

# Substructure and Accessory Proteins in Scallop Myosin Filaments

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**Abstract.** Native myosin filaments from scallop striated muscle fray into subfilaments of  $\sim 100\text{-\AA}$  diameter when exposed to solutions of low ionic strength. The number of subfilaments appears to be five to seven (close to the sevenfold rotational symmetry of the native filament), and the subfilaments probably coil around one another. Synthetic filaments assembled from purified scallop myosin at roughly physiological ionic strength have diameters similar to those of native filaments, but are much longer. They too can be frayed into subfilaments at low ionic strength. Synthetic filaments share what may be an important regulatory property with native filaments: an order-disorder transition in the helical arrangement of myosin cross-bridges that is induced on activation by calcium, removal of nucleotide, or modification of a

myosin head sulfhydryl.

Some native filaments from scallop striated muscle carry short "end filaments" protruding from their tips, comparable to the structures associated with vertebrate striated muscle myosin filaments. Gel electrophoresis of scallop muscle homogenates reveals the presence of high molecular weight proteins that may include the invertebrate counterpart of titin, a component of the vertebrate end filament.

Although the myosin molecule itself may contain much of the information required to direct its assembly, other factors acting *in vivo*, including interactions with accessory proteins, probably contribute to the assembly of a precisely defined thick filament during myofibrillogenesis.

**A**NALYZING how myosin molecules are assembled into filaments is important for understanding the way the activity of individual molecules is modified by intermolecular interactions. In some motile tissues, and perhaps also in certain smooth muscles, assembly and disassembly of filaments appear to be one part of the mechanism of regulating myosin's activity (see for example Atkinson and Korn, 1987; Gillis et al., 1988). Even in striated muscles where native filaments are comparatively stable (Saad et al., 1986), cooperative interactions in the assemblies may add a new level of sensitivity to regulatory switching (Chantler et al., 1981).

A basic mode of myosin assembly was established by studies on purified myosin from vertebrate skeletal myosin, which showed that assembly is initiated by antiparallel packing of myosin rods in a central "bare zone" and that the filament then elongates by parallel packing in each end of the bipolar filament (Huxley, 1963). Kinetic and thermodynamic studies of these processes (Josephs and Harrington, 1968; Reisler et al., 1980; Davis, 1985) confirm the essential difference between the antiparallel and parallel interactions, and have defined the dependence of these processes on ionic conditions and shown that a dimer is the probable unit of assembly. These studies have also established that filament length *in vitro* is roughly determined kinetically, but that the

very precise lengths of native filaments must be specified by additional factors (Davis, 1988).

An important feature of myosin filament structure is that an intermediate level of organization occurs between dimer and full-sized filament: the subfilament (Davis, 1985). Under certain ionic conditions, such structures can be seen by electron microscopy in assemblies of purified vertebrate skeletal myosin (Pinset-Härström, 1985). All native thick filaments from vertebrate skeletal muscles examined so far have a similar structure (i.e., in length, diameter, and screw and rotational symmetries of the cross-bridge surface lattice); and in a few cases, it has been possible to demonstrate directly that they are composed of three subfilaments (Maw and Rowe, 1980; Trinick, 1981).

Invertebrate myosins usually assemble into filaments that are much longer and thicker than those in vertebrate tissues, and which also contain one or more core proteins, often in substantial amounts (Cohen et al., 1971; Levine et al., 1976; Epstein et al., 1985). Very little has been done to characterize the assembly kinetics of these myosins, but some models for filament assembly (e.g., Wray, 1979; Squire, 1981) suggest that, at least in the striated muscles, these myosins too should assemble via a subfilament intermediate.

Here we demonstrate by electron microscopy that native thick filaments from scallop striated muscle (which contain

only  $\sim 10\%$  by weight paramyosin) are indeed composed of subfilaments, and that synthetic filaments assembled from purified scallop myosin also contain subfilaments. We show that synthetic filaments share other important functional properties (ligand-dependent order and disorder of the surface cross-bridge array) with native scallop filaments (cf. Frado and Craig, 1989). We also show that accessory proteins in the form of "end filaments," possibly analogous to vertebrate titin, are present at the ends of some native scallop filaments.

## Materials and Methods

### Fraying

Native filaments were obtained by homogenizing saponin-skinned striated

muscle bundles from the sea scallop, *Placopecten magellanicus*, in relaxing solution (100 mM NaAc, 3 mM MgAc<sub>2</sub>, 2 mM Na<sub>2</sub>ATP, 1 mM EGTA, 2 mM imidazole, 3 mM NaN<sub>3</sub>, pH 7.0). They were frayed either on a carbon-coated EM grid, or in solution, with low ionic strength relaxing buffers (e.g., 3 mM MgAc<sub>2</sub>, 2 mM Na<sub>2</sub>ATP, 1 mM EGTA, 2 mM imidazole, 3 mM NaN<sub>3</sub>, pH >7.5; ionic strength = 18 mM). Synthetic filaments (see below) also frayed readily in these low ionic strength buffers.

### Synthetic Filaments

Scallop myosin (purified from the striated muscle of *Aequipecten irradians*, a gift of A. G. Szent-Györgyi, Department of Biology, Brandeis University) dissolved in high salt was dialyzed against acetate-based relaxing solution (200 mM NaAc, 3 mM MgAc<sub>2</sub>, 2 mM Na<sub>2</sub>ATP, 1 mM EGTA, 1 mM DTT, 2 mM imidazole, 3 mM NaN<sub>3</sub>, pH 7.1). Filaments were activated by washing on an EM grid with a pCa 5.0 solution (relaxing solution plus 1 mM CaAc<sub>2</sub>). Rigor filaments were obtained by extensive washing on the grid with rigor buffer (relaxing solution minus ATP) and waiting 5–10 min (on ice) before staining.

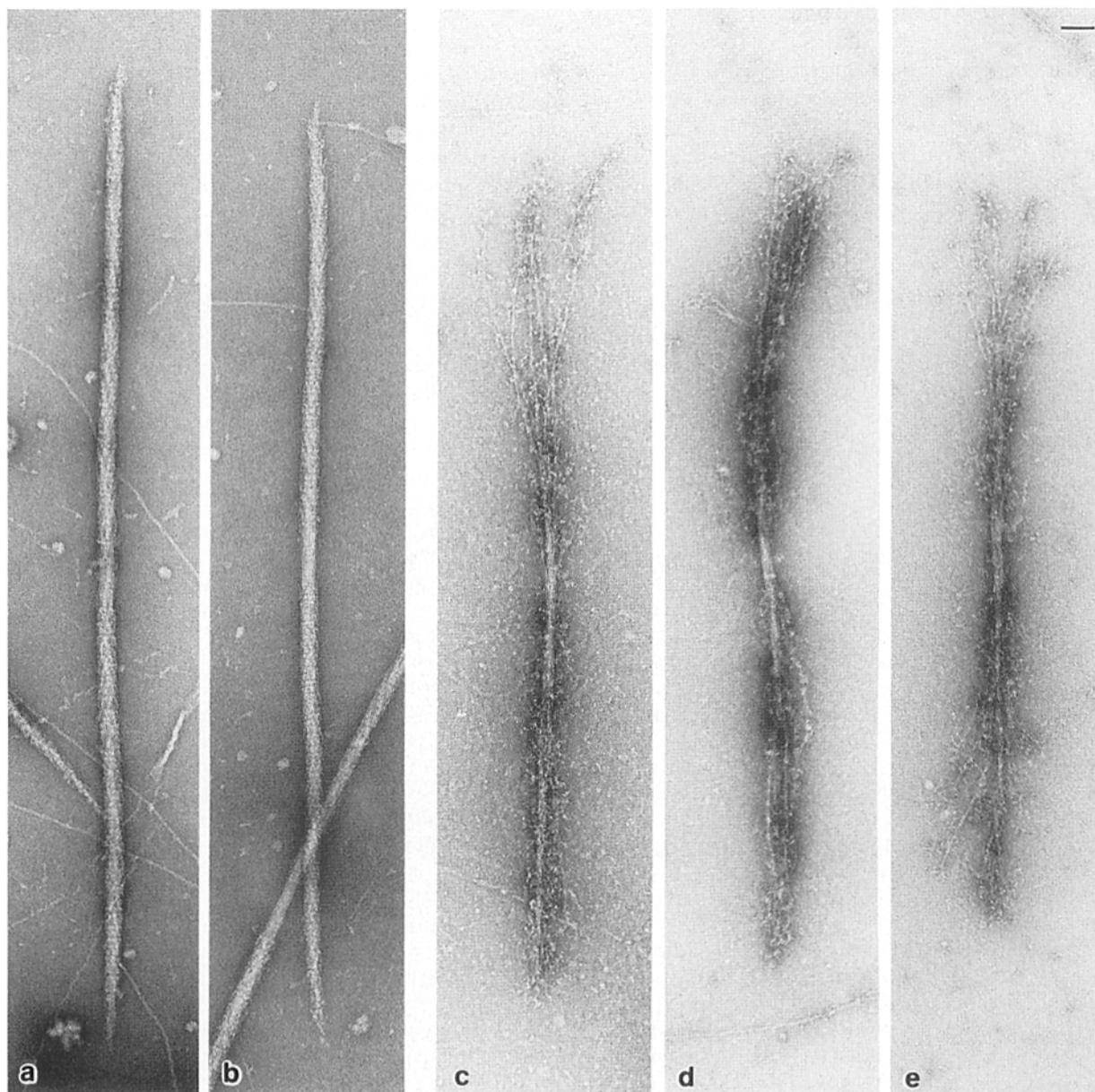


Figure 1. Native myosin filaments isolated from the striated muscles of the scallops (a) *Aequipecten irradians* and (b) *Placopecten magellanicus*, maintained in relaxing solution until negatively stained. (c–e) *Placopecten* filaments frayed into subfilaments by rinsing with low ionic strength relaxing solution at pH 7.7. Bar, 80 nm.

Sulfhydryl modification (performed by M. Titus, Department of Biology, Brandeis University) was achieved by incubating myosin in low salt (40 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 5 mM Na phosphate, 3 mM NaN<sub>3</sub>, pH 7.0) overnight with 5-[2-(2-iodoacetylamino)ethylamino]-1-naphthalene sulfonic acid (IAEDANS)<sup>1</sup> under argon, quenching with a 50-fold molar excess of DTT, and rinsing with low salt solution. For the preparation used for microscopy, the extent of sulfhydryl modification was 0.8 moles/mole. The calcium sensitivity of the Mg-ATPase, both in the absence and presence of actin, was reduced to 35%, primarily by a three- to fourfold increase in the activity in the absence of calcium. The modified myosin was dissolved in high salt and dialyzed against relaxing solution.

### Electron Microscopy

The filaments were visualized for electron microscopy by negative staining with 1% uranyl acetate aqueous solution. For rotary shadowing, the filament suspension was placed on a carbon-coated, glow-discharged glass coverslip. The filaments, after washing with relaxing or fraying solution, were fixed with 1% uranyl acetate, rinsed extensively with 10% glycerol, and excess solution was blotted to a thin film. The samples were visualized by rotary shadowing with platinum at an angle of 15° in an Edwards High Vacuum, Inc. (Grand Island, NY) evaporator. Replicas were floated off in fluoric acid, rinsed extensively with distilled H<sub>2</sub>O, and applied on 400-mesh copper grids. Electron micrographs were recorded in a Philips Electronic Instruments, Inc. (Mahwah, NJ) EM301 or EM420 electron microscope, operated at 80 kV for negatively stained and 60 kV for rotary-shadowed samples. The nominal magnification was 25,000 for the filaments and 51,000 for the end filaments.

### Gels and Blots

Freshly homogenized scallop striated and smooth muscles were dissolved in SDS sample buffer, boiled for 3 min, and run on SDS-polyacrylamide gels (3–13% gradient) in Tris-Bicine running buffer (Trinick et al., 1984). Western blots (not shown) were probed with two monoclonal antibodies to rabbit titin, gifts of J. Trinick and J. Wardale (Muscle Biology Department, AFRC Institute of Food Research, Bristol, England).

1. *Abbreviation used in this paper:* IAEDANS, 5-[2-(2-iodoacetylamino)ethylamino]-1-naphthalene sulfonic acid.

## Results

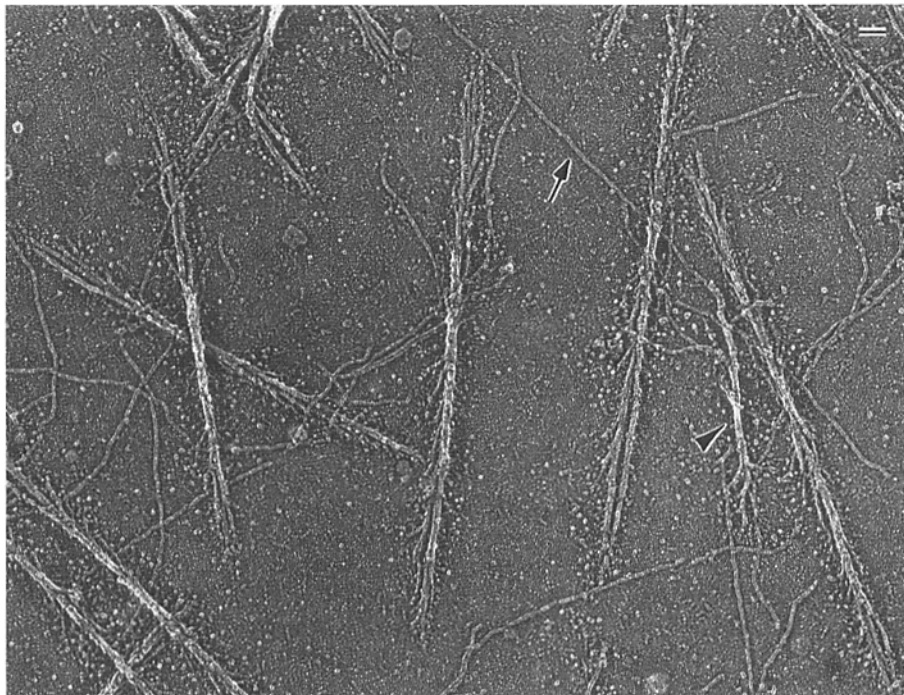
### Substructure of Native Filaments

Native myosin filaments from either *Placopecten* or *Aequipecten* striated muscle are ~2 μm long and 300–400 Å in diameter. In the relaxed state they are characterized by an ordered array of myosin cross-bridges (Fig. 1; see also Vibert and Craig, 1983, 1985). When suspended in relaxing solutions of low ionic strength (<20 mM), they fray into subfilaments (Fig. 1). The amount of fraying (i.e., number of filaments frayed and the length of filament frayed) increases when the pH is elevated to ≥7.5 at low ionic strength, but high pH at normal ionic strength does not induce fraying. Filaments can be frayed either on an electron microscope grid by brief washing, or in solution by diluting a concentrated solution of filaments into a large volume of low ionic strength buffer.

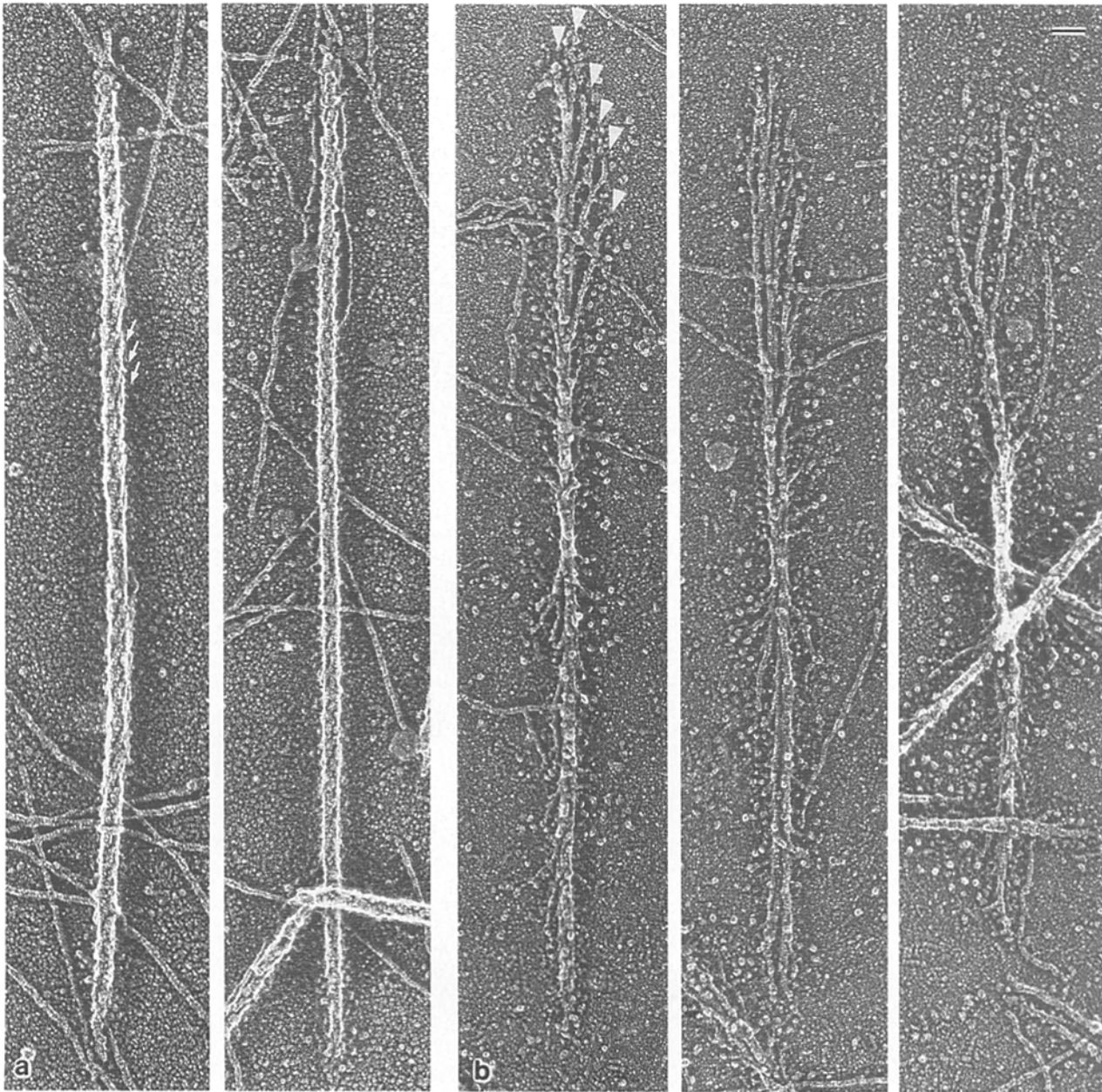
Fraying begins at the filament tips and proceeds toward the bare zone (Fig. 2). The number of subfilaments is not certain, but it appears to be about five to seven. In negative stain or rotary shadow, the subfilaments are of comparable width to native thin filaments (i.e., 80–100 Å) (Figs. 1 and 2). All subfilaments show myosin heads protruding singly or in clusters from their surfaces (Fig. 1, *c–e*), and there is no indication of the presence of paramyosin.

The three-dimensional arrangement of the subfilaments in the native filaments cannot be readily inferred from the images of frayed filaments. In rotary-shadowed filaments (Figs. 2 and 3 *b*), the subfilaments often appear to emerge preferentially on the right-hand side of each half-filament. This appearance suggests that the subfilaments coil around each other, although it is not clear whether the asymmetry about the filament axis reflects left- or right-handed coiling.

Fraying appears to destabilize the subfilament structure.



**Figure 2.** Field of rotary-shadowed myosin filaments from *Placopecten* frayed by rinsing with low ionic strength relaxing solution at pH 7.7. All filaments fray into subfilaments comparable in width to the thin filaments (*arrow*). A filament in which the subfilaments are for the most part depolymerized is indicated (*arrowhead*). Image shown in reverse contrast. Bar, 100 nm.



**Figure 3.** Rotary-shadowed myosin filaments from *Placopecten*: (a) native and (b) frayed into subfilaments. The relaxed filaments in a show the typical right-handed helical tracks of the myosin cross-bridges (white arrows). In one of the frayed filaments in b the subfilaments are indicated by white arrowheads. Also note that two of the filaments in b show the subfilaments splayed to the right-hand side of the half filament. The images are shown in reverse contrast. Bar, 80 nm.

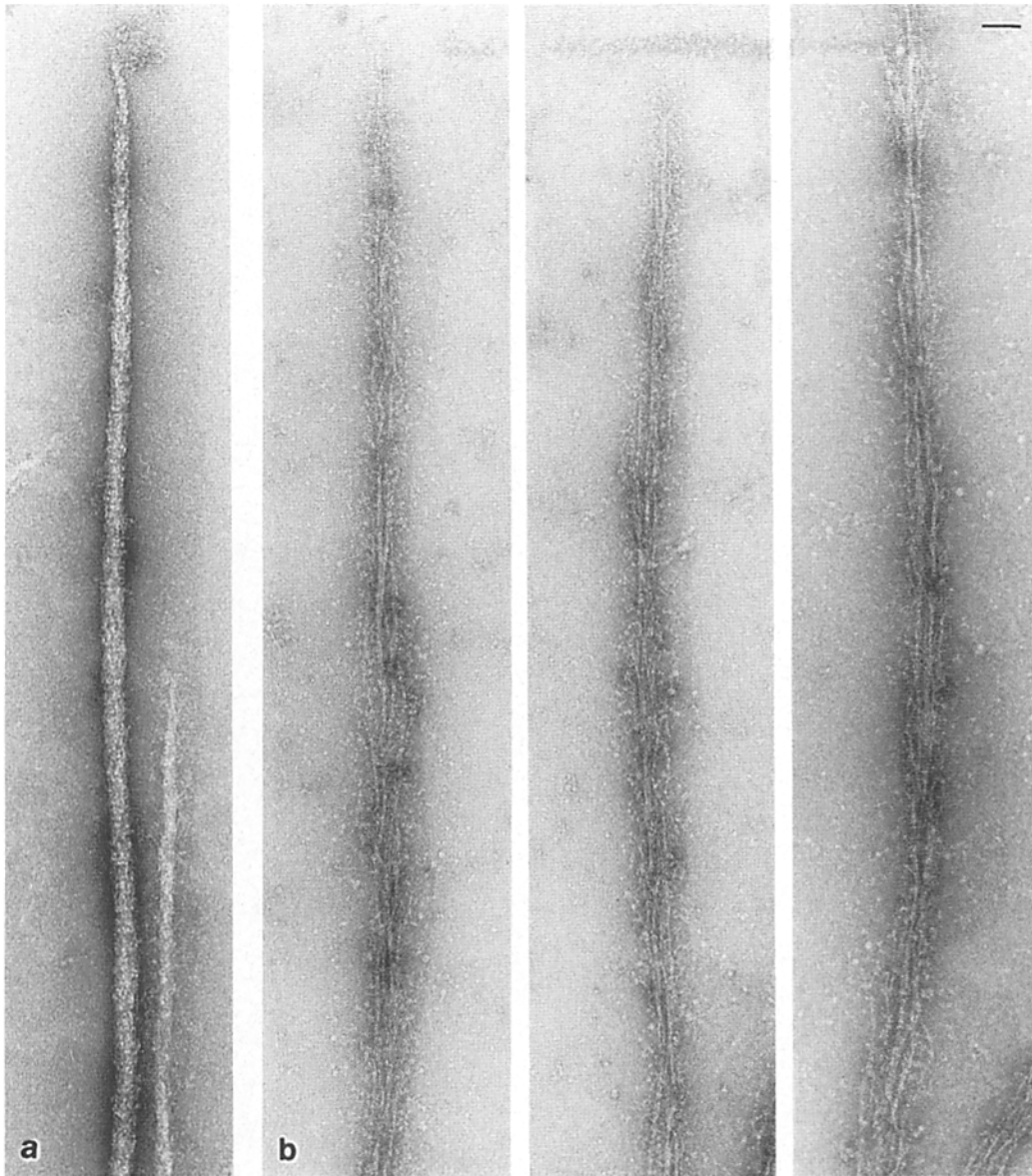
During extended washing with low salt buffers, the subfilaments depolymerize until all that remains of the filament is a short “stub” composed of an apparently undisrupted bare zone with short subfilaments protruding from each end (Fig. 2).

### **Synthetic Filaments**

Purified scallop myosin can be assembled into synthetic filaments that resemble native filaments when the protein is dialyzed against relaxing buffers at ionic strengths of 0.1–0.2 M at pH 7 and 4°C (Fig. 4 a). The filaments assembled at these ionic strengths appear to be of rather uniform diameter

(300–400 Å) but of variable lengths (up to 10 μm). They also show a tendency to aggregate into bundles, which, together with the extended length, make it hard to establish whether they are bipolar. Apparent bare zones are, however, occasionally observed both along the filaments and also at one end. The presence of ATP induces the surface cross-bridges to arrange in a well-ordered helical array comparable to that found on relaxed native filaments, and also reduces the tendency of these filaments to aggregate into bundles.

Synthetic filaments, like native filaments, fray readily into subfilaments in solutions of low ionic strength and high pH (Fig. 4 b). At ionic strengths in the range 10–20 mM, fraying occurs much more readily in ATP-containing solutions than



**Figure 4.** Negatively stained synthetic filaments formed by dialysis of *Aequipecten* myosin against relaxing solution: (a) filaments in relaxing buffer; and (b) frayed into subfilaments by rinsing with relaxing solutions of low ionic strength and high pH. Bar, 80 nm.

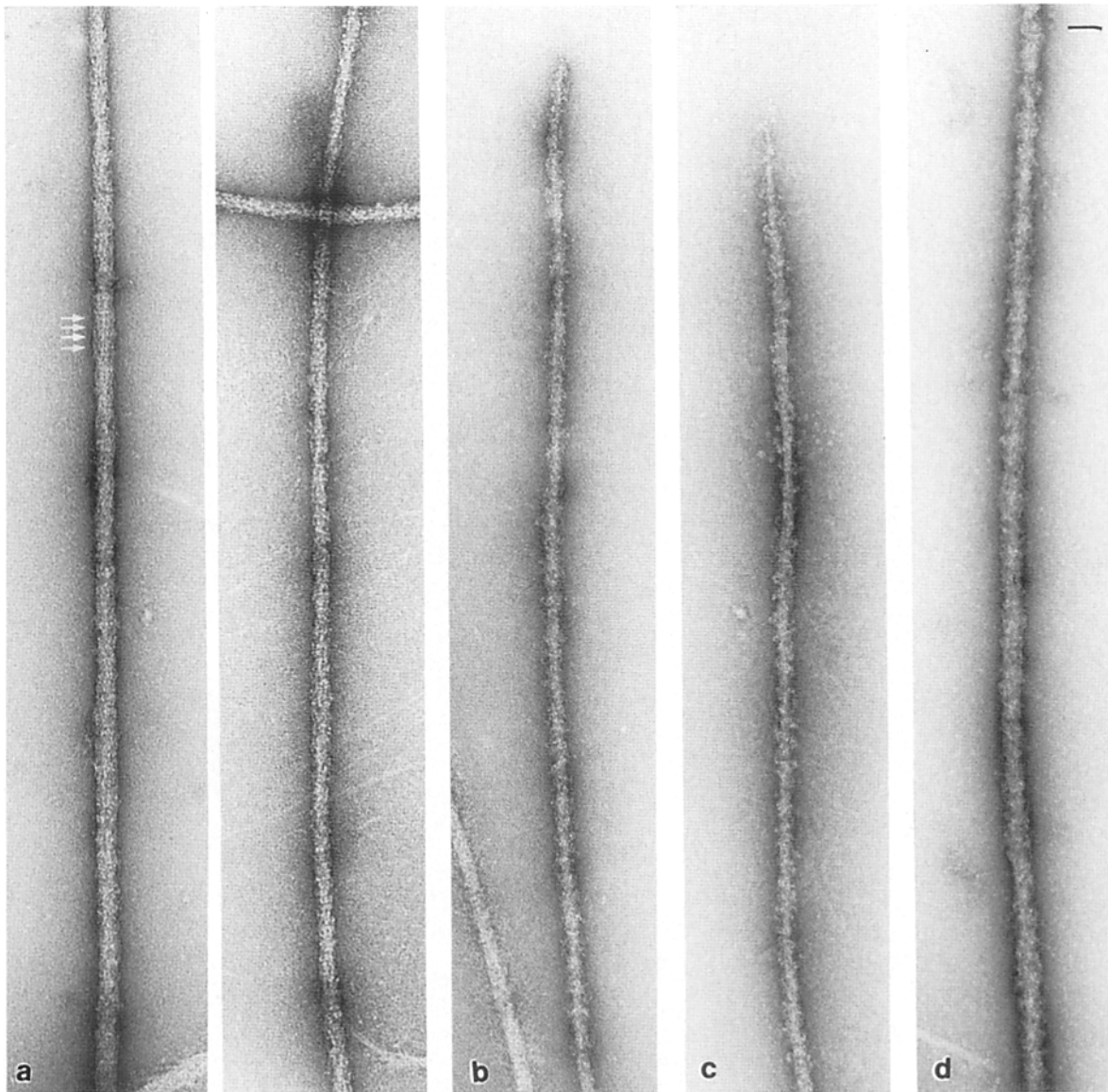
in rigor buffers. At very low ionic strength (<5 mM), however, filaments fray rapidly and completely whether or not ATP is present. The number and three-dimensional organization of the subfilaments within the synthetic filaments are not clear, but it is common to observe four to five subfilaments at any one point along a frayed filament. The fact that the subfilaments are laterally separated for only short distances before crossing over each other suggests that the subfilaments are coiled around one another in the synthetic filaments.

Although synthetic myosin filaments have helically arranged heads when relaxed (Figs. 4 a and 5 a), the order in the surface array is lost when the heads are activated (Fig. 5 b) as observed in native filaments (Vibert and Craig, 1985). Increasing the calcium concentration to pCa 5 causes a rapid disruption of the helical order, which is complete within the few seconds required for preparation of a grid. Removal of

nucleotide (Fig. 5 c) (by extensive washing with rigor buffers) causes a similar transition but with a time scale on the order of 10 min. When synthetic filaments are made from scallop myosin whose most reactive head sulfhydryl has been modified with IAEDANS, the heads do not achieve an ordered helical arrangement in relaxing solutions (Fig. 5 d).

#### **Accessory Proteins**

Some native myosin filaments from scallop muscle show short beaded rods bearing one or more globules protruding from their tips (Fig. 6). These structures appear to be similar to but shorter than the end filaments that have been identified on the thick filaments of vertebrate striated muscle. Well-defined scallop end filaments are seen on only a few thick filaments: more frequently short protrusions with no consistent morphology are visible. Occasionally very fine



**Figure 5.** Examples of synthetic *Aequipecten* myosin filaments formed in relaxing solution in different states: (a) relaxed, (b) activated at pCa 5.0, (c) depleted of nucleotide by extensive rinsing with rigor solution, and (d) in relaxing solution, formed from IAEDANS-modified myosin. Arrows in a point to the 145-Å repeat of the myosin cross-bridges. Bar, 80 nm.

threads appear to extend from the ends of the thick filaments, reminiscent of the fine titin threads sometimes seen at the ends of vertebrate filaments.

SDS gels of scallop striated muscle homogenates reveal several proteins of high chain weight ( $\sim 400,000$ – $700,000$ ) (Fig. 7). These probably include titin-like proteins, which by analogy with vertebrate muscle may be components of the end filaments. Certain protein bands appear to comigrate with bands from the smooth muscle of the scallop. Comparisons with vertebrate striated muscle samples (from rabbit back or chicken breast muscles) show that the prominent scallop bands have higher mobilities than vertebrate titin and nebulin. Western blots probed with monoclonal antibodies to titin from rabbit muscle (gifts of J. Trinick and J. Wardale) show no cross-reaction with scallop bands nor with total scallop muscle homogenates.

## Discussion

Several models for myosin filament assembly invoke subfilaments as an intermediate level of organization between molecule and filament, and in the case of myosin from vertebrate striated muscle, it has been possible to demonstrate directly that both native and synthetic filaments are composed of three subfilaments (Maw and Rowe, 1980; Trinick, 1981; Pinset-Härström, 1985). Here we show that a similar substructure occurs in native filaments from scallop striated muscle. In this case the number of subfilaments (although difficult to count) is probably seven: the rotational symmetry previously determined for native filaments from the sea scallop *Placopecten* (Vibert and Craig, 1983) and also from the bay scallop *Aequipecten* (Vibert, P., L. Castellani, and R. Craig, unpublished observations). Synthetic filaments made

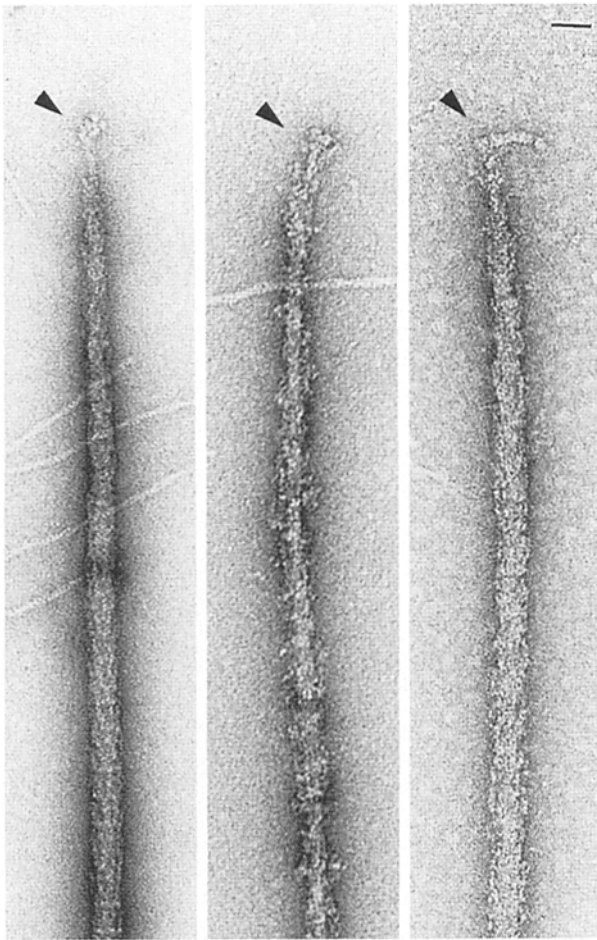


Figure 6. Examples of end filaments protruding from the tips of native myosin filaments from *Placopecten* (arrowheads). Bar, 40 nm.

from *Aequipecten* myosin also contain subfilaments; their number is uncertain, but appears to be five or more. These synthetic filaments have similar diameters to native filaments from *Placopecten* and *Aequipecten*, and may therefore have the same symmetry, although we have not been able to confirm this by image analysis because the degree of order in the helical cross-bridge surface lattice is not good enough.

The subfilaments visualized by fraying isolated filaments in low salt thus appear to be equal in number to the rotational symmetry of the intact filaments (i.e., threefold in vertebrate; probably sevenfold in scallop). X-ray diffraction analysis (Wray, 1979), however, indicates the existence of smaller subfilaments ( $\sim 40$  Å in diameter) in the tubular thick filaments of crustaceans and insects; the diffraction geometry shows that there are three times as many of these 40-Å subfilaments as the rotational symmetry of the cross-bridge array. Microscopy of tubular filaments in sectioned arthropod muscles supports this model (Ashton et al., 1987; Beinbrech et al., 1988). Wray (1979) also suggested that the solid filaments exemplified by vertebrate striated and scallop striated muscle types might be derived from the tubular arthropod design by a collapse of some subfilaments into more central positions. It may be that larger intermediate structures, equal in number to the rotational symmetry, are thereby

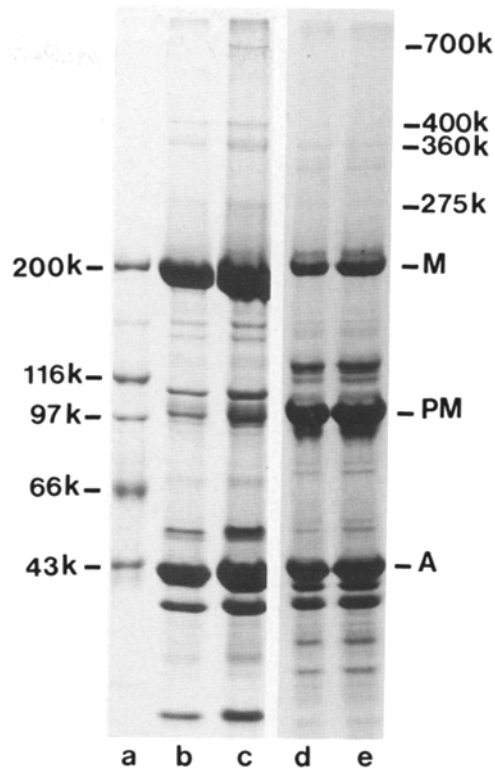


Figure 7. SDS-polyacrylamide gels (3–13% gradient). (Lane a) Molecular weight standards; homogenized muscles from (lanes b and c) striated adductor; and (lanes d and e) smooth adductor of *Placopecten*. M, Myosin; PM, paramyosin; A, actin. Estimated molecular weights of some of the bands with low mobility are indicated.

formed. Certainly the size of the subfilaments seen after fraying (on the order of 100 Å diam in both vertebrate and scallop filaments) must contain several (three to four?) of the 40-Å subfilaments; a view that receives some support from electron microscopy of vertebrate filament cross-sections (Pepe et al., 1986).

One further implication of filament fraying deserves note. Vertebrate striated muscle fibers have been extensively studied in low salt buffers in attempts to characterize the mechanical, kinetic, and structural properties of myosin heads when they are weakly bound to actin. As these conditions also favor fraying of the myosin filaments, loosening of the thick filament backbone structure may contribute, for example, to the enigmatic changes in the x-ray diffraction diagrams that occur when muscle fibers are immersed in low salt buffers (Brenner et al., 1984; Matsuda and Podolsky, 1984; Xu et al., 1987).

Synthetic filaments of scallop myosin assembled under roughly physiological ionic conditions share functional as well as structural resemblances with native filaments (cf. Chantler et al., 1981). In particular, they show a ligand-dependent order-disorder transition in the cross-bridge arrangement (cf. Vibert and Craig, 1985; Frado and Craig, 1989). Moreover, synthetic filaments made from sulfhydryl-modified scallop myosin seem unable to adopt an ordered helical arrangement of heads in relaxing solutions, consistent

with the abolition of the relaxed state indicated by ATPase measurements (Titus et al., 1989), and reinforcing the view that ordering of heads may have a regulatory role in native filaments (Vibert and Craig, 1985; Frado and Craig, 1989).

The major difference between native and synthetic filaments is in their length. Analysis of the amino acid sequence of the rod portion of various myosins (McLachlan, 1984) has revealed a linear periodicity of clusters of charged residues that suggests why certain linear displacements (e.g., 14.5 and 43.5 nm) occur between adjacent rods in various myosin assemblies (probably including 40-Å subfilaments; Wray, 1979). In certain ionic conditions, synthetic filament length is quite closely determined by the kinetics of association and dissociation of myosin dimers at the filament ends (Davis, 1985). This equilibrium depends, however, on complex three-dimensional interactions between the dimer and the growing polymer, and is not simply accounted for by the linear disposition of charge on the myosin rods (Davis, 1988).

To understand myosin assembly will thus require analyzing the full three-dimensional significance of the rod amino acid sequence, which has not yet been achieved for any myosin. Although the distribution of hydrophobic residues favors a coiled-coil structure (McLachlan, 1984), it is not clear, for example, what dictates the pitch of the coiled coil, what interactions between coiled coils give rise to subfilaments, what determines the degree of coiling of the subfilaments (and thereby the pitch of the surface helix of cross-bridges), or what determines the number of subfilaments that form a closed structure laterally. These aspects of myosin assembly, which are very precisely specified for a given myosin but vary widely among different myosins (Wray, 1979), are presumably coded by subtle features of the amino acid sequence that are not yet apparent.

Although the sequence of myosin alone may contain much of the information required to define the form and size of native filaments, it is clear that other cellular factors such as transcriptional control of myosin synthesis (Epstein et al., 1986), and the presence of core proteins (Epstein et al., 1985) or cytoskeletal frameworks (Holtzer et al., 1982; Wang, 1984; Hill and Weber, 1986), are important in specifying the size that filaments reach during myofibrillogenesis. Auxiliary proteins of the thick filaments of vertebrate skeletal muscle are numerous (Starr and Offer, 1971) but are generally present in low stoichiometry, and little is known about their functions. Certain of these may have roles in stabilizing the structure of the assembled myosin filament (e.g., C-protein and possibly end filaments/titin), or in establishing or maintaining the organization of the filaments in the sarcomere (e.g., M-protein and titin).

End filaments were first found on vertebrate muscle filaments, where they appear to be composed of titin and AMP deaminase (Trinick, 1981; Trinick et al., 1984; Fürst et al., 1988). Scallop end filaments appear to be beaded rods bearing one or more globules at their ends, similar to (but shorter than) vertebrate end filaments. SDS gels of scallop muscle homogenates show several bands of higher chain weight than myosin, but monoclonal antibodies to rabbit titin do not cross-react with these scallop bands, or with total muscle homogenates, probably due to the high degree of tissue and species specificity shown by titin antibodies (Hill and Weber, 1986). Locker and Wild (1986) and Maruyama and colleagues (Hu et al., 1986) have shown, however, that high

chain weight proteins are found in most muscles throughout the animal kingdom, with invertebrate titin and nebulin having apparently lower chain weights than their vertebrate counterparts.

Vertebrate titin appears to extend throughout the length of the sarcomere, with an elastic segment in the I band and an inelastic region associated with the thick filament (Wang and Wright, 1988; Fürst et al., 1988). How this distribution in situ is related to the presence of titin in end filaments (Trinick et al., 1984; Hill and Weber, 1986) remains to be established; it is possible that the end filaments seen on isolated thick filaments represent retracted forms of the elastic region of titin filaments torn free from their I band locations. The variable appearances of the protrusions on the tips of scallop thick filaments tend to support this view. The function of titin elasticity in stabilizing the sarcomere arrangement of vertebrate striated muscle (Wang, 1984; Horowitz and Podolsky, 1987) may be a particular adaptation of a more general cytoskeletal role. Significantly, Hill and Weber (1986) have found that titin first appears as elongated threads in chicken myoblasts, suggesting a role in guiding the assembly of the developing myofibrils. The presence of titin-like proteins not only in striated but also in smooth muscles of invertebrates (Locker and Wild, 1986; Hu et al., 1986) supports such a picture.

We thank Drs. Ling-Ling Young Frado and Roger Craig for sharing unpublished results, Drs. Kathy Loesser and Clara Franzini-Armstrong for advice on rotary-shadowing methods, Dr. Carolyn Cohen for continuous support and encouragement during the development of this project, Judy Black for photography, and Beth Finkelstein and Louise Seidel for typing the manuscript.

This work was supported by grants from the National Science Foundation (DMB85-02233 to C. Cohen and P. Vibert); from the National Institutes of Health (AR35829 to L. Castellani, and AR17346 to C. Cohen); and from the Muscular Dystrophy Association (to C. Cohen). Funds to purchase and maintain the Philips EM420T transmission electron microscope were obtained from a Shared Instrumentation grant 1-S10-RR02464-01 awarded to Carolyn Cohen by the National Institutes of Health.

Received for publication 16 December 1988 and in revised form 31 March 1989.

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