

ELECTRON MICROSCOPY OF SYNTHETIC MYOSIN FILAMENTS

Evidence for Cross-Bridge Flexibility and Copolymer Formation

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ABSTRACT

Electron micrographs of negatively stained synthetic myosin filaments reveal that surface projections, believed to be the heads of the constituent myosin molecules, can exist in two configurations. Some filaments have the projections disposed close to the filament backbone. Other filaments have all of their projections widely spread, tethered to the backbone by slender threads. Filaments formed from the myosins of skeletal muscle, smooth muscle, and platelets each have distinctive features, particularly their lengths. Soluble mixtures of skeletal muscle myosin with either smooth muscle myosin or platelet myosin were dialyzed against 0.1 M KCl at pH 7 to determine whether the simultaneous presence of two types of myosin would influence the properties of the filaments formed. In every case, a single population of filaments formed from the mixtures. The resulting filaments are thought to be copolymers of the two types of myosin, for several reasons: (a) their length-frequency distribution is unimodal and differs from that predicted for a simple mixture of two types of myosin filaments; (b) their mean length is intermediate between the mean lengths of the filaments formed separately from the two myosins in the mixture; (c) each of the filaments has structural features characteristic of both of the myosins in the mixture; and (d) their size and shape are determined by the proportion of the two myosins in the mixture.

Myosins from skeletal muscle, (6, 10), smooth muscle (4, 9), and several non-muscle cells (1, 2, 3, 16, 17, 18, 24, 26) spontaneously assemble into bipolar filaments when placed in buffers approximating physiological conditions. All of these "synthetic" myosin filaments have certain features in common, but filaments of each type of myosin have distinctive sizes and shapes. Skeletal muscle myosin filaments are large, smooth muscle myosin filaments are intermediate in size, and most cytoplasmic myosin filaments are very small. These differences in size make it possible to test whether different myosins can copolymerize.

This report describes experiments in which soluble mixtures of skeletal muscle myosin with either human platelet myosin or human smooth muscle myosin were dialyzed to low ionic strength to form filaments. The ultrastructural properties of the resulting filaments suggest that they may be copolymers of the two types of myosin in the mixture.¹

¹ A preliminary account of this work has been presented (19). Independently, Kaminer et al. (11) carried out similar experiments with myosins from rabbit skeletal muscle, chicken skeletal muscle, and chicken gizzard and also conclude that myosin copolymers can form.

A second observation of some interest is that the projections on the surface of myosin filaments appear in two different configurations in negatively stained specimens.

MATERIALS AND METHODS

Myosin Preparations

Myosin from rabbit back and leg muscles was purified by the method of Kielley and Harrington (12). Human platelet myosin and human uterine myosin were purified by the KI-gel filtration method of Pollard et al. (20). All of the myosin preparations were analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate. Densitometry of Coomassie blue-stained gels revealed that the skeletal muscle myosin was contaminated only with a small amount of C protein, accounting for about 1% of the stained protein. The platelet myosin and uterine smooth muscle myosin were both contaminated with 2–5% myosin rod fragment, but otherwise had no single contaminant accounting for more than 1% of the stained protein.

Formation of Synthetic Myosin Filaments

All myosin samples were first dialyzed against 0.5 M KCl, 10 mM imidazole chloride pH 7.0. After their concentrations were measured (5), all samples were diluted to 0.2 mg/ml with the 0.5 M KCl buffer. Filaments were formed from samples of individual myosins and mixtures of skeletal muscle myosin with various proportions of one of the other myosins by dialysis against 0.1 M KCl, 10 mM imidazole chloride pH 7.0 for 2–4 h at 4°C. The half time for KCl diffusion from the narrow (9 mm) dialysis bags used here is 20 min, so that the KCl concentration inside the bags was about 0.11 M after 2 h.

Negative Staining

Filament samples were placed on Formvar- and carbon-coated grids rendered hydrophilic by glow discharge. After about 30 s all but a thin film of sample was removed by contact with filter paper. A drop of 1% aqueous uranyl acetate was applied to the wet grid for about 15 s and then removed by contact with filter paper. The thin film of stain adhering to the hydrophilic surface was then allowed to air dry.

Electron Microscopy

Micrographs were taken at magnifications of 10,000, 20,000 or 35,000 × with a Siemens Elmiskop 101 operating at 100 kV. Magnifications were determined from micrographs of tropomyosin paracrystals taken with each set of negatives. The criteria used in the selection of fields to be photographed were that the filaments be well stained and well separated to allow accurate measurements of their lengths. Every sample

was photographed at 35,000 × so that the presence or absence of small filaments could be determined.

Measurements

After visually inspecting enlarged prints of the micrographs to determine the types of filaments present in a given sample, a few representative prints were chosen for measuring filament lengths. On each of these prints, the length of *all* of the filaments with both ends visible was measured to the nearest millimeter with a ruler. Lower magnification micrographs were used for samples with longer filaments, so that there would not be a systematic bias against long filaments which would be more likely to cross the margin of a micrograph. Magnifications were chosen so that the filaments on the prints were at least 10 mm long and could be measured accurately on the prints. The sample size for each data point varied between 35 and 300 filaments. It was found that duplicate samples of 50 filaments from the same preparation gave virtually identical means and standard deviations. Hence, it is thought that the variation in filament lengths reported below represent the true variation in the size of the filaments, with errors in measurement contributing insignificantly to the variation. The width of the filaments was measured on highly enlarged projections of negatives as described in the accompanying paper (18).

RESULTS

Configurations of Synthetic Skeletal Muscle Myosin Filaments

Synthetic skeletal muscle myosin filaments resemble the thick filaments of skeletal muscle (6, 10, 15; Fig. 1 of this paper). The filaments formed from any given preparation of myosin vary considerably in length (Fig. 2 c). The mean length varies from preparation to preparation (compare Figs. 2, 6, 7 and 8) but generally falls in the range of 0.8–1.5 μm. One preparation formed exceptionally long filaments (Figs. 5 and 7). It is possible that the variation in mean length of the filaments from different myosin preparations was due to differences in the amount of C-protein contamination (14), but this was not investigated systematically.

Even at low magnification (Fig. 1), it is evident that these preparations of skeletal muscle myosin filaments consist of two populations of filaments which have different widths. The plump filaments have a mean width of about 31 nm, while the thinner filaments are about 16 nm wide. Both types of filaments have surface projections, presumed to be the heads of the constituent myosin molecules, but these heads appear to be in different configurations in the plump and thin filaments.

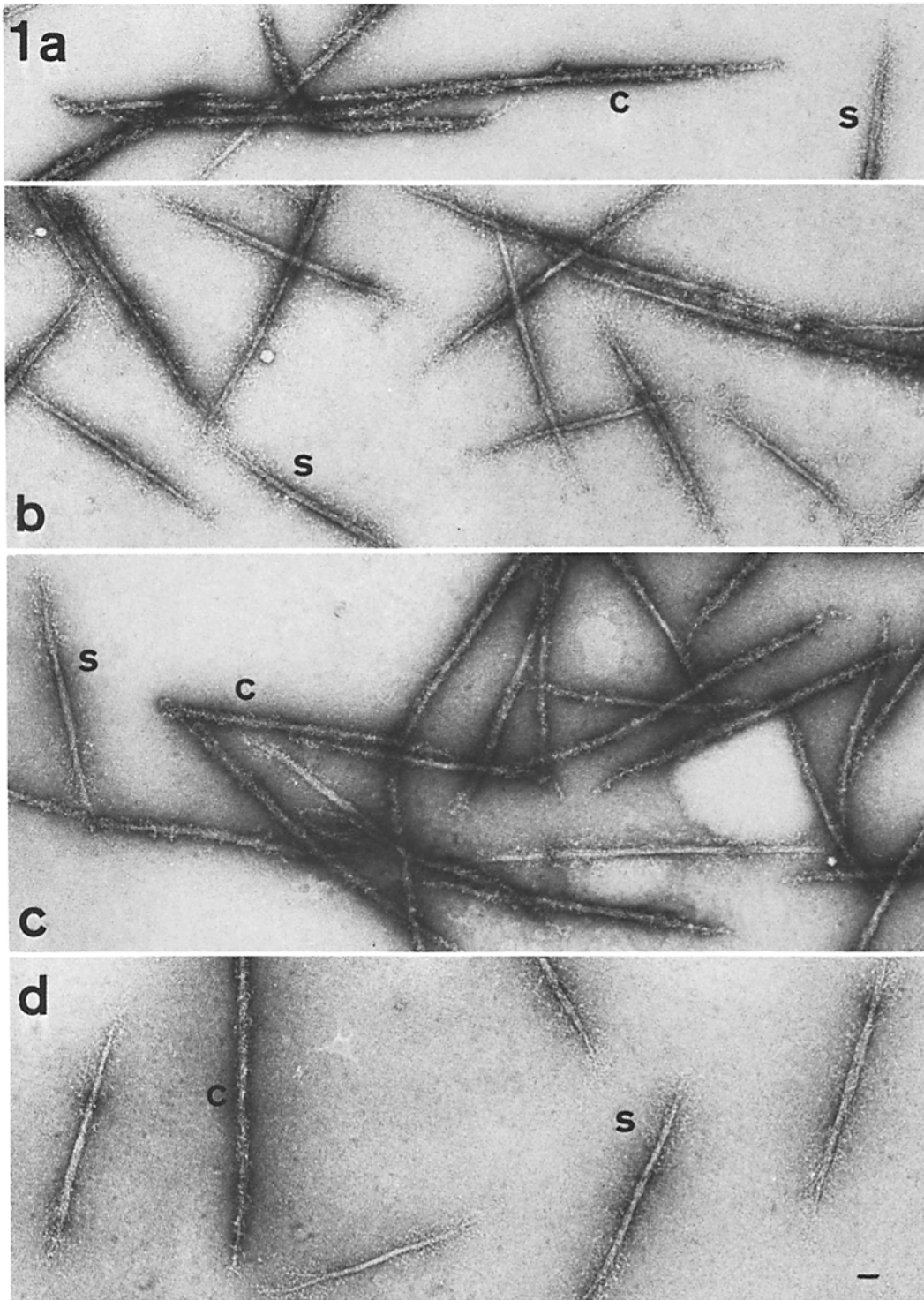
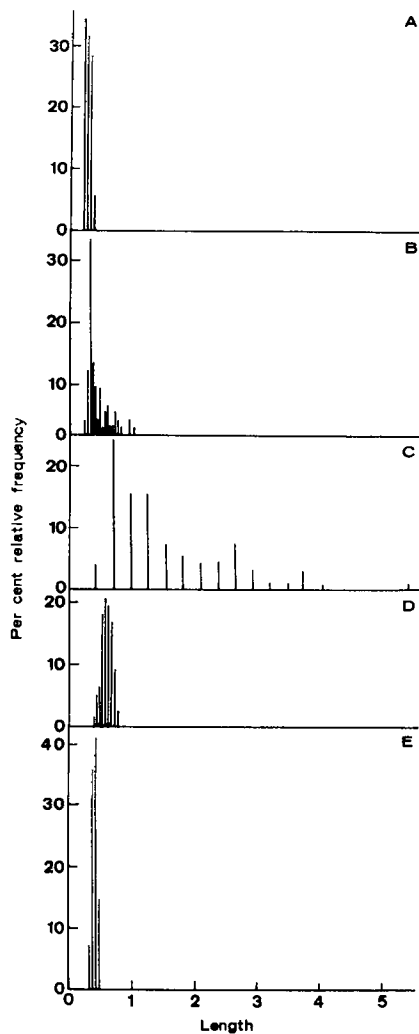


FIGURE 1 Electron micrographs of negatively stained filaments of rabbit skeletal muscle myosin. Filaments were formed by dialysis of purified myosin against 0.1 M KCl, pH 7. The filaments are found in two configurations: either spread (*s*) or compact (*c*). Bar is 0.1 μm . $\times 50,000$.

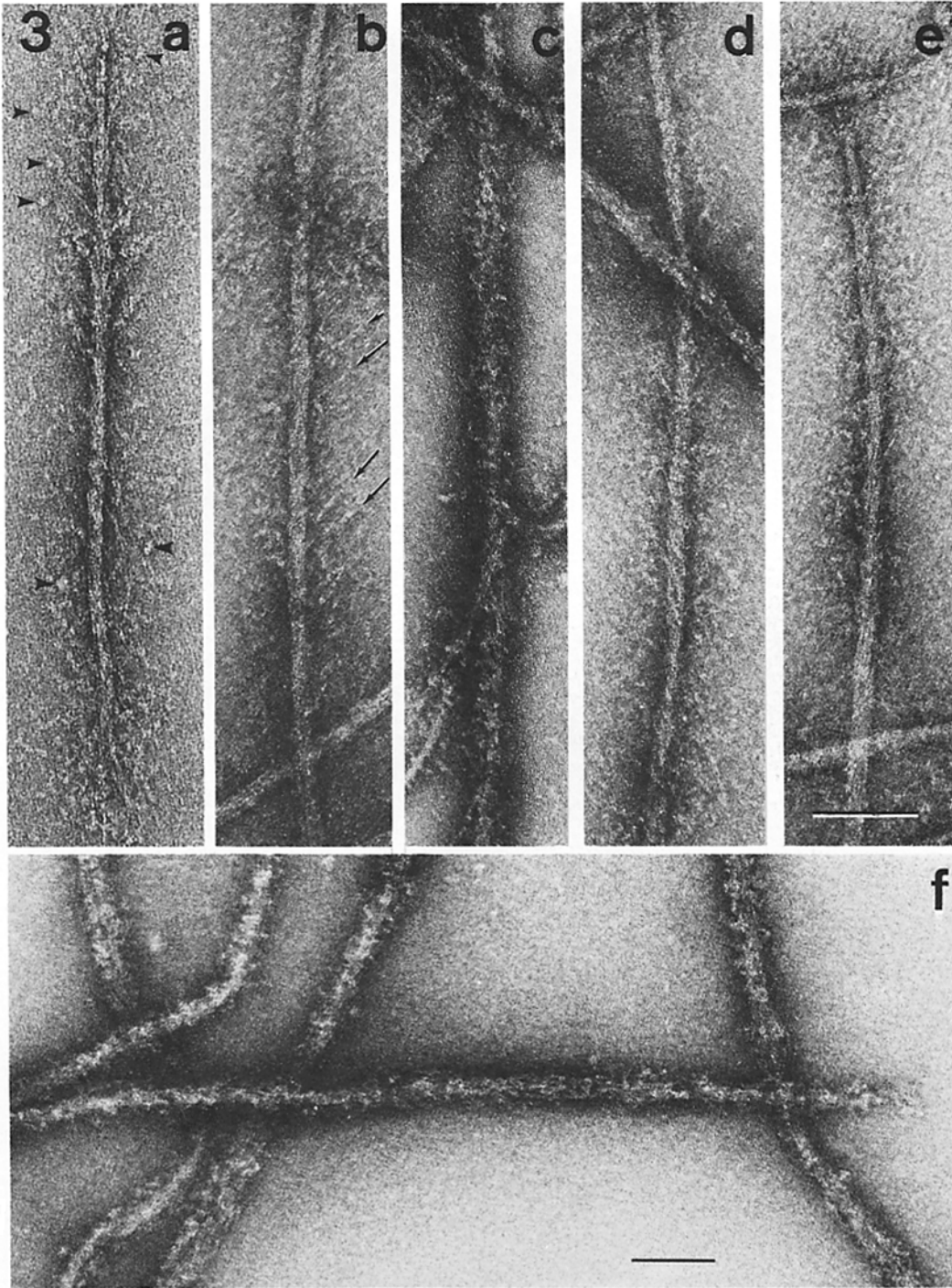


The surface projections on broad (31-nm) filaments lie closely apposed to the backbone of the filament (Figs. 1 and 3). I will refer to this as the compact configuration. Filaments in this configuration occasionally have a smooth central bare zone about 16 nm in diameter, but many of these filaments have surface projections along their entire length. In most cases, inspection of the micrographs reveals little or no order of the surface projections. In a few cases (see Fig. 6 in reference 18 and Fig. 3 of this paper), a faint 15-nm axial repeat has been seen, as expected from X-ray diffraction analysis (8). In fact, most of these negatively stained filaments have a strong 15-nm axial periodicity which is obscured by superimposition and noise, but which is revealed by viewing the micrographs through a diffraction grating.

The thin myosin filaments have a smooth backbone 16 nm wide, which is the only feature of these filaments visible at low magnification and in

FIGURE 2 Length-frequency distributions of various types of myosin filaments. These histograms give the percent relative frequency of various lengths of filaments formed in 0.1 M KCl at pH 7. (A) Purified human platelet myosin. Mean = 297 nm, SD = 36 nm, $n = 35$. (B) 1:1 mixture of platelet myosin with skeletal muscle myosin. Mean = 449 nm, SD = 176, $n = 75$. (C) Purified rabbit skeletal muscle myosin. Mean = 1,530 nm, SD = 1,050, $n = 96$. (D) 1:1 mixture of uterine smooth muscle myosin with skeletal muscle myosin. Mean = 595 nm, SD = 82 nm, $n = 77$. (E) Purified human uterine smooth muscle myosin. Mean = 418 nm, SD = 38 nm, $n = 96$. Length is given in μm .

FIGURE 3 Electron micrographs of negatively stained filaments of rabbit skeletal muscle myosin. The filament in (a) was formed by dilution by myosin in 0.5 M KCl with four parts of water, while the other filaments were formed by dialysis of a soluble myosin solution in 0.5 M KCl against 0.1 M KCl at pH 7. (a) Spread configuration. Except for a central bare zone, the backbone of the filament is surrounded by fine filamentous and globular material. The globular material, marked with arrowheads, may be the heads of some of the myosin molecules. A faint herringbone pattern is visible in the backbone of the upper half of the filament with the point of the pattern facing the central bare zone. (b) Spread configuration. The fine filamentous projections (arrows) extending from the surface of the filament are presumably the subfragment 2 portion of the myosin molecules. (c) Compact configuration. This filament is broader than the spread filaments and has no halo of filaments extending from its surface. Instead, this filament covered with many globular projections disposed directly on its surface. (d) Spread configuration. The staining of the upper section of this filament reveals a ropelike substructure in the light meromyosin backbone with a repeat of about 22 nm. (e) Spread configuration. The surface projections are largely obscured by noise in the surrounding stain. A herringbone pattern, pointing downward, is evident in the backbone. (f) Compact configuration. Like the filament in (c), all of these filaments have projections directly on their surfaces. Bars are 0.1 μm . (a-e) $\times 150,000$, (f) $\times 120,000$.



poorly stained specimens (Fig. 3 *e*), even at high magnification. The smooth backbone is surrounded by a faint halo of very fine filamentous and globular material (Figs. 1 and 3 *a, b, d, and e*), which may correspond to the subfragment 2 and subfragment 1 (head) (13) parts of the myosin molecule, respectively. This fuzzy material extends out up to 60 nm from the backbone of these spread filaments. Central bare zones are difficult to identify. This may be due to the diffuse nature of the projecting material and the fact that the 16-nm wide backbone is the same width as typical bare zones. In spite of the frequent absence of central bare zones, two observations suggest that these spread filaments possess the usual bipolar symmetry: (*a*) there is a tendency for the fuzzy projections to angle away from the center of the filament (Fig. 3 *a and b*); and (*b*) a weak herringbone pattern is seen in the backbone of a few of these filaments (Fig. 3 *a, e*).

All of the projections on each filament tend to be in the same configuration, although a few intermediate filaments can be found.² Both types of filaments were found in all of the preparations of myosin examined. Filaments in the two configurations typically were found mixed together on the grids, where they could be seen lying on top of one another in the same puddle of uranyl acetate. Conditions favoring one or the other configuration have not been investigated.

Synthetic Filaments of Platelet Myosin and Smooth Muscle Myosin

The synthetic filaments formed from purified platelet myosin and smooth muscle myosin are similar, though not identical, to each other (18, and Fig. 4 of this paper) and distinctly different from the skeletal muscle myosin filaments described above. The platelet myosin filaments are described in detail in the accompanying paper (18). In 0.1 M KCl at pH 7, both platelet myosin and smooth muscle myosin form filaments of uniform length (Fig. 2). Under these conditions, the smooth muscle myosin filaments are about one-third longer than the platelet myosin filaments. In both cases, these short bipolar filaments are dominated

² This might mean that the transition from one configuration to the other is a cooperative process with all of the heads on a given filament changing together. The high density of heads and subfragment 2 on the surface of the filaments makes a cooperative transition an attractive approach to avoiding steric interference with each other.

by central bare zones about 160 nm long. At both ends there are projections, which, in the case of the platelet myosin, have been shown to be the heads of the myosin molecules (2, 18). Characteristically, these projections are aggregated into globular clusters, although they have been observed in more spread configurations (18). Both platelet myosin and smooth muscle myosin filaments tend to aggregate end to end, even in the complete absence of actin.

Under the specific conditions chosen for these experiments, platelet and smooth muscle myosin filaments are very small. Under other conditions, these myosins can form larger aggregates including paracrystalline tactoids (19, 22).

Filaments from Myosin Mixtures

To test whether two types of myosin can interact to form a filament, synthetic filaments were formed from mixtures of two types of myosin, which individually would form filaments of different sizes. Mixtures of skeletal muscle myosin with either platelet myosin or smooth muscle myosin dissolved in 0.5 M KCl were dialyzed against 0.1 M KCl at pH 7 and the resulting filaments examined by electron microscopy.

It was anticipated that mixtures of these myosins would form mixtures of long and short filaments, if there were no interaction between the different myosin molecules. Instead, a single population of filaments with intermediate lengths was found in both types of mixtures examined (Figs. 2 and 4). The filaments formed from the mixtures have several features which are consistent with their being copolymers of the two types of myosin molecules. The mixed filaments have a unimodal length-frequency distribution, not the bimodal length-frequency distribution expected for a mixture of long skeletal muscle myosin filaments with short platelet or smooth muscle myosin filaments. The mean length of the mixed filaments is intermediate between the lengths of filaments formed individually by the myosins in the mixture. Each of the mixed filaments has structural features attributable to both of the myosins in the mixture. For example, the filaments prepared from 1:1 mixtures all have prominent bare zones like smooth muscle myosin or platelet myosin filaments. On the other hand, all of these filaments have diameters greater than platelet myosin filaments and have long zones of surface projections at both ends like skeletal muscle myosin filaments. Incidentally, the projec-

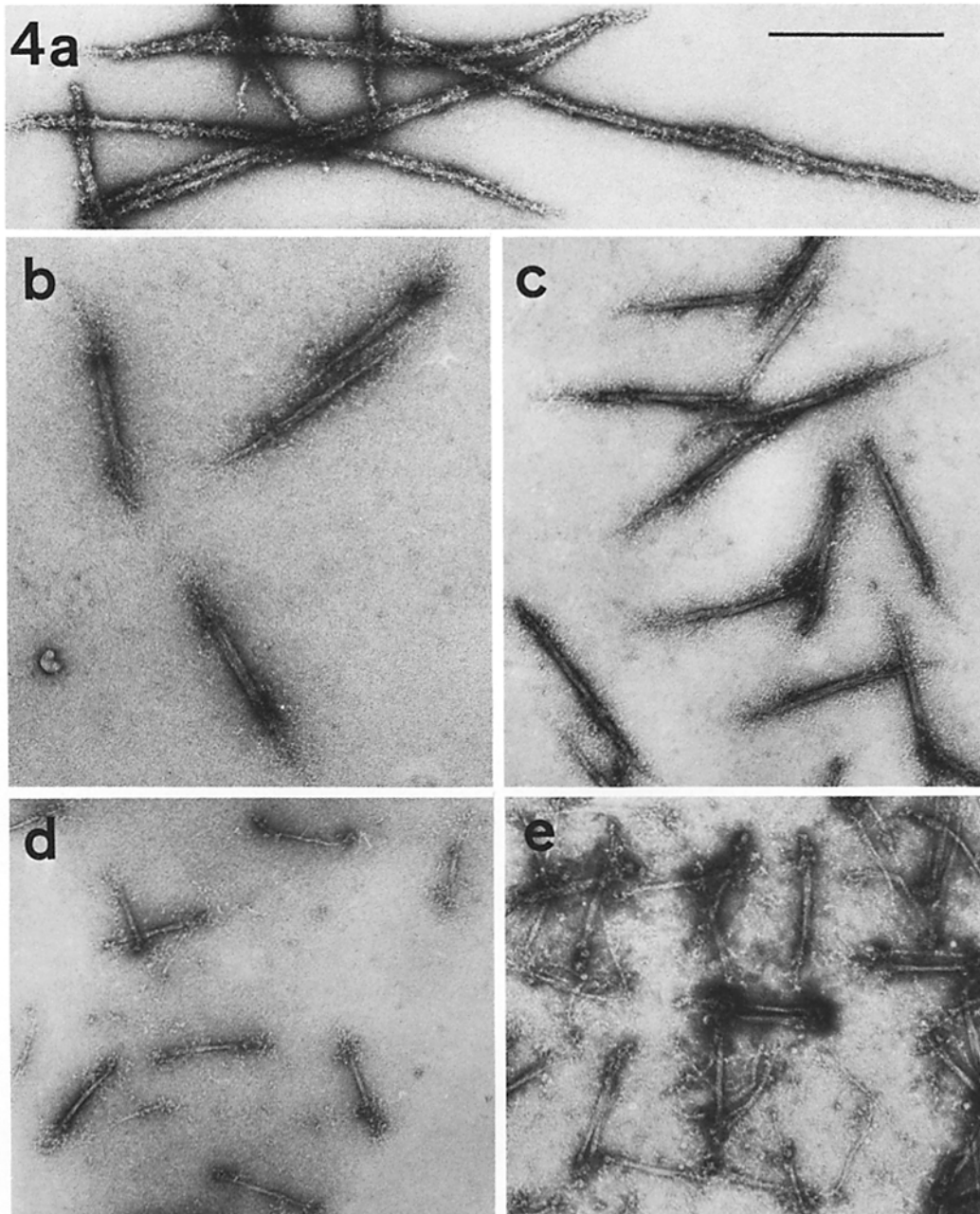


FIGURE 4 Electron micrographs of negatively stained myosin filaments formed by dialysis against 0.1 M KCl at pH 7. (a) Purified skeletal muscle myosin (0.2 mg/ml). (b) Equal concentrations (0.1 mg/ml) of platelet myosin and skeletal muscle myosin. (c) Equal concentrations (0.1 mg/ml) of uterine smooth muscle myosin and skeletal muscle myosin. (d) Purified platelet myosin (0.2 mg/ml). (e) Purified uterine smooth muscle myosin (0.2 mg/ml). Bar is 0.5 μm . $\times 50,000$.

tions on mixed filaments are found in both of the configurations described for skeletal muscle myosin filaments above. The mixed myosin filaments in Fig. 4 are excellent examples of the spread configuration.

The size and shape of the mixed filaments are determined by the proportions of the two myosins in the mixture used to make them (Figs. 5–8). Large proportions of skeletal muscle myosin make the filaments long and variable in length while

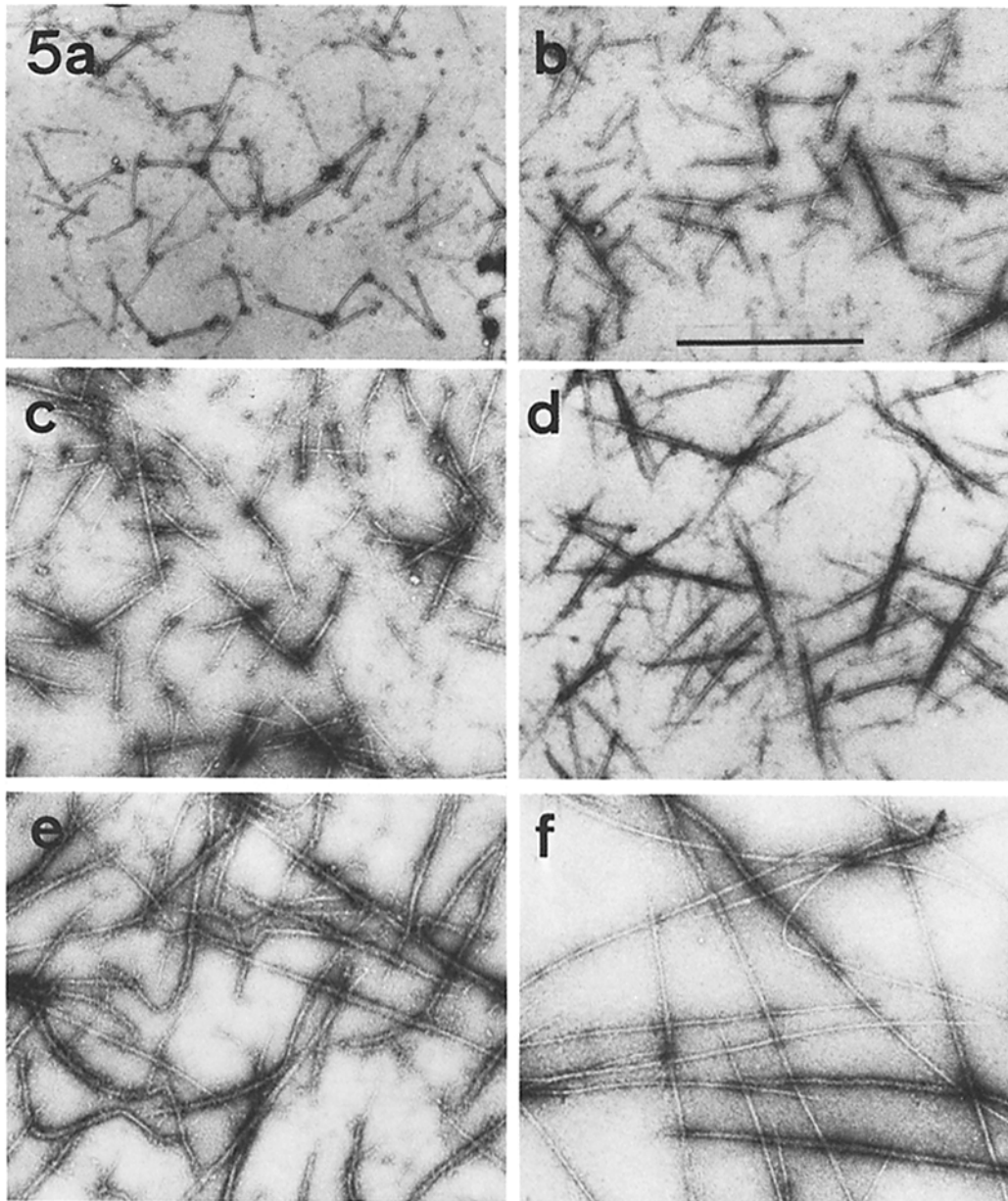


FIGURE 5 Electron micrographs of filaments formed from various proportions of platelet myosin and skeletal muscle myosin by dialysis against 0.1 M KCl at pH 7. Total concentration of myosin 0.2 mg/ml in all cases. (a) 100% platelet myosin. (b) 80% platelet myosin: 20% muscle myosin. (c) 60% platelet myosin: 40% muscle myosin. (d) 40% platelet myosin: 60% muscle myosin. (e) 20% platelet myosin: 80% muscle myosin. (f) 100% muscle myosin. Note the exceptional length of the filaments formed from this preparation of skeletal muscle myosin. Bar is 1 μ m. \times 25,000.

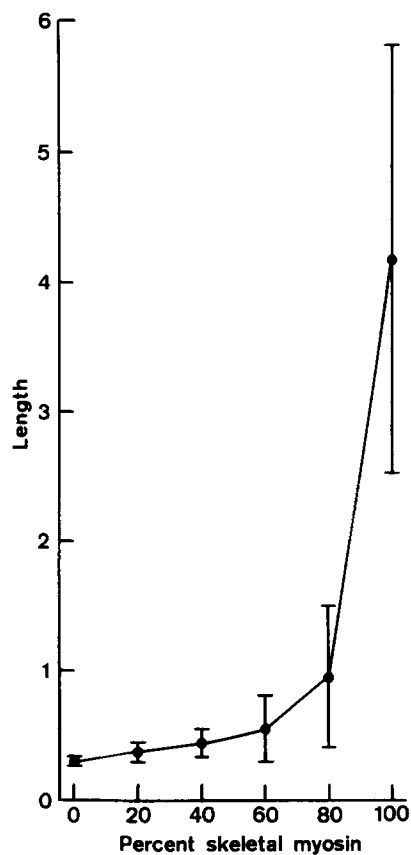


FIGURE 6 Length of filaments formed from various proportions of platelet myosin and rabbit skeletal muscle myosin. Mean length in micrometers is plotted against percent skeletal muscle myosin. The vertical bars represent two standard deviations. This is the same experiment shown in Fig. 5.

filaments with large proportions of platelet myosin or smooth muscle myosin have prominent bare zones and relatively uniform short lengths.

To rule out the possibility that the length of these filaments is influenced by the *concentration* of myosins, rather than their *proportions*, synthetic filaments were formed from several concentrations of skeletal muscle myosin and platelet myosin (Fig. 8). As illustrated, the length of the pure myosin filaments is not altered by a four-fold variation in myosin concentration. Mixtures of the two types of myosin gave the usual unimodal distribution of intermediate size filaments.

DISCUSSION

Myosin Filament Conformations

The electron micrographs presented above show negatively stained skeletal muscle myosin fila-

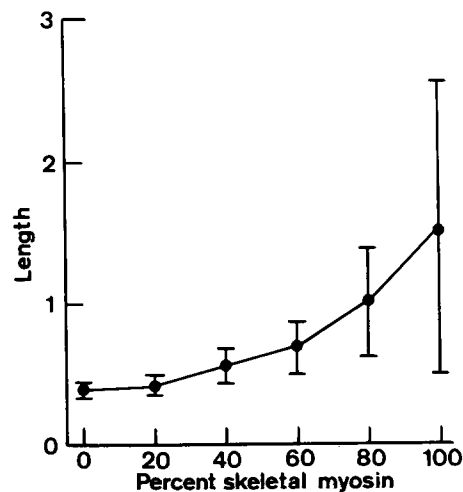


FIGURE 7 Length of filaments formed from various proportions of uterine smooth muscle myosin and skeletal muscle myosin. Mean length in micrometers is plotted against percent skeletal muscle myosin. The vertical bars represent two standard deviations.

ments in two distinctive configurations. Some of the filaments are about 16 nm wide and surrounded by widely spread fine projections. Other filaments are about 31 nm wide and have globular projections directly on their surfaces. If the surface projections are the heads of the myosin molecules (which seems likely), there is a simple, attractive interpretation of the micrographs, which is developed below (Fig. 9).

Presumably, both types of myosin filaments are composed of the same number and type of myosin molecules. In addition, it is thought that all of the filaments have identical cores composed of a specific arrangement of light meromyosin molecules (6, 23). The business end of each myosin molecule, the two globular heads, is joined to the light meromyosin by the subfragment 2 part of the myosin tail. From studies of the isolated fragments, it is believed that myosin heads and subfragment 2 are soluble under physiological conditions and have little tendency to associate with each other or with light meromyosin (13). The region of the myosin tail joining the light meromyosin and subfragment 2 is particularly susceptible to proteolytic digestion and therefore is thought by some to be a flexible hinge in the tail (7, 13). Models of myosin filaments are generally drawn with subfragment 2 lying parallel with the filament backbone and the myosin heads arranged in an orderly helix directly on the surface of the filament. According to X-ray diffraction analysis

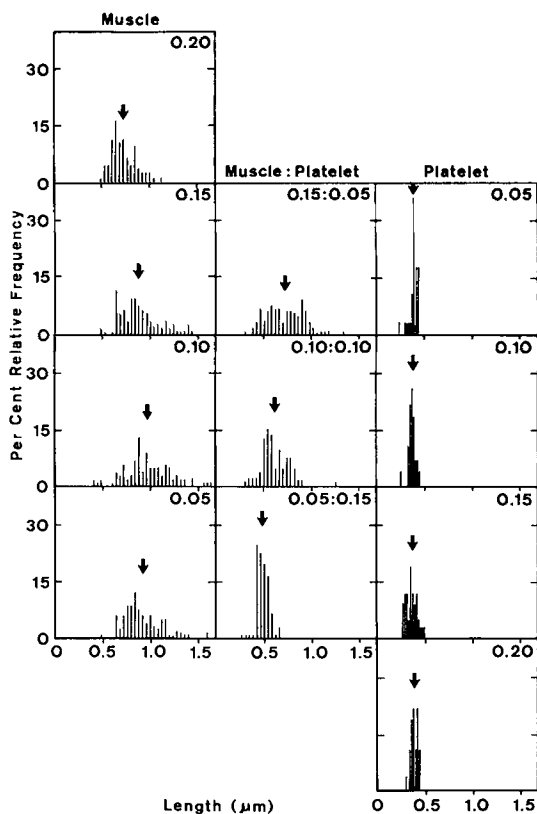


FIGURE 8 Length-frequency distributions of myosin filaments. The length-frequency distributions in the left column are of filaments formed from four different concentrations of skeletal muscle myosin, given in mg/ml in the upper right-hand corner of each histogram. The length-frequency distributions in the right column are of filaments formed from four different concentrations of platelet myosin, given in mg/ml in the upper right-hand corner of each histogram. The length-frequency distributions in the middle column are of filaments formed from mixtures of muscle myosin and platelet myosin in three different proportions, indicated in mg/ml by the ratios in the upper right-hand corner of each histogram. The mean of each distribution is marked with a vertical arrow.

(8), this is the conformation of the filament in resting muscle. I believe that the compact (broad) myosin filaments observed in the negatively stained preparations are in this resting configuration. The globular projections on the surface of the negatively stained filaments do not appear to be as well positioned as the models lead us to believe, but there is probably much more order than is obvious from inspection. Occasionally, the 15-nm axial periodicity of the heads can be seen directly. With

the aid of a diffraction grating, this periodicity is evident on most of the compact filaments. Presumably, a combination of superimposition, noise, and short range disorder obscure this periodicity in the micrographs. I find it impressive that this compact configuration can be preserved and that any order of the heads is detectable, because the solubility of the heads and subfragment 2 suggests that the heads do not have any particular attraction for the light meromyosin backbone and should be free to swing out from the filament backbone, in a more spread configuration.

The narrow myosin filaments appear to have the myosin heads spread away from the filament backbone and tethered to it by the subfragment 2 part of the myosin tail. This exposes the light meromyosin core of these filaments. The core appears to have a fibrous substructure typical of light meromyosin aggregates (13). The faint herringbone pattern seen occasionally in this region may indicate that the light meromyosin molecules may be tilted with respect to the axis of the filament. The fine filamentous material radiating from the backbone is presumably subfragment 2. These minute filaments are poorly resolved in these negatively stained specimens, but they are roughly 2 nm wide and up to 50 nm long, like

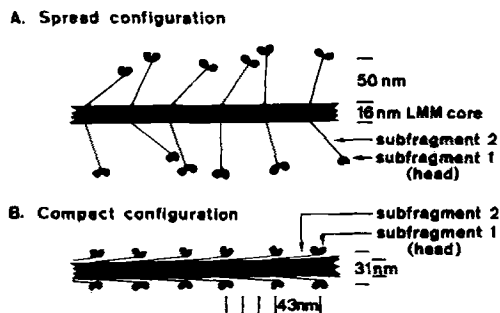


FIGURE 9 A diagrammatic interpretation of the structures of myosin filaments in the spread and compact configurations. The proportions of the myosin molecules and myosin filaments are drawn to scale, but only a few of the myosin molecules are shown in each segment to simplify the drawing. Two myosin molecules are shown in each 43-nm period, while there are actually three to four myosins in each 14.3-nm period, marked with vertical lines at the bottom. Therefore the reader will have to imagine four to six times as many myosin molecules to have a picture of reality. The compact filaments are drawn with the subfragment 2 fibers tilted slightly away from the backbone, for clarity, while in reality they may be disposed directly on the light meromyosin (LMM) backbone.

subfragment 2. The radiating filaments angle abruptly from the backbone, providing direct evidence for a flexible hinge between the light meromyosin and the subfragment 2, as previously postulated from X-ray diffraction analysis (7). The myosin heads do not appear to be well preserved in these negatively stained preparations, but are probably represented by some globular material mixed with the radiating filaments. This spread configuration is that expected from the solubility properties of the various parts of the myosin molecule and may represent the state of the myosin filament in actively contracting muscle.

The dimensions of these filaments are consistent with this interpretation. The ratio of the cross-sectional areas of the two types of filaments, $760 \text{ nm}^2:200 \text{ nm}^2 = 3.8$, is roughly the same as the ratio of the masses of myosin and light meromyosin, $460,000:130,000 = 3.5$. From the staining pattern of these filaments, it appears that the light meromyosin in the core of filaments is packed more tightly than the heads on the surface of the compact filaments, so that the agreement between the cross-sectional areas of the two types of filaments and the masses of the molecules thought to comprise them may be even better if this factor could be taken into account.

The reason why the filaments have two configurations is unknown. At one extreme, it is possible that the spread configuration is simply a drying and compression artifact arising from the negative staining procedure. On the other hand, it is possible that the myosin filaments in solution assume one or the other configuration. It may be relevant that Moós (14) found that myosin filaments had different diameters depending on the presence or absence of C protein, although Niederman³ has observed negatively stained native thick filaments in both configurations. Regardless of the reality of these configurations in solution, their appearance in negatively stained preparations illustrates some new features of myosin filaments and provides some direct evidence for cross-bridge flexibility.

Mixed Filaments

This morphological study provides some evidence that two kinds of myosin can copolymerize. Confirmatory evidence using other techniques is needed, because the finding is somewhat unex-

pected. It was not anticipated, because it is known that the myosins used in these experiments form filaments with different structures. For example, platelet myosin filaments are thin and appear to have two myosin molecules in each 14.5-nm axial period (17, 18) while skeletal muscle myosin filaments are thicker and have three (21, 23, 25) or four (15) myosin molecules in each 14.5-nm axial period. These gross differences in structure might arise from each type of myosin using unique intermolecular bonds in filament assembly. If these bonds were specific for each myosin type, copolymerization would not be expected.

The evidence for copolymerization argues that all of these myosins use some common intermolecular bonds for filament assembly. This idea fits well with the hypothesis of Squire (23) that the myosin molecules in the cores of different types of myosin filaments may be packed in more or less equivalent positions. Each type of myosin may also have some unique intermolecular binding sites which determine the size, shape and myosin packing in the filaments formed from each specific myosin. Because the hybrid filaments have properties intermediate between those of the parents, the function of the unique binding sites of both of the constituent myosins must be expressed in the hybrids. The sites in platelet myosin and smooth muscle myosin which limit the length of those filaments appear to have a particularly strong influence on the properties of the hybrids with skeletal muscle myosin.

It is not known how the two types of myosin molecules are distributed in the hybrid filaments. Various packing schemes have been suggested to me, including: one myosin forming the filament core with the second myosin on the surface; one myosin nucleating assembly by forming the bare zone with the second myosin contributing to growth; and one myosin forming the bulk of the filament with the second myosin blocking the ends. Rather than any of these, I favor the idea that both types of myosin are found throughout the mixed filaments, although the available evidence for this is not strong. The evidence is that all of the filaments from each myosin mixture are similar to each other and all have features attributable to both of the myosins in the mixture. In addition, a preliminary experiment suggests that preformed platelet myosin filaments will not nucleate the assembly of longer filaments upon the addition of skeletal muscle myosin monomers. Experiments with labeled antibodies to each of the myosins in

³ Niederman, R. 1974. Unpublished experiments.

the mixed filaments should clarify this issue in the future.

Regardless of the details of the molecular packing in the mixed filaments, these results raise the possibility that copolymerization of more than one myosin isozyme could conceivably contribute to determining the uniform length of the thick filaments in striated muscles.

Apparently not all myosins can copolymerize, because Nachmias (16) has reported experiments showing that mixtures of *Physarum* myosin and skeletal muscle myosin form separate filaments.

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