UNUSUAL THICK AND THIN FILAMENT PACKING

IN A CRUSTACEAN MUSCLE

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ABSTRACT

The proximal accessory flexor (PAF) of the myochordotonal organ (MCO) in the meropodite of crayfish walking legs contains two populations of muscle fibers which are distinguishable by their diameters. The large accessory (LA) fibers are 40–80 μm in diam and are similar in ultrastructure to other slow crustacean fibers. The small accessory (SA) fibers are 1–12 μm in diam and have a unique myofilament distribution at normal body lengths. There is extensive double overlap of thin filaments at these lengths, and some of them form bundles that may extend the length of the sarcomere. In the middle of the sarcomeres, thick and thin filaments are totally segregated from each other. When the fibers are stretched to lengths beyond double overlap length, the myofilament patterns are conventional. The segregated pattern is reestablished when stretched fibers are allowed to shorten passively. The length-tension relationship of the SA fibers is described by a linear ascending branch, a plateau, and a linear descending branch. The ascending branch encompasses normal body lengths from slack length (L_s) with maximum double overlap to the length at which double overlap ceases (1.8 $\times$ L_s). The descending phase is comparable to that of other skeletal muscles. That is, tension decreases in proportion with the reduction in thick-thin filament interdigitation (2 $\times$ L_s to 3 $\times$ L_s).

KEY WORDS muscle · ultrastructure · myofilaments · crustacean · length-tension

The meropodite segment of the walking limbs of decapod crustaceans contains a proprioceptor, the myochordotonal organ (MCO), originally described by Barth (2). The MCO consists of sensory cells associated with a tendon into which a proximal and a distal accessory flexor muscle insert. Cohen (6, 7) and Dorai Raj and Cohen (9) have studied the function and light microscope structure of this system in crabs. In the crab, two populations of fibers can be distinguished in the accessory flexors on the basis of physiological behavior (1), and appearance in the light microscope (7). Ultra-structural differences between the fiber types in crabs include the size of fibrils, amount of sarcoplasmic reticulum (SR), and ratios of thin and thick myofilaments (8, 12).

In crayfish proximal accessory muscle, we find two populations of fibers which are distinguishable by fiber diameters: we call these large accessory (LA) and small accessory (SA) fibers. The two fiber types are in discrete bundles which can be separated readily.

In addition to diameter, at normal body length the filament lattice structure of the two fiber types differs. This report describes sarcomere length-dependent changes in the structure of the filament lattice and the length vs. tension relationship of

the SA fibers. Unlike the arrangement in other striated fibers, the spatial arrangement of thick and thin filaments, as well as their overlap, is dependent upon fiber length. Rearrangement of filaments occurs between slack length (L_s) and 2 × L_s. At longer lengths, the degree of overlap is as predicted by the sliding filament model (16, 18). A preliminary report of this work has appeared (10).

MATERIALS AND METHODS

Preparation

Crayfish of the genera *Procambarus* and *Orconectes* were obtained from Mogul-Ed (Oshkosh, Wis.). After removing a walking limb, the muscles within the meropodite segment were exposed by cutting away the encompassing chitin exoskeleton, leaving a small chitin bridge on the dorsal edge between the proximal (ischiopodite) and distal (carpopodite) segments. This procedure allowed visualization of the muscles and measurement of length changes when the muscles were extended and flexed by moving the carpopodite. The nerve, main flexor, main extensor, and LA muscles were then removed, leaving the SA muscle and its tendon. After dissection of the small portion of the meropodite from which the SA fibers originate, the chitin bridge and tendon were cut and the SA preparation was transferred to an experimental chamber. The chamber, saline, and methods for recording tension have been described in a previous publication from this laboratory (5). Maximal tensions were evoked either by depolarizing the fibers with isosmotic substitution of 100–200 mM KCl for NaCl or by adding 20 mM caffeine to the saline.

Electron Microscopy

For electron microscopy, muscles were fixed with 0.3% glutaraldehyde in saline or with a mixture of 0.3% formaldehyde (from paraformaldehyde) and 0.2% glutaraldehyde in saline, for 20–30 min. Subsequently, they were transferred to 1% OsO_4 in Veronal acetate buffer, pH 7.4, for 1–2 h, dehydrated in ethanol and propylene oxide, and embedded in Epon-812. In one preparation, 1% tannic acid was applied as a mordant after fixation (19). Sections cut with a diamond knife on a Reichert ultramicrotome (OMU-2) (C. Reichert, sold by American Optical Corp., Buffalo, N. Y.) were mounted on bare grids, stained with uranium and lead salts, and observed and photographed with a Philips EM 200.

RESULTS

Identification of SA Fibers

The bundle of SA fibers lies on the posterior dorsal surface of the proximal accessory flexor (PAF). The SA bundle is 50–90 μm in diam,

comparable to the diameter of a single LA fiber. SA fibers average 4 μm in diam with a range from 1 to 12 μm (Fig. 1). There are about 20 LA fibers and 100 SA fibers in the accessory flexor.

When the PAF tendon is cut, the muscle becomes slack, and the SA bundle separates from the LA fibers at the tendon. The separation occurs because SA fibers are longer than LA fibers.

Filament Distribution at Slack Length (L_s)

In situ, the SA fiber length can be varied between 1.2 and 1.8 × L_s by moving the meropodite-carpopodite (M-C) joint from the fully flexed to the fully extended position. In normal walking, the crayfish maintains the M-C joint closer to the fully flexed position. When the tendon is cut, the SA fibers shorten spontaneously to length L_s. In longitudinal sections of SA fibers fixed at L_s, no I band is visible (Fig. 2), and the ends of the thick filaments are in contact with the Z lines. Sarcomere lengths at L_s range from 5.1 to 6.5 μm, with an average of ~5.5 μm. Thin filaments, many of which are in bundles that vary in width, extend nearly the length of the sarcomeres at L_s (Fig. 2). Z lines are ~200 nm wide with an irregular zigzag course.

Thick filaments appear closer together in the center of the sarcomere than on either side of the center. Thin filaments are excluded from these closely apposed thick filaments, although thin filaments extend through the center of the A band in adjacent regions (Fig. 3). The zone in which some thick filaments are more closely apposed than normal is ~1 μm long.

A striking pattern is present in electron micrographs of transverse sections of myofibrils near the center of the A band where thick and thin filaments are segregated from each other (Figs. 4 and 5). Most of the segregated thick filaments have electron-opaque cores and are larger in diameter by ~5 nm than thick filaments with thin filament orbits around them (Fig. 4). These characteristics of the segregated thick filaments identify the center of the sarcomere and are found in the middle of the A band in other crustacean muscles (13). The center-to-center spacing of thick filaments in the segregated regions can be 30% less than in adjacent areas where thin filament orbits surround thick filaments (Fig. 4).

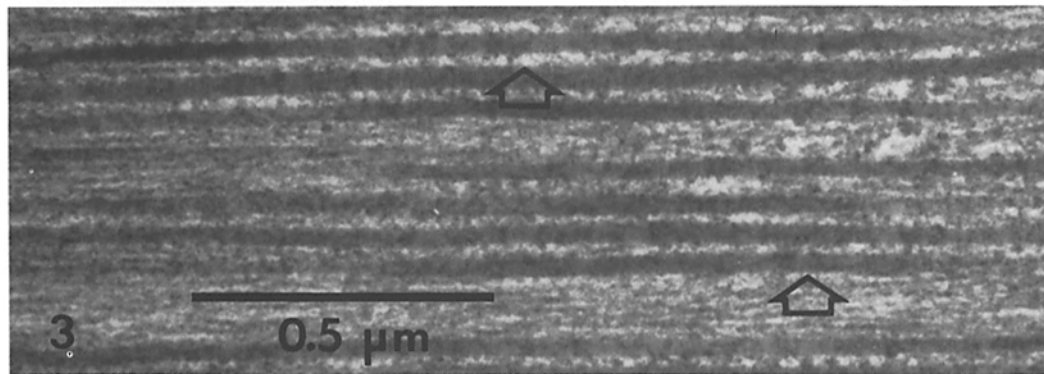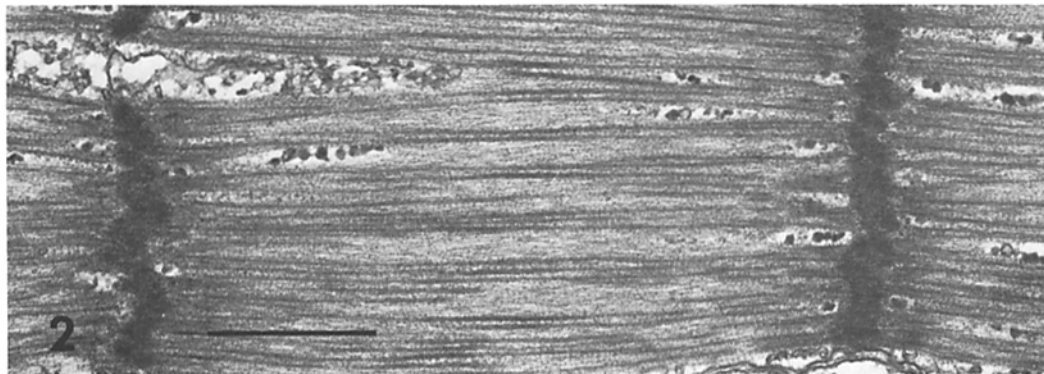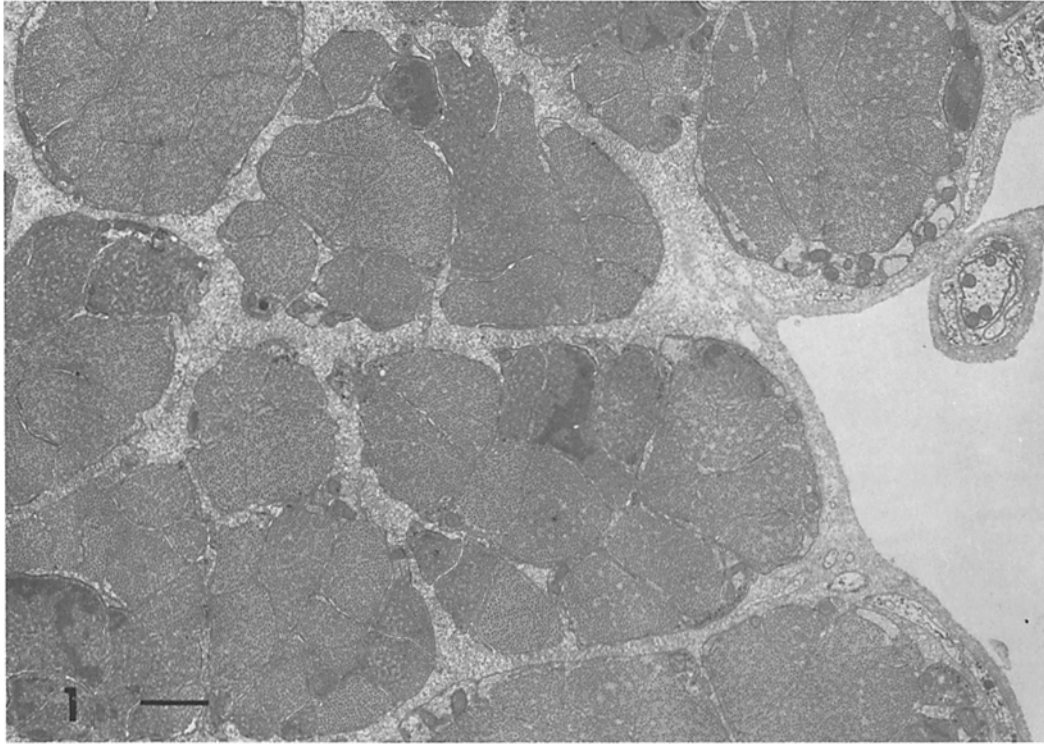The pattern of thick and thin filaments in the central segregated zones is highly ordered, and
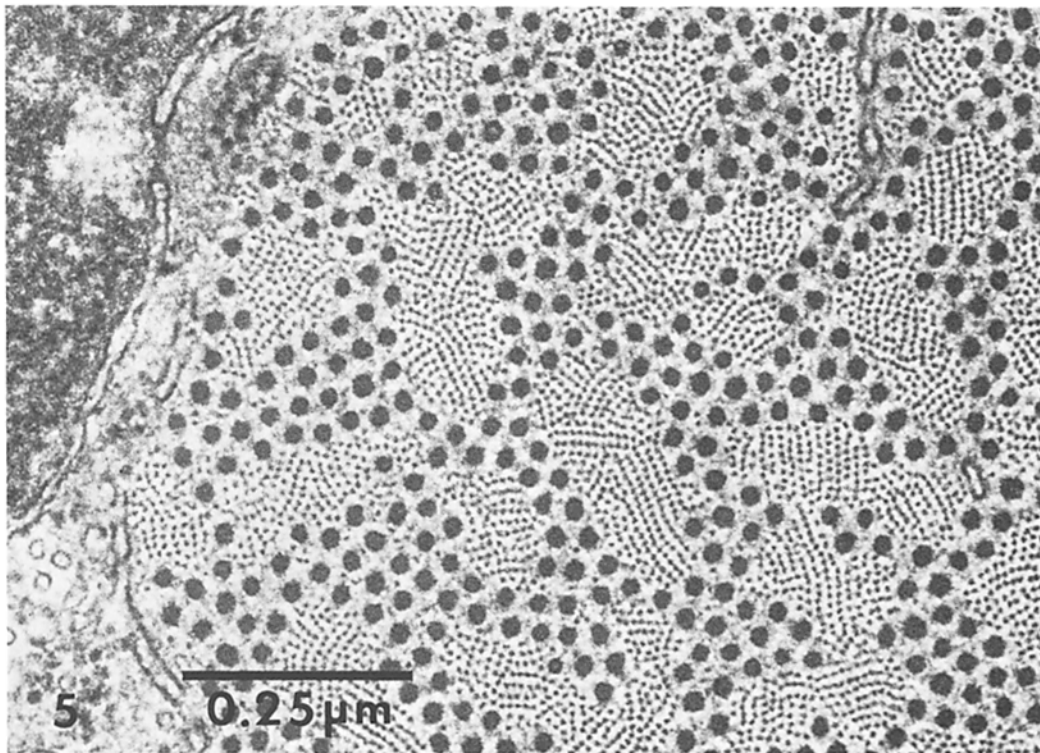
FIGURE 1 Transverse section of fibers from the SA bundle of the PAF of the crayfish. The segregated regions are discernible at this magnification as the patchy appearance in the A-band regions. The extracellular space is filled with collagenlike filaments. Bar, 1 μm. $\times$ 9,000.

FIGURE 2 Longitudinal section of SA fiber fixed at L_s. Thick filaments contact Z lines, and no I band is present. Thin filament bundles, which tend to be larger near the center of the sarcomere, extend throughout the A band. Bar, 1 μm. $\times$ 22,000.

FIGURE 3 Longitudinal section of SA fiber fixed at L_s. This micrograph is from the center of a sarcomere. Thin filaments traverse this entire region. However, there are areas where thin filaments are not seen between adjacent thick filaments (arrows). $\times$ 80,000.
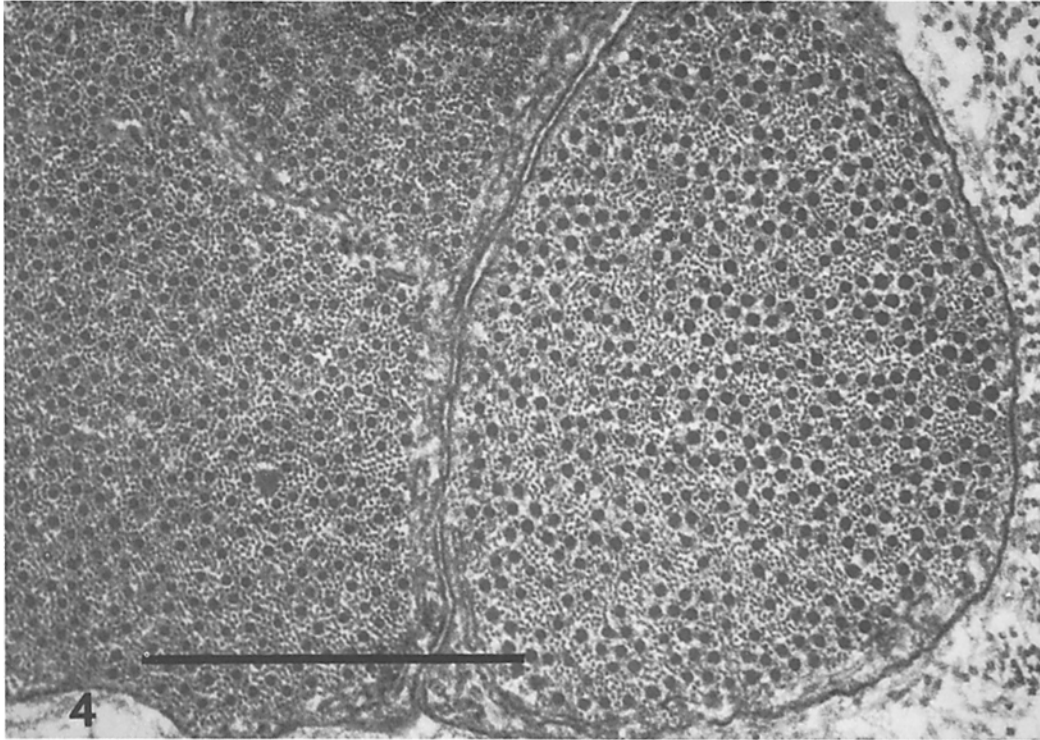
FIGURE 4 Transverse section of SA fiber, fixed at L_s. The fibril on the right side of the micrograph is sectioned near the transition between normal and segregated zones, ~0.5 μm from the center of the sarcomere. Large diameter, thick filaments have few thin filaments in orbits around them. The fibril on the left was sectioned away from the segregated zone, and here thin filament orbits surround the thick filaments. Extra thin filaments are interspersed between the orbital arrays. Bar, 1 μm. × 50,000.

FIGURE 5 Transverse section of SA fibers fixed at 1.2 × L_s. The section passes through the segregated zone in the middle of the sarcomere. Most of the thick filaments are large in diameter (~25 nm) with electron-opaque centers. Thick and thin filaments are almost totally separated. × 120,000.

thin filaments often occur in a crystal-like array (Fig. 5). The ratio of thin to thick filaments in the segregated zones is 10:1. On each side of the center region, thick filaments are surrounded by orbits of 12 thin filaments. In addition, thin filaments which are not associated with orbits are seen in this region (Fig. 4). If the extra-orbital thin filaments are included, the ratio of thin to thick filaments in these regions is 9:1.

The appearance of filaments in transverse sections through different levels in the sarcomere of fibers fixed at L_s may be summarized in this way:

(a) There is a region in the center of the sarcomere where thick and thin filaments are almost completely separated. This region is ~1 μm, and we refer to it as the segregated zone.

(b) Between the segregated zone and each end of the A band is a region where thin filament orbits surround thick filaments and there are "extra" thin filaments between the orbits. We call this the mixed zone.

(c) There is a small region at each end of the A band where normal thin filament orbits surround thick filaments. The extent of this normal zone is difficult to determine, because the Z lines are not straight and intrude on it to varying degrees in adjacent regions. The normal zone is ~0.5 μm at each end of the sarcomere, or ~20% of the sarcomere length, at L_s.

Filament Patterns at Lengths Greater than L_s

If SA fibers are fixed at or beyond 2.0 × L_s, the filament distribution is comparable to that in many other crustacean muscles. There is an H zone which, in transverse sections, is free of thin filaments (Fig. 6). In the A band lateral to the H zone, orbits of 10-12 thin filaments surround each thick filament and there are no extra thin filaments, so the ratio of thin to thick filaments is 5.5:1. SA fibers at 2 × L_s appear nearly identical to LA fibers (Fig. 7), except for the difference in diameters.

The A band has a mean length of 5.5 μm, but ranges from 5.2 to 6.4 μm. It is not known whether this dispersion reflects a dispersion in thick filament lengths. The edges of the A bands are not sharp, so the thick filaments are not aligned precisely. Thin filament length, measured as the distance from the center of the Z line to the edge of the H zone, is ~5 μm.

Between L_s and 2 × L_s, the distribution of filaments varies with fiber length. On the basis of thin filament length, double thin filament overlap should be present until 1.8 L_s and was observed at 1.5 × L_s. No H zone is seen between L_s and 1.5 L_s, and it should be absent until 1.8 L_s. As fiber length increases, the proportion of the region with extra-orbital thin filaments interspersed between thin filament orbits decreases, and the region with no extra thin filaments between the orbits increases. I bands, which are absent at L_s, appear and then increase in length as the fibers are stretched. If SA fibers are stretched to 2.5 × L_s, a length at which they have a conventional appearance, and then are allowed to shorten to L_s before fixation, they have the appearance of unstretched SA fibers. Thus, the pattern of filament segregation and the conventional pattern are interconvertible by changes in fiber length.

General Ultrastructure

The SA fibers, although only 1-12 μm in diam, have an elaborate T-system and well-developed SR, comparable to the organelles of the main walking leg muscles (3, 4, 14). T-tubules arise either directly from the fiber surface or from a complex system of wider-bore clefts similar to those of other crustacean muscles. Frequent dyadic contacts occur between the T-system and SR near the ends of the A bands. Peripheral junctions (11) are present between SR and the membrane of the fiber surface. A large amount of extracellular space filled with collagenlike filaments surrounds the SA fibers (Figs. 1 and 8).

Mitochondria are sparse and scattered (Fig. 8) rather than concentrated in a peripheral ring as in the LA or main flexor fibers. Rough-surfaced endoplasmic reticulum and Golgi regions are common near nuclei.

Neuromuscular junctions (NMJs) appear similar to those of other crustacean fibers. Junction regions are characterized by increased electron opacity in the synaptic gap. Synaptic vesicles are usually round with electron-transparent contents, although a few larger vesicles with electron-opaque cores are present at most axon terminals. No populations of flattened synaptic vesicles were observed in the SA fiber junctions.

Length-Tension Relationship

The position of the passive length-tension curve with respect to that of the active length-tension for the SA bundle is similar to that of single fibers
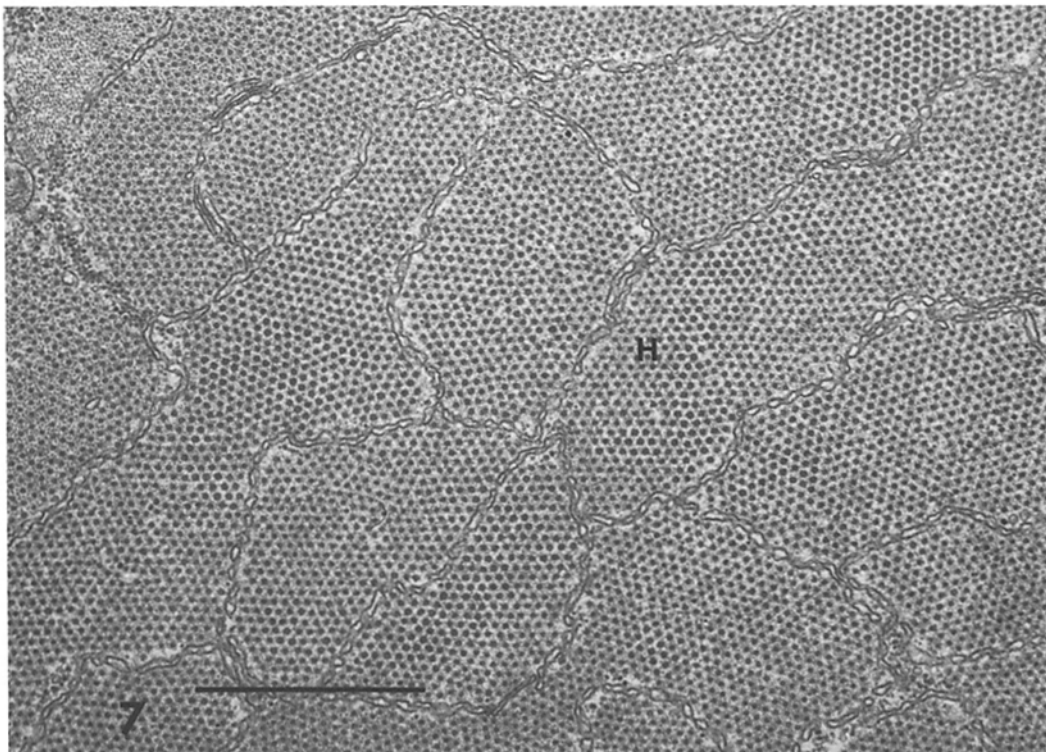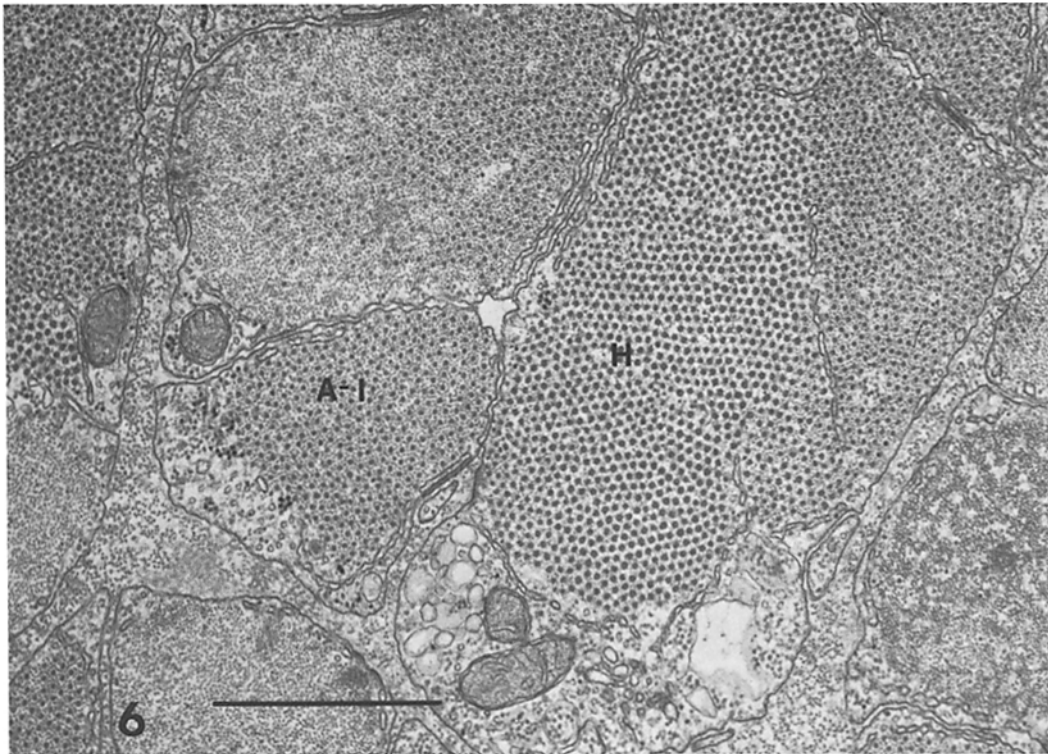
FIGURE 6 Transverse section of an SA fiber fixed at $2 \times L_s$. H zone (H) contains only thick filaments, and no grouping of thick filaments is seen. In the overlap region (A-I), thin filament orbits surround the thick filaments. Bar, 1 μm. $\times$ 30,000.

FIGURE 7 Transverse section of LA fiber from the same block as Fig. 6. The overall appearance of this fiber, and in particular the H zone (H), is quite comparable to that of the SA fiber in Fig. 6. Bar, 1 μm. $\times$ 30,000.
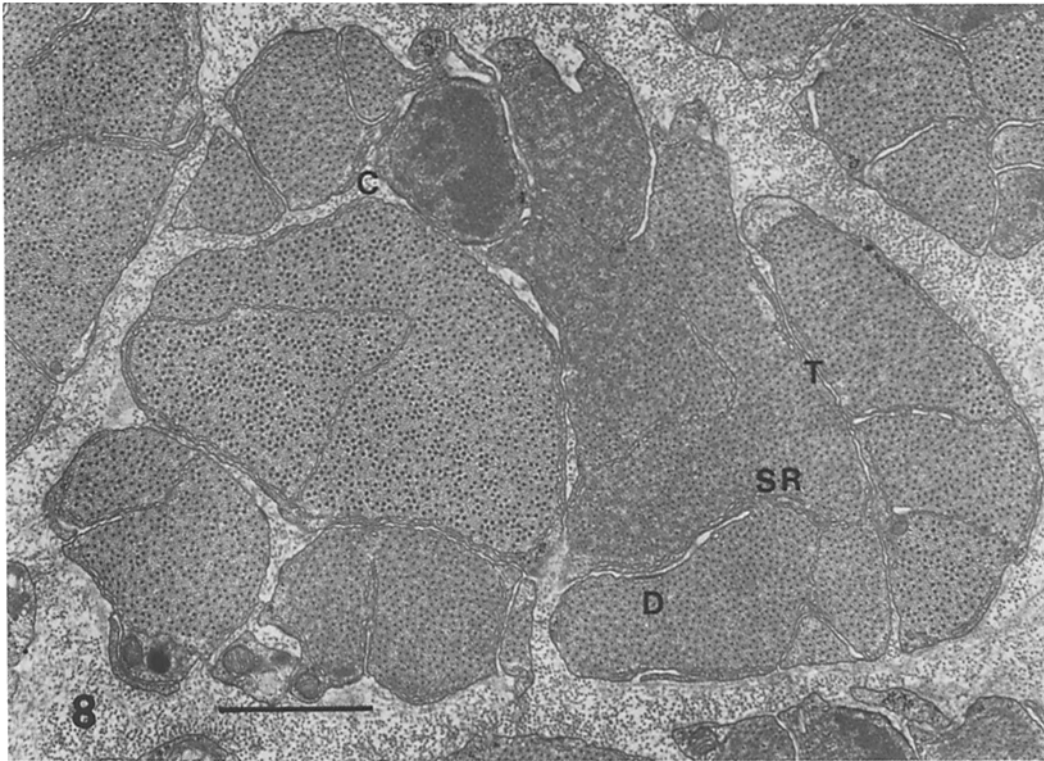
FIGURE 8 Transverse section of SA fiber fixed at 1.2 × L_s. This illustrates the general structure of these fibers. Clefts (C), containing collagenlike filaments, deeply invaginate the fibers. T-tubules (T) originate from either the surface membrane or clefts. The T-system forms dyadic contacts (D) with the sarcoplasmic reticulum (SR). Although not illustrated here, dyads may also be formed between SR and surface membrane or cleft membrane. Bar, 1 μm. × 20,000.

from the main flexor muscle (Fig. 9; see references 20 and 21). Passive tension (recording sensitivity 5 mg/cm) was not observed until the resting fibers were stretched to about 2.1 × L_s. Active tensions were small, but measurable, at lengths near L_s (Fig. 9). Maximum active tension (P_0) was not attained until the bundle was stretched to about 2 × L_s (50-60 mg or about 2 kg/cm^2 of whole muscle, not corrected for extracellular space). The active tensions represented in the curve of Fig. 9 were evoked by application of 20 mM caffeine. Intervals of 20 min between caffeine tensions allowed repriming of the SR (5). The amplitude of the caffeine-induced tensions rose linearly with increasing fiber lengths between L_s and 1.8 × L_s.

After a short plateau the decline of tension with further stretch was also linear and the line extrapolates to zero tension at a fiber length of 3 × L_s, which corresponds to a sarcomere length of ~16.5 μm. This value is close to that predicted by the sliding filament model since both thick and thin filaments are 5.0-6.0 μm long.

Data were obtained for construction of five additional length-tension curves. In two experiments, tensions were elicited by 20 mM caffeine. Three curves were based on tensions evoked by 100 mM potassium. All curves were similar except for the declining phase which was steeper for the tensions induced by high potassium. This might have resulted from an incomplete repolarization of the membrane following repetitive exposures to elevated potassium. Membrane potential was not measured due to the small size of the fibers.

DISCUSSION

Several unique features of the structure and length-tension relationship of the SA fibers have been described. These include: (a) At L_s, sarcomeres are essentially equal in length to the thick filaments. (b) The thick and thin filaments are completely segregated for ~1 μm at the center of
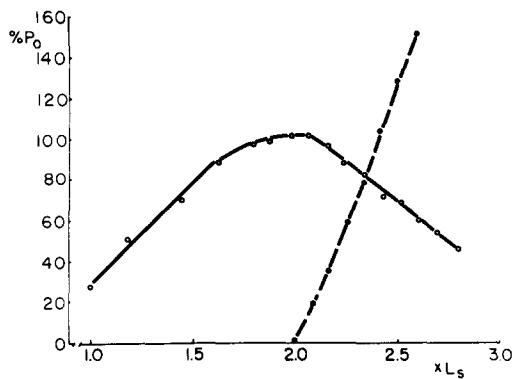
FIGURE 9 Length-tension curve for an entire SA bundle of the crayfish MCO. Length is referred to L_s of the bundle. The active tensions were elicited by application of 20 mM caffeine in crayfish saline. The maximum active tension developed by this bundle was 50 mg. Open circles: isometric active tension; closed circles: passive tension.

spacing between adjacent thick filaments can be 30% less in the segregated zones than in adjacent regions. The offset regions of the thick filaments correspond approximately to the regions where thick and thin filaments are segregated, although whether a causal relationship between these phenomena exists is unknown. The altered center-to-center spacing of the thick filaments could form a "forbidden zone" $\sim$1 μm long from which thin filaments are excluded, and this could result in the observed segregation.
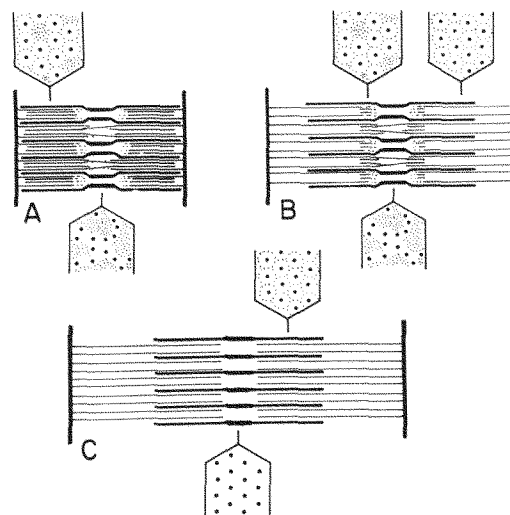

FIGURE 10 Tentative model of filament distributions in the SA fibers. (A) Slack length. There is almost complete double-overlap of thin filaments. No I band is present. Thick filaments are offset in the center of the sarcomere. Although the exact course of thin filaments is unknown, they are assumed to remain in orbits around the thick filaments until they reach the near edge of the central segregation zone. Here they leave the orbits and enter thin filament bundles. The dotted portions of thin filaments indicate that there is uncertainty about their actual behavior and also that they presumably leave the plane of the figure. The filament patterns in transverse section of the central segregated zone and a lateral region are illustrated. (B) 1.5 $\times$ L_s. Double thin filament overlap still occurs for a short distance beyond the central segregated zone. A considerable portion of the thick filaments have normal thin filament orbits around them, and a normal I band is present. The patterns of filaments in transverse sections through the central segregated zone, lateral to the segregated zone where double overlap persists, and in the region with normal orbits are indicated. (C) 2 $\times$ L_s. No double overlap or filament segregation occurs. Filament patterns in transverse sections of a normal H zone and thick-thin filament overlap region are indicated.

the A band at fiber lengths below approximately 1.8 $\times$ L_s. (c) Bundles of thin filaments extend through the sarcomere at L_s. (d) Both thin filament bundles and the segregation of thick and thin filaments at the center of the A band disappear progressively as fiber lengths increase to 1.8 $\times$ L_s. (e) Fibers stretched to 2.5 $\times$ L_s passively return to L_s when released, and the unique filament arrangements reappear. (f) The SA fibers function in the animal at lengths on the rising side of the active length-tension curve, between 1.2 and 1.8 L_s. Except for the unusual filament lattice at fiber lengths between L_s and 1.8 L_s, the ultrastructure (e.g. T-system, invaginations, SR, and NMJs) is similar to that of other fibers in the meropodite.

Filament Distribution and
Sarcomere Length

The diagram of Fig. 10 provides a model which explains the changes in filament distribution as the SA fibers are stretched from L_s to 2.0 $\times$ L_s. The SA fibers are unique among striated muscle in that the sarcomere length at L_s is equal to the length of the thick filaments. Since thin filaments are only slightly shorter, they must extend at L_s from one Z line almost to the next, and there is an extensive double overlap of thin filaments.

In Fig. 10 A and B, the thick filaments are offset in the center, rather like cranks on a crankshaft. The best evidence for this conclusion comes from transverse sections in which center-to-center

At sarcomere lengths near L_s in regions away from the segregated zone, normal thin filament orbits surround thick filaments. In addition, extra thin filaments are found interspersed between the thin filament orbits. The presence of these extra thin filaments is due to the double overlap of thin filaments. When fibers are stretched beyond 1.8 L_s, the ratio of thin to thick filaments is about 5.5 to 1. This is the actual ratio, and the approximate doubling of this ratio at short sarcomere lengths indicates double overlap of thin filaments.

The thin filaments are represented in Fig. 10 A and B as originating at a Z line and extending toward the center of the sarcomere in an orbit around a thick filament until the central forbidden zone is reached. Here, thin filaments are represented as leaving their orbits, passing through the central zone in one of the regions containing only thin filaments, and then continuing as nonorbital thin filaments in the opposite half of the sarcomere. We have no direct evidence that this course is taken by the thin filaments, but the only plausible alternative, given the extensive double overlap, central segregated region, and extra thin filaments outside the segregated regions, is that contralateral thin filaments displace ipsilateral thin filaments from orbits. Since we are unable to follow individual thin filaments for their entire course along the sarcomere, it is impossible to determine whether all of the extra thin filaments seen in conditions of double overlap are from the ipsilateral or contralateral Z line, or some combination of both. However, since the thin filament orbits contain 10-11 thin filaments at long sarcomere lengths, but contain 12 thin filaments at sarcomere lengths near L_s, some of the thin filaments in orbit at short sarcomere lengths originate from the contralateral Z line.

At fiber lengths >1.8 L_s, the filament pattern resembles that of many other crustacean muscles (Figs. 6 and 10 C). Thin filament double overlap ceases at 1.8 L_s and, as the sarcomeres are stretched farther, an H zone appears. Overlap between thick and thin filaments decreases as sarcomeres are stretched beyond 1.8 × L_s and disappears at about 3 × L_s.

The unusual filament pattern at lengths <1.8 × L_s and the fact that this pattern reappears when stretched fibers are allowed to shorten without stimulation, raises several questions regarding the forces of interaction between the filaments. In the central 1 μm of the shortened sarcomere, there are no orbital thin filaments despite the fact that thin filaments traverse this region. It is reasonable to assume that no force bridges are formed in the central segregated region, so this zone is functionally equivalent to the bridge-free L or pseudo H zone of other muscles. Thin filaments originating at Z lines presumably remain in orbit and can participate in bridge formation until they reach the proximal edge of the segregated zone where they enter thin filament bundles.

It is not clear whether the exclusion of thin filaments from orbital positions is a steric consequence of closer spacing of thick filaments, or whether the interfilament forces are altered in that region. Furthermore, the crystalline array of thin filaments sometimes found in the segregated region raises the question of mutual attraction between thin filaments.

We can only speculate about the cause of the spontaneous shortening of sarcomeres to L_s. It is possible that some low level of bridge cycling occurs in the unstimulated muscles, resulting in sarcomere shortening to the point that Z lines abut the thick filaments when the fibers have no external load. It is noteworthy that fibers released after stretch to 2.5 × L_s shorten to L_s and resume the filament pattern of unstretched fibers.

The segregation of filaments that we observed in the SA fibers does not occur in other striated muscles when sarcomeres are shortened actively to produce double overlap. For example, in frog (17) and human (A. B. Eastwood, unpublished observations), the overlapping thin filaments are incorporated into orbits and double the normal orbital numbers. In the main flexor in crayfish meropodite, double overlap produces double orbits but no segregation of thick and thin filaments (unpublished observations).

Length-Tension Relationship

The right-hand branch of the length-active tension relation of the SA muscle resembles that of skeletal muscle fibers from frog (15) and crayfish (21). This similarity is expected since the thick and thin filament relations are similar. Such is not the case for the left-hand branch where the morphological relation of the thick and thin filaments differs markedly from that in frog and ordinary crayfish fibers.

The left-hand branch of the length-active tension relation can be explained if one assumes that double overlap of thin filaments interferes with the normal development of force (15). At L_s, only

~0.5 μm at each end of any thick filament engages a normal orbital array of thin filaments, free of double overlap. The total length, 1.0 μm, is ~20% of the thick filament length, and at L_s the tension developed is ~20% of P_0 (Fig. 10). The length of single overlap of thick and thin filaments increases as the sarcomeres are stretched and tension output rises. P_0 should occur where the region of double-overlap coincides with the segregated zone, since in this latter region no force bonds should occur. Tension will plateau until both sets of thin filaments leave the segregation zone, and thereafter tension will decrease with stretch.

The break in the left-hand branch of the length-active tension curve, which occurs in frog skeletal muscle and which has been correlated with the abutment of the Z lines against the thick filaments (15), does not occur in the length-tension relation of SA fibers. Presumably, this is because the SA Z lines do not contact thick filaments until the fibers are shortened to L_s. Consequently, the relation between length and tension is nearly linear over the normal operating range of the SA fibers *in situ* (between 1.2 and 1.8 × L_s). A linear relation between force output and length could be an important factor in deciphering joint position.

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