

The Effect of Heavy Chain Phosphorylation and Solution Conditions on the Assembly of *Acanthamoeba* Myosin-II

John H. Sinard and Thomas D. Pollard

The Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. At low ionic strength, *Acanthamoeba* myosin-II polymerizes into bipolar minifilaments, consisting of eight molecules, that scatter about three times as much light as monomers. With this light scattering assay, we show that the critical concentration for assembly in 50-mM KCl is <5 nM. Phosphorylation of the myosin heavy chain over the range of 0.7 to 3.7 P per molecule has no effect on its KCl dependent assembly properties: the structure of the filaments, the extent of assembly, and the critical concentration for assembly are the same. Sucrose at a concentration above a few percent inhibits polymerization. Millimolar concentrations of MgCl₂ induce the lateral aggregation of fully formed minifilaments into thick filaments.

Compared with dephosphorylated minifilaments, minifilaments of phosphorylated myosin have a lower tendency to aggregate laterally and require higher concentrations of MgCl₂ for maximal light scattering. Acidic pH also induces lateral aggregation, whereas basic pH leads to depolymerization of the myosin-II minifilaments. Under polymerizing conditions, millimolar concentrations of ATP only slightly decrease the light scattering of either phosphorylated or dephosphorylated myosin-II. Barring further modulation of assembly by unknown proteins, both phosphorylated and dephosphorylated myosin-II are expected to be in the form of minifilaments under the ionic conditions existing within *Acanthamoeba*.

A detailed understanding of the factors affecting the steady-state assembly of nonmuscle myosin-II is an essential prerequisite to deducing the mechanism and regulation of myosin assembly in nonmuscle cells. Myosin-II is the class of myosins with two heads at one end of an alpha-helical coiled-coil tail. These myosins were discovered in muscle but are now known to exist in many, if not all, eukaryotic cells (for review, see Korn and Hammer, 1988). Under physiological conditions, myosin-II polymerizes into bipolar filaments, thereby allowing it to generate force between two antiparallel actin filaments. In skeletal muscle the myosin-II is assembled into stable filaments, but there is some evidence (Yumura and Fukui, 1985) that the assembly of cytoplasmic myosin-II is much more dynamic.

In vitro assembly of various myosin-II's has led to the identification of two major types of filaments: synthetic filaments and minifilaments, the latter also being synthetic but smaller in size. Both the structure and the critical concentration for assembly of skeletal muscle myosin synthetic filaments depend upon the solution conditions during the assembly process (Josephs and Harrington, 1966). Skeletal muscle myosin minifilaments (Reisler et al., 1980) are more homogeneous in structure and have a very low critical concentration for assembly. Addition of KCl transforms minifilaments into the larger synthetic filaments (Reisler et al., 1982), suggesting that minifilaments may comprise part of the core of

the myosin filament. Smooth muscle myosin also forms minifilaments (Trybus and Lowey, 1985), which upon addition of KCl grow to form larger "side polar" filaments (Trybus and Lowey, 1987).

The bipolar filaments formed by *Acanthamoeba* myosin-II at neutral pH and in the absence of Mg⁺⁺ (Pollard et al., 1978) can also be classified as minifilaments. Previously termed "thin filaments" (Pollard, 1982), these minifilaments are bipolar, ~230 nm long, and are composed of eight myosin-II molecules each (Sinard et al., 1989). The structure is very reminiscent of the minifilaments formed by both skeletal muscle myosin and smooth muscle myosin in dilute alkaline buffers. In millimolar concentrations of divalent cations or acidic pH, *Acanthamoeba* myosin-II minifilaments aggregate laterally to form thicker filaments (Pollard, 1982), but do not form longer filaments under any known conditions. These features make *Acanthamoeba* myosin-II ideal for studying the interactions between the molecules in the bare zone without the interference of changes resulting from filament elongation.

Phosphorylation of several smooth muscle and nonmuscle myosin-II's influences their assembly and actin-activated Mg-ATPase. Phosphorylation of the light chains of smooth muscle and vertebrate nonmuscle myosin-II's increases the stability of filaments in the presence of ATP (Onishi et al., 1978; Suzuki et al., 1978) as well as their actin-activated Mg-ATPase (see Adelstein and Eisenberg, 1980). In *Dictyostelium*, phosphorylation of the heavy chain of myosin-II inhibits

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both its ATPase activity and its ability to form filaments (Kuczmarzski and Spudich, 1980). In both cases, phosphorylation may regulate ATPase activity indirectly by controlling the state of assembly.

In *Acanthamoeba*, both dephosphorylation and assembly into filaments seem to be necessary for maximal actin-activated Mg-ATPase activity, but phosphorylation and assembly do not appear to be directly related. Although there is no evidence for light chain phosphorylation, *Acanthamoeba* myosin-II is phosphorylated on the heavy chain in vivo to a maximum of two phosphates per heavy chain (four phosphates per myosin molecule) (Collins and Korn, 1980), and at least one additional site per heavy chain can be phosphorylated in vitro (Cote et al., 1981). The three phosphorylation sites are located in a nonhelical domain (Cote et al., 1984) of 27 amino acids at the tip of the tail (Collins et al., 1982a). Both dephosphorylated and phosphorylated myosin-II forms minifilaments at low ionic strength (Pollard, 1982; Kuznicki et al., 1983), but only the dephosphorylated myosin minifilaments have actin-activated Mg-ATPase (Kuznicki et al., 1983). Although not sufficient, filament formation is necessary for this enzymatic activity since a monoclonal antibody that binds near the tip of the tail concomitantly depolymerizes the filaments and inhibits the actin-activated ATPase (Kiehart and Pollard, 1984). Experiments with heteropolymers (Kuznicki et al., 1983) suggest that phosphorylation at the tip of the tail regulates the ATPase of other molecules within the filament (Atkinson and Korn, 1987). The actin-activated ATPase activity within the cell may thus be regulated independently both by phosphorylation and assembly into filaments.

To understand better the factors governing the assembly of *Acanthamoeba* myosin-II, we have used a sensitive 90° light scattering assay to quantitate not only the magnitude of the effect of phosphorylation and various solution conditions on the assembly process, but also, in several cases, to elucidate the mechanism of that effect. In the accompanying paper (Sinard et al., 1989), we manipulate these conditions to determine the mechanism of minifilament assembly.

Materials and Methods

Myosin Preparation/Dephosphorylation

Sucrose and KCl were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ), and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). *Acanthamoeba castellanii* were grown axenically in aerated 15-liter carboys as described (Pollard and Korn, 1973) except that cells were aerated at a much greater rate increasing the yield to nearly 500 gm packed cells per carboy. Myosin-II was purified by the method of Pollard et al. (1978), except that 5% sucrose was included in the gel filtration buffer to stabilize the protein. Typically, 75–150 mg purified myosin-II was obtained from two carboys. Myosin-II prepared in this way contained three to four phosphates per molecule, as determined by a malachite green assay on ashed samples (Stull and Buss, 1977).

Purified myosin-II at ~5 mg/ml in 25 mM Hepes, pH 8.0, 150 mM KCl, 20% sucrose, 2 mM MgCl₂, 1 mM DTT, 0.5 mM PMSF was dephosphorylated for 3 h at 25°C with 3–5 U of *Escherichia coli* alkaline phosphatase (Sigma Chemical Co.) per milligram of myosin-II. The reaction was stopped by the addition of KCl to 0.5 M and the alkaline phosphatase was removed by gel filtration on Biogel A-15m (Bio-Rad Laboratories, Cambridge, MA). Dephosphorylated myosin-II had 0.6–1.2 phosphates per molecule.

Myosin-II that was gel filtered only once contained a small amount of contaminant. This contaminant voids on the gel filtration column, but when

present in large enough amounts overlaps the myosin peak. Although the contaminant does not absorb significantly at 280 nm, it scatters a tremendous amount of light at 365 nm. Gel filtering the myosin a second time removes the overlapping contaminant. Therefore, even myosin which was not dephosphorylated was gel filtered twice. The chemical nature of the contaminant is not known. It absorbs light equally at 260 nm and 280 nm, does not stain with Coomassie blue or silver on electrophoretic gels, is not significantly extractable by ether, and does not appear to contain organic phosphates.

Myosin-II was stored at 4°C in 10 mM imidazole, pH 7.5, 45% sucrose, 1 mM DTT, 0.02% Na-Azide, and all experiments were performed within 3 wk after the cells were harvested.

Light Scattering Assay

90° light scattering, measured in a fluorescence spectrophotometer (model 650-10S; Perkin Elmer Corp., Norwalk, CT), was used as a sensitive assay for myosin-II polymerization. Excitation and emission wavelengths were both set to 365 nm, and slit widths of 2 and 5 nm, respectively, were used. Samples were prepared by dilution from stock solutions. All buffers were filtered through a 0.22- μ m filter (Sartorius Ministart filter; Vanguard International, Neptune, NJ). Samples typically contained ~1% sucrose resulting from the sucrose in the myosin storage buffer, so an equal concentration of sucrose was included in the blanks. One milliliter samples, prepared in tubes siliconized with dimethyldichlorosilane (Sigma Chemical Co.), were read in a 1 × 1 × 4.5-cm quartz cuvette. Since dust and other particles lead to intermittent noise in the readings, samples were monitored for at least 1 min, and the lowest value read during that period was used. Light scattering intensities are reported in arbitrary units, but absolute values of scattering intensities were reproducible to within 5% from preparation to preparation of myosin-II.

Myosin-II minifilaments scatter about three times as much light as an equal concentration of myosin-II monomers. The signal is maximal at 365 nm (data not shown). Theoretically, there should be no maximum for pure elastic scattering from dimensionless particles, which is an inverse function of the fourth power of the wavelength. The drop in scattering intensity below 365 nm is probably due to decreased light scattering as the wavelength of the light approaches the radius of gyration of the particles and to the somewhat decreased sensitivity of the optics of the fluorescence spectrophotometer at low wavelengths.

Electron Microscopy

For rotary shadowing, samples diluted with an equal volume of glycerol were sprayed onto freshly cleaved mica and dried under vacuum at 23°C. Samples were then rotary shadowed with platinum at an angle of 6° in a Balzers freeze fracture apparatus, followed by evaporation of carbon to stabilize the replicas. For negative staining, samples were applied to glow-discharged carbon films and stained with 1% uranyl acetate. Grids were viewed in a Zeiss EM-10 electron microscope with an accelerating voltage of 80 kV. Micrographs were taken at a magnification of 25,000–50,000, calibrated with tropomyosin paracrystals.

Results

The Light Scattering of Myosin-II Depends Upon the Concentration of Salt

There is a good correlation between the light scattering intensity (Fig. 1 *a*) and the assembly state of the myosin-II (Fig. 1, *b* and *c*) over a range of KCl concentrations. The results are identical for NaCl (data not shown). Above 200 mM KCl, myosin-II is monomeric (Fig. 1 *c*); below 100 mM KCl, myosin-II polymerizes into 230-nm-long bipolar minifilaments (Figs. 1 *b*, and 4 *a*). The intermediate level of light scattering between 100 and 200 mM KCl arises from incompletely assembled minifilaments. These are characterized in the accompanying paper (Sinard et al., 1989).

At very low ionic strength, there is another drop in the light scattering intensity (Fig. 1 *a*). As visualized by electron microscopy, the myosin appears to be in the form of full

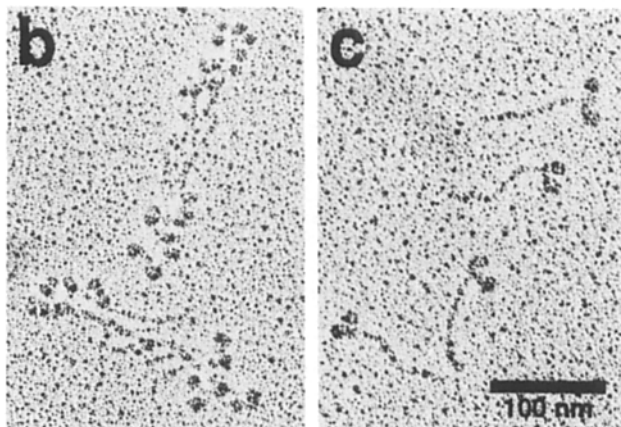
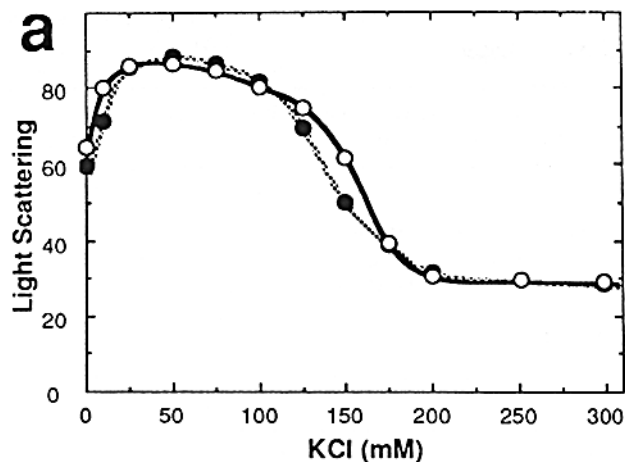


Figure 1. Salt dependence of the light scattering of phosphorylated and dephosphorylated myosin-II. (a) Light scattering of a 100- $\mu\text{g/ml}$ solution of myosin-II in 8.5 mM imidazole, pH 7.0, 1.4% sucrose, and KCl concentrations as indicated. (○) 0.9 phosphates per myosin molecule; (●) 3.7 phosphates per myosin molecule. (b) Electron micrograph of rotary shadowed dephosphorylated myosin-II minifilaments in 25 mM KCl. (c) Electron micrograph of rotary shadowed myosin-II monomers in 300 mM KCl.

length minifilaments indistinguishable from those seen for myosin in 50 mM KCl.

In Low Salt, Myosin-II Assembles with a Critical Concentration Less Than 5 nM

In both 50 and 300 mM KCl, the light scattering intensity is directly proportional to the myosin-II concentration (Fig. 2). These plots are linear out to at least 1 mg/ml (2.5 μM) myosin-II (data not shown). This indicates that at each of these salt concentrations, a homogeneous, non-self-interacting species is present in solution. The slopes of the plots gives some indication of the relative "size" (mass and dimensions) of the species. The linearity of the plot for myosin-II in 50 mM KCl indicates that the minifilament assembly equilibrium is not significantly concentration dependent over this range of myosin-II concentrations. In addition, since the 50-mM KCl plot intersects the plot for high salt (monomers) at approximately the origin, the critical concentration for the assembly of myosin-II minifilaments is $<2 \mu\text{g/ml}$ ($\sim 5 \text{ nM}$).

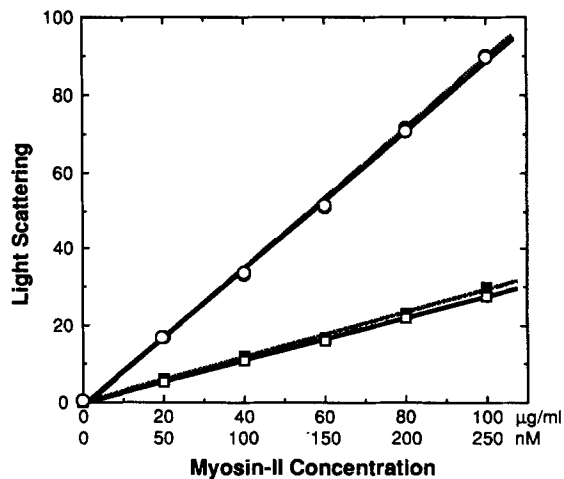


Figure 2. Dependence of light scattering of myosin-II minifilaments and monomers on the protein concentration. Conditions: 8.5 mM imidazole, pH 7.0 1.4% sucrose, and either (○, ●) 50 mM KCl or (□, ■) 300 mM KCl. (○, □) 0.9 phosphates per myosin-II molecule; (●, ■) 3.7 phosphates per myosin-II molecule.

Phosphorylation Does Not Alter the KCl-dependent Assembly Properties of Myosin-II

Heavy chain phosphorylation over the range of 0.6 to 3.7 phosphates per molecule has no effect on the extent of minifilament assembly as a function of the myosin-II concentration nor on the critical concentration for assembly (Fig. 2). The only difference seen is that minifilaments of fully phosphorylated myosin-II depolymerize at a KCl concentration ~ 10 –25 mM lower than minifilaments formed from dephosphorylated myosin-II (Fig. 1 a). The intensity of light scattering of phosphorylated myosin is also slightly lower at 0 mM KCl.

Sucrose Inhibits the Assembly of Myosin-II

Sucrose causes a concentration dependent decrease in the intensity of myosin II light scattering at low salt and a decrease in KCl concentration necessary to depolymerize myosin minifilaments (data not shown). In 50 mM KCl for all sucrose concentrations tested, plots of light scattering vs. myosin concentration are linear and pass through the origin, but have slopes which decrease with increasing sucrose concentration (data not shown). For example, the slope in 20% sucrose is half of that in 1% sucrose. These results suggest that sucrose inhibits the extent of myosin polymerization and decreases the stability of the polymers.

Mg⁺⁺ Induces Lateral Aggregation of Myosin-II Minifilaments

Millimolar concentrations of Mg^{++} significantly alter the light scattering of myosin-II in a complex manner dependent on the concentration of KCl (Fig. 3 a). Above 100 mM KCl, MgCl_2 has little effect on the light scattering of myosin (Fig. 3 a, inset, and Fig. 3 c). Below 100 mM KCl, Mg^{++} increases the equilibrium light scattering of myosin-II. The Mg^{++} concentration required for maximal effect varies with the concentration of KCl. Additional Mg^{++} then decreases the light scattering (Fig. 3 b). The high light scattering in

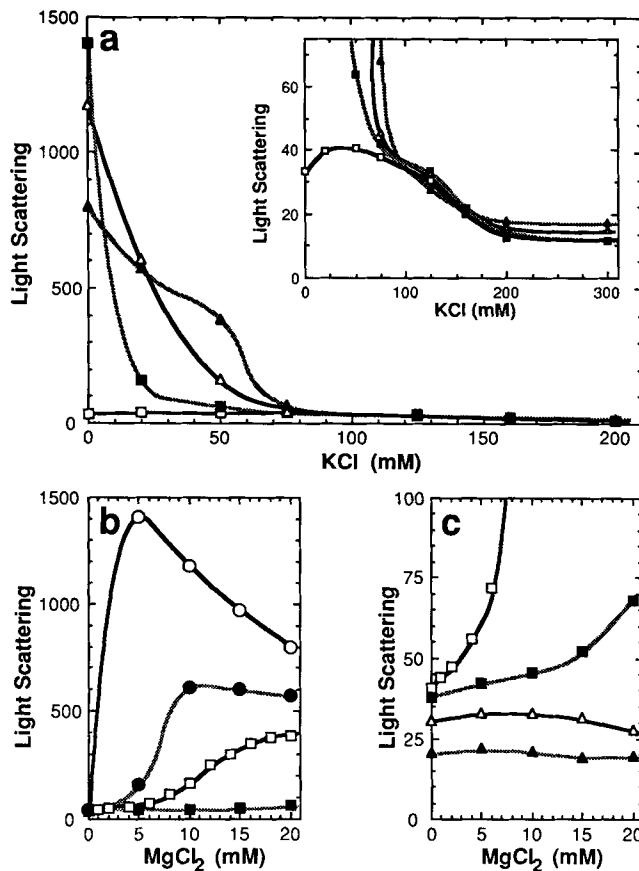


Figure 3. Effect of $MgCl_2$ and KCl on myosin-II assembly. Light scattering intensities are shown for $50 \mu g/ml$ dephosphorylated myosin-II (0.6 phosphates per molecule) in 8.5 mM imidazole, $pH 7.0$, 0.7% sucrose, and ionic conditions as follows. (a) KCl concentration as indicated; (\square) 0 mM $MgCl_2$, (\blacksquare) 5 mM $MgCl_2$, (\triangle) 10 mM $MgCl_2$, (\blacktriangle) 20 mM $MgCl_2$. (b and c) $MgCl_2$ concentrations as indicated; (\circ) 0 mM KCl , (\bullet) 20 mM KCl , (\square) 50 mM KCl , (\blacksquare) 75 mM KCl , (\triangle) 125 mM KCl , (\blacktriangle) 160 mM KCl .

Mg^{++} correlates with the conversion of minifilaments (Fig. 4 a) to "thick filaments" (Fig. 4 b; also see Pollard, 1982). The thick filaments are very heterogeneous in width, are roughly bipolar, and have a length which is one to approximately one and a half times the length of the minifilaments. These structures are believed to represent laterally aggregated minifilaments.

Under a wide variety of conditions, phosphorylation of myosin-II partially inhibits the Mg^{++} induced increase in the light scattering (Fig. 5 a). As with dephosphorylated myosin-II, the effects of Mg^{++} decrease in magnitude as the KCl concentration is increased, and are not seen above 100 mM KCl . At $pH 6.2$, less $MgCl_2$ is needed to induce minifilament aggregation, and the peak light scattering obtained is higher than at neutral pH (Fig. 5 b). Heavy chain phosphorylation has less of an effect on the equilibrium light scattering of the myosin-II as a function of the Mg^{++} concentration at this lower pH than was seen at neutral pH .

Acidic pH also Induces Aggregation of Myosin-II Minifilaments. Basic pH Leads to Depolymerization of the Minifilaments

The light scattering of myosin-II in 50 mM KCl , 0 mM

$MgCl_2$ depends on pH , with high scattering at acidic pH and low scattering at high pH (Fig. 5 c). By electron microscopy the minifilaments aggregate at acidic pH 's, as in Mg^{++} , and depolymerize at high pH (Pollard, 1982; Sinard et al., 1989). Dephosphorylated myosin is slightly more sensitive to aggregation than phosphorylated myosin, whereas phosphorylated myosin is slightly more sensitive than dephosphorylated myosin to the depolymerization induced by basic pH (Fig. 5 c). As with Mg^{++} , the effect of pH is enhanced at lower ionic strength and diminished at higher ionic strengths. pH has no effect on the light scattering of either phosphorylated or dephosphorylated myosin-II in high salt where it is monomeric (data not shown).

ATP Does Not Significantly Alter the Stability of Minifilaments Formed from Either Phosphorylated or Dephosphorylated Myosin-II

Millimolar concentrations of ATP decrease the light scattering of myosin-II minifilaments only slightly beyond that expected from the ionic strength of the ATP. Phosphorylated myosin-II minifilaments are slightly more sensitive to this ATP-induced depolymerization than dephosphorylated myosin-II; addition of 5 mM ATP reduces the light scattering of phosphorylated and dephosphorylated myosin-II minifilaments by 38% and 27% , respectively. Mg -ATP has a similar effect except that it reduces the light scattering only half as much as an equivalent concentration of ATP without Mg^{++} (data not shown).

Discussion

Advantages of the Light Scattering Assay

Light scattering is a very sensitive assay for myosin polymerization since concentrations as low as $10 \mu g/ml$ give very reliable and reproducible signals. The change in the scattering intensity of myosin as a function of its assembly state allows

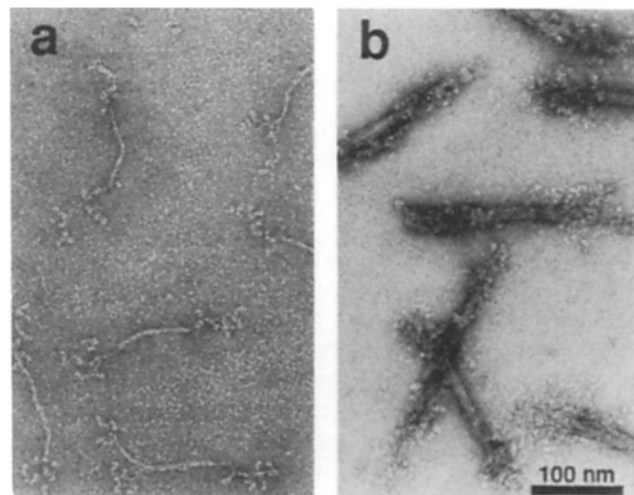


Figure 4. Electron micrographs of myosin-II negatively stained with 1% uranyl acetate, showing the lateral aggregation of minifilaments by $MgCl_2$. (a) Myosin-II in 10 mM imidazole, $pH 7.0$, 50 mM KCl . (b) Myosin-II in 10 mM imidazole, $pH 7.0$, 50 mM KCl , and 10 mM $MgCl_2$.

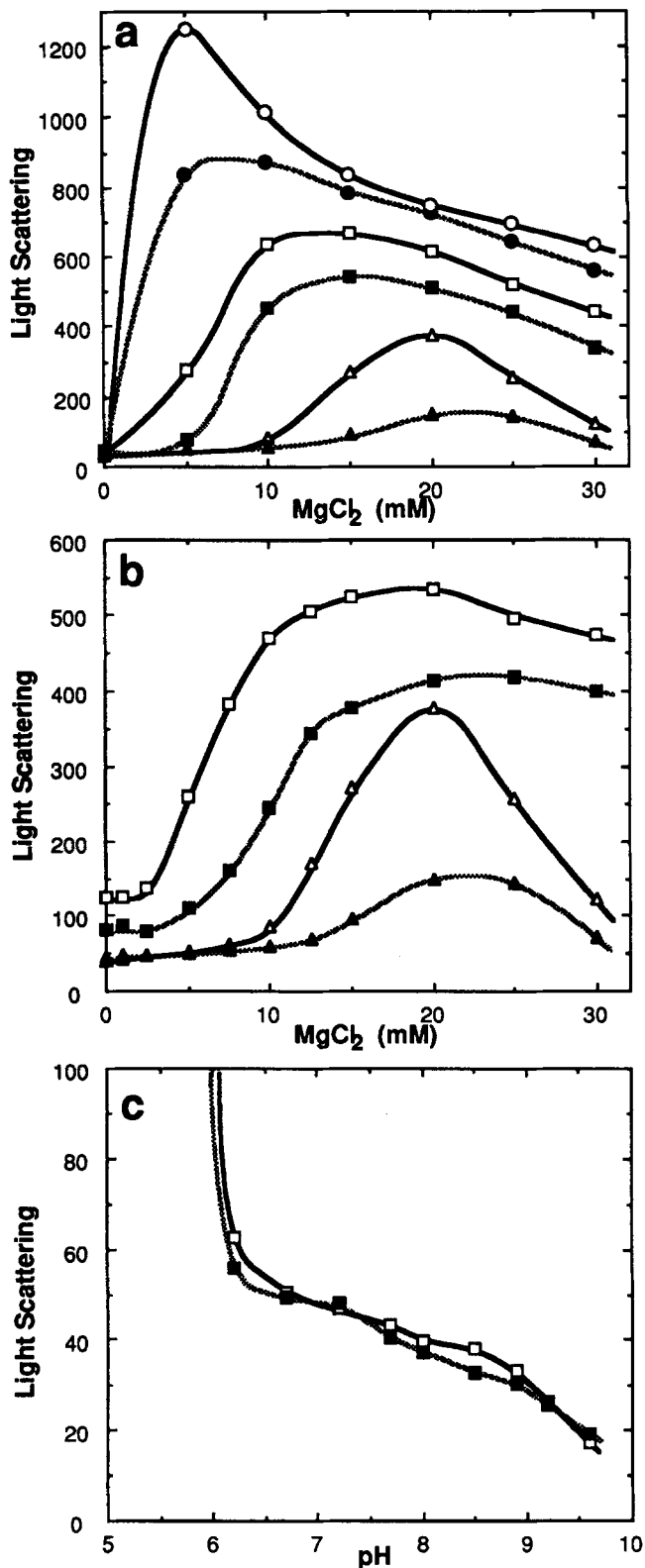


Figure 5. Effect of phosphorylation, MgCl₂, KCl, and pH on myosin-II assembly. All experiments were done at 50 μ g/ml myosin-II, 0.7% sucrose, and other conditions as follows. Throughout, open symbols (\circ , \square , Δ) represent dephosphorylated myosin-II (0.9 phosphates per molecule) and solid symbols (\bullet , \blacksquare , \blacktriangle) indicate phosphorylated myosin-II (3.7 phosphates per molecule). pH values represent the pH of the actual samples and not necessarily of the stock buffers used. (a) 9 mM imidazole, pH 7.0. MgCl₂ concentrations as indicated. KCl concentrations: (\circ , \bullet) 0 mM KCl,

a sensitive, quantitative study of factors affecting myosin assembly. Although the correlation between the light scattering signal and the state of assembly observed by electron microscopy is good, the threefold difference in light scattering between *Acanthamoeba* myosin-II minifilaments and monomers is somewhat less than expected since minifilaments have eight times the mass of monomers (Sinard et al., 1989). The discrepancy is probably attributable to destructive interference of 365 nm light scattered by the two ends of the 230 nm minifilaments, resulting in a lower intensity of light scattering than would be seen if all of the mass were concentrated in a smaller volume.

Light scattering has many advantages over the traditional pelleting method as an assay for polymerization of myosin. For the particular case of *Acanthamoeba* myosin-II, pelleting is a poor assay since the 6S monomers (Pollard et al., 1978) and the \sim 22S minifilaments (Kuznicki et al., 1983; Sinard et al., 1989) are impossible to separate cleanly by pelleting. In general, however, light scattering gives information about the system without disturbing it with a centrifugal field, thus avoiding artifacts which may be introduced by pressure, or by disturbing a system in equilibrium by separating out one or more of the components of that equilibrium. Light scattering also can detect small oligomers (dimers, tetramers) that may be important intermediates in the assembly process but are missed by pelleting since they are too small to be separated from, or are in equilibrium with monomers.

Effect of Solution Conditions on Minifilament Polymerization

The quantitative analysis of assembly by light scattering confirms and extends previous work (Pollard, 1982) showing that *Acanthamoeba* myosin-II, like other myosins, assembles optimally in 5-75 mM KCl. A subtle feature not revealed by pelleting is that in the absence of added salt the light scattering is lower, since the minifilaments partially disassemble into antiparallel tetramers (Sinard et al., 1989), similar to *Dictyostelium* myosin-II which forms monomers, dimers, and tetramers in zero salt (Kuczmarski et al., 1987).

The linear dependence of light scattering on myosin-II concentration and the intersection of such plots for minifilaments and monomers near the origin (Fig. 2) show that the critical concentration for polymerization of myosin-II at 50 mM KCl is very low, $<2 \mu$ g/ml or 5 nM. Similar values are reported for *Dictyostelium* myosin-II (Kuczmarski et al., 1987), skeletal muscle myosin minifilaments (Reisler et al., 1980), and vertebrate gizzard, intestinal brush border, and thymus myosins (Kendrick-Jones et al., 1987).

The inhibition of myosin-II polymerization by sucrose may explain the ability of sucrose concentrations above a few percent to inhibit the actin-activated Mg-ATPase activity of myosin-II (Collins and Korn, 1981). Since plots of light scattering vs. myosin concentration had a lower slope at high sucrose concentrations, but no change in the intercept, sucrose inhibits polymerization by reducing the size of the polymers

(\square , \blacksquare) 25 mM KCl, (Δ , \blacktriangle) 50 mM KCl. (b) 50 mM KCl. MgCl₂ as indicated. 9 mM imidazole, pH 6.2 (\square , \blacksquare) or 7.0 (Δ , \blacktriangle). (c) 50 mM KCl, 0 mM MgCl₂, and 9 mM imidazole or bicine at the indicated pH (imidazole used below pH 8.0, bicine used at pH 8.0 and above).

rather than by changing the critical concentration for assembly. The high sucrose concentrations precluded investigation by electron microscopy of the structures formed.

Aggregation of Minifilaments

The systematic variation of solution conditions has revealed for the first time that Mg^{++} induces the formation of lateral aggregates of fully formed minifilaments, but has no effect on monomers or on the assembly intermediates (see Sinard et al., 1989) present in 100–200 mM KCl at pH 7. Thus, only the complete octameric minifilaments have the properties required to form lateral associations in Mg^{++} . The biphasic dependence of the light scattering on the Mg^{++} concentration can be explained by the fact that since the $MgCl_2$ itself contributes to the ionic strength, high concentrations of Mg^{++} depolymerize the minifilaments thereby precluding aggregation. The occurrence of minifilament aggregation at low pH and the fact that less Mg^{++} is needed to induce minifilament aggregation under acidic conditions suggest that these two processes are not independent and may be the same.

At the molecular level, it is not yet clear how acidic pH and/or divalent cations can produce the same end result. The salt sensitivity of the polymerization suggests that ionic interactions mediate the assembly process. Histidine is the only amino acid side chain whose charge is significantly altered in the pH range of 6.0 to 7.0. The myosin-II tail contains only three histidines (Hammer et al., 1987), a very small number in comparison with the ~ 150 acidic and ~ 120 basic amino acids. The molecular basis of the depolymerization induced by pH > 8.5 , however, is more understandable, as this is beginning to approach the pK of lysine. This would result in a decrease in the net positive charge of these side groups and thus fewer attractive interactions between the molecules in the filament.

Effects of Heavy Chain Phosphorylation

Since phosphorylation is known to regulate the actin-activated Mg-ATPase activity of myosin-II (Collins and Korn, 1980), and since it is known that depolymerization of the myosin filaments with a monoclonal antibody can inhibit the ATPase activity of dephosphorylated myosin-II (Kiehart and Pollard, 1984), it might be expected that phosphorylation would alter the KCl dependent assembly of myosin-II. However, this is not the case. Not only are both phosphorylated and dephosphorylated myosin-II able to form minifilaments, as shown previously with less sensitive assays (pelleting and electron microscopy; Pollard, 1982), but both the critical concentration for minifilament assembly and the extent of assembly as a function of the salt concentration are not affected by phosphorylation of the myosin-II heavy chain. Minifilaments formed from phosphorylated myosin do depolymerize at slightly lower (10–15 mM) salt concentrations than dephosphorylated myosin minifilaments, undoubtedly due to the charge-charge repulsion between the two closely localized phosphates on one myosin molecule with those on other myosin molecules. This charge effect may also explain the lower light scattering intensity for phosphorylated vs. dephosphorylated myosin in 0 mM KCl, where the absence of counter ions in solution would be expected to make the charge effect more significant. Phosphorylation also has no effect on the light scattering of myosin monomers, indicating that no significant conformational change in the monomers occurs as a result of heavy chain phosphorylation.

The lack of an effect of phosphorylation on the assembly of myosin-II seen here in the absence of Mg^{++} differs from the results presented by Collins et al. (1982b) who found a significant effect of phosphorylation on the assembly of myosin-II using a pelleting assay. Our results do agree with later results from the same lab by Kuznicki et al. (1983) who showed that both phosphorylated and dephosphorylated myosin-II in 1 mM $MgCl_2$ have the same sedimentation coefficient.

Unlike the situation in *Acanthamoeba*, phosphorylation of *Dictyostelium* myosin has been shown to alter its assembly properties in the absence of Mg^{++} (Kuczmariski and Spudich, 1980; Kuczmariski et al., 1987). This may be due to the fact that *Dictyostelium* myosin-II is phosphorylated in the alpha-helical coiled-coil region of the myosin tail (Pagh et al., 1984) where it is likely to interfere with the charge-charge interactions necessary for assembly, whereas *Acanthamoeba* myosin-II is phosphorylated in the non-alpha-helical region at the tip of the tail.

Relationship between Minifilament Aggregation and Actin-activated ATPase Activity

Since heavy chain phosphorylation does have an effect on the Mg^{++} or low pH induced aggregation of *Acanthamoeba* myosin-II minifilaments, and since it has been shown that the actin activated MgATPase activity of this myosin is increased by low pH and/or higher Mg^{++} concentrations (Kuznicki and Korn, 1984), the aggregation of myosin minifilaments and the modulation of aggregation by phosphorylation must be considered as a possible mechanism for the regulation of myosin activity in the cell. However, the correlation is poor. The Mg^{++} stimulation of the actomyosin ATPase activity occurs at a lower Mg^{++} concentration (Collins and Korn, 1981) than we find necessary to cause significant minifilament aggregation. Furthermore, it remains difficult to reconcile the in vitro conditions necessary for maximal actomyosin ATPase activity with the in vivo environment. For example, even at high Mg^{++} concentrations, as little as 20 mM KCl inhibits the actomyosin Mg-ATPase activity in vitro by $> 80\%$ (Collins and Korn, 1980). Using atomic absorption spectroscopy, we have measured the soluble K and Mg concentrations in *Acanthamoeba* to be ~ 40 and 4 mM, respectively (Sinard and Pollard, 1989), and the intracellular pH has been estimated to be between 6.0 and 6.5 (Deslauriers et al., 1980). At this pH and these concentrations of KCl and Mg^{++} , there is little difference in the extent of aggregation of the phosphorylated and dephosphorylated myosin-II minifilaments. In fact, there is little aggregation of the minifilaments (Fig. 5 b). Yet, the absence of any effect of phosphorylation on minifilament polymerization, and the consistent, although poor, correlation between the actomyosin-II ATPase activity and the Mg^{++} or pH induced aggregation of myosin minifilaments suggests that not only is polymerization a prerequisite for this ATPase activity (Kiehart and Pollard, 1984) but some higher order assembly of these minifilaments may also be involved. Mg^{++} is not likely to be the inducer of this aggregation in vivo, suggesting that perhaps some other as yet uncharacterized factor is mediating this assembly in vivo which Mg^{++} or low pH mimic in vitro.

Effect of ATP on Minifilament Assembly

In contrast to *Dictyostelium* myosin-II (Kuczmariski et al., 1987), *Acanthamoeba* myosin-II minifilaments are not de-

polymerized by physiological concentrations of ATP. 5 mM ATP reduces the light scattering by only ~30%, and Mg-ATP, the expected form of ATP *in vivo*, has a smaller effect. The effect was somewhat more pronounced for phosphorylated than dephosphorylated myosin-II, but certainly not enough to explain the markedly different enzymatic activities of the two species. ATP concentrations above the physiological range are also required to depolymerize skeletal muscle myosin minifilaments (Reisler et al., 1980).

Conclusions

A large number of different factors can modulate the *in vitro* assembly of myosin-II. The *in vitro* studies described here suggest that at the ionic and pH conditions known to exist *in vivo*, virtually all of the myosin-II in *Acanthamoeba* should be in the form of minifilaments, and these minifilaments should be only minimally aggregated. This would be the case whether or not the myosin-II is phosphorylated. The small size of the minifilaments can account for why they have not been visualized by electron microscopy in the amoeba. Alternatively, there could be other as yet uncharacterized factors within the cell modulating the *in vivo* assembly state of the myosin-II. The cellular mechanisms which regulate the time and place of myosin minifilament formation are far from clear.

In contrast, smooth muscle myosin and skeletal muscle myosin are not expected to be in the form of minifilaments *in vivo*. Physiological conditions in these cells should favor the formation of much larger filamentous structures, and these larger structures have been seen within the cells. Smooth muscle myosin and cytoplasmic myosins-II share the property that the minifilaments seem to represent an assembly unit for the larger filamentous structures, whereas in skeletal muscle, although minifilaments may play a role in the nucleation of larger filaments, they are not likely to be involved in the elongation reactions which convert the bipolar core into longer filaments.

The *in vitro* studies performed to date on all of these myosins have provided a substantial amount of information about the nature of the interactions between the myosin molecules and the types of polymers that can be formed. In the accompanying paper (Sinard et al., 1989) it is shown how manipulation of the assembly conditions investigated in this manuscript have made possible the identification of potential intermediates in the minifilament assembly process. This has allowed us to propose a model for the polymerization of *Acanthamoeba* myosin-II minifilaments and therefore, potentially, for the nucleation of assembly of other myosin filaments.

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