *Lethocerus* flight muscle the ATPase rate is fast enough that the IHM could form every contraction/relaxation cycle.

Somewhat indirect measures of the IHM refolding rate have been obtained from several sources but not from flight muscle. A time resolved, negative stain electron microscopy study of the refolding rate of scallop adductor muscle (a striated muscle) concluded that complete refolding occurred within 50 ms, a rate of 20/sec (Zhao and Craig, 2003), a value not far from that needed for *Lethocerus* thick filaments.

Three types of biochemically distinct relaxed states have been described in the literature, with each state corresponding to different arrangements of the myosin heads. The fully formed IHM is proposed to correspond to the super relaxed state, which has the lowest ATP turnover rate (Stewart et al., 2010). At the other extreme, a disordered relaxed state has been described in which the muscle is relaxed but the heads are disordered and can bind weakly to actin but don't generate force (Wilson et al., 2014). A third, intermediate, state has been

described in Tarantula filaments in which one head of the IHM appears disordered and the other head ordered (Brito et al., 2011; Naber et al., 2011). The state described here in relaxed *Lethocerus* thick filaments appears to correspond to precisely this third state. However, the nature of this intermediate state is the opposite in the two species. In Tarantula, the blocked head is docked to the thick filament and the free head is disordered, whereas in *Lethocerus*, the free head is docked to the thick filament and the blocked head is disordered.

Changes in helical twist

Lethocerus thick filaments were the first to demonstrate changes in helical twist as a result of an externally applied force (Perz-Edwards et al., 2011). The axial spacing changes in *Lethocerus* thick filaments, 0.03–0.05%, correspond to 0.07 Å per crown, which would lead to a cumulative displacement of 0.8 Å after 11 crowns. Potentially more significant are the changes brought on by the reduction in the helical angle. As a percentage of 360°, the 0.16° degree change in the helical angle observed in our disordered-best reconstruction is also rather small, amounting to 0.04%. The cumulative change in helical angle amounts to 1.76° after 11 crowns. The question, for which we have no answer at the moment, only speculation, is how can such small changes in the thick filament backbone impact the stability of the blocked head, when it makes essentially no contact with the backbone other than through the S2 domain. Conversely, the free head makes multiple contacts with the backbone, yet seems unaffected by the change in helical twist. The length of the myosin rod, which extends for 11 crowns, could act as an amplifier of the small change per crown. The displacement of the rod α -helices is largest where the S2 domain exits the backbone and smallest near the C-terminus, which is buried deepest in the backbone. A displacement of ~5 Å is large at the level of protein-protein interactions. However, all the rods are being displaced at the same time and in the same direction so the changes on a per residue basis will still be small. Perhaps, the rod acts as an integrator of a large number of small effects on individual residues.

We can think of two possibilities for how the change in helical twist might affect the blocked head. (1) The most obvious is that the free-head conformation or position is altered, even though it seems to be unaffected at the ~20 Å resolution at which it can be visualized in our reconstructions. There is a small change in the free-head density in the disordered-best reconstruction, but it has not been possible yet to build a model sufficiently convincing to describe the change. Small changes in the free head could have a large effect on the stability of its interaction with the blocked head, which appears to be more dynamic. (2) Small changes in the rod structure within the backbone are passed directly through to the blocked head via its S2 link with the backbone. A potential mechanism for this has previously been described (Tama et al., 2005). In the Tama et al. model, folding of the heads of smooth muscle myosin produces torsional rotations in the individual rod helices, which are transmitted throughout the length of the rod. There is at the moment no direct experimental evidence for the head folding affecting the structure of the rod. To demonstrate an effect will require about twice the resolution achieved in this report.

The phenomenon of stretch activation requires not only that an external stretch activate the myosin heads and/or the thin filament, but also that as the muscle shortens, the reduction in

tension enhances muscle relaxation, a phenomenon known as shortening deactivation. The structure of the IHM reported here in combination with the previous report (Hu et al., 2016) suggests a detailed mechanism for shortening deactivation. Several steps would be involved:

1. The external stretch disorders the blocked head preferentially, which can then bind any target zone where TM movement has moved to the closed position enabling the weak to strong transition.
2. Conversion to strong binding and the following lever arm swing would result in the S2 domain, which is angled azimuthally relative to the filament axis in the relaxed state, becoming aligned with the filament axis thereby ripping the free head from the filament backbone. This mechanism is similar to the force that that was hypothesized to straighten the myosin lever arm azimuthally in contracting muscle (Arakelian et al., 2015).
3. The free head is now literally free to bind actin. The nearest available site would be the target zone actin subunit not occupied by the blocked head within the same target zone.
4. The free head attaches the target zone, executes a power stroke and then detaches after rebinding ATP. The thin filament must move axially by steps of about six actin subunits before either head can reattach during stretch activated cycling and each head must reprime its lever arm by cleaving ATP.
5. If there is no available target zone for reattachment of either head, the free head assumes its docked position on the thick filament resulting in greatly diminished freedom of the blocked head to search for available activated target zones. If none are available, tension would drop.
6. The blocked head reassumes its docked position on the free head thereby reforming the IHM.

We think that the dynamic behavior of the blocked head provides a mechanism whereby the thick filament can sense the activation state of the thin filament as well as alter thin filament activation through interactions with the troponin complex, the so-called troponin bridges (Perz-Edwards et al., 2011; Wu et al., 2010; Wu et al., 2012).

Finally, moving toward higher resolution images of relaxed thick filaments, it may be useful to include the assessment of helical parameters when selecting homogeneous filament segments for reconstruction. Based on our observations here, very small changes in the helical angle, have a large effect on the appearance of the myosin heads.

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Figure 1.

Diagrams of relaxed thick filament structures. (A) The interacting heads motif (IHM) is characterized by an asymmetric interaction between the two myosin heads. One head, the blocked head (red), has its actin-binding interface juxtaposed to the other head (purple), the free-head motor domain (MD) and essential light chain (ELC, green). The free head actin-binding interface is not blocked so that theoretically the complex could bind actin in solution. The regulatory light chains (RLC) are colored yellow (blocked head) and orange (free head) while the blocked-head ELC is blue. The RLC, ELC and part of the myosin

heavy chain form a lever arm, movements of which produce filament sliding. (B) Schematic view of the IHM. (C) Orientation of the IHM in relaxed Tarantula thick filaments. Here the IHM is largely tangential to the surface of the thick filament backbone. This orientation has been observed in all relaxed thick filaments save those from *Lethocerus* flight muscle. (D) Orientation of the IHM in relaxed *Lethocerus* flight muscle thick filaments is nearly perpendicular to the filament axis with the blocked-head SH3 domain at the highest radius. Adapted from (Hu et al., 2016).

Figure 2.

Electron micrograph of relaxed *Lethocerus* thick filaments. Densities transverse to the thick filament axis are called “crowns”. Crowns are spaced axially by 145 Å, and are well defined in ordered filaments and poorly in disordered filaments. However the terms are relative, with no clear division between groups. In any field of view, there are filaments that are better ordered than others. The marked thick filament showed crowns that were less distinctive than those of better-ordered filaments. The better ordered filaments were chosen for 3-D

image reconstruction of the “relaxed” thick filaments. The less-ordered filaments, marked by the black line, were selected later for the present reconstruction.

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Figure 3.

Comparison of the backbone structure of well ordered relaxed thick filaments and poorly ordered relaxed thick filaments. Relaxed filaments are colored yellow, poorly ordered filaments are colored blue. (A) Both reconstructions superimposed at a resolution of 6.4Å. Non-myosin densities, of which four are positioned among the myosin rods do not show well at this resolution. A fifth non-myosin protein, paramyosin, forms the core, but does not follow the myosin helix. Both reconstructions are aligned at the crown position at the top of panel A. (B) A single rod segmented from each reconstruction. When separately aligned, the

structures superimpose well. (C) A cross-section at the top crown illustrating the alignment of the two reconstructions at this level, which corresponds to the C-terminus of the myosin rod. (D) The corresponding rod alignment at the N-terminus of the rod. Here the change in helical structure, multiplied over 11 crowns is visible as an azimuthal offset of a magnitude approximately half the diameter of an α -helix.

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Figure 4.

Comparison of the crown structures. (A) A single crown from relaxed thick filaments of *Lethocerus* flight muscle from (Hu et al., 2016) low pass filtered to 20 Å resolution. Of the four IHMs within the crown, the rightmost is outlined and shown filled with an atomic model of the IHM, displayed as a surface. The coloring scheme for the atomic model is the same as shown in Figure 1. An identical outline of the IHM is superimposed on panels B and C. (B) A single crown from the disordered thick filament structure. There is significant loss of density at the position of the blocked-head motor domain. However there remains significant density for the blocked-head RLC and diminished density for the its ELC. The free-head motor domain also seems to be affected but the free-head ELC and RLC appear

unaffected. The contour thresholds were chosen to match the outlines of the myosin rods, not to match the outlines of the heads. (C) Superposition of the two reconstructions with the relaxed thick filament outline set at a transparency of 50%. The densities match well for the S2 segment and the neighboring RLCs, but less well for the motor domains and blocked-head ELC regions. The arrow points to a region of the map where the contours of the disordered and the ordered free-head motor domain envelopes match poorly indicating that the free-head motor domain may have changed its orientation when the blocked head dissociated or changed its orientation causing the blocked head to dissociate. (D) Longitudinal view of the same maps shown in panel C. Note that the S2 densities overlap very well indicating that the free head has remained bound to the thick filament backbone.

Figure 5.

Model for disorder of the blocked head. The coloring scheme for the atomic model is the same as for Figure 1. The blocked head appears to be prone to dissociation from the IHM leaving the free head attached to the thick filament. The thin filament atomic model from (Wu et al., 2010) is shown in solid view at a 260 Å distance from the thick filament axis with target zone actins colored green and blue, tropomyosin, yellow and troponin, orange. The azimuth of the thin filament model is correct with respect to the inter-thick-filament-axis. In (A) one blocked head in the conventional IHM orientation is depicted with a transparency of

50%. The second blocked head superimposed on the IHM is from the scallop myosin transition state crystal structure, PDB 1DFL, aligned at the N-terminal lobe of the RLC. Folding into the IHM bends the blocked head in order to form the binding interface with the free head. When released from the free head, the blocked head is free to straighten out which is sufficient for the actin-binding domain of the blocked head to contact a target zone actin subunit (green). (B) A 20° anticlockwise change in the thick filament azimuth is all that is needed to place the blocked-head SH3 domain into contact with Tn.

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Table 1

Data Set	# of segments	Helical angle	Axial spacing (unscated)	FSC resolution	Comment
Relaxed-best	~24,000	33.98°	144.0 Å	5.5 Å	(Hu et al., 2016)
Relaxed-worst	~50,000	33.95°	–	~7.2 Å	Similar to relaxed-best except in resolution of the heads, which is lower
Disordered- best	~50,000	33.82°	–	6.4 Å	Reduced density for blocked-head motor domain
Disordered- worst	~50,000	33.85°	–	~7Å	Similar to disordered-best, but contour needed to match backbone envelope is lower