Structure and Polymerization of *Acanthamoeba* Myosin-II Filaments

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**ABSTRACT** *Acanthamoeba* myosin-II forms filaments of two different sizes. Thin bipolar filaments 7 nm wide and 200 nm long consist of 16 myosin-II molecules. Thick bipolar filaments of variable width (14-19 nm) consist of 40 or more myosin-II molecules. Both have a central bare zone 90 nm long and myosin heads projecting laterally at the ends. The heads are arranged in rows spaced 15 nm apart. In the case of the thin myosin-II filaments there are two molecules per row. The thick filaments are formed rapidly and reversibly in the presence of 6-10 mM MgCl₂ (or any of five other different divalent cations tested) by the lateral aggregation of thin myosin-II filaments. Acid pH also favors thick filament formation. Neither the myosin-II concentration (50-1,000 µg/ml) nor ATP has an effect on the morphology of the filaments. The polymerization mechanism was studied quantitatively by measuring the amount of polymer formed (*Cₚ*) under various conditions as a function of total myosin-II concentration (*G*). Above a critical concentration of 15-40 µg/ml, *Cₚ* was proportional to *G* with a slope of 0.5-0.95 depending on conditions. In the range of 0.8-4.9 heavy chain phosphates per molecule, phosphorylation has no effect on the morphology of either the thin or thick myosin-II filaments and only a small effect on the extent of polymerization.

The polymerization of myosin into bipolar filaments has long been studied as a classic example of macromolecular self-assembly (17, 19) and has recently attracted new interest for two reasons. First, recent evidence that light chain (23, 32, 34) and heavy chain (21) phosphorylation can modify polymerization has led to speculation that filament assembly and disassembly might contribute to the control of motile force generation, especially in nonmuscle cells. Second, electron microscopy of nonmuscle cells has failed to demonstrate myosin filaments even with the aid of ferritin-labeled antibodies (5, 36). Although the apparent absence of myosin filaments could be the result of fixation problems, this observation suggests that the myosin of nonmuscle cells is not permanently in the form of filaments.

One approach to learning how a cell regulates myosin polymerization, is to characterize the mechanism by which purified myosin forms filaments under well-defined conditions. Elegant quantitative studies of the polymerization of striated muscle myosin (9, 10, 19) and other cytoplasmic myosins (3, 16, 26, 27, 32) have established that under the conditions in which quantitative measurements can be made by sedimentation velocity ultracentrifugation (alkaline pH and relatively high ionic strength), skeletal muscle myosin forms filaments by a condensation-polymerization mechanism like actin and flagellin. Above a critical concentration a constant monomer concentration is in equilibrium with any concentration of polymer. The critical concentration depends on the salt concentration, pH, and hydrostatic pressure. Smooth muscle myosin polymerization is sensitive to the same solution conditions, but it does not exhibit a critical concentration and the concentration of monomer varies with total protein concentration (25). The theoretical basis of this behavior is not yet understood. Previous studies of cytoplasmic myosin polymerization have emphasized filament structure and simple quantitative analysis of the ionic conditions which support polymerization (3, 4, 16, 23, 26, 27, 32). It is not known, for example, whether cytoplasmic myosins use a condensation polymerization or some other mechanism of assembly.

Of the various cytoplasmic myosins one might use in assembly studies, *Acanthamoeba* myosin-II has some advantages. More is known about its physical properties (29) immunochemistry and enzymatic activity (6-8, 12, 13, 20, 24) than most other cytoplasmic myosins. It consists of two 175,000 molecular weight heavy chains and three or four light chains. It has two heads and a tail ~90 nm long. Compared with other myosins, the tail is shorter by one-third and the heavy chains are correspondingly smaller. Myosin-II can be isolated with 1-3 heavy chain phosphates per molecule and the actin-activated ATPase activity is inversely proportional to the extent of
phosphorylation (6–8). A partially purified kinase will add heavy chain phosphates at three different sites up to a total of six phosphates per molecule (8). All of these phosphates are thought to be located within 5 nm of the end of the myosin-II tail (5).

Myosin-II forms very small bipolar filaments consisting of ~12–16 molecules (29). These filaments are unique because the bare zone is only 100 nm long like the myosin-II tail. All other myosin filaments have bare zones 150 nm long. In the first study of myosin-II filaments it was noted that the filaments were much thicker under some conditions, such as 10 mM CaCl₂ (29). Furthermore, Collins and Korn (7) showed that MgCl₂ in the range of 6–7 mM increased the sedimentation rate of very low (<20 μg/ml) concentrations of both phosphorylated and partially dephosphorylated myosin-II.

The very small size of the myosin-II filaments suggests that the assembly mechanism might be more amenable to quantitative analysis than that of larger myosin filaments. For example, muscle myosin polymerization can only be studied quantitatively under rather restricted, nonphysiological conditions in which the filaments are relatively small.

The goals of this study were to define the structure of myosin-II filaments by electron microscopy and to study their mechanism of assembly looking for possible effects of heavy chain phosphorylation. Myosin-II forms thin bipolar filaments composed of 16 molecules with the heads arranged in rows 15 nm apart and anchored to the backbone by a very short segment of the tail. These thin filaments can rapidly and reversibly aggregate laterally to form thicker filaments. The critical concentration for myosin-II polymerization is much lower than for skeletal muscle myosin due, perhaps, to the relatively minor effect on the polymerization process.

**RESULTS**

Like other myosins, the size of myosin-II filaments depends on the solution conditions. Generally the filaments fall into one of two extreme classes: thin bipolar filaments composed of ~16 molecules and thick filaments that are formed rapidly and reversibly by the lateral aggregation of the thin filaments. I will first describe the structure of the two major filament types and then, quantitative studies of the polymerization reaction and a detailed survey of how solution conditions affect filament structure. The experiments were done with myosin-II with 0.8–4.9 heavy chain phosphates per molecule to evaluate the effect of phosphorylation on filament formation. This is the range of phosphorylation that affects the actin-activated myosin-II ATPase (6).

**Structure of Myosin-II Filaments**

The size of the myosin-II molecule (Fig. 1b) has been re-examined using the replicas from the previous study (29), but with special care to obtain the absolute length of the tail. This was done by recording micrographs of the molecules and a tropomyosin paracrystal standard without changing lens current. The tail lengths had a Gaussian distribution (Fig. 1g) with a mean of 86 nm (SD 12 nm).

In 20 mM KCl at pH 6.8 myosin-II filaments are bipolar with a central bare zone like other myosin filaments, but they are remarkable because they are exceedingly small and, like other cytoplasmic myosin filaments, very uniform in size (Fig. 1; Table I). The overall length is ~200 nm. The bare zone measures ~6 nm wide in its center (Fig. 2; Table I). Although the variation in the measured width is small (SD 15%), this is probably an underestimate of the true width due to stain piling up around these thin myosin-II filaments. The mean length of the bare zone (the distance between the first globular projections on either side) is ~90 nm and the variation in this dimension is considerably larger than the other two dimensions (SD 20%). Globular material, presumably myosin heads, is present at both ends of the filaments. In many cases the heads are clumped together, but in well stained specimens the heads are occasionally spread well enough to be resolved. Up to 12 heads have been counted at one end. Because each molecule has two heads, the thin myosin-II filaments must be composed of at least 12 myosin-II molecules. In the best cases (Fig. 1c) the two heads of individual myosin molecules can be seen attached to the filament backbone by stubby projections ~10 nm long. There are one or two myosin-II molecules visible at any longitudinal position along the backbone. The spacing of these rows of heads is not regular, but is on the order of 15–20 nm.

In 10 mM MgCl₂, 20 mM KCl at pH 6.8 myosin-II filaments are much thicker than in KCl alone (Figs. 2 and 3). Like the thin myosin-II filaments, most of these thick filaments are bipolar with a central bare zone and globular projections at both ends. Compared with the thin myosin-II filaments, the thick myosin-II filaments are much more variable in size and shape (Figs. 2 and 3; Table I). Their mean length is ~250–300 nm. The bare zone is ~90 nm long and ~15 nm wide with a...
TABLE I  
Effect of Solution Conditions and Heavy Chain Phosphorylation on Myosin-II Filament Dimensions

| Conditions          | Bare zone width, mean (SD), nm |
|---------------------|-------------------------------|
|                     | $P_o = 0.8$                   |
| 20 mM KCl, pH 6.8   | 5.8 (0.5)                     |
| 20 mM KCl, 10 mM MgCl$_2$, pH 6.8 | 17.5 (3.4)                 |
| 100 mM KCl, 10 mM MgCl$_2$, pH 6.8 | 9.6 (2.7)                  |
| 20 mM KCl, 0.1 mM EGTA, 0.5 mM ATP, pH 6.8 | 6.5 (0.9)                  |
| 20 mM KCl, 10 mM MgCl$_2$, 0.1 mM EGTA, 0.5 mM ATP, pH 6.8 | 16.7 (4.5)                |
|                     | $P_o = 2.6$                   |
|                     | 5.8 (0.6)                     |
|                     | 15.8 (3.2)                    |
|                     | 8.9 (3.2)                     |
|                     | 6.9 (1.2)                     |
|                     | 14.0 (4.5)                    |
|                     | $P_o = 4.9$                   |
|                     | 6.5 (0.8)                     |
|                     | 15.2 (2.7)                    |
|                     | 9.9 (2.7)                     |

$P_o$ is phosphate content in moles per mole.

**FIGURE 1**  
Electron micrographs of a myosin-II molecule and thin filaments. (a, c-f) Negatively stained filaments formed in 20 mM KCl, 10 mM imidazole, pH 6.8 alone (a), with 1 mM EDTA (c) or with 1 mM MgCl$_2$ (d-f). Individual myosin heads are marked with arrowheads in c-f. (b) A shadowed myosin-II molecule for comparison with the filaments. Note that the shadow gives the illusion that the tail is thicker than in reality (2 nm). (g) Histograms showing the size distribution of the myosin-II tail length, thin filament bare zone length and thin filament total length. Phosphorylation: (a, e, and f) 1.5 phosphates per molecule; (c, d) 0.8 phosphates per molecule. a, x 105,000; b-f, x 155,000.

**FIGURE 2**  
Histogram of the size distributions of the myosin-II filament bare zone widths measured on electron micrographs of negatively stained specimens. Phosphorylation: (A and C) 0.8 phosphates per molecule; (B and D) 2.6 phosphates per molecule. Buffer composition: (A and B) 20 mM KCl, 10 mM imidazole, pH 6.8; (C and D) 20 mM KCl, 10 mM MgCl$_2$, 10 mM imidazole, pH 6.8.

well spread and stained it is impossible to resolve all of the myosin heads at the ends of these thick filaments, but up to 35 have been counted at one end. Therefore, the thick myosin-II filaments must be composed of at least 35 myosin molecules.

More than half of the thick myosin-II filaments have a tightly-packed bare zone with parallel sides and discrete ends like that illustrated in Fig. 3 c, but many of these thick filaments are not as well formed as illustrated in Fig. 3 d-i. In many cases the bare zone is skewed and runs diagonally across the filament (Fig. 3 e and g). The bare zone defects are generally oriented longitudinally and include splits near one end (Fig. 3 d), clefts in the center (Fig. 3 h) and apparent fragmentation into thinner filaments (Fig. 3 i). These defects suggested that the thick filaments might be composed of smaller subunits about the size of the thin myosin-II filaments.

Cross sections of thick myosin-II filaments in pellets which were embedded and sectioned showed that the bare zone profiles are rounded. Some of the thickest filaments stained irregularly, suggesting a subunit structure, but details of the substructure have not yet been resolved.

The possibility that the thick myosin-II filaments are formed by lateral aggregation of thin myosin-II filaments was tested in two ways. First, thin myosin-II filaments were formed in 20 mM KCl at pH 6.8 and then 10 mM MgCl$_2$ was added. After
FIGURE 3 Electron micrographs of negatively stained myosin-II thick filaments formed in 20 mM KCl, 10 mM MgCl₂, 10 mM imidazole, pH 6.8. Phosphorylation: (a–i) 0.8 phosphates per molecule; (j and k) 2.6 phosphates per molecule. The 15-nm periodicity of the side arms is indicated in (j) and (k). a and b, × 105,000; c–h, × 155,000.

60 s in 10 mM MgCl₂ the thin myosin-II filaments were converted quantitatively into thick filaments with the same size, shape, and range of longitudinal defects found in filaments formed by dialysis of myosin-II in 0.5 M KCl (±10 mM MgCl₂) against 20 mM KCl, 10 mM MgCl₂ at pH 6.8 for 5–60 min (Fig. 4a). Second, thick myosin-II filaments formed in 20 mM KCl, 10 mM MgCl₂ at pH 6.8 were adsorbed to electron microscope grids and then washed on the grid with 20 mM KCl, 0.2 mM EDTA. Within 5 s the thick filaments start to break up longitudinally into thin bipolar thin filaments (Fig. 4b). This fragmentation is complete within 15 s, although the heads remain aggregated, holding the thin myosin-II filaments in loose bundles. Together, these experiments indicate that the thick filaments are formed rapidly and reversibly by lateral aggregation of thin filaments.

Quantitative Analysis of the Polymerization Process

The extent of polymerization was measured by sedimentation in an Airfuge. At 23 psi (105,000 g average) myosin-II monomers in 0.5 M KCl sediment out of the upper 60% of the sample at a rate of 0.4% per minute. This corresponds to a sedimentation coefficient of about 6S as expected for myosin-II (29). Both myosin-II thin filaments (in 20 mM KCl) and thick filaments (in 20 mM KCl, 10 mM MgCl₂, are cleared from the top 60% of the airfuge tube in <15 min, leaving a slowly sedimenting species, presumably monomer or dimer, in the supernatant. Thus, during the 15-min centrifugation used to assay polymerization, all of the polymer and ~6% of the slowly sedimenting species are removed from the supernatant (monomer) fraction. No correction was made for this small monomer loss. All of the assays reported here were done using absorbance at 230 nm to measure the concentration of myosin-II in the monomer fraction, but identical results were obtained in control experiments using the Bradford (1) protein assay. Other control experiments established that there was no loss of myosin-II from the monomer fraction due to adsorption to the centrifuge tubes.

The extent of myosin-II filament formation depends on myosin-II concentration and the solution conditions (Fig. 5; Table II; following sections). No myosin-II pellets at very low total myosin concentrations (<15 µg/ml) under any of the conditions tested. At a critical concentration (which varies
FIGURE 4 Electron micrographs illustrating the interconvertibility of myosin-II thin filaments and thick filaments. (a) Thin filaments were formed in 20 mM KCl, 10 mM imidazole, pH 6.8, and then MgCl$_2$ was added to a concentration of 10 mM for 60 s before this specimen was prepared. (b) Thick filaments were formed in 20 mM KCl, 10 mM MgCl$_2$, 10 mM imidazole, pH 6.8, applied to the EM grid and then washed with three drops of 20 mM KCl, 0.2 mM EDTA, 10 mM imidazole, pH 6.8 over a period of 15 s when the grid was stained. Phosphorylation: (a) 1.5 phosphates per molecule; (b) 0.8 phosphates per molecule. $\times$ 105,000.

FIGURE 5 Dependence of myosin-II monomer (C$_m$) and polymer (C$_p$) concentrations on the total myosin-II concentration (G). Conditions: All samples had 20 mM KCl, 10 mM imidazole, pH 6.8. Some samples (O, O) also contained 10 mM MgCl$_2$. Phosphorylation: major plot 2.6 phosphates per molecule; inset 0.8 phosphates per molecule. Filled symbols, C$_m$; open symbols, C$_p$.

TABLE II

| Preparation | Conditions | Phosphorylation | Critical concentration vs. C$_m$ | Slope C$_m$ |
|-------------|------------|-----------------|---------------------------------|-------------|
| AM-58       | 20 mM KCl  | 1.5             | 30                              | 0.83        |
| AM-58       | 20 mM KCl, 10 mM MgCl$_2$ | 1.5             | 35                              | 1.08        |
| AM-60       | 20 mM KCl  | 2.6             | 24, 32                          | 0.83        |
| AM-60       | 20 mM KCl  | 0.8             | 19                              | 0.63        |
| AM-60       | 20 mM KCl, 10 mM MgCl$_2$ | 2.6             | 14, 18, 15                      | 0.96, 0.93  |
| AM-60       | 20 mM KCl, 10 mM MgCl$_2$ | 0.8             | 18                              | 0.91        |
| AM-60       | 20 mM KCl, 10 mM MgCl$_2$ | 2.6             | 20, 15                          | 0.73, 0.71  |
| AM-60       | 20 mM KCl, 10 mM MgCl$_2$ | 0.8             | 29                              | 0.53        |
| AM-60       | 20 mM KCl, 10 mM MgCl$_2$ | 2.6             | 26                              | 0.52        |
| AM-64       | 20 mM KCl  | 4.9             | 16                              | 0.83        |
| AM-64       | 20 mM KCl, 10 mM MgCl$_2$ | 4.9             | <5                              | 1.0         |

Effect of Solution Conditions on Filament Formation

DIVALENT CATIONS: In the absence of divalent cations (1 mM EDTA) in 20 mM KCl at pH 6.8 myosin-II forms thin filaments indistinguishable in size and shape from those in 20 mM KCl without EDTA (Fig. 1c) so that traces of divalent cations are not required for polymerization. MgCl$_2$ in the millimolar range causes a concentration-dependent aggregation of thin myosin-II filaments (Fig. 1) into thick filaments (Fig. 3). This can be assessed by both electron microscopy and a 5-min sedimentation assay (Fig. 6), because the thick filaments sediment somewhat more rapidly than the thin filaments. In 1 mM MgCl$_2$ (Fig. 1d and f) the filaments are identical to those in 1 mM EDTA. The transition from thin to thick filaments is smooth and has a midpoint at 4-5 mM MgCl$_2$. At these intermediate concentrations of MgCl$_2$ the filaments are heterogeneous in size, as indicated by the large standard deviations in bare zone width (Fig. 6). Many are typical thin filaments, some of which are loosely associated in lateral pairs as if caught in the act of aggregating, but some of the filaments are intermediate in size between thin and thick filaments.

All of the divalent cations tested (Ca$^{++}$, Mn$^{++}$, Co$^{++}$, Cu$^{++}$, Zn$^{++}$) at a concentration of 10 mM gave thick filaments very similar to those formed in 10 mM MgCl$_2$. In particular, the mean bare width was 14-18 nm and there were longitudinal clefts in many bare zones. In the two different preparations polymerized in 10 mM CaCl$_2$ the myosin-II heads were polyphosphorylated.
clumped and ordered enough to give a 16-nm axial periodicity (Fig. 7a). In single preparations, none of the other divalent cations gave this periodicity.

**Salt Concentration**: Concentrations of KCl >20 mM inhibit both the extent of myosin-II polymerization and the divalent-cation-induced aggregation of thin myosin-II filaments to form thick filaments. The dependence of polymer concentration on KCl concentration is very similar in the presence of 10 mM MgCl₂ (Fig. 8) or in its absence (not illustrated). Between 20 and 150 mM KCl the polymer concentration falls and at or above 200 mM KCl there are no filaments detectable by electron microscopy. In the absence of MgCl₂, this inhibition of polymerization by KCl takes place without any effect on the size or shape of the filaments that do form (Fig. 9). Thus, up to the point at which no filaments form, the salt concentration has no obvious effect on thin filament structure. In 10 mM MgCl₂ at pH 6.8 normal thick filaments form in 20-80 mM KCl. In 100 mM KCl, 10 mM MgCl₂ there is a high proportion of thin filaments and the thick filaments present are thinner than those at lower KCl concentrations. In 140 mM KCl, 10 mM MgCl₂ only thin filaments form. As in the absence of MgCl₂, no filaments form in KCl concentrations 200 mM (Fig. 9).

In the case of muscle myosin at pH 8.0-8.5 (18, 19) inhibition of polymerization, as observed here by KCl, is caused by an increase in the critical concentration. In contrast, with myosin-II the critical concentration is very low even in 140 mM KCl and varies to only a small extent as a function of KCl concentration (Fig. 10; Table II). Instead the KCl concentration has a large effect on the fraction of myosin-II polymerized above this relatively constant critical concentration. This gives plots of polymer concentration (C_p) vs total myosin concentration (C_t) with slopes <1 (Fig. 10) under inhibitory conditions. The slope of a C_p vs. C_t plot is inversely proportional to the KCl concentration (Table II). The large fraction of myosin-II in the 140 mM KCl supernatant is not due to small filaments that sediment slowly. After 10 min of ultracentrifugation (which pellets the filaments) the myosin-II in the supernatants sedi-

**FIGURE 6** Dependence of myosin-II filament size on the concentration of MgCl₂. Size was measured indirectly by a 5-min centrifugation in a Beckman Airfuge (Beckman Instruments, Inc.) which pellets all of the thick filaments and about half of the skin filaments (O). Bare zone width (mean ± SD) was measured directly from electron micrographs. Phosphorylation: 1.5 phosphates per molecule.

**FIGURE 7** Electron micrographs of myosin-II filaments formed in 20 mM KCl, 10 mM CaCl₂, 10 mM imidazole, pH 6.8. Phosphorylation: (a) 2.6 phosphates per molecule; (b) 0.8 phosphates per molecule. The 15-nm periodicity of the clumped heads is indicated in (a). × 105,000.

**FIGURE 8** Inhibition of myosin-II polymerization by KCl. Polymer concentration was measured by pelleting. The percent polymer was calculated after subtracting the critical concentration in 20 mM KCl (25 μg/ml) from the total myosin-II concentration (100 μg/ml), such that 100% is the polymer concentration in 20 mM KCl. In addition to variable concentrations of KCl the solutions contained 10 mM MgCl₂, 10 mM imidazole, pH 6.8. Phosphorylation: (O), 0.8 phosphates per molecule; (●) 2.6 phosphates per molecule; (□) 4.9 phosphates per molecule. Values plotted are means of three separate determinations.

**FIGURE 9** Effect of KCl concentration on myosin-II filament structure. Plot of mean bare zone width (±1 SD) as a function of KCl concentration. Conditions: 10 mM imidazole, pH 6.8 with (●) varying KCl alone or (O) varying KCl with 10 mM MgCl₂. Phosphorylation: 1.5 phosphates per molecule.
ments no faster than monomer and no filaments are detectable in the negatively stained samples. The fraction of unpolymerized myosin-II in the 140 mM KCl, 10 mM MgCl₂ (pH 6.8) supernatant is competent to polymerize and forms normal thick filaments after 30 min dialysis vs. 20 mM KCl, 10 mM MgCl₂ at pH 6.8.

KCl is the only salt examined in any detail, but inhibition of polymerization is not limited to KCl. Sodium pyrophosphate inhibits myosin-II polymerization at concentrations about 10 times lower than KCl, just as it does with muscle myosin (14).

**pH:** At pH 8 myosin-II forms typical thin myosin-II filaments (Fig. 11a and c). At pH 6 myosin-II forms thick filaments (mean width, 16.2 nm, SD 6.7 nm), which have an even stronger tendency to aggregate in clumps (Fig. 11b) than in 10 mM CaCl₂. Many of these filaments have the heads clumped in rows at 15-nm intervals.

**ATP:** The presence of 0.5 mM ATP has no effect on the morphology of myosin-II filaments. In 20 mM KCl, typical thin myosin-II filaments form in the absence of MgCl₂ (Fig. 11a) and typical thick filaments form in the presence of 10 mM MgCl₂ (Fig. 12b).

**Effect of Heavy Chain Phosphorylation on Myosin-II Polymerization**

Myosin-II has three phosphorylation sites on each heavy chain, all located near the C-terminal end of the polypeptide within 4–5 nm of the tip of the tail (5, 8). My preparations isolated by the method of Pollard et al. (29) had 1.5–2.6 moles phosphate per mole of myosin. After dephosphorylation with potato acid phosphatase, the phosphate content was reduced to 0.8 moles per mole myosin. Dephosphorylation increased the Ca²⁺ ATPase-specific activity about twofold. After dephosphorylation the actin-activated Mg²⁺ ATPase (0.2 mg/ml Acanthamoeba actin, 7.5 mM MgCl₂, 0.5 mM ATP, 0.1 mM CaCl₂, 6 mM KCl, 5 mM imidazole, pH 7, at 29°C) was 240 mmol. min⁻¹. mg⁻¹. In one preparation actin and myosin-II were precipitated by dialysis of the DEAE fractions against 5 mM MgCl₂, 0.25 mM dithiothreitol, 20 mM imidazole, pH 7, instead of the usual hexokinase-glucose treatment. This yielded myosin-II with 4.9 phosphates per mole.

In the range of 0.8–4.9 phosphates per myosin-II, the extent of phosphorylation had no effect on the size of the filaments formed at pH 6.8 in 20 mM or 100 mM KCl, ±10 mM MgCl₂, ±0.5 mM ATP (Table I, Fig. 2). Furthermore, myosin-II with 2.6 or 0.8 phosphates per molecule produced similar filaments over the pH range of 6–8 and in 10 mM concentrations of the various divalent cations tested. The only morphological differences noted were a much stronger tendency of the phosphorylated myosin-II thick filaments to aggregate end to end (compare Fig. 7a and b) and for their heads to clump near the filament backbone (Fig. 3j and k). In 10 mM divalent cations, dephosphorylated myosin-II thick filaments were aggregated on only one out of seven grids examined, and the myosin heads were almost always well spread from the backbone regardless of the solution conditions (Figs. 3a–i, 7b, 12b). In contrast, thick filaments made from myosin-II which had not been treated with acid phosphatase were aggregated end to end on 9 out of 11 grids.

In the quantitative assays for polymerization, extent of phosphorylation had only small effects on the critical concentration (Table II). When compared directly in the same experiment, dephosphorylated myosin-II was slightly more sensitive to
inhibition of polymerization by high KCl concentrations in 10 mM MgCl₂ (Figs. 8 and 10; Table II). For example in Fig. 10, 80 mM KCl inhibited the polymerization of myosin-II with 0.8 phosphates as much as 140 mM KCl with 2.6 phosphates. Likewise, the slope of Cₚ vs. Cᵢ plots in 20 mM KCl (Fig. 5; Table II) was less for dephosphorylated than phosphorylated myosin-II. On the other hand, there is variation in the slopes of these plots from one preparation to the next and my most highly phosphorylated sample (4.9 phosphates per molecule) behaved much like the dephosphorylated sample from another preparation (Fig. 8).

**DISCUSSION**

**Structure of Thin Myosin-II Filaments**

Electron micrographs of thin myosin-II filaments showing the central bare zone and globular projections at both ends clearly indicate that the myosin-II molecules are in a bipolar, antiparallel arrangement like other myosin filaments. Furthermore, these micrographs also contain enough information to determine the number of myosin molecules and their arrangement, at least in two dimensions.

The dimensions of the myosin-II molecule and the thin myosin-II filament put substantial constraints on the number of molecules in the filaments. First, the filaments are slightly more than twice as long as the tail of the molecule, so that except for the terminal myosins at each end, the tails of all of the myosins must overlap in the center of the bare zone. Consequently, the maximum number of molecules in a thin myosin-II filament is established by the diameter of the bare zone. The observed diameter of 6–7 nm in the center of the bare zone is large enough to accommodate ~12 myosin tails in a hexagonal array with center to center spacing of 2 nm and no central hole (27). Second, the heads at the two ends of the thick filaments are arranged in rows spaced at intervals of 15–16 nm (Figs. 3j and k, 7a, 11b). I assume that the same is true of the thin filaments. Given the length of the filament (200 nm) and bare zone (90 nm), there are four rows of heads at 15-nm intervals at each end (Fig. 13). The difficult question is, how many molecules are there in each row? There could be one, giving a total of eight molecules per thin filament. This model is inconsistent with three observations: (a) the bare zone diameter of an 8-molecule filament would be only 5.2 nm; (b) there are up to 12 globular projections at one end (Fig. 1); and (c) two Y-shaped projections are seen directly in some rows (Fig. 1c and f). On the other hand, these observations are consistent with four rows of two molecules each at both ends (Fig. 13). Such a filament should have a bare zone diameter of 7.8 nm, which is well within the experimental error of the measurements of bare zone width, which probably underestimate the true diameter due to shrinkage and piling up of stain. If there were three or four molecules per row the bare zone would have a diameter of 9.6 or 11 nm, which is well beyond the estimate made by electron microscopy. Consequently, the electron micrographs support best a model for the thin filament with 16 myosin-II molecules arranged as shown in Fig. 13a.

In the model of the thin myosin-II filaments the tails of the myosin-II molecules flanking the bare zone extend fully across the bare zone and those in the fourth rows come close to touching end to end, but do not overlap, in the middle of the bare zone. Consequently there are 14 tails in the center of the bare zone and 10 at the edges of the bare zones, accounting for the slight taper in the bare zone diameter observed in the electron micrographs. The model is drawn in two dimensions because nothing is yet known about the (presumably helical) arrangement of the molecules. Given the plane of symmetry in the center of the bare zone and the presumed twofold axial symmetry, there are only four unique myosin positions in these simple filaments. The exploded view (13 b) illustrates the proposed longitudinal stagger of the 10 myosin-II molecules, but is not meant to indicate anything regarding the lateral associations about which nothing is known. This exploded view makes it clear that the myosin-II thin filaments cannot be composed of parallel dimers with a 43 nm stagger, which are thought to be the building blocks of muscle myosin filaments (2, 11). Note also that except for the four myosin-II molecules flanking the bare zone, the heads are not located near the ends of any of the
tails. Consequently, the heavy chain phosphorylation sites at the end of the tail (5) would not appear to be in a position to interact directly with heads and to influence the ATPase activity when the myosin-II is polymerized.

It is interesting that the skeletal muscle myosin "mini-filaments" which form in 10 mM citrate/Tris buffer at pH 8.0 are also composed of 16 myosin molecules (30). The arrangement of the 16 amoeba or muscle myosin molecules in these small filaments is probably an especially stable configuration with limited potential for further elongation.

The micrographs of the filaments also give some indication about the segmental flexibility of the myosin-II molecule. Many well preserved myosin-II filaments have clearly resolved side arms, some of which are Y-shaped. Presumably the arms of the Y are the heads, whereas the base of the Y is comparable to muscle myosin subfragment-2, which links the heads to the light meromyosin backbone of muscle thick filaments (22).

Many of these side arms are perpendicular to the backbone, so at least after drying there can be a 90° bend in the myosin-II tail at the point where the side arm bends away from the backbone.

Compared with the subfragment-2 region of skeletal muscle myosin, which is ~50 nm long (22) and allows the heads to spread up to 60 nm from the filament backbone (28, 35) the corresponding region of the myosin-II tail is quite short. The myosin-II side arms project only 20 nm from the backbone. Given that the heads are ~10 nm long the linker region is no more than 10 nm long. This conclusion is consistent with direct observation of the linker region of individually resolved side arms which is also <10 nm long (Fig. 1). This suggests that a major part of the difference in the length of the tails of myosin-II and muscle myosin could be due to the almost complete absence in myosin-II of a region comparable to skeletal muscle myosin subfragment-2. If the subfragment-2 region of the myosin-II tail is <10 nm long, the remaining part of the myosin-II tail is exactly the same length as muscle myosin light meromyosin (80 nm; reference 22). The idea that the myosin-II tail is all or mostly equivalent to muscle light meromyosin is supported circumstantially by two independent observations. First, the rows of myosin-II molecules in the backbone of the filament are staggered by 15 nm. In muscle thick filaments the 15-nm spacing is determined by the properties of light meromyosin. Second, there are no sensitive proteolytic cleavage sites within the tail of the myosin-II molecule (5). In muscle myosin tail there are, of course, two such sensitive sites, located at the two ends of the subfragment-2 region (22).

**Structure of Thick Myosin-II Filaments**

The thick myosin-II filaments are much more complex than the thin filaments. So far it has been impossible to ascertain their structure by electron microscopy. Their diameter is variable and many are imperfectly formed. The longitudinal clefs in the defective thick filaments and the demonstration that thick and thin filaments are interconvertible in a few seconds by simple changes in the ionic conditions, both argue that the thick filaments are formed by the lateral aggregation of thin filaments. The diameter of the thick filaments is large enough to accommodate 3-6 thin filaments, but unfortunately subunits have not yet been resolved in cross sections of the thick filaments, so that the number and packing of the constituent thin filaments is unknown.

Accepting the interpretation that the thick filaments are composed of thin filaments, there are two observations that suggest that the thin filaments comprising the thick filaments are not in perfect longitudinal register, but rather are staggered longitudinally by multiples of 15 nm. First, many of the thick filaments have skewed bare zones or bare zones with indistinct margins (Fig. 3). Second, the overall length of the thick filaments (300 nm) is 50% greater than the thin filaments. In spite of this longitudinal displacement of the thin filaments, the rows of myosin heads must be in register, because many thick filaments (especially in 10 mM CaCl₂ and at pH 6) have the heads clumped in rows with a 15-nm spacing (Fig. 7a).

**Mechanism of Polymerization**

Myosin polymerization probably involves a number of discrete reversible steps including myosin dimerization, filament nucleation and elongation (10). In the case of myosin-II there is also lateral aggregation of thin filaments to form thick filaments. A quantitative description of the polymerization mechanism will require identification of the reaction pathway and measurement of the kinetic constants for each step. In no case has this been completed, but for muscle myosin Davis (10) has used pressure-jump experiments to show that elongation is very fast, probably diffusion-limited, and that dissociation rates vary by a factor of 500 depending on filament length. Thus polymer length is controlled kinetically by the dissociation reaction.

At equilibrium myosin polymerization systems consist of subunits, which are actually a monomer-dimer equilibrium, and polymer which, in the case of skeletal muscle myosin minifilaments (31) and the myosin-II filaments described, here have a very discrete size distribution. The absence of species intermediate in size between subunits and the polymer demonstrates the high cooperativity of the muscle and *Acanthamoeba* myosin polymerization process.

In the original quantitative analysis of muscle myosin polymerization, Josephs and Harrington (18) introduced as a simplifying assumption, that at equilibrium a skeletal muscle myosin polymerization system consists of only monomer and polymer.

\[
\text{n - monomers} \leftrightarrow \text{polymer}
\]

\[
K = \frac{C_p}{C_m}
\]

\(K\) is the equilibrium constant, \(C_p\) is the polymer concentration, \(C_m\) is the monomer concentration and \(n\) is the number of subunits in the polymer. The dependence of \(C_p\) on total myosin concentration, \(C_t\), is determined by the \(n\) and \(K\). For many values of \(n\) and \(K\) there is a critical concentration below which there is essentially no polymer and above which \(C_m\) is approximately constant. Model calculations show that when \(n\) is small the critical concentration is low and relatively independent of \(K\). When \(K\) is large the monomer-polymer transition around the critical concentration is sharp and the slope of \(C_p\) vs. \(C_t\) is 1. When \(K\) is small the transition from monomer to polymer is smooth and at low \(C_p\), the plot of \(C_p\) vs. \(C_t\) is curved and has a slope <1.

This model must be oversimplified judging from the new work of Davis (10), but under a variety of conditions the behavior of muscle myosin agrees well with the model (18, 31). Over the limited range of concentrations tested *Acanthamoeba* myosin-II polymerization is also consistent with this model. The critical concentration for myosin-II polymerization is one or two orders of magnitude lower than for muscle myosin. This difference may be attributable, at least in part, to the small size of the myosin-II filaments rather than the myosin-II having a
large polymerization equilibrium constant. On the contrary, the fact that plots of $C_p$ vs. $C_t$ are <1, under many conditions (Table II), suggests that the myosin-II equilibrium constant is relatively small, although the data are not yet extensive enough to calculate meaningful values for the equilibrium constant. Smooth muscle myosin polymerization differs from both skeletal muscle and Acanthamoeba myosin-II in having neither a critical concentration nor a linear dependence of $C_p$ on $C_t$ (25). In every case more detailed analysis recognizing the various intermediates in the myosin polymerization reaction will be required to understand the polymerization mechanism. The myosin-II polymerization reactions may be particularly amenable to more detailed analysis, because there are only four unique positions in the filaments and therefore a limited number of reactions and intermediates.

**Form of Myosin-II in the Cell**

Because the concentration of myosin-II in the cell is ~1 mg/ml (29) and the salt concentration is thought to be low, the properties of myosin-II suggest that it should be almost completely polymerized in the cell. The exact cytoplasmic ionic conditions are not known, but need to be established before further conclusions can be drawn. In addition it will be necessary to test for inhibitors of myosin-II polymerization in the cell. Regardless of cytoplasmic conditions and the extent of cytosolic polymerization, it will be very difficult to identify cytoplasmic myosin-II filaments by electron microscopy of thin sections, because the filaments are so small. I hope that ultrastructural localization with highly specific new monoclonal antibodies (20) will help answer the questions about the form of myosin-II in the cell.

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