STRUCTURAL STUDIES OF SYNTHETIC FILAMENTS PREPARED FROM COLUMN-PURIFIED MYOSIN

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ABSTRACT Synthetic filaments prepared from column-purified rabbit skeletal myosin by slow dialysis exhibit characteristic bipolar organization and 14-nm axial subunit spacing. Backbone substructure can be discerned in high resolution micrographs in the form of striations of 3–4-nm width and slight angular tilt from the direction of the filament axis. Filament backbone diameters vary over the population, although remaining relatively constant for a single filament. Approximately 25% of the filaments appear poorly stained and frayed, which may be due to collapse on the electron microscope grid. Optical diffraction studies reveal a 43-nm axial repeat as well as the 14.3-nm subunit repeat, indicating a structural homology with natural filaments. A model for synthetic filament aggregation is presented that is consistent with observations of backbone diameter variation, absence of bare zones, and the presence of fraying filaments.

INTRODUCTION

In vitro aggregates of muscle proteins have played an important role in the elucidation of structural and functional features of muscle contraction. The ease of preparing reconstituted thin filaments with and without regulatory proteins enabled three-dimensional helical reconstruction of these filaments decorated with subfragment-1 (Moore et al., 1970) and regulatory proteins (Spudich et al., 1972; Gillis and O'Brien, 1975; O'Brien et al., 1975). The structure of reconstituted filaments is consistent with x-ray diffraction data on natural thin filaments, allowing functional in vitro results to be applied to understanding in vivo mechanisms.

Extensive studies of synthetic myosin filaments prepared under a variety of conditions (e.g., Huxley, 1963; Kaminer and Bell, 1966; Eaton and Pepe, 1974; Moos et al., 1975) suggest that these filaments do not have the simple relationship to natural thick filaments that reconstituted thin filaments have to natural ones. Synthetic myosin filaments prepared by rapid dilution to low ionic strength are very short, primarily a thick bare zone with dense clusters of heads at either end, whereas filaments prepared by slow dialysis tend to be extremely long (as much as 25 μm), and of disordered appearance in the electron microscope. The length characteristics of a given population of synthetic filaments will, in addition, be affected by pH and final ionic strength, and will even vary from preparation to preparation under the same formative conditions (Pollard, 1975). In contrast, natural thick filaments have a fixed length of ~1.55 μm, a uniform backbone diameter of ~12–13 nm, subunit spacing of 14.3 nm, and an

Some of the results discussed here were presented in abbreviated form at the 22nd Annual Meeting of the Biophysical Society in Washington, D.C., 27–30 March 1978 (Koretz, 1978).

apparent axial repeat of 42.9 nm (Huxley, 1963; Huxley and Brown, 1967; Koretz, unpublished data).

Recent studies of synthetic filaments prepared from column-purified myosin (Moos et al., 1975) suggest that at least some of the disorder observed in previous work is due to the presence of small amounts of other thick filament proteins. Increasing use of synthetic filaments for the study of physical properties of myosin (e.g., Morimoto and Harrington, 1974; Mendelson and Cheung, 1976; Sutoh and Harrington, 1977; Sutoh et al., 1978) makes a further investigation of synthetic filament structural characteristics using column-purified myosin seem essential.

MATERIALS AND METHODS

Protein Preparation

Myosin was prepared as described previously (Offer et al., 1973) from mixed back and hind leg muscles of rabbit, with a final purification step using DEAE Sephadex A-50 column chromatography (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The myosin peak was pooled and precipitated using 20 vol of glass-distilled water, redissolved in unbuffered 0.5 M KCl to a concentration of ~5 mg/ml, and dialyzed overnight against a large volume of 0.5 M KCl. After dilution with KCl to a concentration of 1.0 mg/ml as estimated from the extinction coefficient for column-purified myosin reported by Offer et al., myosin was dialyzed against a covered 0.1 M ammonium acetate solution, pH 7.0, at 4°C for 24 h with gentle stirring. The ratio of the volume of dialysis medium to bag volume was at least 80:1.

Electron Microscopy

Samples were placed on carbon-coated grids, washed with 0.1 M ammonium acetate, and negatively stained with either 1% uranyl acetate or 1% ammonium molybdate. The microscope used was a Philips 200 (Philips Electronic Instruments, Ltd., United Kingdom) with a liquid nitrogen anticontamination device, and micrographs were taken at an accelerating voltage of 80 kV and a magnification of 71,000 ± 5%. This magnification is such that 1 mm on the film represents ~ 14 nm.

Data Analysis

Filament backbone diameters were measured on 3× enlargements of electron micrographs or on 1:1 positive transparencies of micrographs using a 7× magnifier with calibrated graticle. Only those filaments that seemed well-stained and unfrayed were considered, and measurements near the end of a filament or adjacent to the crossover point of two or more filaments were avoided. At least 100 observations were made, and graphed in histogram form.

Optical diffraction patterns were obtained using the apparatus described by O'Brien et al. (1971) and preserved on Ilford Pan-X 35-mm film (Ilford, Ltd., Basildon, Essex, United Kingdom). Noise in the patterns was reduced by placing 1:1 transparencies made from electron microscope negatives in microscope immersion oil between optical flats. Layer line spacings from the film record of the patterns were measured with a Mitutoyo optical comparator (Mitutoyo Mfg. Co., Ltd., Tokyo, Japan) and graphed in modified histogram form from points located in the center of the bins. Because of the spatial relationships of the optical diffraction system and the magnification of electron micrograph transparencies, the 14.3-nm^{-1} reflection was assumed to be that nearest to a 1-mm distance from the equator. Other peaks were assigned reciprocal space distances using the 14.3-nm^{-1} peak as a reference point. The histogram bins were spaced at 0.025-mm intervals, such that the distance from the equator to the 14.3-nm^{-1} reflection is divided into 40 parts; any measurement falling on a boundary between bins was divided between neighboring bins. Small magnification changes from micrograph to micrograph, shrinkage of the filaments upon drying, errors in measurement and, in some cases, proximity of

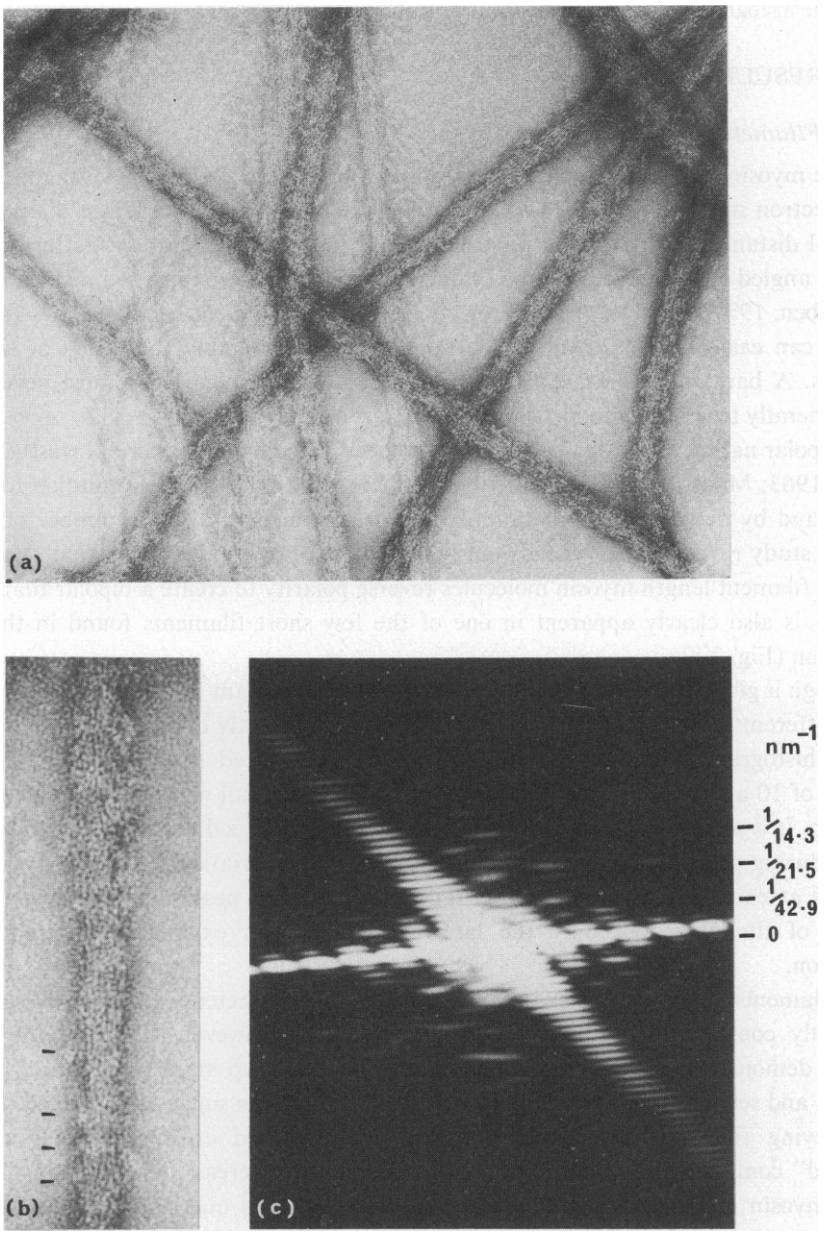

FIGURE 1 Electron microscopy and optical diffraction of synthetic filaments prepared from column-purified myosin. (*a*) A typical electron microscope field (×105,000). Note the variation in diameter from filament to filament, and the presence of a poorly stained, apparently frayed species. (*b*) Enlargement of part of a well-stained synthetic filament from *a* (×210,000). Subunit levels, marked by lines, are clearly visible. One can also discern the backbone striations, with spacing of 3–4 nm, and the occasional absence of a subunit level. (*c*) Optical diffraction pattern of the filament in *b*. The diffraction pattern and spacing of the reflections is similar to what is observed with x-ray diffraction of natural thick filaments *in situ*. The meridional 28.6 nm^{-1} reflection arises from missing subunit levels.

reflections result in a comparatively large background level in the histogram. No differentiation was made in the histogram between meridional and off-meridional reflections.

RESULTS

Filament Appearance

Synthetic myosin filaments prepared from column-purified myosin at pH 7.0 appear regular in the electron microscope (Fig. 1 *a*). Individual filaments have a constant diameter over a long axial distance and gradually tapering ends. The backbone shows a pattern of 3–4-nm striations angled a few degrees off the filament axis, attributable to paired myosin rods (Pepe and Dowben, 1977; Bennett, 1978). Projections from the filament backbone at ~14-nm axial intervals can easily be discerned (Fig. 1 *b*) and are presumably the heads of the myosin molecules. A bare zone, characteristic of natural thick filaments, is almost never seen, as seems generally true for synthetic filaments prepared by slow dialysis.

The bipolar nature of synthetic filaments has been noted by a number of investigators (e.g., Huxley, 1963; Moos et al., 1975) for filaments prepared both by rapid dilution to low ionic strength and by dialysis. Examination of the tapering ends of a large number of filaments from this study reveals myosin heads out to the tip in all cases, indicating that at some point along the filament length myosin molecules reverse polarity to create a bipolar filament. This bipolarity is also clearly apparent in one of the few short filaments found in this type of preparation (Fig. 2 *a*).

Although a given filament generally seems to have a constant backbone diameter along its length, different filaments in the same field of view vary greatly in diameter (Fig. 1 *a*). Fig. 3 shows a histogram of backbone width distribution measured from one preparation, with extremes of 10 and 38 nm. Most filament widths, however, fall within the narrower range of 19–28 nm. It is not clear whether this variability of diameter is due to differential collapse on the grid during preparation for electron microscopy or to variation in the number of myosin molecules at each subunit level during aggregation. Either possibility will account for the majority of the data, but only the latter possibility can explain the extremes of the distribution.

The filaments used for measurements of backbone diameter were all well-stained and individually constant in diameter along their lengths. However, ~25% of the filaments observed demonstrate a different appearance. They take up stain poorly, are variable in diameter, and seem to be frayed (Figs. 1 *a*, 2 *b–e*). It has been suggested (Pollard, 1975) that these fraying filaments are a separate species, composed of myosin molecules in an "activated" configuration for binding to thin filaments, whereas the well-stained filaments contain myosin molecules in the resting configuration. It is questionable whether the two filament types are in fact different. Both show striations along the backbone approximately parallel to the filament axis and 3–4 nm apart and, more important, one occasionally finds filaments where both compact and frayed structures are apparent at different points along their length (Fig. 2 *e*). A more likely explanation is that the fraying filaments arise from collapse on the grid and, near filament ends, from incomplete binding stabilization. This subject, along with variation in filament diameter, will be considered in the discussion section.

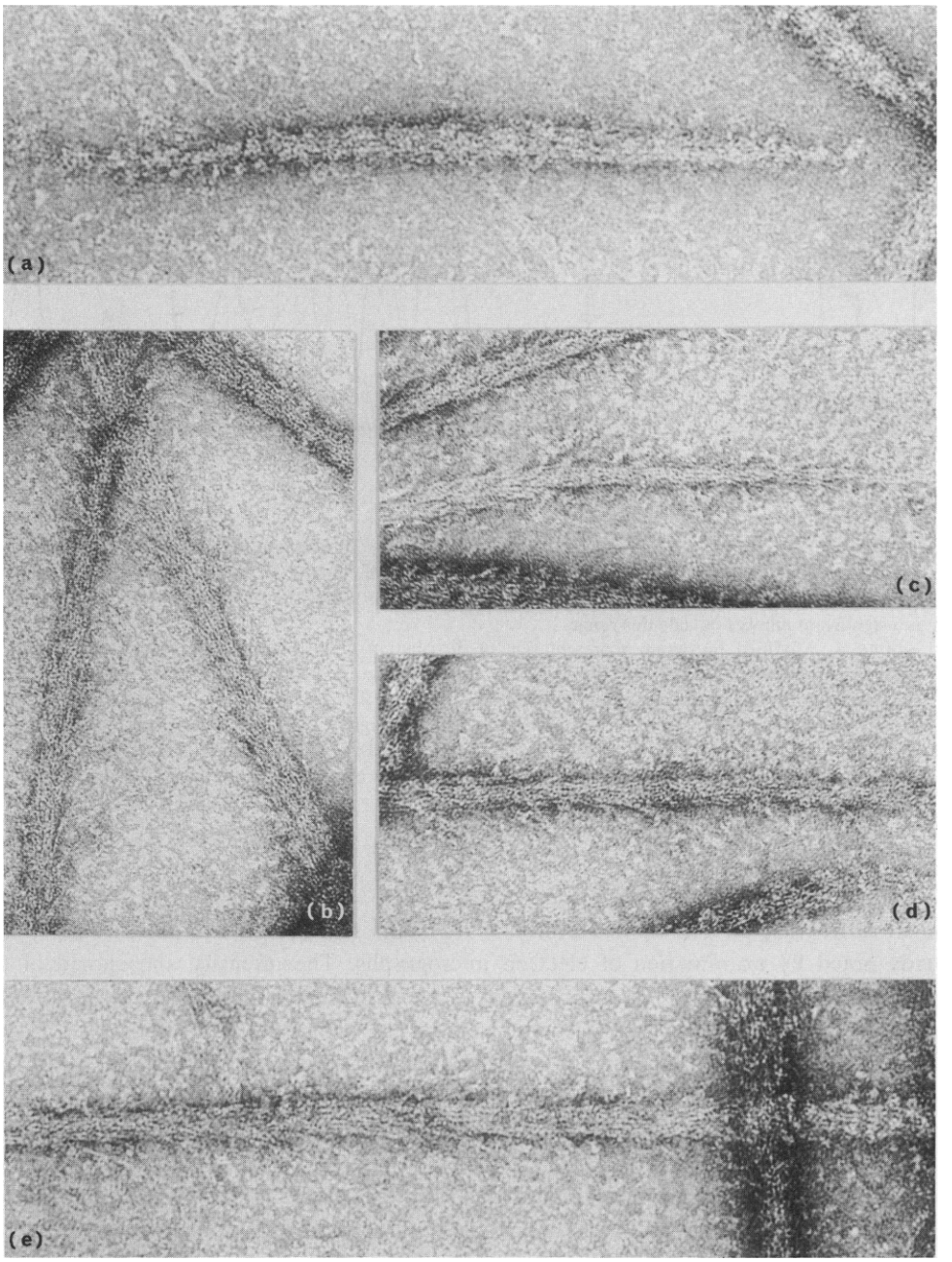

FIGURE 2 Electron micrographs (×210,000). (*a*) A short synthetic filament. No bare zone is visible, as seems generally true for the filaments investigated here. Note that myosin heads are apparent to the tips of the tapering ends, indicating the bipolar nature of the synthetic filaments. (*b–d*) "Fraying" filaments. The myosin heads are at a distance from the backbone, unlike the well-stained regular filaments where the heads appear to rest against the backbone. The polarity of the filament in the fraying areas is apparent. (*e*) A filament which displays both a regular and fraying appearance at different points along its length. This is consistent with the interpretation that fraying filaments may result from collapse on the microscope grid due to poor lateral stabilization.

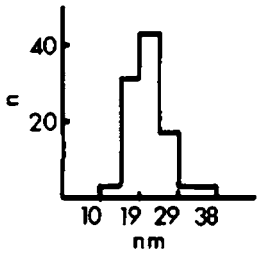

FIGURE 3

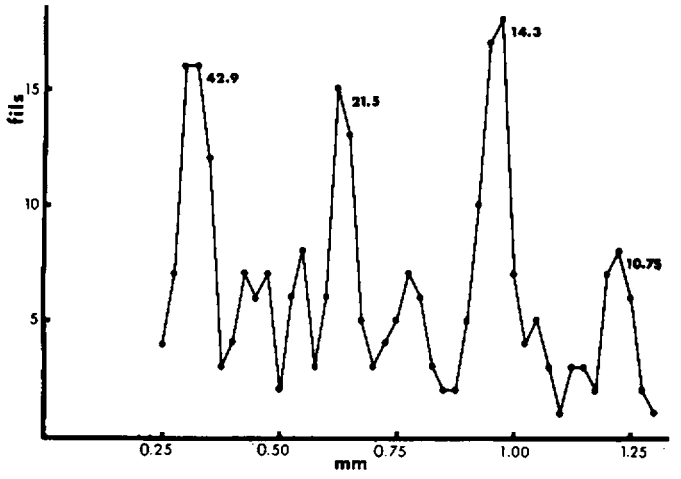

FIGURE 4

FIGURE 3 Distribution of filament diameters plotted in histogram form (N = 108). Abscissa divisions represent millimeters converted to the nearest nanometer, where 1 mm = 4.7 nm, and the ordinate is the number of filaments. Although the majority of the measurements falls within the range of 19–28 nm, there is a significant number outside this range.

FIGURE 4 Modified histogram representation of distribution of reflections from optical diffraction patterns of synthetic filaments (fils) prepared from column-purified myosin, plotted as a function of distance from the equator in millimeters. Assuming that the reflection located at ~1 mm is the subunit spacing of 14.3 nm, the spacings of the other peaks can be assigned. Note that the 43 nm and 21.5 nm peaks are off-meridianal, and that the 28.6 and 14.3-nm peaks fall on the meridian in the original diffraction patterns. The 28.6-nm reflection is attributable to occasional nonsystematic absences of a subunit level (See Fig. 1).

Optical Diffraction Studies

Optical diffraction of well-stained synthetic filaments reveals the 14.3-nm subunit period already noted by examination of electron micrographs. The intensity and spread of this meridional reflection can vary from filament to filament, indicating a tendency to disorder. This is due in part to the relative freedom of movement of the subfragment-1 moiety around its hinge in comparison with natural thick filaments (Mendelson and Cheung, 1976). In some diffraction patterns, a 3.5-nm^{-1} reflection can be observed just off the equator. This is due to backbone striations arising from myosin rod packing (Pepe and Dowben, 1977) and has a slight tilt from the filament axis.

A degree of organization greater than the 14.3-nm subunit repeat is apparent in most diffraction patterns (e.g., Fig. 1 *c*). It is possible to discern off-meridional reflections at axial spacings of ~43 and 21.5^{-1}, as well as the meridional 14.3-nm^{-1} reflection, indicating a periodicity similar to that of natural thick filaments (Huxley, 1963). This can further be seen in the histogram (Fig. 4), which summarizes measurements from 25 optical diffraction patterns of synthetic filaments. Electron micrographs of these filaments are generally one-sided, so that unlike the pattern shown in Fig. 1 *c*, the 21.5-nm^{-1} reflection is often obscured by the mask function. A meridional reflection at ~28.6 nm^{-1} is sometimes visible; this arises from occasional absence of a subunit level.

DISCUSSION

Electron microscope observations of synthetic filaments prepared from column-purified myosin confirm the presence of a regular surface pattern of projections at ~14-nm spacing (Moos et al., 1975), similar to the subunit spacing found in natural thick filaments. Further studies of these filaments using optical diffraction indicate that the organization of the filaments is greater than might be expected from their appearance in the electron microscope, as a 43-nm axial repeat is generally observed. The 43-nm^{-1} off-meridional reflection, with a second order of 21.5 nm^{-1} and a meridional at 14.3 nm^{-1}, is characteristic of natural thick filaments, indicating that synthetic filaments, in the absence of other influences, will aggregate with the period of the natural. These results are consistent with observations of Moos et al.

The clear evidence of a higher level of organization than had previously been reported is most probably due to the different method of myosin purification. The estimated purity using the earlier precipitation-resuspension techniques is ~90%, with the remaining 10% consisting of a mixture of other thick filament proteins. Column purification removes all of these contaminants, except for B-protein, for an estimated purity of 95%. As is seen with synthetic filaments prepared with small amounts of C-protein (Koretz, 1978; Koretz, 1979), the presence of this entity is sufficient to disrupt higher levels of organization at molar ratios as low as 1 C-protein per 13 myosin molecules. Assuming that equal ratios of C-protein to myosin are preserved throughout the precipitation-resuspension method of purification, synthetic filaments prepared from this mixture are approximately composed of 1 C-protein per 6–8 myosin molecules (Morimoto and Harrington, 1973;1974).

Synthetic filaments then have a subunit organization similar to that of natural filaments: a bipolar organization and the 3.5-nm backbone striations previously observed by optical diffraction (Pepe and Dowben, 1977). However, the large variation in filament diameters, the significant percentage of fraying structures, and the broad distribution of lengths (Kaminer and Bell, 1966; Pollard, 1975) suggest that a fundamental difference exists between the two filament types in the mechanism of the aggregation process. Thus any model of synthetic filament formation must be able to explain both the similarities of subunit organization and the differences in gross appearance.

From studies of light meromyosin (LMM) paracrystal structure (Philpott and Szent-Gyorgyi, 1954; Szent-Gyorgyi, 1953; Huxley, 1963), it is apparent that a strong interaction, perhaps the strongest, is the 43-nm stagger of the molecules, which is found in LMM ribbons and many LMM tactoids. A 14-nm period can be observed in some tactoids, and has tentatively been reported for rod paracrystals (Moos et al., 1975), but this interaction will be assumed weaker than the 43-nm stagger. It is also assumed for the purpose of model-building that these two types of molecular associations are the major influences in myosin aggregation. A third influence, of unknown importance, is the heavy meromyosin (HMM) portion of the myosin molecule. Because in the absence of subfragment-1 and -2, and perhaps subfragment-1 alone, aggregation will result in formation of a paracrystalline structure, it is probable that these regions exert a steric directional effect on intermolecular orientation. It will be assumed then that this region affects the 43-nm and 14.3-nm interactions such that myosin molecules all exhibit equivalent relative orientations.

The 43-nm interaction will result in protofilaments, threads of myosin molecules eventually

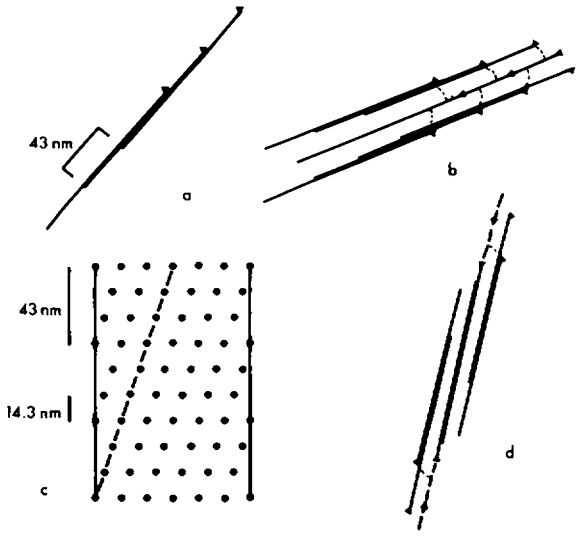

FIGURE 5 Stages in the aggregation model of synthetic myosin filaments. (*a*) The 43-nm longitudinal stagger required for proper head spacing; the striations observed on the filament backbone are consistent with the interpretation of paired myosin rods. Note that all the myosin molecules in the protofilament have the same relative orientation to each other. (*b*) The 14.3-nm staggered lateral binding necessary for creation of the filament backbone. At the same time that this side-to-side binding occurs, with some curvature assumed so that closure takes place, longitudinal addition of myosin molecules continues. (*c*) The helix net of the synthetic filament model presented here. There are 6 helices, each with 18 subunits per turn and an apparent axial repeat every 43 nm. (*d*) A possible nucleus for filament formation. With the antiparallel nucleus formed, aggregation occurs as suggested above in parallel, head-to-tail fashion.

aligning almost parallel to the filament axis, with part or all of the HMM region possibly exerting a steric restriction on rotation around the axis of the protofilament; thus, all myosin molecules in the protofilament have the same orientation (Fig. 5 *a*). The observed 3.5-nm striation would arise from the overlap of the LMM regions of two consecutive myosin molecules in the protofilament. The third myosin molecule in the protofilament binds to the second at approximately the beginning of the subfragment-2 region of the first myosin molecule, so that the protofilament consists of a series of equivalent 43-nm staggered interactions between a given molecule and its two neighbors.

The 14.3-nm interaction, a side-by-side force, acts to bind the protofilaments together (Fig. 3 *b*), and the steric restrictions already presumed cause the growing sheet to curve rather than lie flat. The heads lie on the outer, convex side of the sheet and the light meromyosin portion of the molecules lie on the other side, interacting longitudinally along the protofilament axis, laterally within the sheet, and eventually radially within the synthetic filament backbone. These radial interactions within the backbone, in conjunction with the sheer bulk of myosin tails that must be packed into this portion of the filament, preclude the possibility of a significantly sized hollow core in well-formed filaments.

If the lateral interactions resulting from the 14.3-nm binding force are strong enough and restrictive enough, closure will occur when a specific number of protofilaments have been added to the sheet. For the conditions assumed, this means that synthetic filaments consist of $3n$ protofilaments, where $n = 2, 3, \ldots$ If variation in filament diameter is due to collapse on

the grid, then true filament diameter is at the lower end of the distribution and n is probably equal to 2. (6 myosin molecules per subunit level as shown in Fig. 5 *c* give a loosely packed backbone of ~19 nm.) If the 14.3-nm interactions are not strong enough to force closure, then n can be >2, which alternatively explains larger filament widths.

When synthetic filaments are prepared by rapid dilution to low ionic strength, the structures formed are short and bipolar, consisting mainly of a bare zone with a bunch of myosin heads on either end. It is likely that the seed structure for the longer filaments is some small number of myosin molecules binding in antiparallel fashion and associated with each other by means of the 14.3-nm interaction (Fig. 5 *d*). As molecules add to this structure by the mechanism described earlier, the initial bare region is obscured and eventually disappears. Filament length will be a function of the number of these seed structures formed and their strength and stability relative to other possible myosin-myosin interactions.

Fraying filaments can be explained in terms of this model in a straightforward fashion. Closure will not occur if the number of protofilaments is not equal to $3n$, because the stagger between the two edges is not 14.3 nm. Alternatively, closure might occur but the boundary would consist of a weaker or more distorted set of bonds than if the stagger were 14.3 nm. Finally, reaching the critical concentration—the equilibrium between aggregates and monomers for given protein and salt concentrations—would result in mismatched edges in the regions of monomer addition; at this point no further polymerization occurs, leaving an incompletely stabilized region. Application of filaments containing nonstabilized or poorly stabilized areas to an electron microscope grid would result in collapse on the grid. It is then probable, if this explanation is correct, that the frayed areas are flat myosin aggregates of one or two layers that lack the radial stabilization of the well-stained regions.

The model of myosin aggregation is also consistent with, and explains, the 28.6-nm^{-1} meridional reflection often visible in optical diffraction patterns of these filaments. The reflection is attributed to the occasional nonsystematic absence of a subunit level. If addition of myosin molecules is occurring both laterally and longitudinally, then it is possible for closure to occur laterally at a certain point before all six molecules of a given subunit layer have been added longitudinally. It is also possible that this closure might prevent more than one layer from being formed, but the mask function of the optical diffraction patterns prevents resolution of meridional reflections closer to the equator.

Synthetic myosin filaments differ from natural filaments in protein composition, length, diameter, and gross appearance. However, they have in common several factors: bipolarity, backbone striations, and most important, subunit periodicity. The latter in particular enables synthetic filaments to be used as an in vitro model system for the study of thick filament protein interactions. In the current work, results about subunit periodicity confirm the importance of interactions found with myosin fragments for myosin itself. Further research on the effects of other thick filament proteins on these interactions is in progress, and some results have been reported for C-protein (Koretz, 1978; 1979).

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