ASSEMBLY OF SMOOTH MUSCLE MYOSIN INTO SIDE-POLAR FILAMENTS

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ABSTRACT

The in vitro assembly of myosin purified from calf aorta muscle has been studied by electron microscopy. Two types of filament are formed: short bipolar filaments similar to those formed from skeletal muscle myosin, and longer "side-polar" filaments having cross bridges with a single polarity along the entire length of one side and the opposite polarity along the other side. Unlike the case with skeletal myosin filaments, antiparallel interactions between myosin molecules occur along the whole length of side-polar filaments. The side-polar structure may be related to the in vivo form of myosin in vertebrate smooth muscle.

KEY WORDS myosin · smooth muscle · thick filaments · self-assembly · side-polar filaments

The structure of myosin filaments in vertebrate smooth muscle has long been a puzzle. While it is generally agreed that long (>2 μm) filaments are present, there have been differing reports on the shape of these filaments in cross section and on their mode of assembly. Small and Squire (18) described wide ribbonlike structures in which all cross bridges on one face exhibited a single polarity, and those on the other face had the opposite polarity. They suggested that the ribbons had a core of nonmyosin protein ("lento-filaments") that determined the polarity of the myosin molecules arrayed on the surface, and pointed out that this "face-polarity" was well suited to producing the large degrees of shortening characteristic of smooth muscle. The specific polar interactions between myosin molecules that were deduced from these ribbons form the basis for Squire's general model of thick-filament structure (25). Jones et al. (9), Shoenberg and Haselgrove (16), and Somlyo et al. (23, 24) have shown that the large width of the ribbons is an artifact produced by the lateral aggregation of narrower in vivo filaments during fixation. However, the hypothesis that there is a single cross-bridge polarity along the entire "face" of a thick filament has not been disproven. With myosin purified from calf aorta muscle, we have obtained in vitro filaments whose structure reconciles a number of these earlier results. The filaments, which are formed from myosin alone, are narrower than ribbons, but appear to have two surfaces with opposite and uniform polarity along their entire length. They show a mode of assembly for myosin molecules that has not previously been described.

MATERIALS AND METHODS

Actomyosin was extracted from calf aortas, which had been stripped of their adventitia, in 25 mM potassium phosphate, 0.5 mM dithiothreitol (DTT), pH 7, and was precipitated by reducing the pH to 6.4 and adding KCl to 0.15 M (13). After dissolving the actomyosin in 0.6 M KCl, 10 mM potassium phosphate, 10 mM MgATP, 0.5 mM DTT, pH 7.5, an enriched myosin fraction was obtained between 33% and 66% ammonium sulfate saturation. This fraction was chromatographed on 4% agarose (1), in 0.6 M KCl, 15 mM potassium phosphate, 1 mM MgATP, 0.5 mM DTT, pH 7.5, and the myosin peak was concentrated by the addition of ammonium sulfate. Nonmyosin contamination was limited to high molecular weight proteins and totalled less than 10% as determined by densitometry of sodium dodecyl sulfate (SDS) gels.[1] No actin or tropomyosin was detected with myosin loadings beyond

[1] The presence of some fibroblast myosin could not be ruled out, but it should be no more than a minor component since the method of preparation, including removal of the adventitia, should not select for it.

the linear range of staining with Coomassie blue. The myosin was monodisperse in the analytical ultracentrifuge, and the value of ATPase activity was typical of published values for smooth muscle myosin. A detailed characterization of this myosin will appear elsewhere.[2]

Myosin rod was obtained as a biproduct of subfragment 1 preparation, by papain digestion of purified myosin (12).

Myosin or myosin rod, at a concentration of 3 mg/ml or less, was polymerized by dialysis for a minimum of 24 h to desired conditions (14). All buffers contained 1 mM EDTA and 0.5 mM DTT in addition to 2 mM potassium phosphate (pH 6 and 7) or 2 mM veronal (pH 8). Substituting phosphate for veronal at pH 8 gave the same results.

Negative staining: a drop of the filament suspension was placed on a grid coated with an intact or holey carbon film. After a brief rinse, those filaments adhering to the film were fixed for 60 s with a drop of 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7. (This fixation step produced a marked improvement in filament preservation.) The fixative was rinsed off with 0.1 M ammonium acetate, pH 7, and the filaments were negatively stained with 1% uranyl acetate or uranyl formate. All measurements were made on filaments not overlying a hole in the carbon film. *Sectioning:* filaments that were formed at 0.3 M KCl, pH 6, were centrifuged for 1.5 h at 40,000 rpm in the Beckman SW65 rotor. The pellet was fixed with glutaraldehyde, postfixed in osmium tetroxide, dehydrated in ethanol, and embedded in Araldite. Sections 30–120 nm thick were cut on a diamond knife and stained with potassium permanganate and lead citrate Grids were examined in a Philips EM 301 electron microscope.

RESULTS

Monomeric myosin can assemble into two types of filament. One type is a short bipolar filament (maximum length 0.5 μm) similar to that observed in the early stages of assembly of striated muscle myosin (8). Cross bridges are seen at each end of a central bare zone, about 180 nm long, which is formed by the antiparallel overlap of myosin tails running approximately parallel to the filament axis (Fig. 1*a*). Such filaments have also been reported with myosins from other vertebrate smooth muscles (6, 10, 19, 27). The second type of filament is distinctly different; the myosin tails pack with antiparallel overlaps but run at angles of 5–20° to the filament axis (Fig. 1*b* and *c*; Fig. 2; and Fig. 4*a*). The array of cross bridges on one side of the filament is thus offset with respect to the array on the other side, giving the filament an oblique appearance. Asymmetric, tapering "bare zones" occur at the ends of the filament, but there is no central bare zone where the polarity of the cross bridges might reverse as it does in skeletal myosin filaments. As a result of

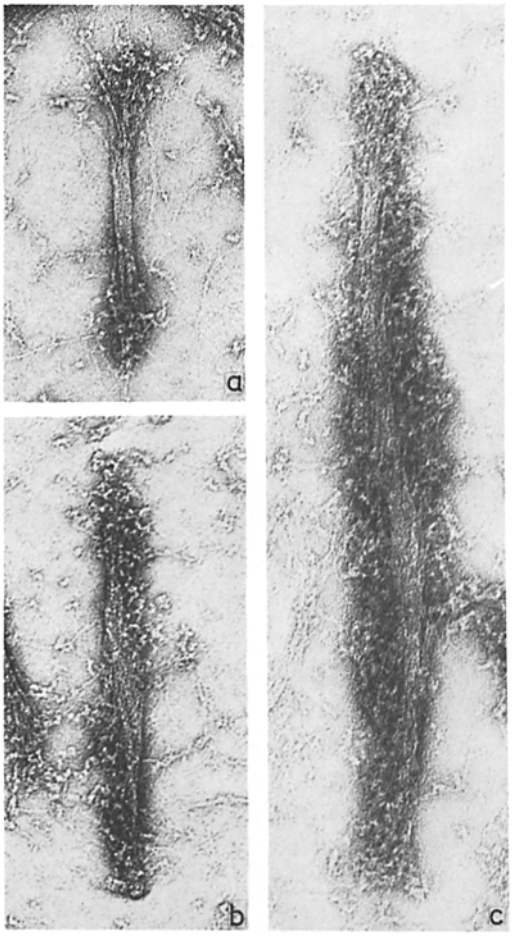

FIGURE 1 Filaments formed from purified calf aorta myosin. The bipolar filament (*a*) and the side-polar filament (*b*) are from the same grid and were formed by dialyzing monomeric myosin against 0.2 M KCl, pH 7. The side-polar filament (*c*) was formed by dialysis against 0.3 M KCl, pH 6. The bipolar filament has a central bare zone that is parallel to the filament axis, while the side-polar filaments have terminal bare zones lying at an angle to the axis. Micrographs in this and later figures have been printed so that the bare zones appear on the left at the top of each side-polar filament and on the right at the bottom. The filaments shown in this figure are embedded in a film of stain over a hole in the carbon support film, a procedure that gives very good contrast and detail (8). (*a* and *b*) × 190,000; (*c*) × 250,000.

[2] Megerman, J., and S. Lowey. Manuscript in preparation.

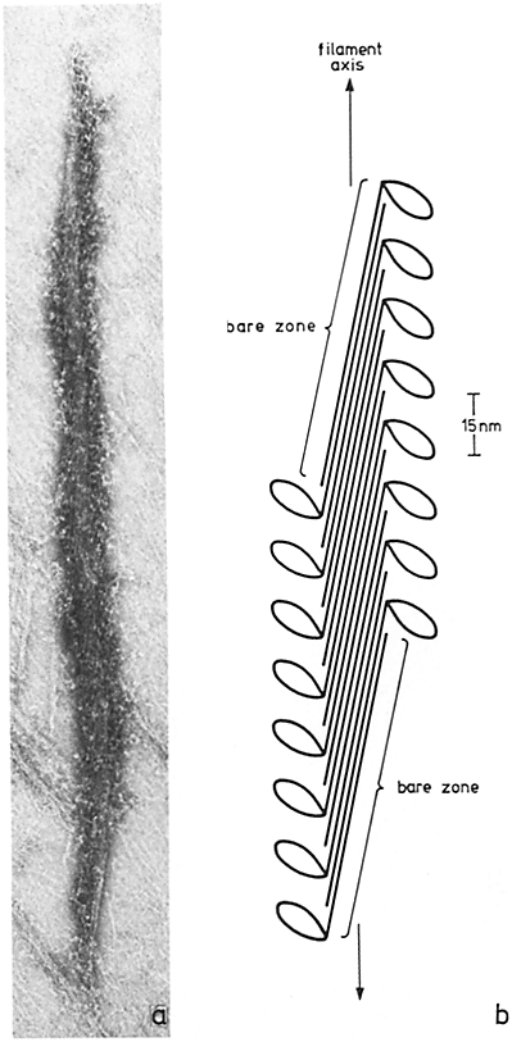

FIGURE 2 (*a*) 1.7-μm long side-polar filament formed from purified myosin at 0.3 M KCl, pH 6. × 86,000. (*b*) Schematic diagram of side-polar filament for comparison with Fig. 2*a*. Only two-dimensional details of those features thought to be fundamental to the side-polar mode of assembly are shown. The real structure is thicker than the single layer of myosin molecules shown, but the full three-dimensional packing of the myosin molecules is not yet understood. (For example, parallel interactions may also be present, generating the 15-nm cross-bridge repeat, and the antiparallel interactions may not occur in the plane of the page.) For the sake of clarity, certain aspects of the structure have been exaggerated. The myosin tails have arbitrarily been drawn to overlap almost completely. The specific interactions actually involved remain to be determined.

this mode of packing, the cross bridges on opposite sides of the filament are oppositely directed; we have therefore termed these filaments "side-polar" to distinguish them from the bipolar filaments of skeletal muscle myosin.

The type of filament formed depends on the conditions of dialysis. Only the short bipolar filaments occur at 0.2 M KCl, pH 8, while they coexist with side-polar filaments in approximately equal amounts at 0.2 M KCl, pH 6 or 7. Dialysis to 0.3 M KCl, pH 6, at concentrations less than 3 mg/ml, yields 90% side-polar filaments, while at 10 mg/ml about equal quantities of the two types are formed. More than 80% of the filaments are bipolar when formed by dialysis from the monomer to "physiological" conditions (0.14 M KCl, pH 7), but more than 80% remain side-polar when they are first formed in 0.3 M KCl, pH 6 and then placed in the physiological medium. In all cases, more than 80% of the total protein occurred in filament form (14).

This polymorphism of smooth muscle myosin filaments is not due to the presence of two kinds of myosin nor to an additional "core" protein. Side-polar filaments obtained at 0.3 M KCl, pH 6, were dissolved in high salt to give a monodisperse myosin; when repolymerized in 0.2 M KCl, pH 8, they formed short bipolar filaments. Conversely, short bipolar filaments could be reformed into side-polar filaments. The assembly of myosin into side-polar or bipolar filaments is thus reversible, and the two modes of assembly are the property of a single myosin species. Further, the levels of nonmyosin contaminants in the myosin appear to be too low to provide a core for the resultant filaments. Nor is the assembly influenced by divalent cations. All filaments shown in the figures were formed in the presence of 1 mM EDTA; the substitution of 5 mM Ca^{2+} or 5 mM Mg^{2+}, with or without 2.5 mM ATP, in 0.3 M KCl, pH 6 had no effect on filament structure.

The unusual distribution of cross bridges in side-polar filaments is most evident in the longer specimens. Such filaments, which grow up to 6 μm in length, frequently exhibit one or more twists of 180° about the filament axis (Fig. 3*c*). The twists are recognized by a reversal in the angle that the tails make with the filament axis (seen clearly in filaments made from myosin rod, Fig. 4*b*), and by a narrowing of the filament due to the disappearance of cross bridges. The narrowing is accompanied by an increase in stain density on either side of the filament, which is consistent with a structure of greater depth in this region. These observations imply that the cross bridges lie above and below the filament backbone where a twist occurs, while elsewhere they lie on either

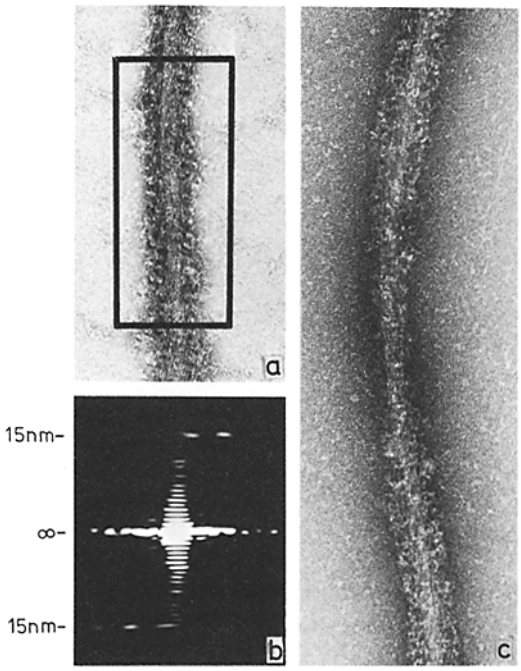

FIGURE 3 (*a*) Portion of a 6-μm long side-polar filament formed at 0.3 M KCl, pH 6, showing the 15-nm repeat of the myosin cross bridges. × 120,000. (*b*) Optical diffraction pattern of the region of the filament indicated in Fig. 3*a*. This pattern is due mainly to the myosin cross bridges since little change occurs if the backbone is masked out. Note the similarity to optical diffraction patterns of edge-views of ribbons (18). The filament in Fig. 3*a* was formed from myosin that had not been column chromatographed. It was chosen simply for the purpose of clarity. Filaments having a similar 15-nm repeat and giving similar optical diffraction patterns are also formed with chromatographed myosin, but these features are then not so striking. (*c*) Portion of a 3.4-μm long side-polar filament showing a twist. At the top and bottom of the micrograph, cross bridges are seen on either side of the filament backbone. In the middle, the filament looks narrower in projection (cross bridges are no longer apparent at the sides), and there is an increase in density of stain along the edges. Analysis of numerous filaments that show one or more "bridge-free" patches at random positions along their length demonstrates that these regions are indeed twists and not conventional bare zones: a twist could always be correlated with a reversal of both the angle of the tails on either side of it, and of the bare zones at the ends of the filament. The filament was formed from purified myosin at 0.3 M KCl, pH 6. × 97,000.

side in the plane of the microscope grid. This finding seems to eliminate the possibility that cross bridges above and below the filament backbone might have been pulled to the sides, during drying of the negative stain, to produce a filament that simply appears to have bridges on only two sides. We conclude that cross bridges are not cylindrically distributed about the axis of side-polar filaments.

Preliminary results showing side-polar filaments in cross-section (Fig. 5) are consistent with this interpretation. The backbone is seen to be approximately square, and many filaments show cross bridges that project from only two opposite sides of the square, the other two sides being bare (Fig. 5*b* and *c*).

Side-polar filaments bear cross bridges with an axial spacing of about 15 nm along the entire

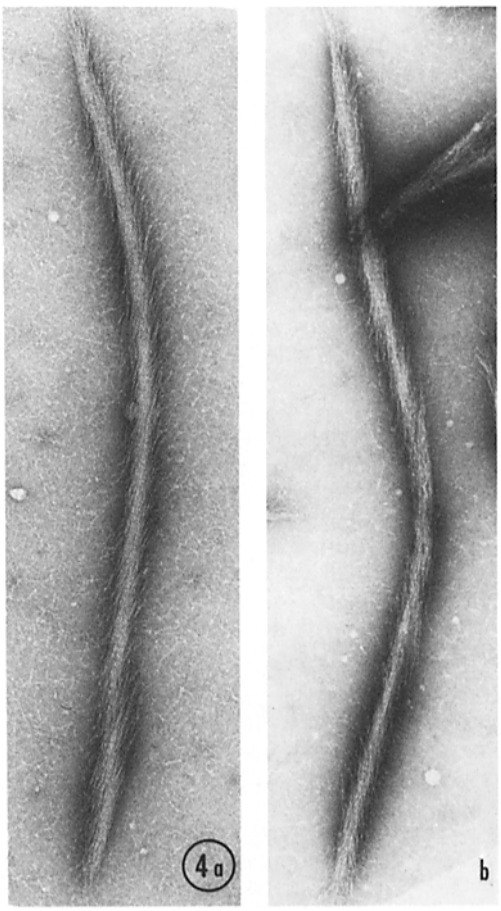

FIGURE 4 Side-polar filaments formed from smooth muscle myosin rod by dialysis against 0.3 M KCl, pH 6. The polarity at any position is clear from the angle of the myosin rods at the edges of the filament. (*a*) No twist present; Fig. 4*b* shows a twist about halfway along. Note how the angle between the rods and the filament axis changes from one end of the twist to the other. × 64,000.

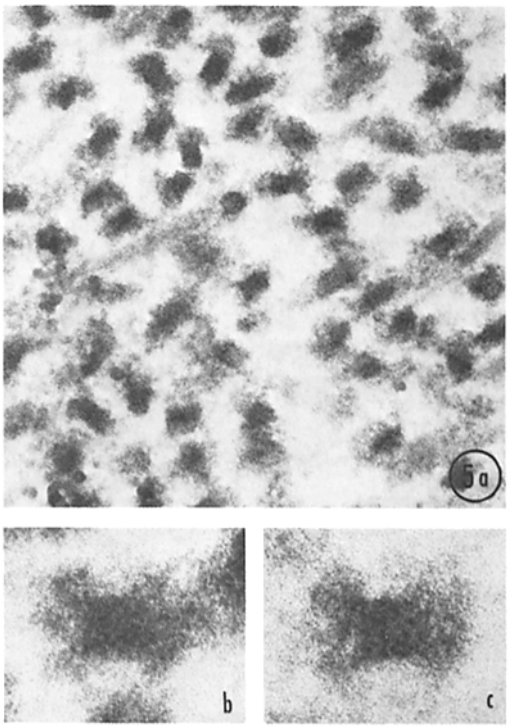

FIGURE 5 Section of pelleted side-polar filaments. Of the variety of filament orientations seen in Fig. 5*a*, some are approximately transverse and show a roughly square profile. (*b* and *c*) Transverse sections of side-polar filaments at higher magnification. Cross bridges originate from the vertical sides of the squares only. When sectioned longitudinally, the filaments appear similar to those seen by negative staining. The structure therefore does not appear to have been significantly modified by centrifugation. (*a*) × 90,000; (*b* and *c*) × 300,000.

length of each side except for the terminal bare zones (Figs. 2*a* and 3*a*). The cross bridges on opposite sides of the filament are parallel to each other, an arrangement which is not consistent with a helical distribution, and lie at angles of about 70° to the filament axis (Figs. 2*b* and 3*a*). Optical diffraction patterns of side-polar filaments show a 15-nm layer line (coming from the cross bridges), which is asymmetric about the meridian (Fig. 3*b*), further supporting the side-polar model we propose.

DISCUSSION

The assembly of side-polar filaments differs in some respects from other modes of assembly currently known for myosin. The initiation of striated muscle thick filaments depends on the antiparallel (tail-to-tail) interaction of myosin molecules. Growth beyond a length of about 0.5 μm involves the addition of myosin molecules with exclusively polar (head-to-tail) interactions (8). In the side-polar filaments, however, antiparallel interactions occur along the entire length of the filament. That is, the filament can be thought of as growing by the polar addition of bipolar myosin units, producing a structure that appears to have uniform packing throughout its length (Fig. 2*b*), unlike the case with skeletal muscle thick filaments. The necessity of specific antiparallel contacts for the growth of smooth muscle myosin filaments is further suggested by the failure of our bipolar filaments to grow longer than 0.5 μm. The length of the smooth muscle myosin molecule is about 0.17 μm (4, 11); 0.5 μm may thus be the maximum length of a bipolar filament in which all the myosin molecules have some antiparallel overlaps (15). We conclude that antiparallel overlaps between myosin tails are probably essential for the initiation and growth of smooth muscle myosin filaments.

With myosin prepared from taenia coli, gizzard, and vas deferens muscles, Sobieszek (19, 20) and Sobieszek and Small (21, 22) reported the formation of "cylindrical" filaments, also bearing cross bridges along their entire length except for a bare zone at each end, and inferred that these filaments were constructed from bipolar myosin units. On the basis of a 14.5-nm repeat, which appears to be superimposed on the backbone of the filament, and the optical diffraction patterns of their filaments, these authors concluded that the bipolar myosin units were helically arranged, that their filaments "do not appear to possess any polarity" (22), and therefore that they did not represent the in vivo state of myosin in vertebrate smooth muscle.

Although our filaments are similar to those of Sobieszek and Small, they exhibit some clear differences. Cross bridges are confined to two sides of the filament, are not superimposed on the backbone (Fig. 1*c* and 3*a*), and are parallel to each other on opposite sides of the filament axis (Fig. 2*b*). This cross bridge arrangement and its asymmetric optical diffraction pattern show that side-polar filaments do not possess helical symmetry. They do, however, possess a very distinct polarity that makes them suitable as in vivo elements in a sliding filament system.

There are several reasons for considering the

possibility that the in vivo state of myosin in vertebrate smooth muscle is a filament constructed from antiparallel myosin units and having terminal bare regions: (*a*) central bare zones have not been demonstrated in sectioned smooth muscle thick filaments (2); (*b*) Ashton et al. (2) report that thick filaments are about 2.2 μm long. This is consistent with the structure we propose (since side-polar filaments and the filaments of Sobieszek and Small grow up to 6 μm in length), but not with our bipolar filaments, which are never longer than 0.5 μm; (*c*) their diameters, and the lengths over which these filaments taper, are consistent with observations from sectioned muscle (2); and (*d*) the cross-sectional shape of our filaments is consistent with the approximately square profiles seen in some sections (17). In addition, we find that side-polar filaments are stable in 0.14 M KCl, pH 7, conditions that are similar to physiological. The formation of filaments with terminal bare zones from several smooth muscle myosins (19–22) and from amoeba and slime mold myosins (3, 7) suggests that this may be a general mode of assembly for myosins from nonstriated contractile systems.

It is not clear whether side-polar or cylindrical filaments would be the more likely in vivo form. Thick filaments which appear similar to the cylindrical form have been extracted from isolated taenia coli cells (17). However, it is not clear (22) how such filaments could account for the unique polarity of face-polar ribbons seen in sectioned taenia coli (18). Even though ribbons have been shown to be aggregates of narrower filaments (9, 16, 23, 24), these filaments should themselves reflect the polarity observed within the ribbons (26). Side-polar filaments could, however, provide a straightforward explanation of the ribbon polarity. Lateral aggregation of side-polar filaments through their bridge-free sides, i.e., aggregation perpendicular to the plane of the micrographs, could produce ribbons having cross bridges with uniform polarity on one face and opposite polarity on the other. The strong similarity between the appearances of side-polar filaments and of edge-views of ribbons, and between their respective optical diffraction patterns (Fig. 3*a* and *b*; cf. Small and Squire [18], Plate XII) supports this possibility.

There is one major difference, however, between the structure of our filaments and that proposed for the ribbons. Since the side-polarity of the filaments is not dependent on the presence of a nonmyosin core, we suggest that the in vivo structures might similarly have no core. This is contrary to Small and Squire's model of the ribbon, in which a nonmyosin core interposes between the myosin molecules on opposite faces so that there are no antiparallel myosin overlaps. We find, in contrast, that an essential feature of side-polar filaments is the requirement for antiparallel contacts along the whole length of the filament. Finally, we note that the specific polar interactions in Squire's general model of the myosin filament were deduced from the face-polar ribbons assumed to have no antiparallel interactions between myosin tails (25); some of the interactions deduced by Squire may, nevertheless, still be correct.

Filaments with cross bridges having a single polarity along the entire length of one side could readily explain the ability of smooth muscle to undergo extreme shortening (5), in a manner similar to that suggested by Small and Squire for their face-polar ribbons (18). During contraction, a thin filament could slide unimpeded along a thick filament "side" until the end of the thin filament was reached. If the thick filament were bipolar, sliding would be inhibited when the thin filament encountered cross bridges of opposite polarity. The side-polar mode of assembly could thus confer a distinct structural advantage on a sliding filament system required to shorten by large amounts.

We thank Drs. Carolyn Cohen, Peter Vibert, and Susan Lowey for valuable discussions. This work was done during the tenure of a National Institutes of Health postdoctoral fellowship (J. Megerman) and of a Research Fellowship of the Muscular Dystrophy Association (R. Craig), and was supported by National Institutes of Health grants AM17346-05 to C. Cohen, and AM17350 to S. Lowey; National Science Foundation grants PCM76-10558 to C. Cohen and GB-38203 to S. Lowey; and Muscular Dystrophy Association grants to S. Lowey and C. Cohen.

Received for publication 11 July 1977, and in revised form 20 September 1977.

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