Structure of myosin filaments from relaxed _Lethocerus_ flight muscle by cryo-EM at 6 Å resolution

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We describe a cryo–electron microscopy three-dimensional image reconstruction of relaxed myosin II–containing thick filaments from the flight muscle of the giant water bug _Lethocerus indicus_. The relaxed thick filament structure is a key element of muscle physiology because it facilitates the reextension process following contraction. Conversely, the myosin heads must disrupt their relaxed arrangement to drive contraction. Previous models predicted that _Lethocerus_ myosin was unique in having an intermolecular head-head interaction, as opposed to the intramolecular head-head interaction observed in all other species. In contrast to the predicted model, we find an intramolecular head-head interaction, which is similar to that of other thick filaments but oriented in a distinctly different way. The arrangement of myosin’s long α-helical coiled-coil rod domain has been hypothesized as either curved layers or helical subfilaments. Our reconstruction is the first report having sufficient resolution to track the rod α helices in their native environment at resolutions ~5.5 Å, and it shows that the layer arrangement is correct for _Lethocerus_. Threading separate paths through the forest of myosin coiled coils are four nonmyosin peptides. We suggest that the unusual position of the heads and the rod arrangement separated by nonmyosin peptides are adaptations for mechanical signal transduction whereby applied tension disrupts the myosin heads as a component of stretch activation.

INTRODUCTION

Muscle contraction is driven by the motor protein myosin II, which pulls on and translates actin filaments (I). The basic structure of myosin II (henceforth referred to simply as “myosin”) has been known for decades. The molecule has two heads, called subfragment 1 (S1), and a long tail, called the rod domain (Fig. 1A). When myosin polymerizes, the rods pack together to form thick filaments, which are bipolar in all striated muscles. The heads are the motors that move actin (I), and they project from the surface of the filament (Fig. 1, B and C). There have been two long-standing questions: How do the rods pack together to form the filament backbone? How are the heads docked on the surface of the filament so as to be ready to bind actin when needed and inactive when not needed?

The docking of the heads onto the thick filament backbone may be directly relevant to the physiology of how muscles most efficiently maintain the relaxed state (2, 3). Several published three-dimensional (3D) reconstructions of relaxed myosin thick filaments (4–8) indicate that the heads are docked in an asymmetric structure known as the interacting heads motif (IHM) (Fig. 1D). The IHM was first observed in smooth muscle myosin and explained how smooth muscle myosin is switched on and off by phosphorylation (9). In the IHM, one head is called blocked because its actin-binding domain contacts the adjacent head and is therefore unavailable to bind actin. The other head is called free because its actin-binding domain is not obstructed and could theoretically bind actin. The IHM was originally thought to be specific to smooth and nonmuscle myosin but was later observed in thick filaments from tarantula (4, 10), scallop (8), _Limulus_ (5), and human cardiac muscle (6, 7), leading to the suggestions that the IHM is a universal feature of all thick filaments (11) and that it provides a mechanism for maintaining the so-called superrelaxed state (2, 3).

Our experience with flight muscle from the giant water bug, _Lethocerus_ spp., led us to question the IHM’s universality for two reasons. First, images of relaxed thick filaments from _Lethocerus_ flight muscle show the myosin heads extending perpendicular to the filament axis to form distinct, narrow shelves of density (12), which first led to the term “crowns” to describe where the myosin heads emerge from the thick filament backbone (13). This appearance is quite distinct from that of other relaxed thick filaments, such as those from tarantula. Our second reason came from modeling the thick filament structure from low-resolution x-ray fiber diffraction patterns. In the preferred model, there was no intramolecular head-to-head contact as seen in the IHM. Instead, the two heads splayed away from one another to contact neighboring molecules (14). Thus, we did not expect to see evidence of the IHM in _Lethocerus_ filaments.

The structure of the myosin head has been well characterized by x-ray crystallography (15), and several quasi-atomic models of the IHM exist (4, 9, 10). However, comparatively less is known about the detailed structure of the rod domain or how the rods pack together to form the thick filament backbone. It has long been known that the rod is an α-helical coiled coil, based on sequence analysis as well as x-ray fiber diffraction (16–19). Only a few crystal structures of short disconnected myosin rod segments have been published (20–23). Sequence analysis of the rod shows the heptad repeat characteristic of coiled coils, four skip residues where an extra amino acid interrupts the heptad repeat, and a 28-mer repeat giving rise to periodic, alternating regions of positive and negative charge. The charge profile suggests favorable ionic interactions between rods axially staggered by odd multiples of 145 Å, with 435 Å being most probable (18, 24, 25). Several published models predict how the rods might...
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Published 3D reconstructions tend to support the subfilament model (40 Å subfilaments possibly associating to form larger subfilaments. Other to make a helical subfilament ~40 Å in diameter, with several 100 Å subfilaments wrapping around the thick filament backbone (26, 27). The most widely accepted model envisions a few rods wrapping around each other to make a helical subfilament ~40 Å in diameter, with several 40 Å subfilaments possibly associating to form larger subfilaments. Published 3D reconstructions tend to support the subfilament model but lack the resolution to see individual rods to clarify the packing (4, 5, 8, 28). Modeling of the high-resolution x-ray fiber diffraction pattern from vertebrate muscle supported a different arrangement, with side-to-side association of rods into layers, but could not rule out subfilaments (19).

Thick filaments contain additional proteins besides myosin. In insects, these include projectin, kettin, flightin, myofilin, and paramyosin. Projectin and kettin connect the thick filament to the Z-band of the sarcomere and are thought to provide the high passive stiffness in insect flight muscle (29). The exact function and structure of myofilin and flightin are unknown (30, 31), but flightin deletion mutants in Drosophila have abnormal thick filaments and are flightless (32, 33). Paramyosin is an α-helical coiled-coil protein with homology to the rod domain of myosin and is found in the core of invertebrate thick filaments in variable amounts, depending on species (34). There are no known analogs to paramyosin, flightin, or myofilin in vertebrates; conversely, there is no known analog in insects to myosin-binding protein C found in vertebrate thick filaments (35). Projectin and kettin are considered mimittins, similar to vertebrate titin but shorter. They connect only to the ends of the thick filament in insect flight muscle (36). None of these accessory thick filament proteins have been imaged at high resolution, although densities imaged at low resolution have been assigned to titin- and myosin-binding protein C in vertebrate thick filaments (6).

Here, we report a 3D image reconstruction obtained by cryo-electron microscopy (EM) of relaxed thick filaments from an asynchronous flight muscle of the giant water bug, Lethocerus indicus. The reconstruction reveals a novel arrangement of the heads, unprecedented resolution of the rods and details of their packing in the backbone, additional nonmyosin proteins embedded in the filament backbone, and a central core of protein, presumably paramyosin.

RESULTS

Cryo-EMs of filaments show regularly spaced, distinct crowns (Fig. 2A), similar to those seen by conventional or negative-stain EM (12, 37, 38). We computed a 3D density map (Fig. 2, B and C) from ~24,000 particles, that is, ~450 Å–long filament segments windowed from the EMs and processed using the RELION software package (39). The global resolution of the reconstruction is 6.2 Å (fig. S1A). However, resolution varies by region, and a ResMap calculation (40) indicates a poorer and variable resolution for the myosin heads, ~12 to ~21 Å (fig. S1B). The region where the myosin rods are packed is much better defined, at 5.5 Å (fig. S1, C and D). Further inside the filament, the contrast is weak and the resolution estimate is ~10 Å. The Fourier transform of our reconstruction shows excellent correspondence to the x-ray diffraction pattern from whole muscle (fig. S2), supporting the validity of this structure.

The myosin interacting heads motif

When the map is low-pass–filtered to 20 Å, to account for the worse resolution in the region of the heads, the crown structure can be clearly visualized (Fig. 2B). Helical myosin filaments are described by their rotational symmetry, that is, the number of head pairs in each crown, the axial spacing, and the relative rotation between successive crowns. The axial spacing is ~145 Å in all species, whereas the rotational symmetry and the rotation per crown vary by species. The known fourfold rotational symmetry of Lethocerus filaments (38, 41, 42) is the only symmetry imposed in the reconstruction, whereas the axial repeat and the rotation per crown are allowed to freely vary and are refined and reported during the iterative reconstruction process. The final axial repeat value was 144.0 Å; therefore, the map was rescaled to the known value, 145 Å (43). This rescaling corresponds to changing the image pixel size from 1.215 to 1.223 Å per pixel, well within our magnification uncertainty. The final rotation per crown is 33.98°, which is similar to the previously reported value, 33.9° (44). Stacking multiple copies of the map together creates a pseudo filament, provided that each copy is translated and rotated by a multiple of 145 Å and 33.98°, respectively (Fig. 2C).
portion of subfragment 2 (S2) that connects the myosin heads to the thick filament backbone. The length of the tether, ~110 Å, agrees with previous efforts to visualize S2 in *Lethocerus* by pulling it away from the backbone (45).

The crown structure is composed of four separate blobs that are highly asymmetric when seen in either longitudinal or cross section. We docked the IHM structure PDB (Protein Data Bank) 1I84 into the map as a rigid body and found that it fit reasonably into the map in only one orientation (Fig. 2, D to F, and movie S1; see also fig. S3). When so placed, the S1-S2 junction points directly into the rodlike tethers (Fig. 2F, red arrow), confirming our identification of the tether as S2.

At the chosen contour level, parts of 1I84 stick out of the map, especially for the blocked head (Fig. 2, D to F, red), whereas other parts fit loosely within the envelope. The variable fit is partly an effect of the variable resolution, which is worst for the blocked head and better for the free head (fig. S1B). A flexible fitting of the atomic model would presumably improve the fitting for both heads. However, we elected not to do one, given that we cannot resolve secondary structure in the heads and a flexible fitting might not give meaningful results. The rigid-body docking of 1I84 is sufficient to conclude that *Lethocerus* thick filaments do have an IHM-like structure, in contrast to the model predicted from low-resolution x-ray diffraction patterns (14).

In sharp contrast to other muscle types, the *Lethocerus* IHM is oriented perpendicular to the filament axis (Fig. 2D and fig. S4). Also in contrast to other muscle types (4–6), the IHM appears to be freestanding, with no stabilizing contacts between neighboring IHMs, either within the crown or between successive crowns. The position of the IHM points the free head toward the filament backbone and away from where an adjacent actin filament would be positioned in the muscle sarcomere and, in this sense, is similar to other filament types (4–6, 46). However, the IHM-backbone contacts are unique.

The free head (Fig. 2, D to F, purple) appears to make three contacts with the thick filament backbone. One is via the motor domain, another via the ELC, and a third via the RLC (Fig. 2E, arrowheads). At the current resolution, the motor domain and ELC contacts must be considered speculative. The RLC contact is not speculative, because it is well ordered, as described later. The RLC contacts the S2 tether from the crown below, very near the region where that S2 merges into the filament backbone. This region of S2 is known as Ring 3, a concentrated region of negatively charged residues in cardiac β-myosin (21).

In contrast to the free head, the blocked head (Fig. 2, D to F, red) appears to make no contact with the backbone or S2, which may explain why the blocked head is more disordered and, hence, has lower resolution in the reconstruction (fig. S1B). The SH3 domain of the blocked head fits poorly to the map, as shown (Fig. 2E). However, it is interesting to note that the SH3 domain is thus positioned farthest from the thick filament center and therefore closest to where an adjacent actin filament would be located in the muscle sarcomere.

The entire myosin molecule

When the map is low-pass–filtered to 5.5 Å, the heads and the S2 tethers become noisy because of their poorer resolution and effectively disappear from the map, but a forest of α-helical coiled-coil rods that make up the backbone is clearly revealed for the first time (Fig. 3, A and B, and movie S2). Small, disconnected densities within the head region correspond to the RLC of the free head (Fig. 3, A to C, orange).

Note that the pseudofilament does not represent the central, head-free bare zone or the tapered ends of the filament, which were excluded from the reconstruction.

Four thin rods (Fig. 2B, red arrow) emerge from each crown and appear to tether the crown to the backbone. These tethers bend 18° to the right and 11° inward to merge with the backbone near the crown above. The obvious conclusion is that the tether must be the N-terminal part of subfragment 2 (S2) that connects the myosin heads to the thick filament backbone. The length of the tether, ~110 Å, agrees with previous efforts to visualize S2 in *Lethocerus* by pulling it away from the backbone (45).

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The 20 Å filtered map can be combined with four copies of the 5.5 Å map to make a pseudofilament from which a continuous density representing an entire myosin molecule may be traced and segmented (Fig. 3D). The center molecule in Fig. 3D is seen en face and shows that the rod meanders from side to side azimuthally but runs approximately parallel to the filament axis. The side molecules are turned 90° and show that the rod also meanders in and out radially but is approximately straight and tilted inward by ~1.5°, in agreement with the tilt value predicted from modeling the high-resolution x-ray diffraction pattern from vertebrate muscle (19). The actual path of the rod is somewhat tortuous, which can best be appreciated in an axial view (Fig. 3E and movie S3), although once the tether joins the backbone at ~100 Å radius, the variations are small relative to the ~20 Å width of the coiled coil (Fig. 3E, circles).

The contour length of the rod, including the tether, is 1598 Å. With a rise per residue of 1.485 Å (47), this length corresponds to 1077 amino acid residues, spanning from R843 near the head-rod junction to F1919 of the Drosophila sequence (accession no. NP_724008.1), used here because no Lethocerus sequence has been published to date. By this reasoning, the last 17 residues (R1920 to I1936) do not appear in the map, presumably because they are disordered, and indeed, a MARCOIL (48) analysis of the sequence indicates that the probability of forming a coiled coil drops to <50% for the last 15 residues. Although using the Drosophila sequence hampers this analysis, there is high conservation of the rod sequence among representative insects, with >88% identity (table S1). Therefore, we tentatively conclude that we are imaging all regions of the molecule that we expect to be structured, implying that we may use the contour length as an approximation of the sequence.

Fig. 3. Myosin filament backbone. (A) The 5.5 Å map illustrates the myosin rod density within the 20 Å envelope. One myosin rod is colored blue and the free-head RLC densities are orange. (B) A portion of (A) magnified ×2 and rotated 34° shows the unwound coiled coils in the Skip 1 region of the rod (blue). The free-head RLC density (orange) is closely juxtaposed to the S2 of another myosin molecule. (C) An M-ward view of the IHM juxtaposed with myosin rods shows the free-head heavy chain and RLC contacts to the rods. (D) Three symmetry-related molecules in one crown are shown. The arrows on the right mark the coiled-coil crossovers. (E) Z-ward view of the path of a single rod in axial projection, color-coded red to blue by Z-height. Large circle, 100 Å radius. Two 10 Å-diameter circles surrounded by a 20 Å circle are placed where the clipping plane intersects the S2 tether and represent the coiled coil of the rod. (F) Skip region atomic structures fitted to the rod density. Skip residues are colored red; red spheres indicate their predicted positions from the contour length of the rod. Arrowheads indicate bridging densities in the map filled by bulky side chains.
The skip residues
Within the rod, the crossover period (that is, half of the coiled-coil pitch; Fig. 3D, arrows) varies widely from 60 to 126 Å, whereas the mean, 86.4 Å, is within the range expected based on x-ray diffraction, 89 ± 5 Å (17). One notable region of especially long pitch can be seen on the surface of the filament backbone where the rod bends to the right and the coiled coil locally unwinds so that the α helices are parallel rather than wrapping around each other (Fig. 3B, blue). By contour length, this region corresponds to the first myosin skip residue (18), as well as the S2–light meromyosin junction and a bending point in the rods of isolated molecules (49). Crystal structures of short rod segments surrounding the skip residues show an extended unwinding of the coiled coil into parallel helices for Skips 1 to 3 (22), similar to what we see in the map for the Skip 1 region. We used the Drosophila sequence and the skip residue crystal structures to build homology models using SWISS-MODEL (50). The Skip 1 model fit into the map remarkably well, with the skip residue aligned very close to its location predicted by contour length (Fig. 3F). The automatic rigid-body fitting by Chimera (51) appears to be dominated by two pairs of stacked histidines (Fig. 3F, arrowheads), which are the only residues in the model with sufficient electron density to show some fingerprint in our map.

In contrast to the good match between the atomic model and the map Skip 1 region, the remaining models match poorly to their corresponding skip regions, although there are some similarities. In the original crystal structures, Skips 1 to 3 were virtually identical unwound coiled coils, whereas Skip 4 showed a bent molecule with normal coiled coils on either side of the bend. In contrast, we find that the Skip 2 region appears to be normally coiled or even hypercoiled (½ pitch, ~60 Å), whereas both Skips 3 and 4 showed some local unwinding (½ pitch, ~100 Å). We see a sharp bend in the Skip 4 region, reminiscent of two crystal structures for that region; however, in the crystal structures, both chains are bent, whereas we see a kink in only one helix. A similar, single-chain bend is also seen in the Skip 3 region of the map. Neither bend is well fit by the Skip 4 crystal structures as a single chain. Similar to Skip 1, the fittings for Skips 2 and 3 aligned the skip residues close to their predicted positions, and the fittings appeared to be dominated by bulky side groups falling into bridging densities. In the case of Skip 4, there are no bulky side groups in the model or bridging densities in the map, and the fitting is dominated by the bend.

Myosin rod packing within the backbone
How the rods might pack together in a thick filament was previously the subject of intense speculation (4, 26, 45, 52, 53), but high-resolution data have been lacking until now. A cross section through the map with the rods numbered according to their crown level immediately suggests how the rods are arranged (Fig. 4A), as the pattern (0, 3, 6 …), (1, 4, 7 …), and (2, 5, 8 …) emerges. Any rod is thus laterally associated with the rods ±3 crowns away, that is, 435 Å, as predicted (18). When examined in 3D, the ±3 contact is essentially maintained for the entire length of the rods (Fig. 4B and movie S4). Rods do not wrap around one another to form 40 Å subfilaments (Fig. 4A, inset), as previously proposed (26); instead, they remain parallel and form a ribbon-like structure (Fig. 4, B and C), nearly equivalent to the “curved molecular crystalline layer” model, first proposed by Squire 43 years ago [compare especially Fig. 4A to Fig. 9 of the work of Squire (54)]. We refer to these layers as “ribbons” to distinguish them from helical subfilaments.

The ribbons insert into the filament backbone tangentially, similar to the blades of a turbine (Fig. 4A, lines). There are 12 ribbons in the filament, and adjacent ribbons are axially offset by one crown repeat, 145 Å. The heads all project from one side of the ribbon, and the rods cross the ribbon to end with their C termini on the other side, closest to the filament core (Fig. 4B). Depending on where it is sectioned, a ribbon contains either three or four molecules. Successive molecules in a ribbon are ~12° apart (3 crowns × 33.98° per crown...
- $90^\circ \approx 12^\circ$, with the $-90^\circ$ due to the fourfold rotational symmetry). Thus, the ribbons slowly wrap around the filament axis, following a steep helix (Fig. 4C) with a pitch of $\approx 13,000$ Å ($435$ Å $\times 360^\circ/12^\circ$). Because there are 12 ribbons, the vertical distance between ribbons, that is, the axial repeat, is $\approx 1100$ Å.

To gauge the extent of potential contacts in the ribbon, we used the spine function in Chimera (51) to define the path of all rods and analyzed the distances between them (Fig. 4, D to G). Tracing from N terminus to C terminus, our reference, Molecule 0, joins the backbone and contacts Molecule +3, staying mostly within 17 to 20 Å (Fig. 4E, blue trace), which is the range expected for uniform coiled coils touching one another (52). There is a notable hump in the graph (Fig. 4E, arrow), where a visible split in the ribbon corresponds to the Skip 1 region of Molecule 0. In this region, Molecule 0 transiently breaks its contact with Molecule +3 and instead contacts Molecule $-3$ as it is joining the ribbon. A subsequent bend in Molecule +3 corresponds to that molecule’s Skip 3 region and serves to bring it back into contact with Molecule 0 (Fig. 4E, double arrowheads). The C terminus of Molecule +3 approaches within 13.5 Å of Molecule 0 (Fig. 4E, single arrowheads). Because all molecules are equivalent, the distance between Molecules +3 and 0 is the same as the distance between Molecules 0 and $-3$. That is, the red trace in Fig. 4E is identical to the blue trace, only shifted axially by $3 \times 145$ Å.

Excluding the tether region, the rod is within 20 Å of either neighbor in the ribbon for 75% of its length. Averaged over all of its length, it is $19 \pm 2$ Å from its nearest neighbor within the ribbon and $20 \pm 3$ Å from both neighbors. The rods can be seen to gently undulate parallel to one another within the ribbon, with a 435 Å periodicity (Fig. 4B). We conclude that the rod thus shows a striking 3D shape complementarity to maintain the 435 Å stagger between rods within the ribbon.

In contrast, potential contacts between a given molecule and the adjacent ribbons are restricted to small local patches (Fig. 4, F and G). Molecule 0 transiently approaches Molecules $\pm 1, \pm 2,$ and $\pm 5$ and does not approach closer than 25 Å to any other molecule. The restricted nature of these contacts is explained by the observed undulations of the rod, because the adjacent ribbons undulate out of phase. Excluding the tether region, the rod is within 20 Å of some molecule in the neighboring ribbon for only 44% of its length. Averaged over all of its length, it is $34 \pm 12$ Å from all $\pm 1, \pm 2,$ and $\pm 5$ neighbors, and $22 \pm 5$ Å from the nearest of those neighbors. Thus, as judged by the distance between rods, the potential binding within ribbons involves closer contacts than the binding between ribbons.

**Nonmyosin densities among the rods**

Surprisingly, extra densities that cannot be assigned to myosin are seen snaking among the myosin rods (Fig. 5 and movie S3). Because their identity is currently unknown, we refer to them by their color in the figures: red, yellow, blue, and green. They appear as four unconnected densities, axially positioned between crowns (Fig. 5A and movie S5). Their stoichiometry is 1:1 with myosin; that is, below each crown composed of four myosin molecules, there are four copies of each of the four densities (Fig. 5B). The combined volume of one set of the extra densities, 24,000 Å$^3$, corresponds to $\approx 180$ residues or $\approx 20$ kDa of polypeptide chain (see Materials and Methods). Although appearing as separate features, it is possible that they represent a single polypeptide with disordered connecting segments. They are mostly extended structures but also have folded, globular domains. They are mostly located inside the filament among the rods.

The extra densities all pass between adjacent ribbons, bridging them (Fig. 5C). The yellow density, in particular, contacts three adjacent ribbons as if to stitch them together (Fig. 5C). To varying extents, the extra densities also pass between adjacent rods within a ribbon and therefore split the ribbon. The red density is noteworthy because it has a tail passing through the rod region that is visible from the outside of the filament (Fig. 5, A and B). The tail penetrates directly through the ribbon near the top of the previously described split formed at the Skip 1 region (Fig. 5D, star). The blue and green densities are comparatively small and contact only the rods, whereas the red and yellow densities also contact the filament core. From the perspective of any one density, it contacts several rods as it wends among them (Fig. 5, B and C). From the perspective of one of the rods, multiple copies of each density contact it at various points along its length (Fig. 5E).

**The paramyosin core**

Inside the rods, within a radius of 42 Å, is a 26 Å-thick cylindrical shell of density (Fig. 6, purple, and movie S3). Paramyosin is well

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**Fig. 5. Nonmyosin densities.** (A) Nonmyosin densities in the region between crowns are colored red, yellow, blue, and green. (B) A Z-ward axial view of the 5.5 Å map shows the extra densities tightly packed among the rods. (C) This longitudinal view looks from inside the filament, toward the rods from four different ribbons, which are alternately colored light or dark gray. The individual densities are inserted between the rods of a given ribbon but also contact and therefore bridge adjacent ribbons. (D) A single ribbon is shown with the extra densities that contact it. The red density penetrates near the top of a large split in the ribbon (star). (E) A single myosin molecule is shown in orthogonal views with the extra densities that contact it. Numbers represent the predicted amino acid residue of the *Drosophila* myosin sequence (see text) at 145 Å intervals. Double arrows indicate the predicted location of the four myosin skip residues.
known to be present in the core of *Lethocerus* and other invertebrate thick filaments (30, 34); thus, we call this shell of density the paramyosin core. Both the red and yellow extra densities appear to make contact with the outer part of the core, but the resolution within the core is insufficient to determine whether they penetrate into it. The core density varies within each crown repeat, consisting of a ring of continuous density positioned near the level of the crowns (Fig. 6, solid line) and a region between the crowns (Fig. 6, dashed line) with eight rodlike features suggestive of coiled coils seen at low resolution. However, the core structure seen here is unlikely to be an accurate representation of paramyosin. Reported arrangements of paramyosin (47, 55–58) suggest that they do not match myosin (34) and will therefore be poorly represented in our reconstruction, which is refined to the helical symmetry of the myosin heads (that is, a fourfold rotational symmetry, a 145 Å axial rise, and a 34° rotation per crown). Attempts to model the paramyosin core based on previously proposed paramyosin arrangements are discussed in the Supplementary Materials, including figs. S5 and S6. The modeling supports the interpretation that regular arrangements of paramyosin can explain the density variation within the paramyosin core.

**DISCUSSION**

**Comparison to previous efforts**

Regarding our unexpected observation of the IHM in *Lethocerus* filaments, our structure differs from an earlier image reconstruction of *Lethocerus* filaments (38), principally because of the increased resolution, which clearly resolves the paired myosin heads. A resolution of ~25 Å or better is required to determine with certainty whether or not the heads are arranged similar to the IHM (4, 9). This point is illustrated by two lower-resolution image reconstructions of scallop thick filaments (46, 59) that were subsequently proven wrong by a higher-resolution reconstruction (8). Likewise, a resolution of ~25 Å or better is necessary to resolve S2 (4), which we think is a necessity for correct interpretation of the map. The orientation of S2, and its connection to the backbone, places strong constraints on how the myosin heads must be positioned within the EM density, as was seen in the first reconstruction to demonstrate the IHM in thick filaments (4).

The issue of insufficient resolution also applies to the previously published x-ray structure (14), which was modeled from the low-resolution diffraction pattern using data to only 72 Å. A more critical failure of the x-ray modeling is that it constrained both heads of the molecule to have the same shape (14). This constraint alone guaranteed that the modeling could not find the IHM as a possible solution, because the lever arms of the free and blocked heads have different orientations in the IHM (9).

**Subfilaments versus ribbons**

Two general models for myosin rod packing proposed either 40 Å–diameter subfilaments made of three rods (26) or side-by-side association into layers (54). Either model can accommodate the diversity of thick filament structures observed in different species by changing the rotation per crown and/or the number of heads per crown (26, 54). Moreover, the high sequence conservation within the rod domain (table S1) suggests that whatever the packing scheme, it is likely to be similar in all filament types, as previously proposed (26, 54). In *Lethocerus* thick filaments, the 12 ribbon-like structures that make up the backbone (Fig. 4, A to C) are clearly the layer type. Because this is the first example to resolve the rod α helices and unambiguously demonstrate the packing, we explore how general the ribbon structure might be and what evidence there is for subfilaments.

Previous x-ray diffraction of crustacean muscle (26) was interpreted in terms of 40 Å subfilaments. However, no direct evidence of subfilaments was given, and Wray’s entire analysis is consistent with the structure that we see here if we reinterpret what he suggested were subfilaments as ribbons (see Supplementary Materials). Previous thick filament reconstructions were also interpreted in terms of 40 Å subfilaments (4, 5, 28, 60, 61). However, these lack sufficient resolution to visualize individual rods and so do not unambiguously clarify the packing. Higher-resolution structures may reveal them to have a ribbon arrangement too. Likewise, the large-diameter bundles seen in scallop thick filaments (8) or similar bundles seen in frayed vertebrate thick filaments (62, 63) might be composed of ribbons, not subfilaments as they have been previously interpreted. One problematic facet of the 40 Å subfilament model is that it involves contact between two rods that are six crowns apart (Fig. 4A, inset), whereas analysis of the charge distribution in the rod suggests that this interaction will be disfavored (18, 24, 25). Overall, we think that the evidence for subfilaments is not especially compelling. We ourselves previously misinterpreted structures in *Lethocerus* thick filaments as subfilaments (45), which, with hindsight, we must now recognize as ribbons. It is conceivable, though unproven, that the ribbon structure is a feature of many, or even all, thick filaments. These issues are discussed in greater depth in the Supplementary Materials.

We find it remarkable that a myosin-packing scheme proposed 43 years ago based on a myosin assembly found in vertebrate smooth muscle (54) could so accurately predict the myosin rod arrangement in insect flight muscle, a muscle about as different functionally from vertebrate smooth muscle as evolution could have produced.
Nonmyosin densities
What proteins make up the nonmyosin densities? The obvious candidates are the insect muscle thick filament proteins flightin and myofilin (30, 31, 64). No other good candidates emerge. The minititins projectin and kettin are short, and antibody labeling suggests that they connect only to the ends of the thick filaments (36), which were intentionally excluded from the reconstruction. As described in Results, we believe that paramyosin is restricted to the filament core, consistent with antibody labeling (30). Moreover, the 8:1 stoichiometry of myosin to paramyosin (56) is wrong for the observed 1:1 ratio of myosin to the extra densities.

Myofilin (~30 kDa) alone could account for all the extra densities (~20 kDa total), whereas flightin (19 kDa) by itself is probably too small if disordered segments not seen in the map are required to link the four disconnected densities. The overall stoichiometry of myosin to either protein, ~2:1 (30), is not quite right compared to our observed 1:1 ratio. However, antibody labeling indicates that these proteins are restricted to ~70% of the filament (30), which reduces the stoichiometry to ~1.4:1 in the labeled region of the filament, which also corresponds to the region that we used for the reconstruction. Antibody labeling indicates a flightin epitope on the outside of the filament (30), potentially identifying the red density as flightin. In contrast, myofilin appears to be located inside the filament (30), potentially identifying the yellow density as myofilin. The assignment of the red density to flightin is supported by the Drosophila flight muscle myosin rod mutation E1554K, because this mutation eliminates flightin’s in vivo incorporation into thick filaments (65), and it is located at one position where the red density contacts the myosin rod (Fig. 5E).

If the extra densities are neither flightin nor myofilin, then they may be what they appear to be: small, possibly not yet identified, separate peptides incorporated within the thick filament backbone. Hence, they may modulate thick filament function in some way, thereby imitating small peptides that function within membranes, such as phospholamban (66).

Orientation, disruption, and reformation of the IHM
The IHM was first observed in smooth muscle myosin (9) but is now understood as a general mechanism for sequestering the heads and reducing the basal adenosine triphosphatase rate in a relaxed muscle (3, 11). Conversely, the IHM must be disrupted to free the heads for binding to actin during contraction. Here, we discuss potential mechanisms for the disruption and reformation of the IHM and the unique IHM orientation.

In smooth and nonmuscle myosin, disruption/reformation of the IHM is tightly controlled by RLC phosphorylation (67). For tarantula filaments, which are modulated but not strictly controlled by phosphorylation, Alamo et al. proposed a detailed mechanism in which the free head is preferentially phosphorylated and dynamically docking and undocking from the IHM because of its greater accessibility compared to the blocked head (10). In contrast, the unique orientation of the IHM seen here, coupled with the poorer resolution and absence of stabilizing contacts for the blocked head, suggests that the blocked head is more accessible to phosphorylation and preferentially docking and undocking from the IHM in the Lethocerus thick filaments.

The unusual orientation of the IHM suggests possible significance for stretch activation, which is especially strong in Lethocerus muscle.

Stretch activation is a feature of all striated muscles whereby stretching a partially activated muscle results in greater active force (68, 69). Proposed mechanisms suggest that stretch either activates the thin filaments (44) or activates the thick filaments (68), although both mechanisms may operate simultaneously. The observation of the IHM in Lethocerus thick filaments suggests specific details for thick filament activation. Normal mode analysis of the myosin molecule indicates that formation of the IHM necessarily distorts and stores elastic energy throughout the entire the rod (70). Reversing that logic, it is possible that distortions to the rod caused by tension on the thick filament disrupt the IHM. In this light, the IHM/rod structure is spring-loaded. The paucity of stabilizing contacts in the Lethocerus IHM makes it a hair trigger, which, in turn, may partly explain why stretch activation is larger in insect flight muscle than in other striated muscle types (68). Stretch-induced changes to the thick filament crown structure have already been reported by x-ray diffraction of insect (44) and vertebrate (71) muscles. These ideas and others are developed more fully in the Supplementary Materials.

Historical perspective
This work stands on deep historical groundwork. Early x-ray diffraction work on keratin also included muscle or myosin, often dried (72, 73), and was the framework for the description of the α helix by Pauling et al. (74). Discrepancies between the expected and observed x-ray reflections later informed the independent descriptions of the α-helical coiled coil by both Pauling and Corey (75) and Crick (16, 76). Confirmation of the coiled-coil structure in living muscle by Cohen and Holmes followed a decade later (17). Although numerous coiled-coil structures of variable length have been determined by crystallography, nothing approaching the 1600 Å length of myosin’s coiled coil has been visualized at this level of detail in its native environment. It is remarkable that so many key predictions of the early literature are confirmed, such as the average coiled-coil pitch, the 435 Å offset between adjacent molecules, and the packing of the rods into ribbons. Yet, there are many unexpected new details, such as the lack of symmetry between the two chains in the coiled coil, the variation in coiled-coil pitch, the twists and turns of the rod within the filament backbone, the apparent changes in skip residue structures in their native environment, and the presence of nonmyosin proteins threaded among the myosin rods.

Materials and methods
Myofibrils
Live L. indicus were imported from Thailand. The dorsal longitudinal flight muscles of 10 insects were dissected, minced, placed in 50 ml of cold relaxing buffer [5 mM MgAcetate, 5 mM NaATP, 5 mM NaPO4 (pH 6.80), made by Cohen and Holmes followed a decade later (17)]. Although numerous coiled-coil structures of variable length have been determined by crystallography, nothing approaching the 1600 Å length of myosin’s coiled coil has been visualized at this level of detail in its native environment. It is remarkable that so many key predictions of the early literature are confirmed, such as the average coiled-coil pitch, the 435 Å offset between adjacent molecules, and the packing of the rods into ribbons. Yet, there are many unexpected new details, such as the lack of symmetry between the two chains in the coiled coil, the variation in coiled-coil pitch, the twists and turns of the rod within the filament backbone, the apparent changes in skip residue structures in their native environment, and the presence of nonmyosin proteins threaded among the myosin rods.

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Native thick filaments
We diluted 0.1 ml of myofibrils with 0.3 ml of filament buffer [5 mM MgAcetate, 5 mM NaATP, 5 mM EGTA, 20 mM MOPS, and 150 mM NaCl (pH 7.0)], centrifuged for 4 min at 4500g to pellet the myofibrils, resuspended in 0.25 ml of filament buffer with 5.2 mM CaCl$_2$ added, and incubated for 30 min on ice with 2 µg of μ-calpain. The μ-calpain was a gift from the laboratory of the late D. Goll. Calpain digested the Z-band (77) and was necessary to obtain sufficient yield of intact filaments. Calpain-digested myofibrils were pelleted as before and resuspended in 0.1 ml of shearing buffer [5 mM MgAcetate, 5 mM NaATP, 5 mM EGTA, 20 mM MOPS, 100 mM NaCl, 5 mM DTT, and 10 mM Na-PO$_4$ (pH 7.0)], and sheared by pulling the suspension through a 26-gauge needle with a syringe 5 to 10 times to release filaments. The preparation was centrifuged for 2 min at 3500g to pellet unsheared fibrils, leaving a mix of thick and thin filaments in the supernatant. To remove actin filaments (78), the supernatant was incubated for 20 min on ice with 20 µg of a His-tagged, Ca$^{2+}$-insensitive gelsolin fragment (residues 25 to 406 of plasma gelsolin), which we expressed in *Escherichia coli* and purified by standard techniques. Expression vector was a gift from M. Briggs. After gelsolin treatment, the filament suspension was checked by conventional negative-stain EM for quality and yield and then used directly for cryo-EM specimens. At no point in the procedure was blebbistatin used.

Cryo-EM sample preparation
R2/1 Quantifoil grids were cleaned using a Gatan Solarus 950.M plasma cleaner in argon gas for 22 s. Samples were vitrified on a Vitrobot Mark IV (FEI) with the environmental chamber set to 100% relative humidity and 22°C. The crude filament preparation (4 µl) was applied to the grid directly after cleaning, incubated for 60 s, blotted once, and then plunged into liquid ethane to vitrify.

Data collection
Low-dose images were automatically collected using the Leginon software package (79) on a Titan Krios microscope (FEI) equipped with a field emission gun and operated at 300 keV. Images were recorded with a DE-20 Direct Electron detector. The defocus range was from 1.5 to 3 µm and the pixel size was 1.215 Å, as calibrated by FEI. Each micrograph consisted of a 48-frame movie, with a total dose of 65 e$^-$/Å$^2$.

Image data processing
We used the Appion software package (80) to manage the data, perform damage-compensated motion correction, contrast transfer function determination, and for particle picking. The damage-compensated motion correction process (81) aligned each of the 48 frames in the movie to the integrated image, low-pass–filtered them to a resolution that changed as a function of integrated dose, and then summed their Fourier transforms. The summation was reweighted so that low-resolution Fourier coefficients, which had more measurements, would have the same final weight in the sum as high-resolution Fourier coefficients with fewer measurements. Defocus was initially searched using ACE (82) and then refined by CTFIND3 (83). The filaments were manually selected, divided into 432 × 432–pixel boxes, and then extracted and normalized using Appion. Each “particle” consisted of a filament segment masked to a length of 450 Å, slightly more than three crowns. Adjacent particles overlapped by 290 Å (two crowns). A total of ~72,000 filament particles showing clear crowns were transferred to RELION (39) along with their defocus information. The specific version used was RELION 1.2 implemented by Clemens et al. (84), which included the Iterative Helical Real Space Reconstruction package (85).

We used STARTCSYM in the EMAN package (86), which implements the common line method (87) and the known fourfold filament symmetry, to create an initial, reference-free, reconstruction low-pass–filtered to 60 Å resolution. This low-resolution reconstruction was used as a starting model for the iterative 3D classification scheme in RELION to select for the best-ordered particles, as described below.

To reduce computational burden, data were binned by a factor of 2 for particle selection by iterative classification. The first round randomly divided the 72,000 particles into four groups, calculated four reconstructions, generated projections for each reconstruction, and then reassigned each of the 72,000 starting particles to one of the four groups according to which projection it most closely resembled. If the data set came from a single structure, subsequent rounds of reconstruction/classification should yield four similar reconstructions, or one reconstruction would have the large majority of the individual segments, whereas heterogeneity in the data set would reveal itself in subsequent rounds as a divergence of the four structures. Twenty-one cycles of autorefinement were sufficient to classify the 72,000 particles into two groups with clearly defined heads and S2 tethers, and two groups with poorly defined heads and no visible S2 tether (fig. S7). The ~24,000 particles autoassigned to the first two groups were then combined and used to calculate the final reconstruction, unbinned. One of the good reconstructions from the classification scheme was low-pass–filtered to 60 Å and used as the starting model for the final reconstruction. For the final reconstruction, we used the “gold standard” procedure (88), which involves computing two independent reconstructions in parallel. The final map was sharpened using EM-BFACCTOR (89), which reweights the reconstruction transform based on the Fourier shell correlation curve and then low-pass–filters it to a corresponding resolution to show each part’s structure.

Although the resultant 3D map was clearly polar, we still needed to determine which end corresponded to the filament bare zone. To accomplish this, three filaments with visible bare zones were selected and divided into particles. Because the orientation of these particles was known, projections of the reconstruction could then be compared to them using the program align2d in EMAN (86) to determine the orientation of the reconstruction.

Maps, models, and volume segmentations
We used Chimera (51) to create the images. Longitudinal views are presented with the long axis of the filament vertical in the plane of the paper, with the bare zone toward the top. The filament bare zone corresponds to the M-band of the sarcomere and the C terminus of the myosin molecules. Axial views are specified as M-ward when looking toward the filament bare zone, that is, toward the M-band. Conversely, they are specified as Z-ward when looking away from the bare zone, that is, toward the end of the filament and the Z-band of the sarcomere. Figures are presented in orthographic projection, whereas movies are presented in perspective. Maps are displayed with the Chimera Volume Viewer contour level set to 0.4 for the 20 Å low-pass–filtered map, 3.9 for the 5.5 Å map, and 1.1 for the 10 Å map. Volume segmentation was done using Chimera’s Segment Map tool (90) with default parameters, followed by manually grouping connected densities. We used a BLAST (Basic Local Alignment Search

Hu et al. Sci. Adv. 2016;2:e1600058 30 September 2016
Tool) alignment (91) of the Drosophila (NP_724008.1) and human cardiac (P12883.5) myosin sequences to identify corresponding regions and generated Drosophila homology models for the myosin skip regions using SWISS-MODEL (50) and the published crystal structures (PDB 4XAJ, 4XAK, 4XAA, and 4XAD). Atomic structures were manually docked into a map and refined as a rigid body using Chimera’s Fit in Map tool with default parameters. The atomic model of the IHM was a version of 1184 that included the RLC and ELC side chains (provided by I. Rayment).

Contour length, sequence, distances between rods, and volume
We applied Chimera’s measure spine function to volume segmentations of the rods and S2 tethers. This defined the path of each rod’s centroid as a series of points in 3D, spaced at ~10 Å intervals along the filament (Z) axis. Path points at ~1 Å intervals in Z were generated by a LabVIEW script using linear interpolation in Z and cubic Hermite interpolation for the X and Y coordinates. Starting from where these spines intersected with the S1-S2 junction of 1184 as it fit in the map, the point-to-point distances were summed to give the contour length as a function of Z and the total contour length of the rod. The sequence was related to the contour length (and hence Z) by assuming a rise of 1.485 Å per residue relative to the coiled-coil axis (47). A second LabVIEW script determined the distance between rods as a function of Z, as follows. From each point on one rod, the adjacent rod was scanned to find the two closest points. The adjacent rod was then linearly interpolated between those points at ~0.1 Å intervals in Z, and the interpolated points were scanned to find the minimum distance to the point on the first rod. Average distances are reported as means ± SD. To estimate the number of residues contained by the extra densities, the volume of the rod segments was measured in Chimera and divided by the number of residues determined from the contour length analysis to give a calibrated factor of ~135 Å³ per residue, for that contour level, which was then applied to the measured volume of the extra densities.

Resolution determination
The resolution (fig. S1A) was calculated at the 0.143 threshold of the gold-standard Fourier shell correlation, which is based on the consistency between two reconstructions calculated from the data randomly split into two groups (88). We computed the local resolution in two steps using ResMap (40). The resolution from 10 to 30 Å was calculated with a step size of 1 Å with the map binned by a factor of 2 (fig. S1B). The resolution from 5.5 to 10 Å was calculated with a step size of 0.5 Å using the unbinned map (fig. S1, C and D).

Supplementary Materials
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/9/e1600058/DC1

Table S1. Sequence identity among representative myosins.

Modeling the paramyosin core

In-depth discussion of subfilaments versus ribbons

The IHM and possible relevance to stretch activation

Fig. S1. Resolution of the reconstruction.

Fig. S2. Comparison of the Fourier transform of the map to the x-ray pattern of whole muscles.

Fig. S3. Poor fitting of the IHM with the blocked head contacting the backbone.

Fig. S4. Orientation of the IHM in Lethocerus filaments compared to tarantula filaments.

Fig. S5. Schematic illustration of the paramyosin modeling.

Fig. S6. Model of the paramyosin core.

Fig. S7. Illustration of particle classification scheme.

Fig. S8. Key to movie S3.

Movie S1. Fitting of IHM structure into the 20 Å map.

Movie S2. Myosin molecule within the filament.

Movie S3. Cross-sectional thick filament fly-through.

Movie S4. Ribbon structure.

Movie S5. Nonmyosin densities.

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Necessity for a myosin rod.

Spare the rod, spoil the regulation: The authors declare that they have no competing interests.

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