

Effect of Heavy Chain Phosphorylation on the Polymerization and Structure of *Dictyostelium* Myosin Filaments

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Abstract. In *Dictyostelium* amebas, myosin appears to be organized into filaments that relocalize during cell division and in response to stimulation by cAMP. To better understand the regulation of myosin assembly, we have studied the polymerization properties of purified *Dictyostelium* myosin. In 150 mM KCl, the myosin remained in the supernate following centrifugation at 100,000 *g*. Rotary shadowing showed that this soluble myosin was monomeric and that ~80% of the molecules had a single bend 98 nm from the head-tail junction. In very low concentrations of KCl (<10 mM) the *Dictyostelium* myosin was also soluble at 100,000 *g*. But rather than being monomeric, most of the molecules were associated into dimers or tetramers. At pH 7.5 in 50 mM KCl, dephosphorylated myosin polymerized into filaments whereas myosin phosphorylated to a level of 0.85 mol P_i/mol heavy chain failed to form filaments. The phosphorylated myosin could be induced to form filaments by lowering the pH or by in-

creasing the magnesium concentration to 10 mM. The resulting filaments were bipolar, had blunt ends, and had a uniform length of ~0.43 μm. In contrast, filaments formed from fully dephosphorylated myosin were longer, had tapered ends, and aggregated to form very long, threadlike structures. The *Dictyostelium* myosin had a very low critical concentration for assembly of ~5 μg/ml, and this value did not appear to be affected by the level of heavy chain phosphorylation. The concentration of polymer at equilibrium, however, was significantly reduced, indicating that heavy chain phosphorylation inhibited the affinity of subunits for each other. Detailed assembly curves revealed that small changes in the concentration of KCl, magnesium, ATP, or H⁺ strongly influenced the degree of assembly. Thus, changes in both the intracellular milieu and the level of heavy chain phosphorylation may control the location and state of assembly of myosin in response to physiological stimuli.

IN both striated and smooth muscle, myosin molecules are assembled into stable filaments that slide relative to the actin-containing thin filaments to produce force. It has been suggested that nonmuscle motility involves a sliding filament mechanism, and the fact that isolated nonmuscle myosins retain the ability to self-assemble (39) strongly suggests that filaments are required for some aspects of cell motility. Indeed, Langanger et al. (25) showed that myosin was assembled into bipolar thick filaments which were distributed along the length of stress fibers, and studies by Yumura and Fukui (51) demonstrated that in *Dictyostelium* amebas myosin formed discrete structures having the dimensions of thick filaments.

Recent studies have revealed that the state of myosin assembly can directly regulate ATPase activity. For example, dephosphorylated smooth muscle (47, 48) and thymus (46) myosins do not form filaments and have low actin-activated ATPase activity. Phosphorylation of the light chain, however, induces myosin assembly concomitant with an increase in ATPase activity. The ability of *Acanthamoeba* myosin II to form filaments was inhibited by either removing a small piece from the tip of the tail (24) or by adding monoclonal antibodies (19). In both cases, filament disassembly was ac-

companied by a loss of actin activated ATPase activity. Studies with *Dictyostelium* myosin showed that monoclonal antibodies that bind to the tail inhibit force generation (10), and that the myosin must be assembled into thick filaments before cytoskeletons can contract (41).

Additional studies with *Dictyostelium* have shown that myosin filaments can relocalize within the cell (51). Filaments were restricted to the uropod of actively locomoting cells and were transiently associated with the contractile ring during cytokinesis. When starved cells were stimulated with the chemotactic molecule cAMP, the myosin filaments rapidly moved between the endoplasm and the cortical region. Such dynamic behavior suggests that there are mechanisms for controlling the assembly and translocation of myosin filaments. We have shown that the *Dictyostelium* myosin heavy chain is phosphorylated in vivo and that this phosphorylation inhibits both assembly and actin-activated ATPase activity (23). Transient changes in the intracellular concentrations of H⁺, K⁺, and other small ions are known to occur (1, 26) and this flux may influence *Dictyostelium* myosin polymerization.

To better understand the regulation of thick filament formation, the assembly of purified myosin was studied. Assembly of the *Dictyostelium* myosin was quite sensitive to

relatively small changes in pH and the concentrations of KCl, MgCl₂, and ATP. Heavy chain phosphorylation significantly suppressed filament formation under a variety of conditions and also appeared to regulate filament length.

Materials and Methods

Myosin Purification

Dictyostelium amebas were cultured in HL/5 media supplemented with glucose. The cells were harvested during late logarithmic growth (i.e., 5–8 × 10⁶ cells/ml) and myosin was purified as previously described (20) and stored on ice. Muscle myosin was prepared from rabbit back muscle (27) or chicken gizzard (2) and stored in 50% glycerol at –20°C.

Myosin Dephosphorylation

To remove phosphate from both the 18,000-D light chain and the heavy chain, a partially purified *Dictyostelium* phosphatase was used (DMP-II, see Kuczmariski and Pagone [21]). Purified *Dictyostelium* myosin in 0.2 M KCl, 0.5 mM dithiothreitol (DTT), 7.5 mM MgCl₂, 10 mM Tris-Cl, pH 7.5, was incubated with the phosphatase at a 1:30 weight ratio (phosphatase/myosin) for 2 h at 25°C. Separate experiments using in vivo-labeled myosin confirmed that these conditions removed >95% of the phosphate from both the heavy and the light chain. After dephosphorylation the myosin was separated from the phosphatase by gel filtration (1.6 × 81-cm column of Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in 0.5 M KCl, 1 mM EDTA, 1 mM DTT, 1 mM NaN₃, 10 mM triethanolamine, pH 7.5). The myosin peak was placed in a dialysis bag, concentrated with Aquacide IIa (Calbiochem-Behring Corp., San Diego, CA) and finally dialyzed against the Sephacryl column buffer.

Heavy Chain Phosphorylation

The myosin tail was phosphorylated with a heavy chain kinase purified from vegetative amebas (peak II kinase, reference 20). The reaction contained 9.0 mg of dephosphorylated myosin, 1.0 mg of kinase, 7.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM NaN₃, and 2.0 mM [³²P]ATP (100 cpm/pmol) in a final volume of 3.0 ml. After 0.5–2.5 h at 25°C, KCl was added to a final concentration of 0.5 M and the kinase was removed by gel filtration. The myosin was concentrated as described above.

Protein Determination

Protein concentrations were determined by two dye-binding assays (36, 40) with bovine serum albumin as a standard.

Assembly Assays

Unless otherwise indicated myosin, at a concentration of 1.0 mg/ml, was extensively dialyzed against 10 mM triethanolamine, 0.5 mM DTT, and 0.01% NaN₃, pH 7.5 (buffer 1) and diluted into the appropriate buffer to give a concentration of 0.2 mg/ml in a final volume of 100 μl. The myosin was allowed to assemble for 10 min at 22°C and then centrifuged in an Airfuge (Beckman Instruments, Inc., Fullerton, CA; 10 min at 23 psi, room temperature) (23). To eliminate nonspecific protein binding, the polypropylene centrifuge tubes (Robbins Scientific, Palo Alto, CA) were preincubated with 1.0 mg/ml BSA for 60 min, washed three times with 0.5 M KCl, three times with distilled water, and then air-dried overnight.

Electron Microscopy

Rotary Shadowing. Purified myosin in buffer 1 was diluted to a final concentration of 0.05 mg/ml, 50% glycerol, 5 mM triethanolamine (pH 7.5) and 0–150 mM KCl. The diluted myosin was immediately sprayed onto freshly cleaved mica, dried under vacuum, rotary shadowed at an angle of 6° with platinum, and carbon-coated (49). Images of the myosin molecules and of a calibration standard (diffraction grating replica, catalogue no. 1020, Ernest F. Fullam, Inc., Schenectady, NY) were taken at a magnification of 40,000 on a JEOL 100 CX electron microscope. Contour lengths were measured from micrographs using a digitizing pad interfaced with a personal computer.

Negative Stain. A drop of myosin solution (0.1–0.2 mg/ml) was applied to a carbon-over-Formvar copper grid. After 30 s the grid was rinsed with

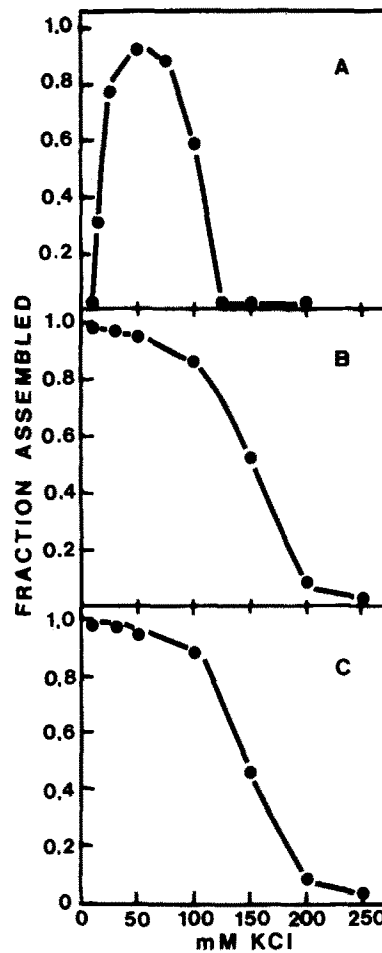


Figure 1. Effect of salt on myosin assembly. (A) *Dictyostelium* myosin, (B) rabbit skeletal muscle myosin, and (C) chicken gizzard myosin were assayed for assembly in the Airfuge in buffer 1 containing the indicated concentrations of KCl. The muscle myosins remained assembled <50 mM KCl whereas the *Dictyostelium* myosin was soluble.

four drops of the myosin buffer and stained with 1.5% aqueous uranyl acetate.

Determination of Isoelectric Point

The isoelectric point (pI) was estimated from the ability of myosin to bind to Sephadex SP ion exchange resin as a function of pH (50). Buffers ranging from pH 5.2 to 6.0 were prepared from stock solutions of 0.1 M citric acid and 0.2 M Na₂HPO₄.

Results

Effects of Salt on Assembly

Using ultracentrifugation to assay polymerization, *Dictyostelium* myosin assembled maximally in 40–60 mM KCl but was soluble at KCl concentrations <10 mM and >150 mM (Fig. 1 A). This behavior was not dependent on potassium ion, because identical curves were obtained with LiCl or NaCl. Similar assembly curves were observed with seven independent myosin preparations, and the maximum extent of salt-induced polymerization varied from 61% to 90%. This variability may reflect differences in the extent of heavy chain phosphorylation (see below). In contrast, both rabbit skeletal

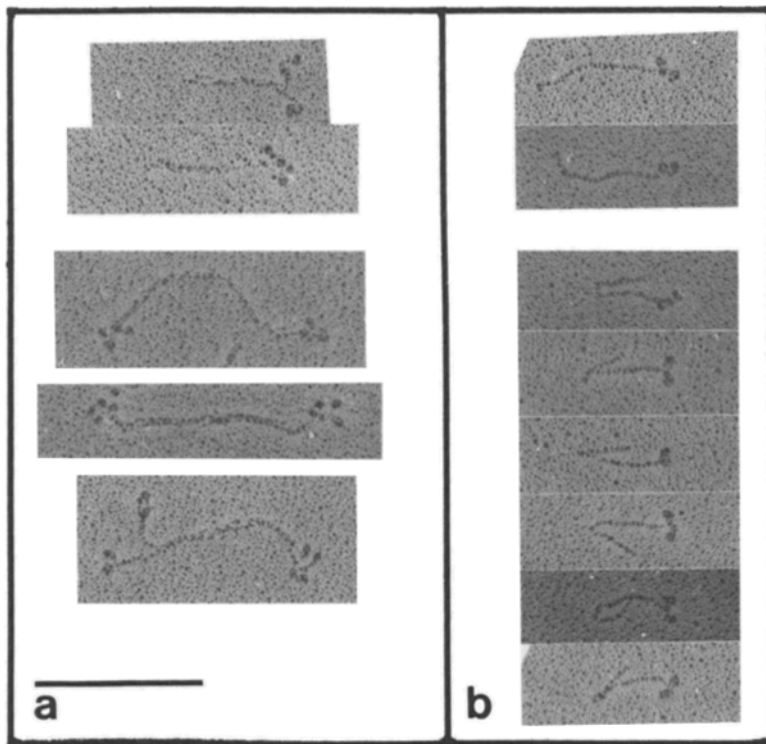


Figure 2. Effect of ionic strength on molecular interactions. Dictyostelium myosin was diluted into 5 mM triethanolamine (pH 7.5), 50% glycerol, and either no KCl or 150 mM KCl and then processed for rotary shadowing. (a) Dimers and tetramers were prevalent in the absence of KCl. (b) In 150 mM KCl only monomers were observed, and a majority of these had a sharp bend in the tail. Bar, 0.25 μ m.

myosin and chicken gizzard myosin remained partially assembled above 150 mM KCl and fully assembled in KCl concentrations as low as 10 mM (Fig. 1, B and C). Even after 48 h of dialysis against buffer 1, the muscle myosins remained assembled, and there was no preferential low ionic strength solubility over a pH range of 5.0–9.0 (data not shown).

Examination in the electron microscope revealed that at pH 7.5 in 10, 50, or 100 mM KCl the *Dictyostelium* myosin assembled into loose filamentous structures. When the soluble myosin (either no KCl or 150 mM KCl) was examined by the negative-stain technique, no macromolecular structures could be discerned. To study the conformational state under these salt conditions, the myosin was rotary shadowed with platinum. In 150 mM KCl, only monomers were present (Fig. 2 b) and $\sim 80\%$ of these had a single sharp bend ($\geq 90^\circ$) in the tail which was located 98 (± 9.6) nm from the head-tail junction ($n = 266$). In contrast, in low ionic strength the majority of the myosin molecules were assembled into dimers or tetramers (Fig. 2 a). Although most of the dimers were oriented in a parallel fashion, some antiparallel dimers were observed (see Table I). Tetramers were always bipolar and were composed of two pairs of parallel dimers associated via their tails. Thus, in the absence of other factors known to affect assembly, ionic strength was an important determinant of myosin conformation.

Effect of Heavy Chain Phosphorylation on Myosin Assembly

Consistent with our previous studies (23), removal of phosphate enhanced myosin assembly whereas heavy chain phosphorylation inhibited assembly (Fig. 3 A). Although the absolute extent of assembly varied with the degree of phosphorylation, in four separate experiments dephosphorylated

myosin had maximum assembly, phosphorylated myosin showed the least assembly, and the partially phosphorylated, untreated myosin always had an intermediate level of assembly.

Electron microscopic examination of negative-stained preparations revealed that in 50 mM KCl the dephosphorylated myosin formed filamentous structures (Fig. 4 a). No filaments were seen with the phosphorylated myosin although amorphous aggregates, which presumably represented the small amount of sedimentable material, were observed (Fig. 4 b). In high ionic strength both the dephosphorylated and phosphorylated myosins were monomeric by rotary shadowing. The level of phosphorylation had no apparent effect on myosin conformation because for both myosins $\sim 78\%$ of the molecules had a bend in the tail at the same position as illustrated in Fig. 2 b. At lower ionic strength both myosins associated into dimers and tetramers.

Effect of Magnesium and ATP on Myosin Assembly. The addition of 2 mM $MgCl_2$ strongly enhanced assembly of dephosphorylated myosin in low ionic strength, and nearly 100% of the myosin sedimented in 5–20 mM KCl (Fig. 3 B). In contrast, assembly of the fully phosphorylated myosin was only slightly enhanced. Magnesium shifted the solubility

Table I. Molecular Species in Buffer 1

Species	Percentage
Monomer	15.8
Antiparallel dimer	8.6
Parallel dimer	38.2
Tetramer	31.6
Other*	5.8

* Species which could not be clearly identified because of overlap or head-to-head interactions.

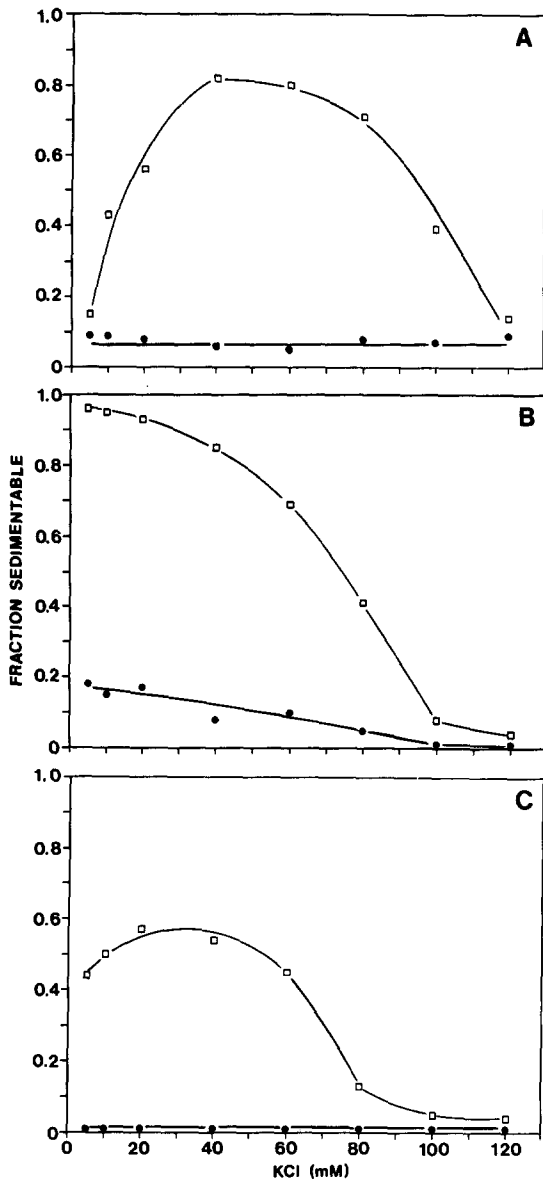


Figure 3. Heavy chain phosphorylation inhibited salt-induced assembly. Assembly was measured in the Airfuge in buffer 1 with the indicated concentrations of KCl and either (A) no magnesium, (B) 2 mM MgCl₂, or (C) 2 mM Mg-ATP. Purified *Dictyostelium* myosin was fully dephosphorylated (□) and a portion of this myosin was phosphorylated to a level of 0.85 mol P_i/mol heavy chain (●).

curve to lower KCl concentrations. For example, in the absence of magnesium ~100 mM KCl was required for half-maximal disassembly (Fig. 3 A), while only ~75 mM KCl was required in the presence of magnesium (Fig. 3 B). This was a consistent finding and was seen with three separate myosin preparations. In 2 mM MgCl₂ the dephosphorylated myosin formed discrete, tapered thick filaments (Fig. 4 c), while the small amount of phosphorylated myosin that did assemble contained loose filamentous strands similar to those illustrated in Fig. 4 a.

In the presence of 2 mM ATP or sodium pyrophosphate, neither myosin preparation was sedimentable at KCl concentrations ranging from 0 to 150 mM and no filaments were seen in the electron microscope (data not shown). If both

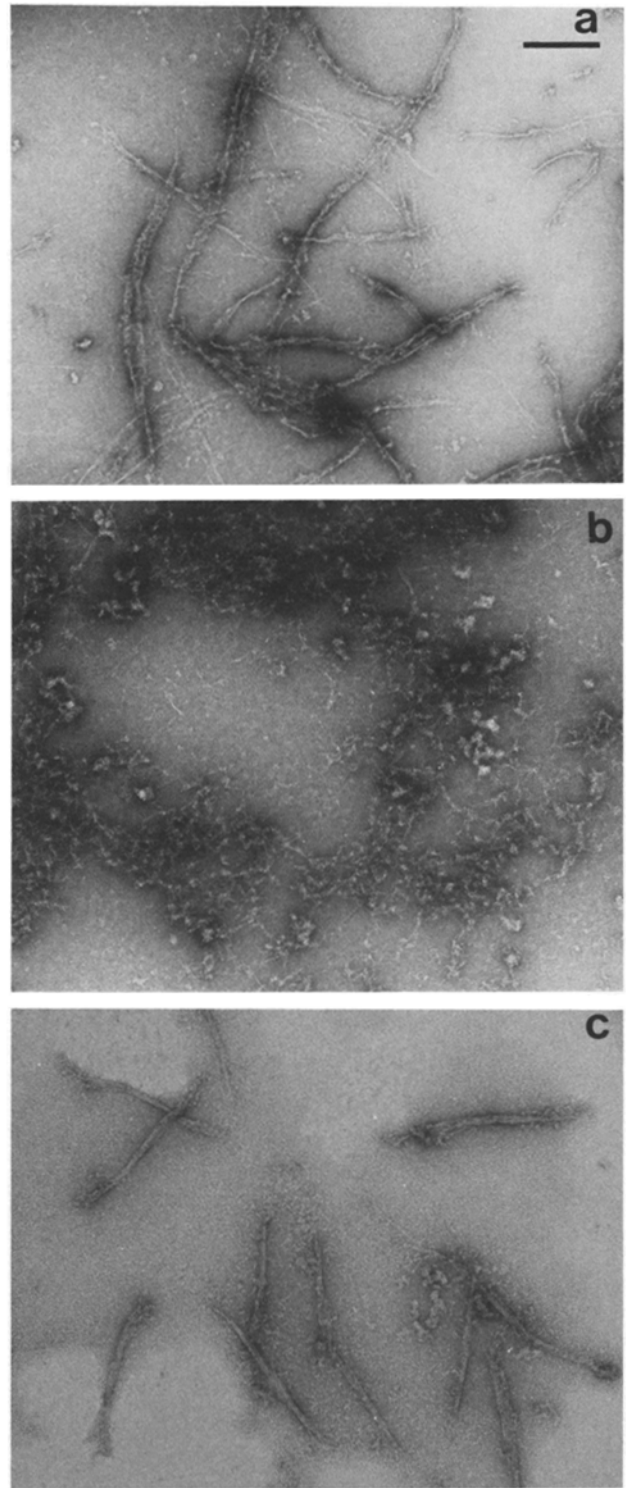


Figure 4. Negative stain of myosin at pH 7.5. In a simple buffer containing 50 mM KCl and 10 mM Tris, pH 7.5, the dephosphorylated myosin formed loose filaments (a) whereas the fully phosphorylated myosin contained only amorphous aggregates (b). In 2 mM MgCl₂ the dephosphorylated myosin molecules assembled into well-formed filaments with tapered ends (c). Bar, 0.25 μm.

magnesium and ATP were added (2 mM Mg-ATP), the phosphorylated myosin remained unassembled while the dephosphorylated myosin exhibited reduced assembly (Fig. 3 C), although half-maximal disassembly still occurred at 75 mM

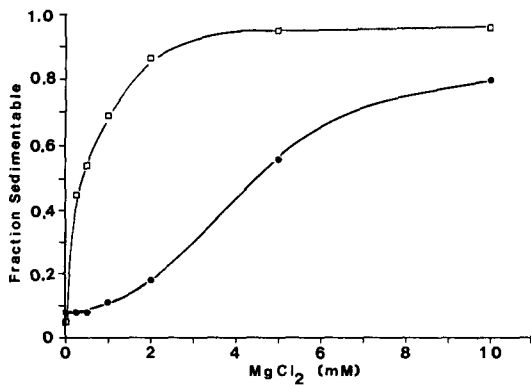


Figure 5. Heavy chain phosphorylation suppressed magnesium-induced myosin assembly. The myosin used for these studies was the same as described for Fig. 3. The dephosphorylated myosin (□) readily assembled at low concentrations of magnesium whereas the phosphorylated myosin (●) required much higher magnesium concentrations for maximal assembly.

KCl. The filaments formed from dephosphorylated myosin resembled those illustrated in Fig. 4 c.

Nearly full assembly of both phosphorylated and dephosphorylated myosin could be induced by magnesium chloride. The dephosphorylated myosin, however, required much

lower concentrations, and 50% of the myosin polymerized at a MgCl₂ concentration of only 0.4 mM, whereas 10 times as much magnesium was required for the phosphorylated myosin (Fig. 5). In 10 mM MgCl₂ the dephosphorylated myosin formed individual filaments with tapered ends (Fig. 6 a, arrows) and these often associated to form threadlike filaments several micrometers in length. In contrast, the phosphorylated myosin formed shorter filaments with blunt ends and these filaments did not aggregate linearly (Fig. 6 b; Table II).

Effects of pH on Myosin Assembly. The assembly of both phosphorylated and dephosphorylated myosin was strongly enhanced at acid pH irrespective of the presence of divalent cations or ATP (Fig. 7). In all cases the dephosphorylated myosin assembled more readily than did the phosphorylated myosin. Magnesium significantly enhanced the assembly of dephosphorylated myosin but had relatively little effect on the phosphorylated myosin (Fig. 7 B). Although the addition of 2 mM Mg-ATP suppressed assembly of both myosins (Fig. 7 C), it is interesting to note that in conditions which may approximate those in situ (pH 6.6, 2 mM Mg-ATP), the heavy chain phosphorylated myosin showed significantly less assembly. When examined in the electron microscope, the filaments resembled those induced by magnesium; the dephos-

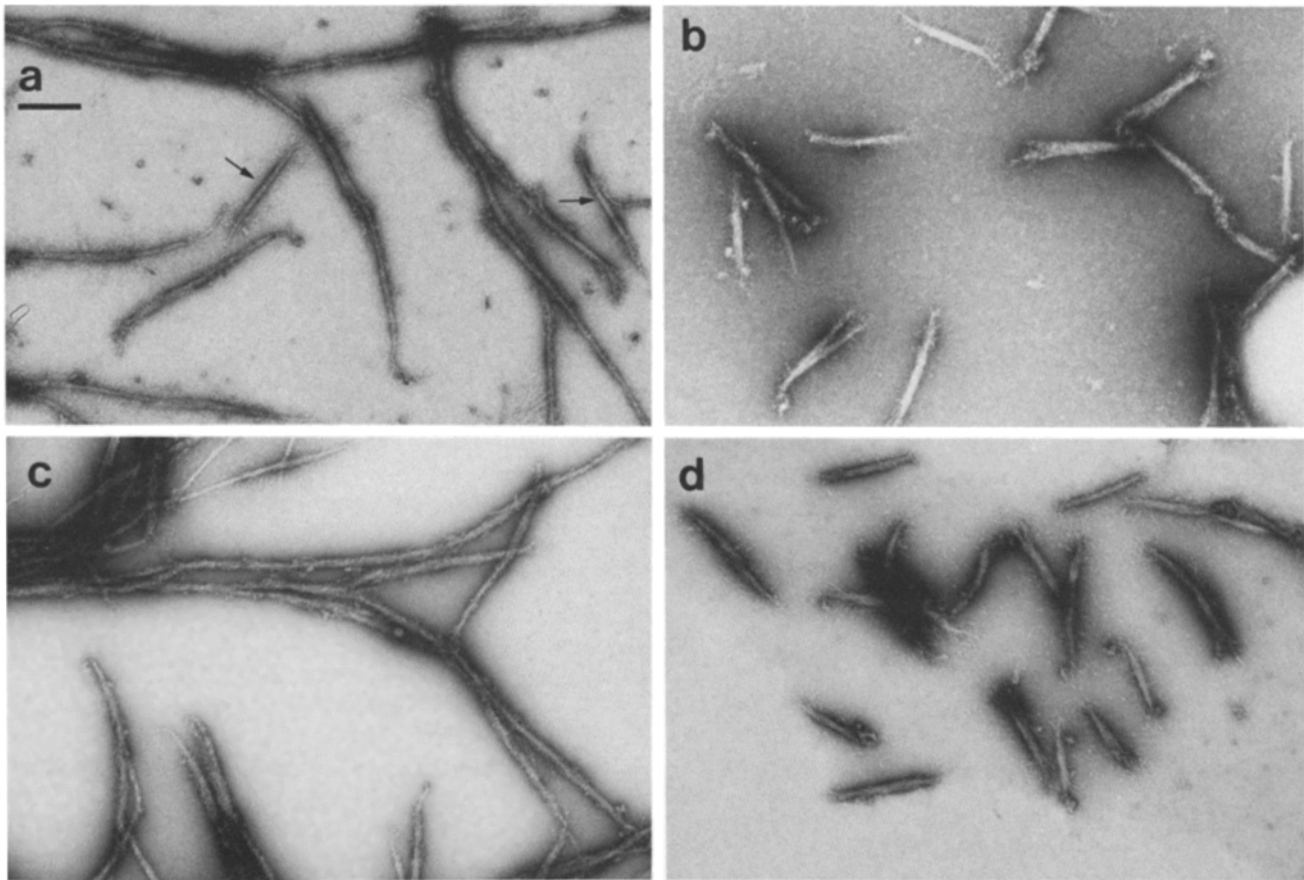


Figure 6. Heavy chain phosphorylation altered myosin filament length. Myosin filaments were negative-stained with uranyl acetate. In buffer 1 plus 10 mM MgCl₂, dephosphorylated myosin formed individual filaments with tapered ends (arrows in a) and these often aggregated to form long, threadlike filaments. In contrast, the phosphorylated myosin formed short filaments with blunt ends (b). In 10 mM MgCl₂, 10 mM 2-(N-morpholino)ethane sulfonic acid (MES) pH 6.6, only the long, threadlike filaments were seen with dephosphorylated myosin (c) whereas the phosphorylated myosin still formed individual, short filaments (d). Bar, 0.25 μm.

Table II. Effect of Heavy Chain Phosphorylation on Myosin Filament Length

Conditions	Filament length	
	Dephosphorylated*	Phosphorylated‡
	μm	μm
pH 6.6, 10 mM MgCl ₂	N.D.§	0.44 (0.08) [n = 87]
pH 7.5, 10 mM MgCl ₂	0.74 (0.18) [n = 38]	0.43 (0.082) [n = 68]
pH 7.5, 5 mM MgCl ₂	0.56 (0.07) [n = 72]	0.42 (0.12) [n = 88]

Values are given as mean with standard deviation in parentheses.

* Phosphate was removed from both heavy and light chains (see Materials and Methods).

‡ 0.85 mol P_i/mol heavy chain.

§ At pH 6.6, end-to-end association prevented measurement of the length of individual tapered filaments.

|| n, number of filaments measured.

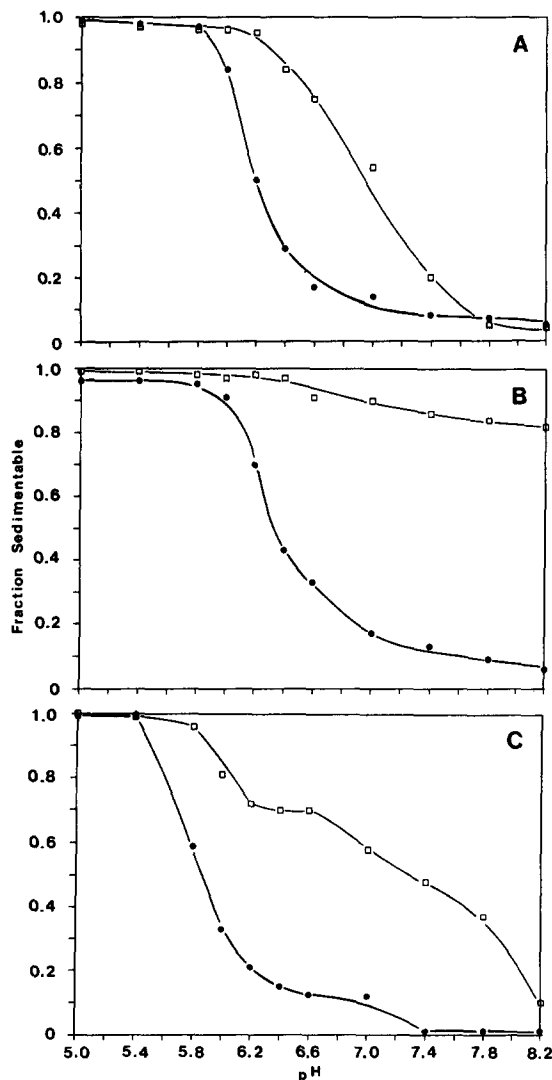


Figure 7. Effect of heavy chain phosphorylation on pH-induced myosin assembly. Assembly was measured as a function of pH using either 10 mM MES (pH 5-7.4) or 10 mM Tris (pH 7.4-8.2) with either (A) no additions, (B) 2 mM MgCl₂, or (C) 2 mM Mg-ATP. The myosin was the same as described for Fig. 3. In the pH range of 6.0-8.0 assembly of phosphorylated myosin (●) was suppressed relative to that of the dephosphorylated myosin (□).

phorylated myosin formed spindle-shaped filaments which aggregated at lower pH to form long, threadlike structures whereas the phosphorylated myosin always formed short, stubby thick filaments with only a slight tendency to aggregate, even at low pH in the presence of 10 mM MgCl₂ (Fig. 6, c and d).

Phosphorylation Lowers the pI of the Myosin. The observed shift in the pH-induced assembly curve (Fig. 7 a) suggested that phosphate groups increased the net negative charge on the myosin molecule and that more H⁺ ions were required to neutralize the charge. The pI was measured and it was found that heavy chain phosphorylation lowered the pI from 5.60 to 5.46 (data not shown).

Critical Concentration. To study the effect of phosphorylation on the assembly process, the extent of polymerization at equilibrium was measured as a function of total myosin concentration. When assayed with either 2 or 10 mM MgCl₂ almost all of the dephosphorylated myosin formed polymer and consequently the plot of myosin sedimented vs. total myosin had a slope of ~1 (Fig. 8). In contrast, in 2 mM magnesium a large fraction of the fully phosphorylated myosin remained soluble at equilibrium. For example, at a total

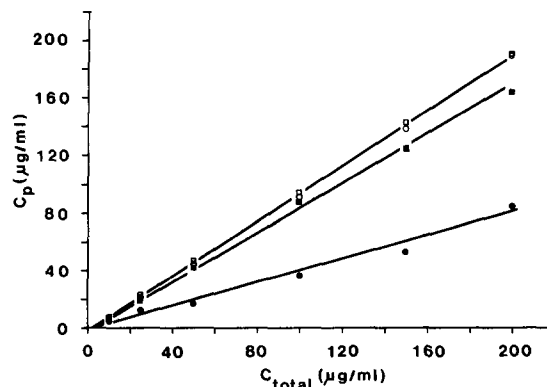


Figure 8. Dependence of polymer concentration on total myosin concentration. Assembly was assayed in 15 mM MES (pH 6.6), 20 mM KCl, and either (○, ●) 2 mM MgCl₂ or (□, ■) 10 mM MgCl₂. Both unphosphorylated (open symbols) and heavy chain phosphorylated myosin (closed symbols) showed a low critical concentration for assembly of ≤ 5.0 µg/ml. In 2 mM MgCl₂ heavy chain phosphorylation significantly reduced subunit affinity, as indicated by the lowered slope of the curve. C_{total}, total myosin concentration; C_p, myosin polymer concentration. For clarity, the concentration of monomer (C_{total} - C_p) is not shown on the graph.

concentration of 200 $\mu\text{g/ml}$ 60% of this myosin remained soluble. Thus, at equilibrium, heavy chain phosphorylation appeared to lower the affinity of subunits for each other. However, the critical concentration for assembly (as determined by the intercept of the polymer curve with the x -axis) was very low (0–5 $\mu\text{g/ml}$) and was not influenced by the level of phosphorylation. As would be predicted from results presented earlier, the equilibrium was sensitive to solution conditions. Consequently, raising the magnesium concentration to 10 mM increased the slope of the phosphorylated myosin plot to a value much closer to 1.0 (Fig. 8) whereas 2 mM ATP (in the absence of magnesium) completely prevented assembly of both myosins at concentrations as high as 400 $\mu\text{g/ml}$ (data not shown).

Discussion

Effect of Solution Conditions and Phosphorylation on Myosin Assembly

The interactions between individual *Dictyostelium* myosin molecules were found to be dependent on solution conditions and, in some respects, these interactions differed from those seen with muscle myosin. For example, in very low ionic strength, the *Dictyostelium* myosin formed soluble dimers and tetramers whereas muscle myosin remained fully polymerized. This most likely reflected differences in degree rather than in the basic assembly mechanism inasmuch as Reisler et al. (44) showed that in very low ionic strength the most stable muscle myosin aggregate was a small “minifilament” composed of only 16 to 18 monomers. This species was quite sensitive to solution conditions because increasing the ionic strength to 80 mM caused the filaments to grow in length, while raising the pH converted the minifilaments into monomers (43).

Both *Dictyostelium* and muscle myosins were soluble in high ionic strength, but the salt concentration required for half-maximal assembly differed by about 50 mM (Fig. 1). This may be physiologically important in that the intracellular ionic strength of *Dictyostelium* is quite low; the K^+ concentration is only 48 mM for the amoeba (1) compared with ~ 150 mM for vertebrates (8). The solubility properties of other nonmuscle myosins were also variable. Purified human platelet myosin exhibited half-maximal assembly at 240 mM KCl (32), and in 10 mM MgCl_2 , half-maximal assembly of *Acanthamoeba* myosin II occurred at a KCl concentration of 90–100 mM (38). In lower ionic strength (20 mM KCl and no magnesium), this myosin formed thin bipolar filaments composed of ~ 12 molecules which were sedimentable in the Airfuge (15 min at 100,000 g). Physarum myosin appeared to be soluble in all concentrations of KCl, and filaments could only be formed by adding divalent cations (31). Brain myosin, on the other hand, was quite resistant to salt solubilization in that 25% remained sedimentable even in 0.4 M KCl (28).

Simultaneous removal of phosphate from both the heavy chain and the light chain increased *Dictyostelium* myosin assembly. This was most likely due to removal of heavy chain phosphate since phosphorylation of the light chain did not affect the KCl assembly curve (Tafari and Kuczmariski, unpublished observations; 12), and subsequent addition of phosphate to the heavy chain depressed assembly (Fig. 3). This inhibition was dose-dependent because the higher the

level of phosphorylation, the greater the inhibition of polymerization. Millimolar amounts of ATP or sodium pyrophosphate completely solubilized *Dictyostelium* myosin regardless of the state of phosphorylation, and similar effects of polyanions have been reported for muscle myosin (13). Within the cell, however, ATP should be complexed with magnesium, and under these more physiological conditions the dephosphorylated myosin assembled, while heavy chain-phosphorylated myosin remained completely soluble (Fig. 3 C).

In addition to magnesium and ATP, another physiologically important variable is pH. It has been demonstrated that the intracellular pH of *Dictyostelium* amoebae is ~ 6.6 (9) and this value increases transiently during the chemotactic response (26) and development (16). It was possible to induce full assembly of myosin by lowering the pH in vitro (Fig. 7 A). Again, heavy chain phosphorylation exerted an inhibitory effect and significantly higher concentrations of H^+ were required to induce maximal assembly compared with dephosphorylated myosin. Together, these results provide strong support for the suggestion that heavy chain phosphorylation may suppress myosin assembly within the living cell.

Mechanism of Myosin Assembly

Although the details of filament formation have not been clarified, it is believed that parallel myosin dimers interact via their tail regions to form an antiparallel tetramer (35) and that once such a nucleus has formed, growth proceeds by the bilateral addition of subunits. The exact nature of these subunits is not known, but work by Davis et al. (7) and Reisler et al. (42) suggested that in the case of skeletal muscle myosin it was the parallel dimer. The visualization of dimers and tetramers (Fig. 2) suggests that *Dictyostelium* myosin assembly involves a similar sequence of events.

Growth of the filament continues until an equilibrium is reached between a length-independent “on” rate and a length-dependent “off” rate (6). Because many different rate constants are involved and because they can be influenced by solution conditions, analysis of filament formation is quite complex. By studying the polymerizing system at equilibrium, however, it is possible to describe the overall assembly process. Using the analytical ultracentrifuge, Josephs and Harrington (17) concluded that above a “critical concentration,” a constant amount of skeletal muscle myosin monomer was in equilibrium with any amount of polymer. In contrast, smooth muscle myosin formed polymer even at very low protein concentrations and the amount of monomer increased with the total protein concentration (29). In studies with *Acanthamoeba* myosin II, polymer was measured directly after sedimentation in the Airfuge. In this system, monomer concentration was essentially constant over a range of myosin concentrations and the polymer curve extrapolated to a critical concentration of 15–40 $\mu\text{g/ml}$. Neither this value nor the extent of polymerization were significantly affected by the addition of up to 5 mol of phosphate per mol of myosin (38). Under optimum conditions *Dictyostelium* myosin exhibited a critical concentration of ~ 5 $\mu\text{g/ml}$, and this also was unaffected by the level of heavy chain phosphorylation. It is important to note, however, that the extent of polymerization could be strongly inhibited by heavy chain phosphorylation, as can be seen from the decreased slope of the polymer plot in 2 mM MgCl_2 (Fig. 8). This reduction in the amount of

polymer formed at equilibrium implies that phosphorylation lowered the overall affinity between subunits within the thick filament.

The forces holding skeletal muscle myosin filaments together are mainly ionic, and it has been estimated that only about 25 bonds are formed per mole of monomer incorporated (18). Phosphorylation of *Dictyostelium* myosin appears to exert its primary effect via an electrostatic mechanism in that filament formation could be induced by lowering the pH or by adding Mg^{2+} . In skeletal (33), smooth (5), and *Acanthamoeba* (24) myosins only small regions of the heavy chain located near the COOH terminus of the tail are essential for myosin polymerization. The site of *Dictyostelium* myosin heavy chain phosphorylation has been localized to a region roughly 35 kD from the tip of the tail (22, 34), and it appears that this is near a region of the tail which is essential for assembly (unpublished observations).

In low pH the dephosphorylated myosin filaments had a strong tendency to aggregate, and in the presence of magnesium they formed very long, threadlike structures. Similar filaments measuring over 10 μm and having a constant diameter of 15–18 nm have been obtained with skeletal muscle myosin (30, 37). Hinssen et al. (15) also obtained long filaments from smooth and some nonmuscle myosins by carefully controlling the rate of dialysis. Although the details of how such structures form have not been elucidated, such studies point out the plasticity that is possible for subunit association.

Form of Myosin in the Cell

Attempts to visualize myosin filaments in nonmuscle cells at the ultrastructural level (14) have met with limited success. Calculations suggest that only a few filaments could be expected in a given thin section (32) and this problem is compounded by the fact that nonmuscle myosin filaments are readily destroyed by osmium fixation (51). Immunofluorescent staining of cells fixed in cold methanol or formaldehyde, however, reveals that myosin is organized into discrete structures. In fibroblasts, antimyosin staining is periodic along the length of stress fibers (11) and in *Dictyostelium*, myosin is disposed in rodlike structures having the dimensions of synthetic thick filaments (51). In addition, biochemical evidence suggests that filaments are required for the contraction of *Dictyostelium* cytoskeletons (41). Myosin relocates when cells divide (11, 51) or respond to external stimuli such as concanavalin A (4) or cAMP (51), and it has been shown that the level of heavy chain phosphorylation increases concomitant with relocation to the cytoskeleton (3).

A major unanswered question is whether the myosin filaments are moved intact or if the structures are first disassembled and then reassembled in a new location. The results presented here suggest that unmodified *Dictyostelium* myosin is quite sensitive to small ions present in the intracellular milieu. Thus, slight changes in the concentrations of H^+ , K^+ , Mg^{2+} , or ATP could contribute to the regulation of myosin structure within the cell. In conjunction with these changes, heavy chain phosphorylation could lead to transient filament disassembly. However, it may be that within the cell, filaments are relatively stable structures, and that heavy chain phosphorylation regulates the organization of myosin subunits within a given filament. Rapid exchange of subunits between muscle myosin filaments has been demonstrated by

fluorescence energy transfer (45), and it should be possible to use this technique to further characterize the effect of heavy chain phosphorylation on the assembly and stability of *Dictyostelium* myosin filaments.

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