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Comparison of the Actin Binding and Filament Formation Properties of Phosphorylated and Dephosphorylated *Acanthamoeba* Myosin II†

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ABSTRACT: Dephosphorylated (less than 0.5 mol of phosphate/mol of heavy chain) and maximally phosphorylated (3 mol of phosphate/mol of heavy chain) *Acanthamoeba* myosins II were compared. Actin-activated Mg^{2+}-ATPase activity was highest for dephosphorylated myosin II at 4-4.5 mM Mg^{2+} and at 7 mM Mg^{2+} for phosphorylated myosin II. At all concentrations of Mg^{2+}, activity was higher for the dephosphorylated enzyme. Assays of the actomyosin ATPase activities as a function of F-actin concentration showed that this higher enzymatic activity is attributable to the higher V_{max} value of the dephosphorylated form of myosin II; in fact, the K_{ATPase} value was higher for the more active, dephosphorylated form than for the less active, phosphorylated form. Both forms of myosin II bound essentially completely to F-actin in the presence of ATP, but the dephosphorylated form of the enzyme may have a greater affinity for F-actin, although insufficient to account for the higher actin-activated ATPase activity of dephosphorylated myosin II. Both dephosphorylated myosin II and phosphorylated myosin II formed bipolar filaments in 10 mM $MgCl_2$-20 mM KCl, but the filaments of the dephosphorylated form were larger and less readily dissociated by approximately physiological concentrations of MgATP. The dephosphorylated enzyme formed filaments, in the absence of Mg^{2+}, at KCl concentrations as high as 200 mM while phosphorylated myosin II was fully soluble at 200 mM KCl and 85% soluble at 25 mM KCl. Therefore, dephosphorylation of the heavy chains of *Acanthamoeba* myosin II increases its self-association, possibly its binding to F-actin, and the V_{max} of its actin-activated ATPase activity. There is no evidence that these three effects of dephosphorylation of myosin II are causally related but it seems likely that all are the result of structural changes in the myosin filaments.

Acanthamoeba myosin II contains a pair of heavy chains of about 185 000 daltons and two pairs of light chains of about 17 500 and 17 000 daltons (Maruta & Korn, 1977; Pollard et al., 1978; Collins & Korn, 1980, 1981). Collins & Korn (1980) showed that myosin II contains about 1.2-1.5 mol of phosphate/heavy chain when isolated from cells by their procedure (Collins & Korn, 1981) and that it contains about 2 mol of phosphate/heavy chain when isolated by the procedure of Pollard et al. (1978). Myosin II can be maximally phosphorylated by a partially purified kinase to the extent of 3 mol of phosphate/heavy chain, all esterified to serine residues (Cote et al., 1981). Phosphorylation of the light chains of

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Acanthamoeba myosin II has not been detected either in enzyme isolated from cells grown on radioactive inorganic phosphate (Collins & Korn, 1980) or in myosin II phosphorylated by crude or partially purified kinase preparations in vitro (Cote et al., 1981).

Purified myosin II with an average of 2 or 3 mol of phosphate/heavy chain was found to have little or no actin-activatable Mg^{2+}-ATPase activity, but purified myosin II with an average of about 1.2 mol of phosphate/heavy chain had appreciable activity (Collins & Korn, 1980). Maximal actin-activated Mg^{2+}-ATPase activity was obtained when the enzyme was dephosphorylated to less than 0.5 mol of phosphate/heavy chain by potato acid phosphatase (Cote et al., 1981). The actin-activated ATPase activity of partially or totally dephosphorylated myosin II also requires Ca^{2+}, at appropriate concentrations of Mg^{2+} (Collins & Korn, 1981). On the other hand, the Ca^{2+}-ATPase and K^+-EDTA-ATPase activities of myosin II are unaffected by the state of its phosphorylation (Collins & Korn, 1980).

Evidence from limited proteolysis of myosin II by trypsin or chymotrypsin (Collins et al., 1982) demonstrated that the three phosphorylatable sites on each heavy chain are contained within a peptide segment no larger than about 9000 daltons located near the C terminus of the heavy chain (about 100 000 daltons in peptide sequence away from the actin-binding and ATPase sites in the head region of the molecule). To help understand the mechanism by which the state of phosphorylation of the heavy chains regulates actomyosin II ATPase activity, we have now compared in greater detail some of the enzymatic and physical properties of maximally phosphorylated and maximally dephosphorylated *Acanthamoeba* myosin II.

Experimental Procedures

Amoebae were grown as described by Pollard & Korn (1973) and Collins & Korn (1981). *Acanthamoeba* myosin II was isolated according to Collins & Korn (1981) and then either phosphorylated with *Acanthamoeba* myosin II heavy chain kinase (Cote et al., 1981) or dephosphorylated with potato acid phosphatase (Collins & Korn, 1980). The myosin II was then repurified from the kinase or phosphatase by chromatography on Bio-Gel A-15m (Collins & Korn, 1980; Cote et al., 1981). In one experiment, myosin II prepared according to the procedure of Pollard et al. (1978) was used. Rabbit skeletal muscle actin was prepared by the procedue of Eisenberg & Kielley (1974) and the F-actin was treated with Dowex 1 (Cl form) to remove excess ATP.

The phosphate content of the myosin samples was determined chemically as previously described (Collins & Korn, 1980) and, in samples that had been phosphorylated by kinase and [γ-^{32}P]ATP, by radioactivity measurements. Peptide maps or radioactive, phosphorylated myosin II were prepared as described by Cote et al. (1981).

Ca^{2+}-ATPase and actin-activated Mg^{2+}-ATPase activities were assayed by incubating myosin II with [γ-^{32}P]ATP as described by Collins & Korn (1980) at 35 °C for 30 min and measuring release of radioactive P_i according to the procedure of Pollard & Korn (1973). Protein concentrations were measured by the colorimetric assay of Bradford (1976) using bovine serum albumin as the standard.

[γ-^{32}P]ATP was purchased from New England Nuclear. Potato acid phosphatase, bovine serum albumin, ATP, and imidazole (Grade III) were purchased from Sigma. The reagents for the Bradford assay and Bio-Gel A-15m were obtained from Bio-Rad, and sucrose (enzyme grade) was from Bethesda Research Laboratories. $MgCl_2$ solutions were prepared by diluting 1 M $MgCl_2$ from Fisher.

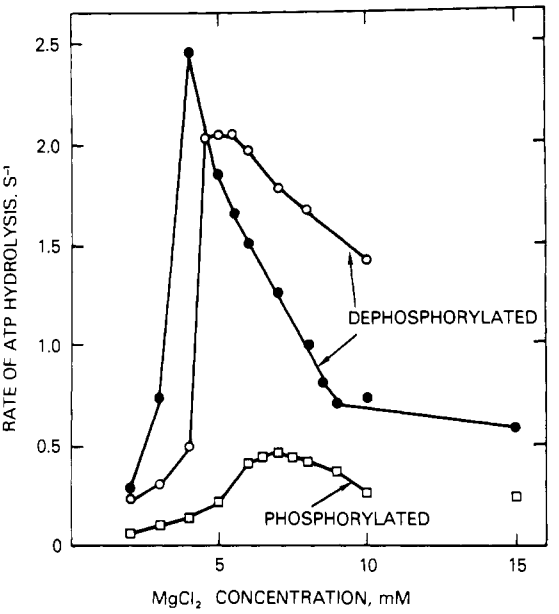

FIGURE 1: Effect of Mg^{2+} concentration on the actin-activated ATPase activities of phosphorylated and dephosphorylated myosin II. Myosin (5.5 μg/mL dephosphorylated and 4.3 μg/mL phosphorylated) was incubated for 30 min at 35 °C with 0.5 mg/mL F-actin in buffer containing 10 mM imidazole, pH 7.0, 1 mM [γ-^{32}P]ATP, 0.1 mM $CaCl_2$, and 2–15 mM $MgCl_2$ in a total volume of 250 μL and assayed for the production of [^{32}P]P_i as described by Pollard & Korn (1973). In two experiments (●, ×), F-actin and $MgCl_2$ were mixed before the addition of the other components, while in one experiment (○), F-actin was the last component added to the incubation mixture.

Results

Phosphorylation and Dephosphorylation of Myosin II. As isolated by the procedure of Collins & Korn (1981), *Acanthamoeba* myosin II contains 1.2–1.5 mol of phosphate/mol of heavy chain. The preparations used in the experiments described in this paper were phosphorylated with partially purified myosin II heavy chain kinase and [γ-^{32}P]ATP. As determined by the incorporation of ^{32}P, an additional 1.5 mol of phosphate were added per mol of heavy chain for a total of 2.7–3 mol of phosphate/mol of heavy chain. Autoradiography of peptide maps prepared after exhaustive trypsin digestion demonstrated that all three phosphorylation sites described by Cote et al. (1981) were labeled. The same preparations of *Acanthamoeba* myosin II were dephosphorylated by potato acid phosphatase under conditions previously shown to reduce the total esterified phosphate to less than 0.5 mol/mol of heavy chain (Collins & Korn, 1980). This was confirmed by parallel dephosphorylation of myosin II previously phosphorylated with [γ-^{32}P]ATP where the loss of phosphate could be followed by the decrease in radioactivity.

Dependence of Actomyosin II Activity on Mg^{2+} Concentration. Previously, we had observed that the actin-activated ATPase activity of partially phosphorylated myosin II [the enzyme as isolated by the procedure of Collins & Korn (1981)] was maximal at 5.5 mM Mg^{2+}, at which concentration more fully phosphorylated myosin II (either the enzyme as isolated by the procedure of Pollard et al. (1978) or after phosphorylation by myosin II heavy chain kinase (Cote et al., 1981) had very little activity. We have now demonstrated (Figure 1) that the optimal Mg^{2+} concentration (at pH 7.0) for maximally dephosphorylated myosin II is 4–4.5 mM (curves A and B, for two different preparations of dephosphorylated myosin II) and that maximally phosphorylated myosin II

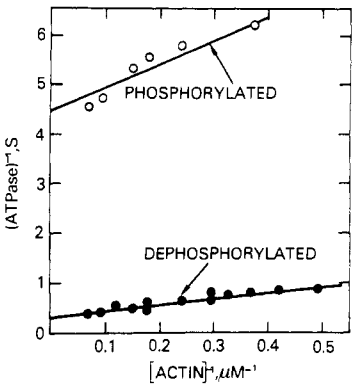

FIGURE 2: Double-reciprocal plots of the ATPase activities of phosphorylated and dephosphorylated myosin II as a function of F-actin concentration. Incubation conditions were identical with those described in the legend to Figure 1 except that the $MgCl_2$ concentration was constant at 5 mM, the concentration of F-actin was varied as indicated, and all samples contained 0.5 mg/mL bovine serum albumin. Bovine serum albumin had no effect on the enzymatic activities and was necessary for the binding studies done in parallel in Figure 3.

Table I: Kinetic Data for Actin-Activated ATPase Activity of Phosphorylated and Dephosphorylated *Acanthamoeba* Myosin II[a]

myosin	Mg^{2+} concn (mM)	V_{max} (s^{-1})	K_{ATPase} (μM)	$K_{binding}$ (μM)
dephosphory-	5	3	4	0.3
lated	4	3.7	4	
phosphory-	5	0.22	1	1.6
lated	7.5	0.4	1.6	

[a] The values for 5 mM Mg^{2+} were calculated from the experiments shown in Figures 2 and 3. The values for 4 and 7.5 mM Mg^{2+} were calculated from identical experiments (not shown) except for the concentration of Mg^{2+}. V_{max} is calculated per head by using a molecular weight of 440 000 for the two-headed molecule. V_{max} and K_{ATPase} values at 5 mM Mg^{2+} for myosin II as isolated (1.1 s^{-1} and 4 μM) were similar to those reported previously by Collins & Korn (1981) (0.77 s^{-1} and 0.8 μM).

(curve C) also has detectable actin-activated ATPase activity but at a much higher concentration of Mg^{2+} (7 mM). The activity of dephosphorylated myosin II was always considerably higher than the activity of phosphorylated myosin II, although the ratio of their activities varied with the Mg^{2+} concentration. The two curves for dephosphorylated myosin were obtained with different preparations, but the difference between them was probably caused by differences in the ways the reaction mixtures were prepared and not to differences in the myosins. The binding data presented later were not affected by the order of addition of F-actin.

Kinetic Analysis of the Actomyosin II ATPase Activity. Double-reciprocal plots of the rates of ATP hydrolysis by actin-activated phosphorylated and dephosphorylated myosin II are shown in Figure 2. For this experiment, 5 mM Mg^{2+}, which lies between the optimal Mg^{2+} concentrations for phosphorylated and dephosphorylated myosin II, was used because dephosphorylated myosin II was partially sedimentable at higher concentrations and it was important to be able to correlate, under identical conditions, the actin-activation data with actin-binding data that were obtained by a sedimentation assay. The kinetic constants calculated from the experimental data are shown in Table I. It appears that the difference in actomyosin ATPase activity between dephosphorylated and phosphorylated myosin II at 5 mM Mg^{2+} is primarily an effect of the V_{max} values. In fact, the K_{ATPase} values for F-actin are the reverse of the relative ATPase activities of the two forms

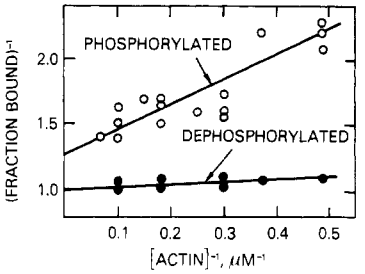

FIGURE 3: Double-reciprocal plot of the fractions of phosphorylated and dephosphorylated myosin II bound to F-actin in the presence of ATP as a function of actin concentration. The reaction mixtures were identical with those described in the Legend to Figure 2. Samples were mixed at 0 °C, incubated at 35 °C for 5 min, and then centrifuged in the Beckman airfuge at 30 psi for 30 min at room temperature to sediment F-actin and bound myosin. The concentration of unbound dephosphorylated myosin II in the supernatant solutions was determined by measuring the actin-activated Mg^{2+}-ATPase activity. The concentration of unbound phosphorylated myosin II was determined by measuring the radioactivity remaining in the supernatant solution (^{32}P-labeled myosin II was used in this experiment). All values were corrected for the very small fraction of myosin II that sedimented in the absence of F-actin. Bovine serum albumin was present to reduce nonspecific losses by adsorption of protein onto the walls of the centrifuge tube.

of the enzyme; i.e., the less active, phosphorylated enzyme reaches half-maximal activity at a lower concentration of F-actin than the more active, dephosphorylated enzyme. The same conclusion is reached from the kinetic data obtained separately for each form of the enzyme at its optimal Mg^{2+} concentration rather than at a Mg^{2+} concentration taken between their respective optima (Table I). The V_{max} value for the dephosphorylated enzyme at 4 mM Mg^{2+} is 9 times the V_{max} value for the phosphorylated enzyme at 7 mM Mg^{2+} while the K_{ATPase} value is lower for the phosphorylated form of myosin II. Muscle actin was used in these experiments because Collins & Korn (1981) obtained linear reciprocal plots with partially phosphorylated myosin II using muscle actin but not with *Acanthamoeba* actin.

Binding of Dephosphorylated and Phosphorylated Myosin II to F-Actin in the Presence of ATP. The K_{ATPase} values for actin calculated from the enzymatic data are kinetic constants and not binding constants. Binding was measured directly as the fraction of myosin that sedimented in the presence of F-actin in 5 mM Mg^{2+}. Under these conditions neither phosphorylated nor dephosphorylated myosin II sedimented in the absence of F-actin although, as will be discussed later, both forms of myosin II were present as small bipolar filaments. Dephosphorylated myosin II was partially sedimented in the absence of F-actin at higher concentrations of Mg^{2+}, and therefore, binding could not be studied under those conditions. Apparent binding constants were calculated from the reciprocal plots of the fraction of myosin bound as a function of the concentration of F-actin (Figure 3). True dissociation constants can be obtained only for soluble, monomeric myosin. It should be noted that the binding data were obtained under the conditions of the enzymatic assay, i.e., in the presence of 1 mM ATP and 5 mM Mg^{2+}, the only difference being temperature (room temperature for binding and 35 °C for ATPase, but this does not affect the interpretation because the relative enzymatic activities of phosphorylated and dephosphorylated myosin II are the same at the lower temperature). Less than 10% of the ATP was hydrolyzed during the binding assay which was much too little to affect the results. By extrapolation of the lines in Figure 3, all of the dephosphorylated myosin II would have been bound at infinite concentration of F-actin, but only about 82% of the phosphorylated

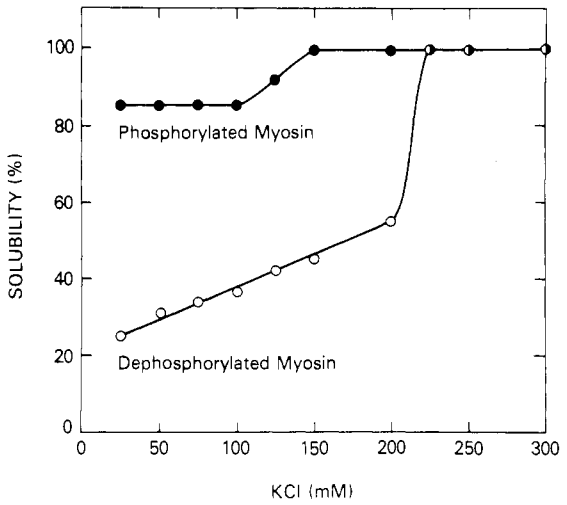

FIGURE 4: Effect of KCl concentration on the solubility of filaments of phosphorylated and dephosphorylated myosin II. Myosin was dissolved (0.2 mg/mL) in 10 mM imidazole, pH 7.5, 0.3 M KCl, 10% sucrose, and 1 mM dithiothreitol, and aliquots were then dialyzed against solutions of 10 mM imidazole, pH 7.5, and 1 mM dithiothreitol containing 25–300 mM KCl at 2 °C for 24 h. Samples (50 μL) of each suspension were centrifuged in a Beckman airfuge at 22 °C for 20 min at 30 psi, and the concentration of soluble myosin was determined by measuring the Ca^{2+}-ATPase activities of the supernatant solution. The Ca^{2+}-ATPase activities of sedimentable and nonsedimentable myosin II were identical.

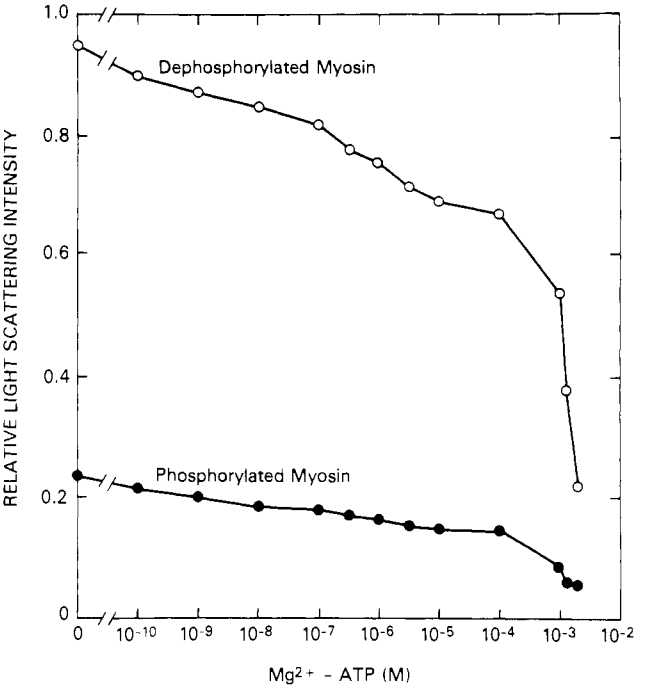

FIGURE 5: Effect of MgATP concentration on the stability of filaments of phosphorylated and dephosphorylated myosin II. Myosin filaments were formed by dialysis of both preparations of myosin (0.7 mg/mL) against a solution containing 10 mM imidazole, pH 7.0, 20 mM KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol at 4 °C. Samples (1 mL) were then diluted to 0.2 mg/mL with the dialysis buffer and light scattering measurements made in an SLM 4000 spectrofluorometer at 22 °C using an excitation and emission wavelength of 340 nm. Successive additions of 4-μL aliquots of appropriate stock solutions of MgATP were made at intervals of 5 min. Changes in light scattering intensity were complete within 5 min; no changes in scattering occurred for at least 3 h in the absence of MgATP.

myosin II would have sedimented with the F-actin at infinite actin concentration. This may imply that perhaps as much as 18% of the phosphorylated myosin II was denatured and unable to interact with actin in which case the V_{max} for the active enzyme would have been correspondingly higher than indicated in Table I.

The $K_{binding}$ values for phosphorylated and dephosphorylated myosin II (Table I) are in the opposite relationship of their K_{ATPase} values (Table I); dephosphorylated myosin II binds about 5 times more tightly to F-actin than does phosphorylated myosin II. Even so, the enhancement of actin-activated ATPase activity by dephosphorylation is mainly due to an effect on V_{max}. For example, at 10 μM F-actin, 68% of the phosphorylated myosin (83% of that which can bind at infinite F-actin concentration) and 98% of the dephosphorylated myosin were bound to F-actin (Figure 3), but the actin-activated ATPase activity of phosphorylated myosin was only 9% of the activity of dephosphorylated myosin (Figure 2). At 2 μM F-actin, 50% as much phosphorylated myosin II was bound to F-actin as dephosphorylated myosin but it had only 13% of the enzymatic activity.

The competitive binding of ^{32}P-labeled phosphorylated myosin II and dephosphorylated myosin II was compared in 0.5 M KCl and the absence of ATP, conditions under which both forms of the enzyme exist as soluble monomers. Both forms of myosin II bound to F-actin with a stoichiometry of one myosin head per actin subunit and their affinities were indistinguishable (data not shown). Neither phosphorylated nor dephosphorylated myosin II has actin-activated Mg^{2+}-ATPase activity at concentrations of KCl greater than about 25 mM (Collins & Korn, 1981) so K_{ATPase} cannot be compared to $K_{binding}$ under these conditions.

Effects of KCl and Mg on Filament Formation by Phosphorylated and Dephosphorylated Myosin II. The solubilities of phosphorylated and dephosphorylated myosin II as a function of KCl concentration are compared in Figure 4. Both forms of myosin II were 100% soluble when dialyzed overnight against 300 mM KCl in the absence of Mg^{2+} and were monomers when examined by sedimentation-equilibrium analysis. Each was then dialyzed against KCl solutions of varying concentrations. Although some of the phosphorylated myosin II became sedimentable when the KCl concentration was lowered from 150 to 100 mM, about 85% of the phosphorylated enzyme did not sediment even at KCl concentrations as low as 25 mM. In a parallel experiment, dephosphorylated myosin II was only about 55% nonsedimentable at 200 mM KCl and only about 25% did not sediment at 25 mM KCl. The dephosphorylated myosin II became 100% soluble again when readjusted to 300 mM KCl (data not shown). The solubility of myosin II isolated by the procedures of Pollard et al. (1978), which has 2 mol of phosphate/mol of heavy chain, is essentially the same as that of myosin II phosphorylated by kinase to contain 3 mol of phosphate/mol of heavy chain (data not shown).

In a similar experiment, filaments of mysoin II were first formed by dialysis against a buffer containing 20 mM KCl and 10 mM $MgCl_2$ and their solubility was then determined as a function of MgATP concentration (Figure 5). As determined by light scattering measurements, the initial suspension of dephosphorylated myosin II was much more aggregated than the suspension of phosphorylated myosin II. Addition of MgATP to a final concentration of 3 mM caused a steady decline in the light scattering of the suspension of phosphorylated myosin II to a very low value. Addition of MgATP to the suspension of dephosphorylated myosin II caused a steady decrease in light scattering to about 0.1 mM MgATP followed by a sharper decrease in light scattering when the MgATP concentration was raised to 3 mM. But even in 3 mM MgATP, the suspension of dephosphorylated

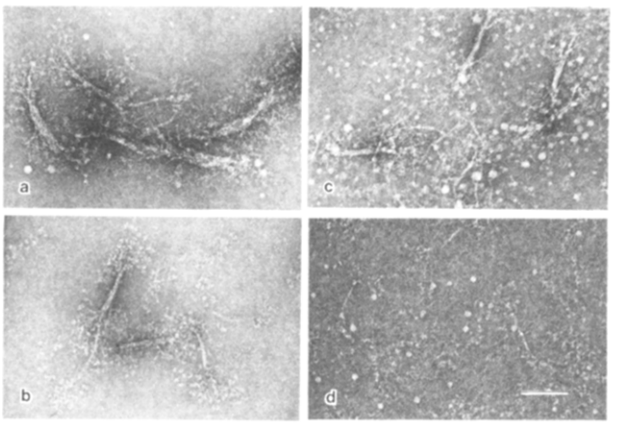

FIGURE 6: Effect of MgATP on the electron microscopic images of filaments of phosphorylated and dephosphorylated myosin II. One drop of the solutions studied in Figure 5 was placed on a grid with a carbon-coated parlodion substrate film. After 2 min the grid was rinsed successively with 1 drop of buffer, 2 drops of deionized water, and 3 drops of 1% uranyl acetate, then drained, and air-dried. The negatively stained specimens were photographed in a Siemens Elmiskop 101 at 100 kV. (a) and (c) are dephosphorylated myosin II in the presence of 0 and 3 mM MgATP, respectively, and (b) and (d) are phosphorylated myosin II in the presence of 0 and 3 mM MgATP, respectively. The bar is 100 nm.

myosin II was as aggregated (as measured by light scattering) as the suspension of phosphorylated myosin II had been in the absence of MgATP.

Negative-staining electron microscopy extended the light scattering data. The suspension of dephosphorylated myosin II in the absence of MgATP consisted mostly of large aggregates of thick bipolar filaments (Figure 6a). In contrast, the phosphorylated myosin II, in the absence of MgATP, consisted of much smaller aggregates of thinner bipolar filaments (Figure 6b). The dephosphorylated myosin in 3 mM MgATP (Figure 6c) resembled the phosphorylated myosin in the absence of MgATP while the phosphorylated myosin, in 3 mM MgATP, contained relatively few, dispersed small filaments (Figure 6d). The diameter of the bare zone of the myosin filaments was typically about 20 nm for the sample shown in Figure 6a, 12 nm and smaller for the samples shown in Figure 6b,c, and less than 6 nm for the sample shown in Figure 6d. Filaments with bare zone diameters of about 6 nm contain about 8–10 myosin molecules.

Circular Dichroism. Spectra obtained with myosins at about 0.1 mg/mL were essentially indistinguishable for phosphorylated and dephosphorylated enzymes and indicated about 60% α helix for both (data not shown).

Discussion

The effect of the Mg^{2+} concentration on actomyosin II ATPase activity is striking. As shown in Figure 1 and by Collins & Korn (1981), a relatively high concentration of Mg^{2+} is required to activate dephosphorylated myosin II, and we now find significant enzymatic activity for maximally phosphorylated myosin II when the Mg^{2+} concentration is further increased. Previously, Collins & Korn (1981) found that Ca^{2+} sensitivity of actomyosin II ATPase activity could be detected only over a very narrow range of Mg^{2+} concentration, and in both studies the effect of Mg^{2+} appears to be highly cooperative. The variable inhibition of activity at very high Mg^{2+} concentrations, probably because of effects on F-actin and not on myosin II, increases the complexity of the Mg^{2+} effects.

From the kinetic data (Figures 2 and 3; Table I), the difference in the actin-activated ATPase activities of dephosphorylated and phosphorylated myosin II appears to be due to the difference in their V_{max} values. The dephosphorylated form of the enzyme, which has the higher actin-activated ATPase activity at all concentrations of Mg^{2+}, has the higher V_{max} but the less active phosphorylated form of the enzyme has the lower K_{ATPase} for F-actin (Table I). Therefore, the greater catalytic activity of dephosphorylated myosin II must be due to a faster turnover number at the catalytic site.

Although not explicitly demonstrated in this paper, both dephosphorylated and phosphorylated myosin II form filaments similar to those in Figure 6 under conditions of enzymatic assay. This fact (which will be the subject of a later paper) may help explain the highly cooperative effects of Mg^{2+} concentration [Figure 1 and Collins & Korn (1981)] and myosin concentration (Collins & Korn, 1981) on the actin-activated ATPase activity. Despite the presence of myosin filaments, apparently normal linear reciprocal plots were obtained for ATPase activity as a function of actin concentration (Figure 2), in contrast to the observations of Pope et al. (1981) for rabbit skeletal muscle myosin. They found a biphasic dependence of ATPase activity on actin concentration extrapolating to a V_{max} of about 8 s^{-1} and a K_{ATPase} of 2.5 μM for actin concentrations between 1 and 20 μM and a V_{max} of about 7 s^{-1} and K_{ATPase} of about 40 μM at actin concentrations between 20 and 100 μM (at the lowest ionic strengths tested). The values we find for dephosphorylated *Acanthamoeba* myosin II between 2 and 15 μM F-actin (Table I) are quite similar to the values found by Pope et al. (1981) for rabbit skeletal muscle myosin between 1 and 2 μM F-actin; however, we found no evidence for biphasic behavior with F-actin at concentrations as high as 50 μM (data not shown).

For kinetic studies with muscle myosin, it has often been found useful to prepare heavy meromyosin or subfragment 1, both of which are unable to form filaments. Unfortunately, we have not yet been able to prepare an analogous derivative from *Acanthamoeba* myosin II (Collins et al., 1982). In any case, we would not be able to study the regulation by phosphorylation of such a derivative because the phosphorylation sites of myosin II are located at the end of the tail portion that would be lost upon cleavage.

Even though the myosin did not sediment under the conditions of the assay described in Figure 3, the fact that the myosin was present as filaments creates a potential problem in the interpretation of the binding studies. Binding to F-actin of just a fraction of the myosin heads in a filament might be sufficient to cause the entire filament to sediment. Therefore, the lower $K_{binding}$ found for dephosphorylated myosin II (Table II) might reflect its presence in larger filaments (as suggested by Figure 6, under somewhat different conditions) rather than tighter binding to F-actin.

Binding studies under assay conditions, i.e., in the presence of ATP, are further complicated by the fact that the myosin heads will be present in multiple conformations reflective of the steady-state ATPase cycle. At a minimum, one would expect myosin·ATP, myosin·ADP·P_i, myosin·ADP, and myosin to be present, each of which might have a different binding constant for F-actin. The relative concentratins of each species might be different for phosphorylated and dephosphorylated myosin II, further complicating a direct comparison. It can still be concluded, however, that, even in the presence of ATP, both phosphorylated and dephosphorylated myosin II can bind essentially quantitatively to F-actin. Moreover, although the simplest interpretation of the enzymatic data (as discussed in the Results) is that phosphorylation does not regulate actin-activated ATPase of myosin II primarily by controlling its binding to F-actin, the enzymatically more active, dephos-

phorylated form may have a higher affinity for actin than the less active, phosphorylated form.

Kuczmarski & Spudich (1980) had previously reported an effect of phosphorylation on the solubility of *Dictyostelium* myosin in solutions of KCl quite similar to that shown in Figure 4 for *Acanthamoeba* myosins. In their experiments, *Dictyostelium* myosin with about 0.6 mol of phosphate/mol of heavy chain was 50% soluble in 50 mM KCl and about 80% soluble in 80 mM KCl while myosin with only about 0.1 mol of phosphate/mol of heavy chain was essentially insoluble at both these KCl concentrations and required about 110 mM KCl to reach 50% solubility. Also in their experiments, as in ours (Figure 4), the increase in solubility of the dephosphorylated myosin occurred over a rather narrow range of concentration of KCl.

MgATP has also previously been shown to affect differentially the solubility of filaments of phosphorylated, vertebrate myosins. Phosphorylation of one pair of light chains of smooth muscle myosin (Suzuki et al., 1978) and thymus myosin (Scholey et al., 1980) makes the filaments more resistant to solubilization by MgATP than filaments of myosins in which the light chains are not phosphorylated. In these cases, however, the differential solubilization was observed between 10^{-7} and 10^{-5} M ATP and the soluble myosin was probably monomeric, not filamentous as in the present studies. With *Acanthamoeba* myosin II, the most dramatic effects of MgATP were seen at approximately millimolar concentrations (Figure 5) and MgATP affected primarily the size of the filaments (Figure 6), there being little soluble myosin present at any MgATP concentration with either phosphorylated or dephosphorylated myosin II.

It is intriguing that, with the four myosins thus far examined, the form of the myosin that has the highest actin-activated ATPase activity forms the more stable and highly aggregated filaments. For the two vertebrate myosins, the enzymatic activity is greater and the solubility less when the 20 000-dalton light chain is phosphorylated, while for the two amoeba myosins, the activity is greater and the solubility less when the heavy chains are dephosphorylated. Others (Suzuki et al., 1978; Scholey et al., 1980; Kuczmarski & Spudich, 1980) have raised the possibility that only the enzymatically functional forms of the myosins may be filamentous in the cell.

On the other hand, there is some evidence that nonphosphorylated myosin (i.e., the enzymatically inactive state) is filamentous in smooth muscle (Somlyo et al., 1981) while there is still no direct evidence that myosin filaments are ever present in nonmuscle cells. For example, we have not yet seen filaments such as those in Figure 6 in *Acanthamoeba*. Therefore, the physiological role of the effects of phosphorylation on myosin filament formation has not been established. The mechanism of regulation of filament formation in vitro by phosphorylation also remains to be determined. It is presumably different for vertebrate and amoeba myosins because phosphorylation occurs at very different regions of the molecules and has an opposite effect on solubility as well as on enzymatic activity. For *Acanthamoeba* myosin II, the two sites known to be phosphorylated in vivo are sufficient to regulate filament assembly as well as actin-activated ATPase activity (Cote et al., 1981). The physiologic role, if any, of the third site that is phosphorylated by the heavy chain kinase in vitro (Cote et al., 1981) remains to be determined.

Although they are affected in parallel by phosphorylation, filament formation and actomyosin ATPase activity are not necessarily causally related in any of the systems that have been studied. Preparations of heavy meromyosin (which are unable to form filaments) from smooth muscle (Seidel, 1978; Ohnishi & Watanabe, 1979) and *Dictyostelium* (Peltz et al., 1981) myosins have actin-activated ATPase activity while filaments of phosphorylated *Acanthamoeba* myosin II have very little actomyosin ATPase activity. Moreover, the actin-activated ATPase activity of smooth muscle heavy meromyosin can still be regulated by the state of phosphorylation of its light chains (Seidel, 1978; Ohnishi & Watanabe, 1979). As mentioned before, however, the cooperativity in the actin-activated ATPase activity of myosin II as a function of Mg^{2+} and myosin concentrations suggests that myosin–myosin interactions may play an important role in the actin-activated ATPase activity of *Acanthamoeba* myosin II. Because both phosphorylated and dephosphorylated myosin II are filamentous under conditions of the enzyme assay, the differences in their enzymatic activities may be the result of a subtle effect of the state of phosphorylation on the interaction of myosin molecules within the filaments.

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