

Rigor Crossbridges are Double-headed in Fast Muscle from Crayfish

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Abstract. The structure of rigor crossbridges was examined by comparing rigor crossbridges in fast muscle fibers from glycerol-extracted abdominal flexor muscle of crayfish with those in "natively decorated" thin filaments from the same muscle. Natively decorated thin filaments were obtained by dissociating the backbone of the myosin filaments of rigor myofibrils in 0.6 M KCl. Intact fibers were freeze-fractured, deep-etched, and rotary shadowed; isolated filaments were either negatively stained or freeze dried and rotary shadowed. The crossbridges on the natively decorated actin maintain the original spacing and the disposition in chevrons and double chevrons for several hours, indicating that no rearrangement of the actomyosin inter-

actions occurs. Thus the crossbridges of the natively decorated filaments were formed within the geometrical constraints of the intact myofibril. The majority of crossbridges in the intact muscle have a triangular shape indicative of double-headed crossbridge. The triangular shape is maintained in the isolated filaments and negative staining resolves two heads in a single crossbridge. In the isolated filaments, crossbridges are attached at uniform acute angles. Unlike those in insect flight muscle (Taylor et al., 1984), lead and rear elements of the double chevron may be both double-headed. Deep-etched images reveal a twisted arrangement of subfilaments in the backbone of the thick filament.

THE junction between subfragments 1 and 2 (S1 and S2) of the myosin molecule is a region of high flexibility, which allows the two myosin heads to take variable angles relative to the rod portions and to participate in various types of interactions with the thin filaments (Slayter and Lowey, 1967; Elliott and Offer, 1978). Negative staining of *in vitro* actomyosin interactions in the absence of ATP has shown that the two heads of a single myosin molecule can either cross-link separate actin filaments (double filament interaction; Trinick and Offer, 1979) or interact with a single filament (single filament interaction; Craig et al., 1980). The crossbridges resulting from the two types of interactions contain either a single or a double head, respectively, and are thus called single- and double-headed crossbridges.

The nature of the crossbridges produced under rigor *in vivo* conditions (i.e., under the geometrical constraints imposed by the three-dimensional arrangement of the filaments, the helical arrangement of crossbridges on the thick filaments, and the availability of target zones on the thin filaments) has been more difficult to establish. The highly regular arrangement of attached crossbridges in rigor insect flight muscles has been the object of the most extensive and close analysis (Reedy, 1967, 1968; Taylor et al., 1984; Reedy and Reedy, 1985). However, even these detailed images do not directly solve the question of crossbridge nature, since the flared X configuration has been successfully modeled on the basis of both single- (Offer et al., 1981) and double-headed

(Squire, 1972, 1981) crossbridges. Similarly, several indirect arguments have been used to support double filament interactions in myofibrils (Offer and Elliott, 1978; Borejdo and Oplatka, 1981), but no direct evidence has been obtained. On the basis of crossbridge size, it has recently been proposed that *in vivo* rigor crossbridges in fish (Franzini-Armstrong et al., 1983; Varriano-Marston et al., 1984) and in insect muscles (Taylor et al., 1984; Reedy and Reedy, 1985) are a mixture of single- and double-headed ones. The presumed double-headed crossbridges have a distinctive triangular shape; i.e., they have a narrow neck at their origin from the thick filament and are much wider in proximity of the thin filament. Neither of these studies could resolve the two heads of the crossbridges because of limitations inherent in the techniques.

In this study, we compare the structure of rigor crossbridges in glycerol-extracted fast abdominal flexor muscle from the crayfish (using deep-etching, rotary shadowing) with those in "natively decorated" thin filaments isolated from the same fibers (using either negative staining or freeze-drying on mica followed by rotary shadowing). Natively decorated thin filaments were obtained by dissociating the backbone of the myosin filaments of rigor myofibrils in 0.6 M KCl, as first done in rabbit muscle (Ip and Heuser, 1982). Crayfish myofibrils dissociated much more readily than those from rabbit under these conditions. This may be due to either of two reasons: (a) the Z-lines dissolve more

readily, and (b) there is less cross-linking of actin filaments by myosin (see Discussion, and Borejdo and Oplatka, 1981). The experiments took advantage of stability of rigor crossbridges (White, 1970; Guth and Kuhn, 1978; Yamamoto and Herzig, 1978), which we found to be maintained in the *in vitro* situation. We could thus compare crossbridges in the myofibrils with those on isolated filaments, which maintained the same disposition, using high resolution morphological techniques. We also report on several details of thick filament structure.

Materials and Methods

Crayfish (*Procambarus clarkii*) were anesthetized on ice and quickly decapitated. The fast, deep abdominal flexor muscle was exposed and rigor was obtained by extracting with cold rigor solution (15 mM potassium phosphate buffer, 100 mM potassium acetate, 5 mM K₂EGTA, pH 6.3 [the low pH was suggested by R. Padron]) in 50% glycerol at 4°C for 4 d. Rigor muscle was rinsed several times in rigor solution, and then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.1) at room temperature for 3 h. For thin sectioning, muscles were postfixed in 2% OsO₄ in 0.1 M cacodylate buffer for 30 min, dehydrated in alcohol, and embedded in Spurr's medium. For deep-etching, fixed muscle pieces were infiltrated in 40% methanol with 3% glycerol, and frozen in freon 22. They were fractured at -110°C and etched for 15 min at -100°C and 2.10⁻⁶ Torr. After deep-etching, the specimens were cooled to -150°C, and rotary shadowed at angles of 25 or 45°.

To obtain "natively decorated" thin filaments, superficial bundles of fibers were rinsed in rigor solution, finely minced, and homogenized on ice with three 3-s bursts (separated by 30 s) of a Sorvall OmniMixer (Omni Corp., Waterbury, CT) using the 5-ml cup. Myofibrils were washed three times and finally resuspended in 0.6 M KCl to dissociate the thick filaments. Extent of dissociation was checked using phase-contrast microscopy. Usually, 30-45 min were required for complete dissociation and filaments were used for up to 3.5 h after that. In some samples, the solution was diluted to 0.3 M KCl before negative staining. Negative staining was done on either carbon-collodion- or carbon-coated grids. The filaments were rinsed on the grid sequentially with eight drops each of 0.6 M KCl, 0.1 M ammonium acetate, and 1% uranyl acetate with bacitracin (20 µg/ml) (Gregory and Pirie, 1973). Some filaments were exposed to eight drops of 0.1% tannic acid in 0.1 M ammonium acetate before the ammonium acetate rinse (Kensler et al., 1985).

For freeze-drying, droplets of suspension were placed on sheets of freshly cleaved mica. They were sequentially rinsed with several drops of 0.6 M KCl, 0.1 M ammonium acetate, 1% uranyl acetate, and 10% glycerol. A thin layer of solution was frozen in liquid nitrogen. The mica sheets were freeze dried for 60 min at -100°C and under 2.10⁻⁶ Torr vacuum. The specimens were rotary shadowed with platinum at a 15° angle, and replicated with carbon. Replicas were floated on water, and collected on uncoated 300-mesh grids. Alternatively, filaments were adsorbed onto finely homogenized mica, and then rapidly frozen by contact with a copper block maintained at helium temperature (Heuser, 1983). Frozen specimens were processed for electron microscopy as described by Ip et al. (1985). Fracture and replication were performed in a 400D unit (Balzer Corp., Hudson, NH). Grids were examined in a Philips 410 or JEOL 100 CXII electron microscope.

Results

Description of Intact Muscle

Resting sarcomere length in the superficial abdominal flexor muscle of crayfish is ~3 µm. At that length, there is almost complete overlap of thin and thick filaments (Fig. 1). Straight Z-lines and sharp edges of H-zones indicate that good alignment is maintained during rigor induction. As in all fast arthropod muscles (Jahromi and Atwood, 1967; Reedy, 1967; Hayes et al., 1971), the thin filaments are disposed halfway between two adjacent thick filaments (dyadic disposition).

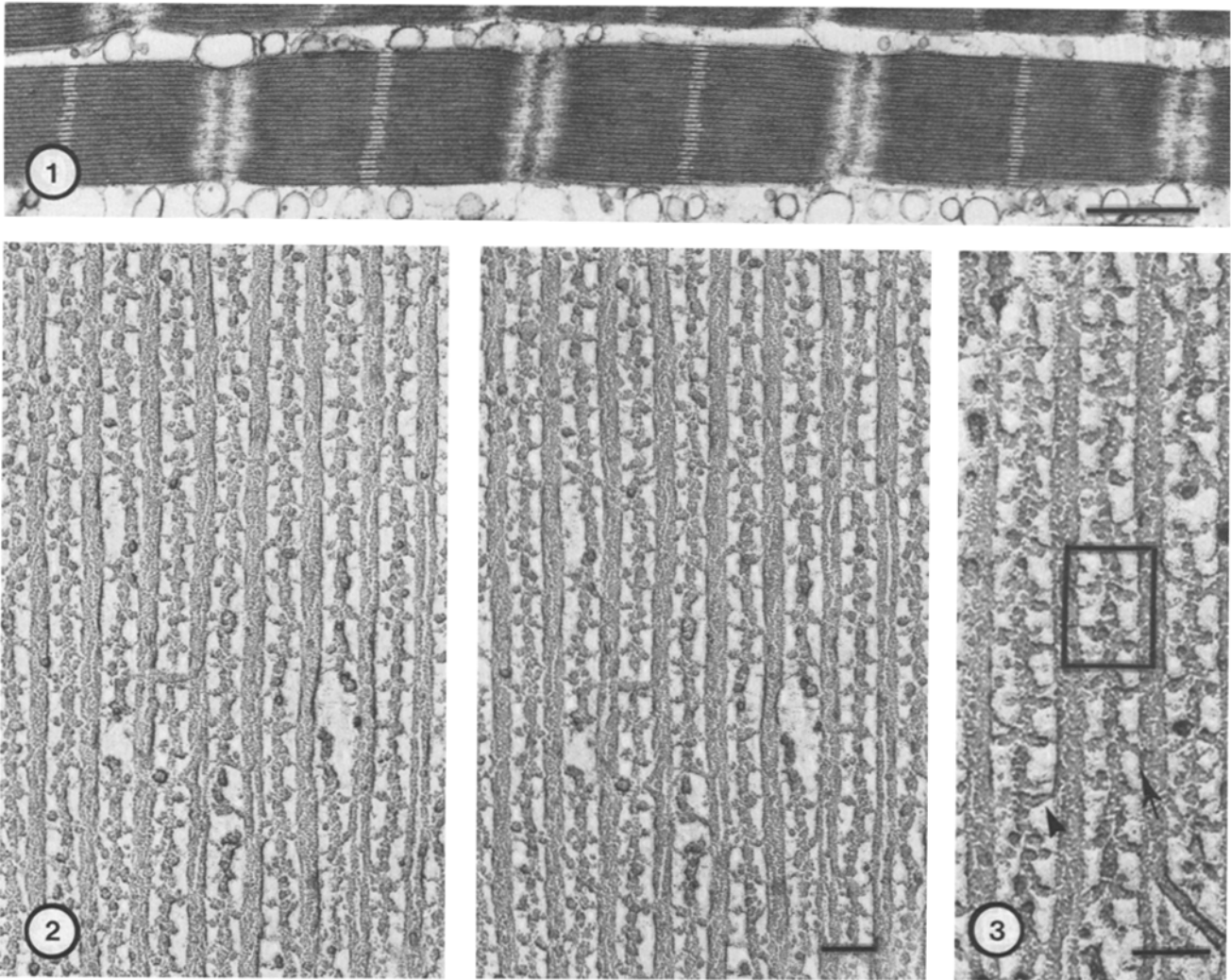
Fractures along the 1,0 plane expose a single layer of thin

and thick filaments (myac layer, Figs. 2-5; see Reedy, 1967). Individual thin filaments are flanked by two thick filaments, which are at a center-to-center spacing of 50-55 nm. Shadowing at 45° and 25° angle results in different visibilities and appearances of crossbridges. At 25° (Figs. 2 and 3) recessed crossbridges receive much less platinum than those closer to the fracture plane, so that some crossbridges are almost invisible. A shadowing angle of 45° (Figs. 4 and 5) results in complete shadowing of crossbridges which are recessed relative to the fracture plane, and therefore in more faithful imaging of crossbridges disposition (cf. Figs. 2 and 5). Basic crossbridge disposition is similar to that described for insect flight muscle, and we follow the terminology established for that muscle (Reedy, 1967, 1968). Actin target zone is the segment of thin filament which has the appropriate azimuthal orientation for crossbridge attachment from one myosin filament. At each target zone either a single crossbridge (singlet) or two crossbridges (doublet) contact the thin filament. The target zones for the two myosin filaments flanking one thin filament are at the same axial level, and the resulting crossbridges disposition is called chevron. Two aligned doublets form a double chevron.

The two crossbridges of a doublet have different azimuthal angles and are called lead and rear elements (see Taylor et al., 1984). This is particularly visible in the boxed area of Fig. 3, where the low angle (25°) shadow emphasizes the lead element and barely reaches the rear element. Notice that in each double chevron the two more heavily shadowed crossbridges (lead bridges) attach to the filaments in a staggered position, and their position relative to the rear elements results in an apparent left-handed crossbridge helix (see Fig. 9 in Heuser, 1983). Azimuthal angle difference between rear and lead elements is not very large and sometimes a rear crossbridge is heavily shadowed, so that two heavily shadowed crossbridges are attached at the same level on the actin filament. At a 45° shadowing angle, lead and rear elements are almost equally coated with platinum and stereo images must be used to detect azimuthal angle differences (Fig. 5).

Most chevrons are complete; i.e., only occasionally a single crossbridge is missing from a chevron. No globular structures analogous to those seen in relaxed muscle and representing detached crossbridges are visible close to the shaft of the thick filaments. Since individual myosin heads are clearly resolved in shadowed replicas, we conclude that there are no detached myosin heads in crayfish muscle in rigor. Crossbridges are periodically disposed along the thin filaments and chevrons are aligned across the sarcomere. This is best seen by holding a single micrograph (Fig. 4) close to eye level, and glancing at a right angle to the filament axis. Alignment across the sarcomere is most obvious where doublets in several adjacent filaments are equally distinct (see boxed area in Fig. 4). Optical diffraction patterns from the electron micrographs (Fig. 4, *inset*) confirm preservation of the basic periodicity of crossbridge attachment: the pattern has a strong second order of the 76-nm actin repeat. Layer lines are sampled by the filament spacing.

The crossbridges have somewhat variable shapes, but two predominate. Most crossbridges have a very narrow diameter at their site of origin from the thick filament and a much wider diameter in proximity of the thin filaments (triangular shape, Figs. 3 and 6, *a-j*). Other, less frequent cross-



Figures 1-3. (Fig. 1) Longitudinal section of crayfish abdominal flexor muscle fiber in rigor. Z-line and bridge-free regions are fairly straight, showing that myofilaments remain well aligned. Short H-bands indicate an almost complete overlap of thin and thick filaments. Bar, 1 μm . (Fig. 2) Stereo and flat views, respectively, of myac layer (alternating thick and thin filaments) in rigor muscle shadowed at 25°. M-line is at bottom. Bar, 50 nm. (Fig. 3) is a high magnification showing details of crossbridge shapes. (*Arrowhead*) Triangular-shaped crossbridges. (*Arrow*) Straight and narrow crossbridge. Triangular crossbridges predominate. At a shadowing angle of 25°, recessed crossbridges are poorly visible and their shape cannot be precisely determined. Bar, 50 nm.

bridges have approximately the same narrow diameter over their length (Fig. 6 *c*, *arrowhead*). The triangular shape is particularly evident in some of the crossbridges shadowed at 25° (Figs. 3 and 6 *j*). In the great majority of doublets the crossbridges of the lead and rear chevron are both triangular (Fig. 6, *a-i*). The gallery of images in Fig. 6, *a-j*, were selected from micrographs tilted by at least 15° about the filament axis (see legend to Fig. 5). As a result of tilt, shadow of the thick filament shaft is darker on one side. As predicted by Taylor et al. (1986; Fig. A), the triangular shape of the crossbridges is most clear in tilted images. Notice that in Taylor et al. (1986), only one element in each doublet is triangular, while in our images both elements have a triangular shape.

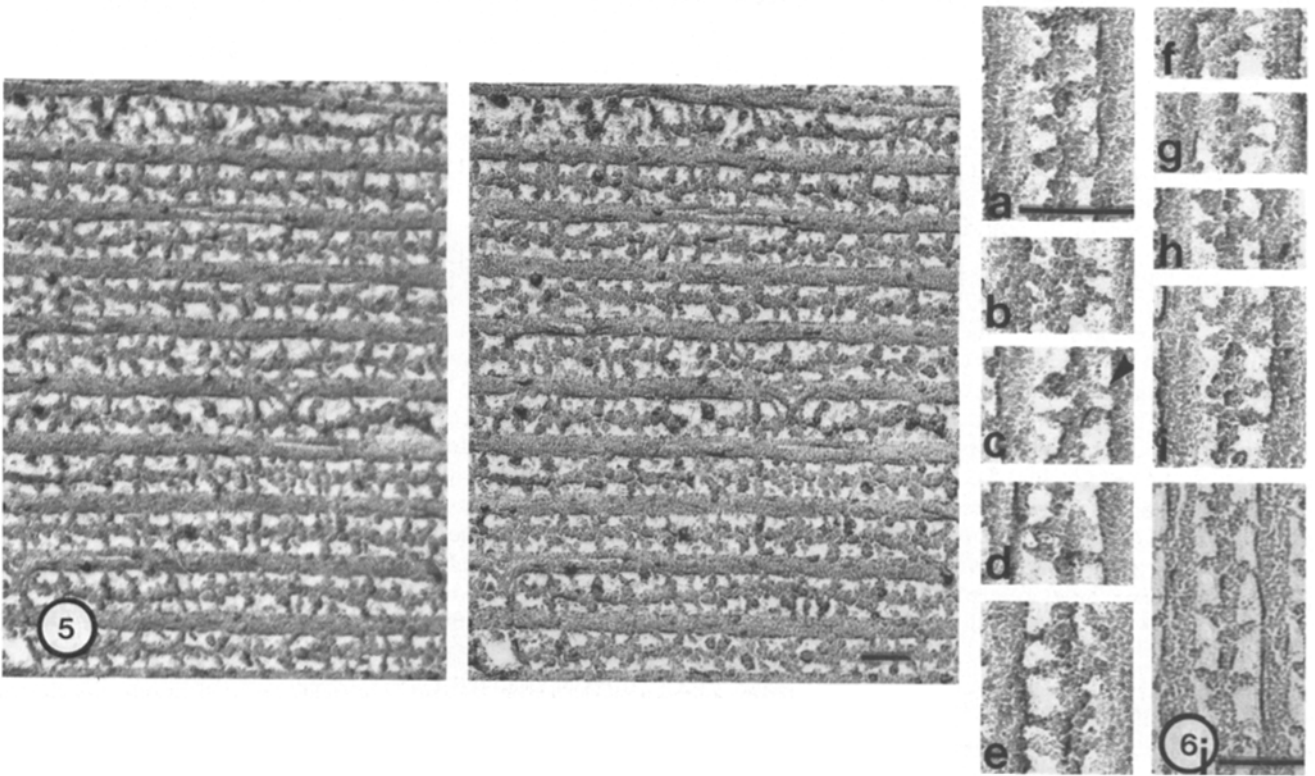
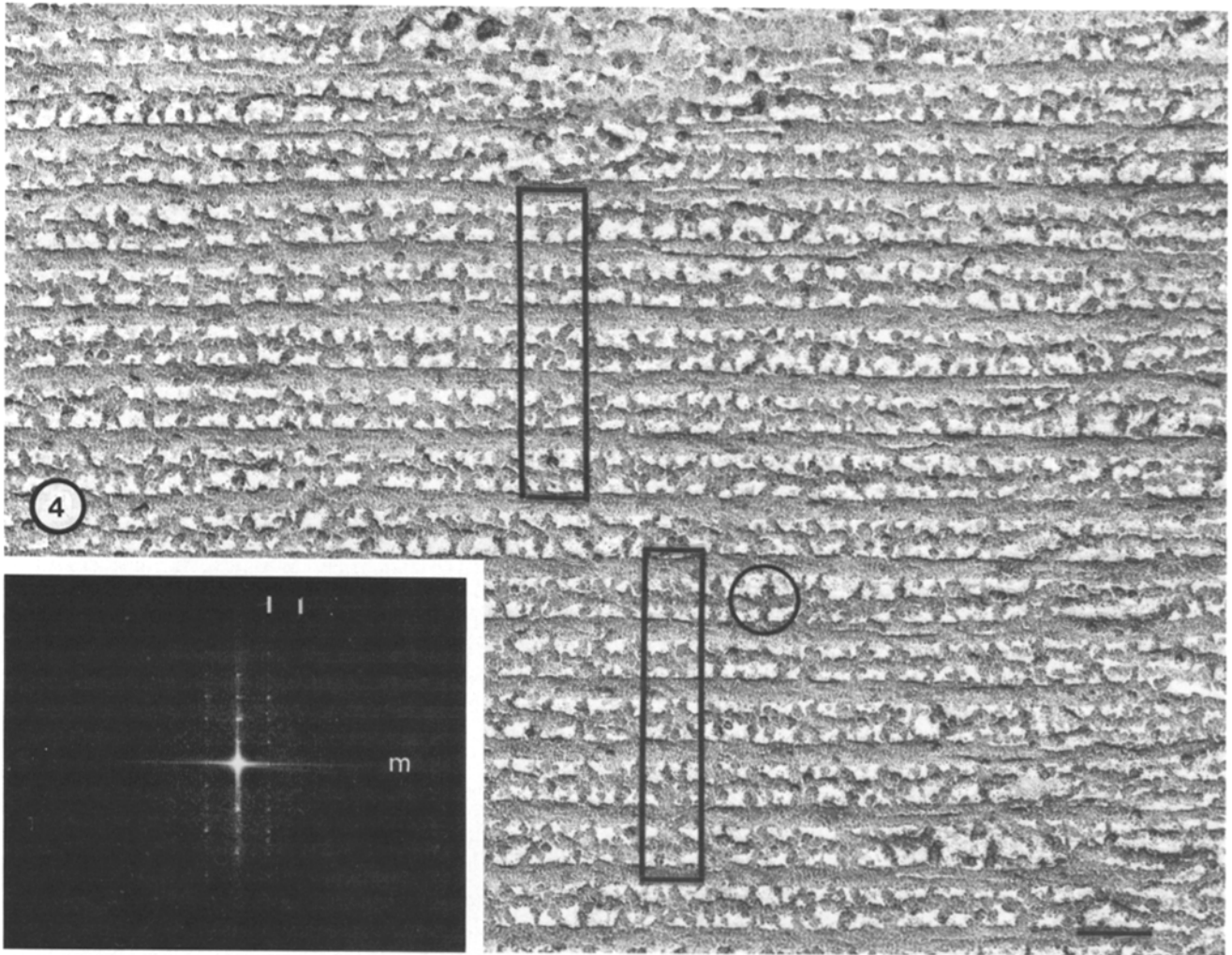
Measured Parameters of Intact Muscle

Average Center-to-Center Spacings between Target Zones. This was obtained by measuring the length of thin filaments

segments and dividing by the number of target zones. Groups of 6-14 target zones were measured. 120 spacings from seven micrographs at a magnification of 125,000 gave a range of 35-38 nm and an average of 37 nm. This indicates little shrinkage during preparation for EM.

Percentage Occupancy of Actin Target Zones by Singlets or Doublets. Crossbridges were counted on three micrographs at a magnification of 125,000 from fractures shadowed at 45° (see above), which showed well-visible 37-nm spacing along the micrograph. The relative frequencies of singlets and doublets vary along adjacent thin filaments. Counts were made on 5-7 segments from adjacent thin filaments in each micrograph. 759 target zones were counted, and on the average, 58% are occupied by doublets.

Axial Distance between Attachment Sites of the Crossbridges in a Doublet (Intradoublet Spacing). The distance between the center of adjacent crossbridge profiles at the periphery of the thin filament was measured in well-recogniz-



able doublets. Doublets fall into two categories. In some, the edges of the two crossbridges are contiguous (Fig. 6, *b, d*, and *f-i*); in others they are separated by a gap (Fig. 6, *a, c, e, i*, and *j*). Out of 71 measured doublets (in two micrographs), 41 (58%) fall in the first category and have an average intradoublet spacing of 11.7 ± 0.78 nm (mean \pm SD); 27 (38%) fall into the second category and have an average intradoublet spacing of 16.2 ± 1.5 nm; 3 (4%) of the doublet fall into the first category, but have a very short intradoublet spacing of 7.7 nm.

Width of the Crossbridge at the Periphery of the Thin Filament. The width was measured in 23 triangular and nine thin crossbridges. The averaged widths were 10.6 ± 1.5 nm, and 7.8 ± 1.4 nm, respectively. Comparison of the size of shadowed crossbridges (Fig. 6) with those of negatively stained images (Fig. 13) shows that overall contribution of platinum shadow to the diameter of the crossbridge is ~ 2 nm. Thus diameters of small and large crossbridges, in proximity of the actin filament, would be ~ 9 and 6 nm, respectively.

Distance between Periphery of Myosin Filament and Center of Thin Filament. This was obtained by halving the spacing between the surfaces of the two adjacent thick filaments. Thus inaccuracy due to irregularity in the position of the thin filament was avoided. The average spacing between 12 sets of filaments in three micrographs is 17 ± 1.9 nm.

Description of Natively Decorated Filaments

Thin filaments isolated from the rigor myofibrils after 0.6 M KCl extraction are decorated by periodically disposed crossbridges (Figs. 7–12). Occasionally there is a short segment, free of crossbridges, either intercalated between two decorated portions or at one end of the filaments. Transition between decorated and naked segments is abrupt (Fig. 8). The naked segments probably originate from the I-band of the myofibrils. Decorated thin filaments are often much longer than thin filaments in the intact sarcomere, which is ~ 1.5 μ m long (see Fig. 7). This indicates annealing of the filaments in solution and is probably responsible for the intercalation of naked segments in the decorated filaments. Polarity of crossbridge attachment is unidirectional along each filament, but changes where filaments are still attached to remnants of the Z-lines. The crossbridges form a constant acute angle with the actin filament.

In most negatively stained images, myosin tails are either barely or not at all visible. However, we often find images

showing aggregation of the myosin tails into subfilaments accompanying the thin filaments (Figs. 7 and 10). This occurs in samples which were exposed only to 0.6 M KCl before negative staining as well as in those exposed to 0.3 M KCl.

The disposition of crossbridges along the actin filament is unaltered from that in the intact muscle. Specifically, the crossbridges on either side of the filaments are at the same level, thus forming the characteristic chevron configuration; target zones are occupied by either singlets or doublets; periodic disposition and original spacings (see below) are maintained; adjacent segments of the same filament are decorated by a predominance of either singlets or doublets (Fig. 9). Fourier transform of negatively stained filaments shows strong reflections at ~ 38 and 5.9 nm (Fig. 7, *inset*). The latter reflection is quite strong, and probably corresponds to the enhanced 5.9-nm actin layer line seen in contracting muscle (Matsubara et al., 1984).

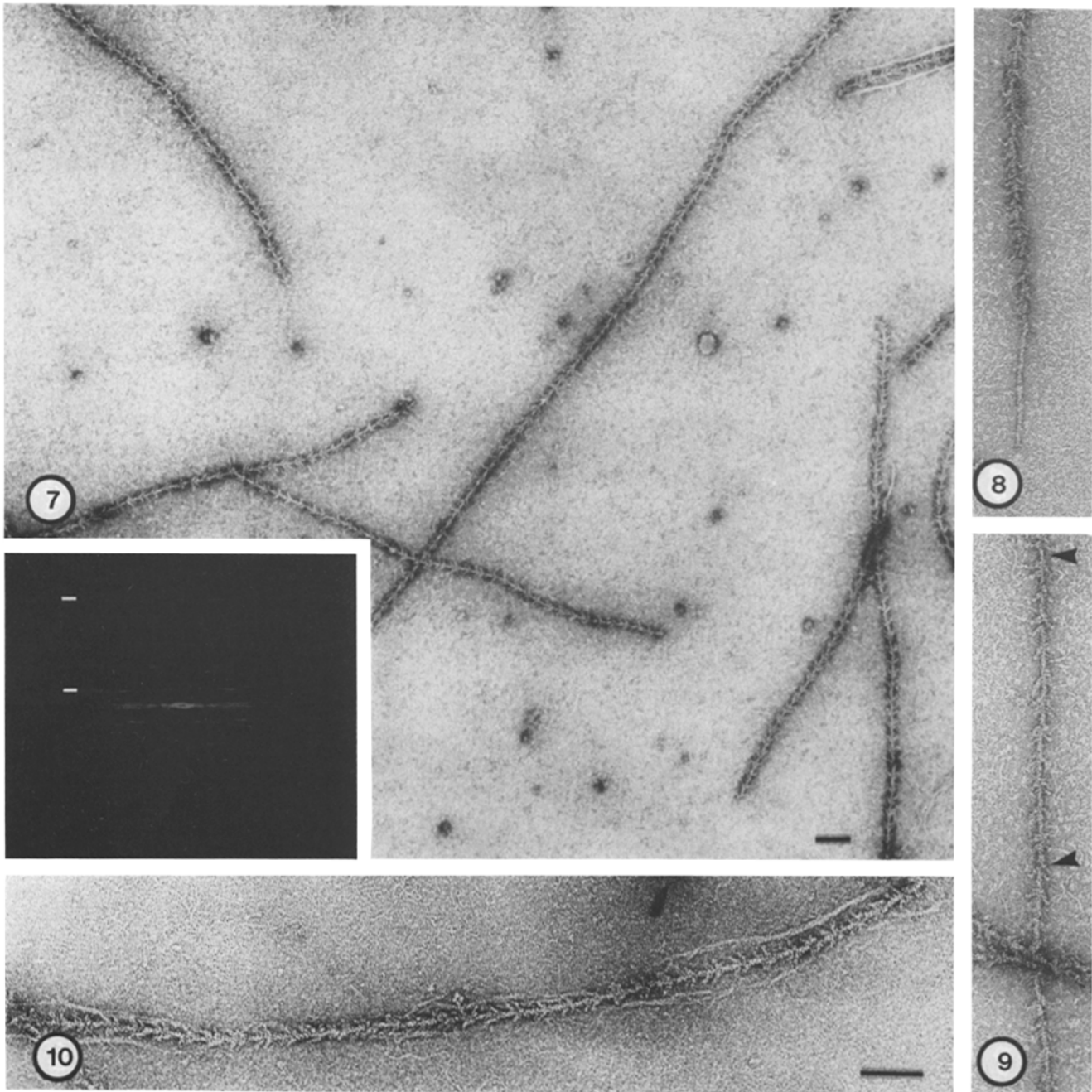
Crossbridges decorating isolated filaments maintain the triangular shape, but differ in one significant respect from the in situ filaments: the crossbridges are at constant acute angles (Fig. 11). The wider and more variable angles of crossbridge in the intact sarcomere are thus imposed by geometrical constraints.

Most of the crossbridges in the negatively stained images do not show a clear substructure. However, appropriately oriented crossbridges show two distinct components (Fig. 12), of sizes and shapes corresponding to single S1 subfragment, as shown by Craig et al. (1980). The two heads are rarely resolved in both components of the doublet, but Fig. 12 (*a* and *c*) shows four heads each for two separate doublets. Since in the intact muscle most crossbridges are triangular, including the two adjacent crossbridges forming most doublets, we conclude that rear and lead elements are both double-headed in crayfish muscle. The size of a crossbridge with the two heads resolved is comparable to that of a triangular crossbridge in the intact muscle, with the allowance of an ~ 2 -nm addition to the diameter due to platinum shadow.

Measured Parameters of Natively Decorated Filaments

The following parameters were measured in a manner similar to that used for the intact muscle: (*a*) Spacing between target zones: the weighted average from 336 spacings on 21 filament segments (12 micrographs) is 37.3 nm. (*b*) Occupancy of target zones: 576 target zones from seven filaments, two micrographs were counted. 54% are occupied by crossbridge doublets. (*c*) Apparent length of crossbridge: the

Figures 4–6. (Fig. 4) Myac layer in rigor muscle shadowed at 45°. The M-line is on the right of the image. The image seems more crowded than in Fig. 2, because all crossbridges received sufficient platinum and are clearly visible. The periodic disposition of crossbridges is best visualized by holding the micrograph at eye level and glancing across the filaments (see also Fig. 5, left). Chevrons (*circle*) and double chevrons (*rectangular boxes*) decorate thin filaments. Doublets within rectangular boxes are particularly well aligned across the sarcomere, indicating similar azimuthal orientation of filaments. Bar, 50 nm. (*Inset*) Optical diffraction pattern of selected area from the micrograph showing a strong second order of the actin 76-nm repeat. A faint higher order is also visible. Note that meridian (*m*) is horizontal, so that pattern and micrograph have the same orientation. (Fig. 5) Different azimuthal angles of crossbridges in doublets are clearly visible in this stereomicrograph from a replica shadowed at 45° (cf. Fig. 2). Hollow core of myosin filament backbone is visible where thick filament has been fractured. Twisted cable-like structure of backbone is seen. Bar, 50 nm. (Fig. 6) Gallery of doublets from shadowed muscle. (*a-i*) Shadowed at 45°. (*j*) Shadowed at 25°. *a-i* are obtained from images tilted by 15–30° along the filament axis. The apparently uneven shadow of the thick filament backbone indicates the direction of tilt: where the shadow is darker on the right side, the tilt was in the clockwise direction. Crossbridges forming doublets are either contiguous (*b, d, e, f, h*, and *i*) or separated by a small gap (*a, c, e, g*, and *i*). The majority of the crossbridges, including the two elements of each doublet, are wide and have a triangular shape (*a-j*). Very few are thin and straight (*c*, *arrowhead*). Bars, 50 nm.



Figures 7-10. Negative staining of isolated natively decorated thin filaments. (Fig. 7) A low magnification electron micrograph showing the periodic decoration of thin filaments with crossbridges. One filament (*center*) is very long, illustrating the capacity of the filaments to anneal. (*Inset*) Calculated Fourier transform of the segment between arrowheads showing a strong reflection at actin's 38-nm and 5.9-nm layer lines. Bar, 100 nm. (Fig. 8) Short segment free of crossbridges located at the end of a decorated filament. Transition between bridge-free segment and decorated segment is abrupt. Note a similar transition in Fig. 7. Bar, 100 nm. (Fig. 9) Crossbridges are periodically disposed along the thin filament and maintain the characteristic chevron and double chevron pattern observed in intact muscle, for several hours after isolation. Bar, 100 nm. (Fig. 10.) Aggregation of myosin to form subfilaments along the sides of the thin filaments. Notice the high percentage of doublets on this filament. Bar, 100 nm.

length of crossbridges was measured as the distance between the center of the thin filament at the site of crossbridge attachment, to the visible tip of the crossbridge. From 60 clearly visible crossbridges, from 11 filaments (five micrographs), the average crossbridge length is 18.80 nm. Obviously, the measurements are not very precise because in negatively stained images there is some uncertainty on structural boundaries.

Calculations

Using the data from deep-etched micrographs, and assuming 38.0-nm spacing for actin target zones and 14.3-nm spacing for subunit axial translation (crown) in the myosin filament, we can calculate the average number of crossbridges contributed by each crown of the myosin filament to the six surrounding actin filaments, as follows. 100 actin target zones,

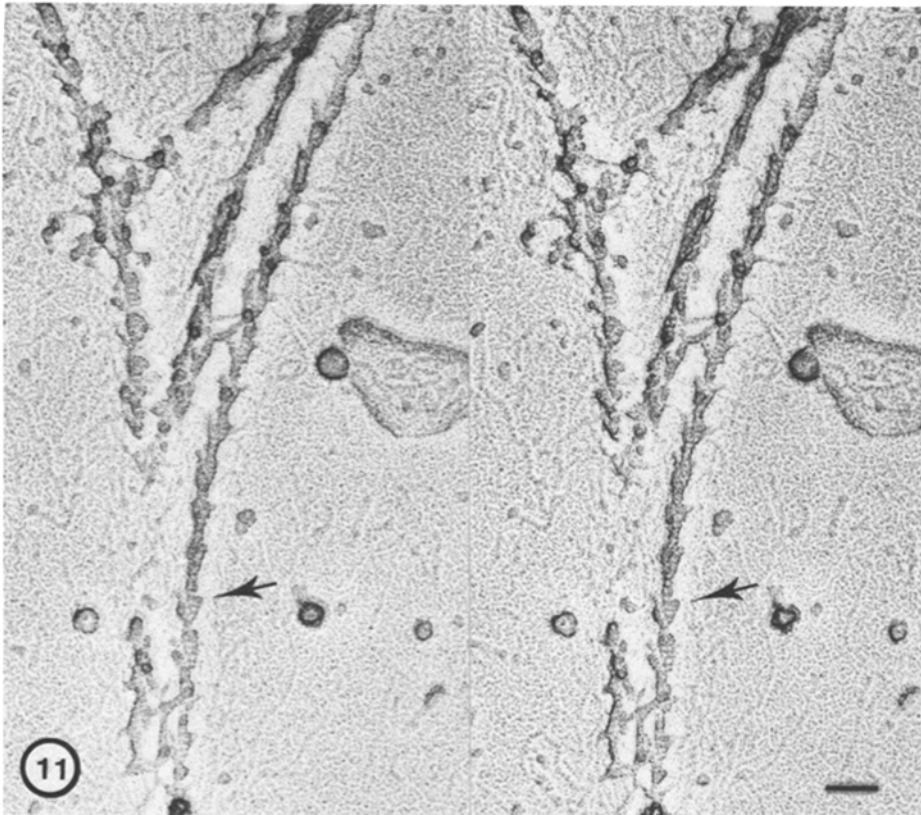


Figure 11. Stereomicrograph of isolated filaments prepared with the mica technique and then shadowed. The triangular shape of the crossbridges is clearly visible (arrows). Doublets are less clearly visible than in negatively stained filaments, probably because crossbridges are closely apposed to the thin filaments. Note long, lightly shadowed titin molecules on the background. Bar, 50 nm.

available to one myosin filament, occupy a length of $100 \times 38.0 = 3,800$ nm on the actin filament. Since 58% of the target zones are occupied by doublets, the number of crossbridges occupying 100 target zones is $58 \times 2 + 42 = 158$ crossbridges. One myosin filament contributes at least to $6 \times 158 \times 14.3/3,800 = 3.6$ crossbridges per crown. Note that these numbers are likely to be underestimates, since profiles which do not clearly reach the thin filaments or which have an odd shape were not counted as crossbridges. Since the majority of crossbridges are triangular and, presumably, double-headed, this means that the thick filament is most likely to be four-stranded.

Intact Muscle, Thick Filament Backbone

Over most of the A bands, the thick filament backbones have a hollow core, which is visible wherever the fracture follows

the center of the filament (Figs. 2, 4, and 13). The peripheral portion of the backbone is composed of subfilaments with a diameter of 3.8–4.8 nm which follow a right-handed twisted path (Fig. 13). At each level, three subfilaments are visible and two others are sometimes resolved at the periphery. Considering that we see only one side of the filament and that the two edges of the cylinder are not well shadowed, this is consistent with 12 subfilaments in the wall of the hollow cylinder forming the thick filament backbone (see Discussion). The diameter of the thick filament backbone is somewhat variable along the length of the filament, ranging between 17 and 22 nm.

Discussion

The main conclusion of this work is that the majority of rigor

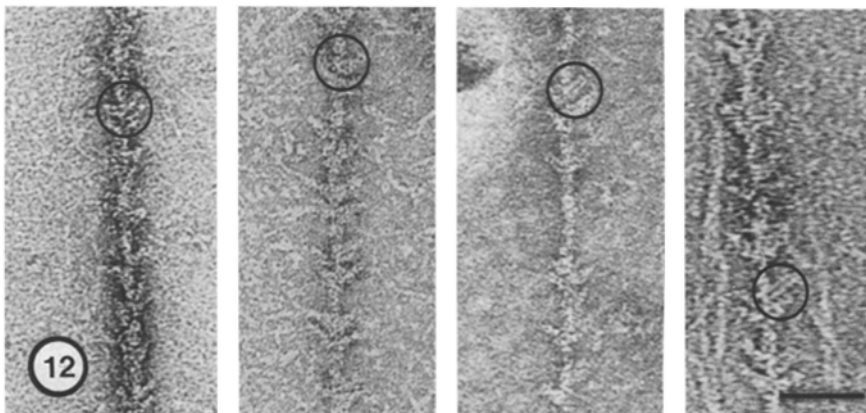


Figure 12. Negatively stained isolated native filaments. The two heads forming each crossbridge are resolved (circles). Notice that both elements of a doublet have two heads. Bar, 50 nm.

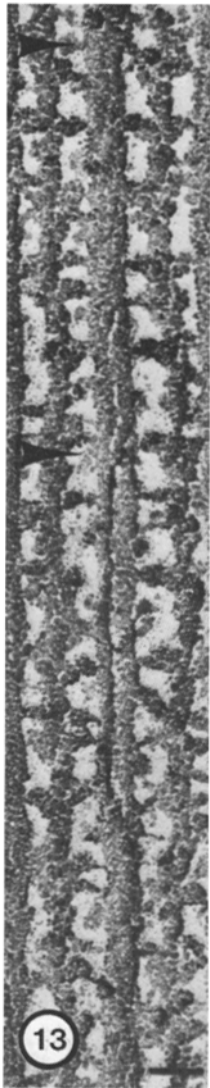


Figure 13. High magnification of the thick filament backbone in intact muscle. The backbone has a hollow core and a right-handed helical pattern of subfilaments, particularly visible between arrowheads, 45° shadow. Bar, 25 nm.

crossbridges in crayfish muscle are composed of two myosin heads. The evidence for this comes from the large size of the majority of crossbridges in the intact muscle and the direct visualization of the two heads on isolated natively decorated filaments. The conclusion depends heavily on the presumption that the crossbridges on the isolated filaments are the same as those in the intact muscle. The presence of an abrupt transition between bridge-bearing and bridge-free actin segments in natively decorated filaments, and the persistence of periodicity and chevron pattern of the attachments of crossbridges, indicate that there is no rearrangement of the actomyosin interactions during dissolution of the myosin backbone and in the subsequent time interval before negative staining. A randomization of the crossbridge disposition would undoubtedly occur if crossbridges were free to detach and reattach in significant number during the exposure to high molar KCl.

A second, although indirect, demonstration that the majority of crossbridges are double-headed and that myosin does not form double filament interactions can be derived from the very close correspondence in crossbridge density along intact and isolated thin filaments. If the thin filaments

were extensively cross-linked in the myofibril by single-headed crossbridges participating in double-filament interactions (Borejdo and Oplatka, 1981), breakup of myofibrils into isolated actin filaments would depend on dissociation from actin of approximately half of the crossbridges, and we would find a reduced density of crossbridges on the isolated filaments. This does not exclude that a few double-filament interactions occur and indeed we do see a minority of thin, presumably single-headed crossbridges. We do not know whether dissociation rate of the myofibrils in high KCl is limited by these few cross-links or by Z-lines.

Further evidence for the double-headed nature of most crossbridges comes from the calculation that the density of crossbridges along thin filaments corresponds to approximately four crossbridges per crown of the myosin filament. Thick filaments derived from several arthropod muscles are four-stranded (Stewart et al., 1981; Kensler and Levine, 1982; Levine et al., 1983; Crowther et al., 1985; Kensler et al., 1985) and each crossbridge is composed of two myosin heads (Stewart et al., 1985). There is no reason to believe that crayfish thick filaments should differ from those of other arthropods.

The diameters of the crossbridges that we interpret as single- and double-headed are not consistent with the ~10-nm diameter of S1 obtained from single myosin molecules deposited on mica (Heuser, 1983). However, 10-nm diameter for a single S1 is not consistent with the ability of S1 to decorate actin filaments by attaching in a 1:1 ratio to actin monomers spaced at ~5-nm intervals. Our values are entirely consistent with the best current estimate of S1 diameter (6 nm at its widest) from crystallized fragments (Winkelman et al., 1985). The length of the crossbridge is similar to the most accepted length for S1 (see Craig et al., 1986).

In crayfish muscle, lead and rear elements of the double chevrons are both double-headed crossbridges. In the insect muscle, instead, the rear element is thought to be single-headed (Taylor et al., 1984, 1986). It is not clear whether this difference is real or a result of the imaging approaches used. Although shadowed images do not have the detail of the reconstructed images, overall profiles of lead elements look remarkably similar by the two approaches. Rear elements, on the other hand, are wider in the shadowed images.

The permanence of the native crossbridge pattern on the isolated filaments is consistent with the slow decay of force of muscle fibers in the absence of ATP (Schoenberg and Eisenberg, 1984), which indicates a dissociation rate constant for the intact crossbridge much smaller than that found for S1 *in vitro* (Marston, 1982). Permanence of the rigor crossbridge has been explained on the basis of cooperative interactions (Kuhn, 1978) whose anatomical basis may very well be the double-headed nature of the crossbridge.

Crossbridge doublets in our photographs show either contiguous attachment of the two crossbridges, or intercalation of an actin monomer between the two. Since each crossbridge is double-headed, the total length of the target zone is either four or five actin monomers. This is consistent with the conclusions obtained from modeling of the insect rigor muscle based on a four-stranded myosin filament and a 45° azimuthal range around actin (Haselgrove and Reedy, 1978, 1984). An intercalated actin monomer was also found between the crossbridges of a doublet in insect flight muscle (Taylor et al., 1984), thus making the target area either four

or five monomers long depending on the composition of the two crossbridges. The very short intradoublet spacing found for a small percentage of doublets indicates that one of the two crossbridges is sometimes single-headed. In vertebrate muscles, separation between crossbridges of a doublet is more variable than in the crayfish, and target zones may be as long as six monomers. This contributes to the less orderly distribution of rigor crossbridges in those muscles (Varriano-Marston et al., 1984).

Helical symmetry of the crayfish thick filament is likely to be very different from that of the insect flight muscle (Wray, 1979). This is confirmed by the lack of an 1,160-Å reflection in X-ray diffraction of crayfish muscle in rigor (Wray et al., 1978). In insect muscle, the 1,160-Å reflection and its higher orders are thought to be due to the coincidence of myosin and actin repeats at multiples of that length.

Limited layer line sampling in the X-ray diffraction of crayfish muscle indicates some randomness in the orientation of thick filaments. However, we find rows of doublets aligned across the sarcomere, demonstrating similar orientation of thick filaments in some direction, perhaps as a result of a superlattice similar to that postulated for vertebrate muscles (Squire, 1981).

Our images of the thick filament backbone are consistent with the subfilament structure demonstrated by Ashton et al. (1987) and the model proposed by Wray (1979). Wray proposes that the filament is a tube, whose wall is formed by 12 subfilaments oriented at a small angle to the filament axis. The size and the number of the subfilaments in our images are consistent with this model. Note that since our images are from rigor myofibrils, and the crossbridges are formed only by the S1 portion of the myosin molecule (Varriano-Marston et al., 1984), the subfilaments in the backbone must be composed of S2, as well as light mesomyosin. In the negatively stained images of natively decorated actin filaments, we often noticed myosin subfilaments accompanying the actin filaments and joined to them by crossbridges. We do not know whether these represent one of the original subfilaments, or a reaggregation of the tails of the dissolved myosin. We note that such subfilaments were seen in material which was negatively stained from the 0.6-M KCl solution, which should not permit reaggregation. In the intact filament backbone, subfilaments follow a right-handed twist, and this is in agreement with the right-handed crossbridge helix seen in relaxed muscle and in isolated filaments (Ip and Heuser, 1982; Vibert and Craig, 1983; Kensler et al., 1985), and with the model of Wray (1979). In rigor muscle, on the other hand, the crossbridge helix is apparently left-handed (Reedy, 1968; Heuser, 1983) and this is confirmed in our study. Interaction with the actin helix may be responsible for this discrepancy.

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