Proc. Natl. Acad. Sci. USA
Vol. 77, No. 12, pp. 7292–7296, December 1980
Cell Biology

Regulation of myosin self-assembly: Phosphorylation of *Dictyostelium* heavy chain inhibits formation of thick filaments

(cell movement/nonmuscle contraction/myosin ATPase/protein kinase)

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Communicated by I. R. Lehman, September 2, 1980

ABSTRACT *Dictyostelium* myosin is composed of two heavy chains and two pairs of light chains in a 1:1:1 stoichiometry. Myosin purified from amoebae grown in medium containing [^{32}P]phosphate had two of the subunits labeled (0.2–0.3 mol of phosphate per mol of 210,000-dalton heavy chain and ≈0.1 mol of phosphate per mol of 18,000-dalton light chain). Kinase activities specific for the 210,000-dalton and for the 18,000-dalton subunits have been identified in extracts of *Dictyostelium* amoebae, and the heavy chain kinase has been purified 50-fold. This kinase phosphorylated *Dictyostelium* myosin to a maximum of 0.5–1.0 mol of phosphate per mol of heavy chain. Heavy chain phosphate, but not light chain phosphate, can be removed with bacterial alkaline phosphatase. Actin-activated myosin ATPase increased 80% when phosphorylated myosin was dephosphorylated to a level of ≈0.06 mol of phosphate per mol of heavy chain. This effect could be reversed by rephosphorylating the myosin. The ability of myosin to self-assemble into thick filaments was inhibited by heavy chain phosphorylation. For example, in 80–100 mM KCl, only 10–20% of the myosin was assembled into thick filaments when the heavy chains were fully phosphorylated. Removal of the heavy chain phosphate resulted in 70–90% thick filament formation. This effect on self-assembly could be reversed by rephosphorylating the dephosphorylated myosin. These findings suggest that heavy chain phosphorylation may regulate cell contractile events by altering the state of myosin assembly.

The contractile proteins actin and myosin, first studied in muscle, have been identified in various nonmuscle cells. Investigations using fluorescent probes have suggested that these proteins are involved in many cell functions including cell division, amoeboid movement, maintenance of cell shape, and translocation of cell surface proteins (for review, see refs. 1–3).

In contrast to the highly stable myofibril structure in striated muscle, the assembly and disassembly of the contractile apparatus of nonmuscle cells is extremely dynamic. For example, when a fibroblast attaches to a substrate, it flattens out, and stress fibers subsequently appear. These structures contain bundles of actin filaments with which myosin is associated in a periodic manner (4). When the fibroblast rounds up for division, the stress fibers are disassembled and a contractile ring containing actin and myosin is transiently assembled during cytokinesis (5, 6). Other examples include the rapid formation of actin-containing microspikes (7, 8) and the petaloid to filopodial transformation of sea urchin coelomocytes (9).

Although little is known about the mechanisms by which nonmuscle cells regulate the assembly and disassembly of the contractile apparatus, some progress has recently been made in understanding the regulation of assembly of one of its components, actin. Profilin, a protein isolated from spleen, binds actin monomers stoichiometrically and inhibits their polymerization into filaments (10). Other factors isolated from blood and brain depolymerize existing actin filaments (11), and a protein from *Physarum* causes fragmentation of actin filaments in a calcium-dependent manner (12). In addition, there are various gelation factors that reversibly crosslink filaments (13, 14).

In contrast to the situation for actin, much less is known about the control of nonmuscle myosin assembly. Although most nonmuscle myosins self-assemble *in vitro* to form thick filaments, nothing is known about the control of assembly *in vivo*. In this paper we report that the heavy chain of *Dictyostelium* myosin is phosphorylated *in vivo* and that heavy chain phosphorylation inhibits self-assembly of the myosin and alters its ATPase activity *in vitro*.

MATERIALS AND METHODS

Cell Culture. *Dictyostelium* strain Ax3 was grown as described (15). Cells were harvested in late logarithmic phase. For *in vivo* labeling studies, cells were grown for about 4 generations in [^{32}P]orthophosphate (15–20 μCi/ml; 1 Ci = 3.7 × 10^{10} becquerels) in the defined medium of Franke and Kessin (16), with total phosphate decreased to 0.4 mM.

Myosin Purification. *Dictyostelium* myosin was purified as described (17), with minor modifications which will be detailed elsewhere.

Actin Purification. *Dictyostelium* actin was prepared by the method of Uyemura *et al.* (18).

Heavy Chain Kinase Purification. Purification of a specific heavy chain kinase from *Dictyostelium* will be described in detail elsewhere. Briefly, amoebae were disrupted by sonication in a buffer containing sucrose and sodium pyrophosphate and centrifuged at high speed (18). The high-speed supernate was subjected to ammonium sulfate fractionation, affinity chromatography (Affi-Gel Blue, Bio-Rad), and molecular sieve chromatography (Bio-Gel A-1.5 m). These steps resulted in a 50-fold purification and a 10% recovery of kinase activity. When incubated with purified *Dictyostelium* myosin, the kinase did not cause proteolysis, as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis, even after incubation at 22°C for 24 hr.

***In Vitro* Phosphorylation of *Dictyostelium* Myosin.** Purified *Dictyostelium* myosin (3–4 mg/ml) was incubated for 5–6 hr at 22°C with the partially purified heavy chain kinase (0.5 mg/ml) in 30 mM KCl/3% (wt/vol) sucrose/0.5 mM dithiothreitol/10 mM $MgCl_2$/1.5 mM [γ-^{32}P]ATP (10–20 cpm/pmol)/0.02% sodium azide/12 mM Tris·HCl, pH 7.5. To separate the myosin from the kinase and [γ-^{32}P]ATP, the reaction mixture was chromatographed on a 1.5 × 70 cm Bio-Gel A-0.5 m column equilibrated in storage buffer (0.4 M KCl/1 mM dithiothreitol/0.02% azide/10 mM Tris·HCl, pH 7.5). Myosin, which eluted in the void volume, was induced to assemble by dialysis against 50 mM KCl/10 mM $MgCl_2$/1.0 mM

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dithiothreitol/10 mM Tris maleate buffer, pH 6.5. The assembled myosin was collected by centrifugation and resuspended in a small volume of storage buffer. More than 75% of the myosin loaded onto the column was recovered.

Dephosphorylation of *Dictyostelium* Myosin. Bacterial alkaline phosphatase was found to remove heavy chain phosphate, but not light chain phosphate, from myosin phosphorylated either *in vivo* or *in vitro*. To dephosphorylate the heavy chain, myosin (3–4 mg/ml) was incubated for 24 hr at 22°C with bacterial alkaline phosphatase (0.05 mg/ml; BAPF, Worthington) in storage buffer plus 5.0 mM $MgCl_2$. Myosin was separated from the phosphatase by Bio-Gel A-0.5 m chromatography, as described above.

Phosphate Measurement. To determine the specific activity of phosphate, cells were centrifuged from the ^{32}P-containing medium and washed twice with 10 mM Tris·HCl at pH 7.5. An aliquot of cells was assayed for radioactivity in a scintillation counter and an equal volume was assayed for inorganic phosphate by the method of Chen *et al.* (19). The molar ratio of phosphate per subunit of myosin was determined by cutting the appropriate band from a $NaDodSO_4$ gel, digesting with 0.5 ml of 30% H_2O_2 for 16 hr at 90°C, and assaying in 10 ml of ACS scintillation fluid.

Protein Determination. Protein was measured by either the method of Schacterle and Pollack (20) or the method of Bradford (21).

Assembly Assay. Thick-filament formation was measured by incubating 100 μl of myosin solution (0.2 mg/ml) in the buffer conditions stated in the figure legends in a Beckman Airfuge tube. After incubation at 22°C for 20 min, filaments were sedimented by centrifugation at 120,000 × *g* for 6 min in a Beckman Airfuge. The supernate was removed and the pellet was sonicated in 100 μl of 0.4 M KCl. The protein concentration of both supernate and pellet was then determined. Separate experiments established that assembly was complete in 20 min and that centrifugation for up to 30 min was no more effective than centrifugation for 6 min for sedimenting assembled myosin. Electron microscopic analysis confirmed that sedimentable myosin was in the form of individual thick filaments in all cases.

ATPase Assay. ATPase activity was measured by using [γ-^{32}P]ATP as described (15). Activity of myosin alone was measured in 0.6 M KCl/10 mM $CaCl_2$/1.0 mM ATP/10 mM Tris·HCl, pH 8.0. Actin-activated myosin ATPase was measured in 15 mM KCl/5 mM $MgCl_2$/0.1 mM $CaCl_2$/1.0 mM ATP/25 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 7.0, with myosin (0.06 mg/ml) and *Dictyostelium* actin (0–0.4 mg/ml). The actin-activated ATPase activity was calculated by subtracting the activity found for myosin alone.

Electrophoresis and Autoradiography. $NaDodSO_4$/polyacrylamide gel electrophoresis was as described by Laemmli (22). Gels were stained with either Coomassie blue (23) or Fast Green (24), dried on a Hoefer slab drying apparatus, and subjected to autoradiography with Kodak X-Omat R x-ray film with a DuPont Cronex intensifying screen.

RESULTS

***In Vivo* Phosphorylation of *Dictyostelium* Myosin.** Purified *Dictyostelium* myosin is composed of pairs of three subunits: a 210,000-dalton heavy chain and two light chains, 18,000 and 16,000 daltons (15). These subunits are present in a 1:1:1 molar ratio as determined by densitometry of $NaDodSO_4$ gels stained with Fast Green (data not shown). A similar ratio was observed when the protein stain was Coomassie blue. Myosin isolated from cells grown in media containing [^{32}P]orthophosphate had phosphate covalently associated with the 210,000-dalton heavy chain and the 18,000-dalton light chain but not with the 16,000-dalton light chain (Fig. 1). The stoichiometry for *in vivo* labeling using two separate myosin preparations is shown in Table 1. An average of 0.24 mol of phosphate per mol of heavy chain and 0.10 mol of phosphate per mol of 18,000 dalton-light chain was observed. The phosphorylated residues apparently are phosphoserine or phosphothreonine as judged from their lability in 1 M NaOH for 16 hr at 22°C and their stability in 1 M HCl for 10 min at 100°C.

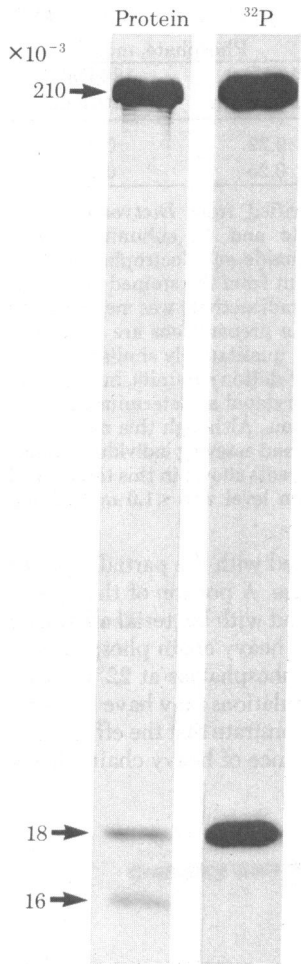

FIG. 1. $NaDodSO_4$ gel (12%) and its autoradiograph, showing *in vivo* phosphorylation of *Dictyostelium* myosin. Myosin purified from amoebae grown in [^{32}P]orthophosphate-labeled medium contained heavy and light chains as judged by gel electrophoresis (Protein). The corresponding autoradiograph (^{32}P) demonstrates that the 210,000-dalton heavy chain and the 18,000-dalton light chain, but not the 16,000-dalton light chain, were phosphorylated *in vivo*.

***In vitro* Phosphorylation of *Dictyostelium* Myosin.** A high-speed supernate of a *Dictyostelium* extract was found to have both heavy chain kinase and 18,000-dalton light chain kinase activities. These two activities could be separated by ammonium sulfate fractionation, and the heavy chain kinase was further purified by affinity chromatography and molecular sieve chromatography. With this partially purified heavy chain kinase, purified *Dictyostelium* myosin was phosphorylated maximally, to <1 mol of phosphate per mol of heavy chain, suggesting a single specific phosphorylation site. The kinase phosphorylated only the heavy chain and was free of proteolytic activity (Fig. 2).

Effect of Heavy Chain Phosphorylation on ATPase Activity. To determine if heavy chain phosphorylation modified the *Dictyostelium* myosin ATPase activity, purified myosin

Table 1. *In vivo* phosphorylation of *Dictyostelium* myosin

Exp.	Phosphate, mol/mol subunit: 210 kDal heavy chain	18 kDal light chain	16 kDal light chain
1	0.22	0.08	0
2	0.25	0.11	0

Myosin was purified from *Dictyostelium* amoebae grown in [^{32}P]orthophosphate and its subunits were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis. The heavy and light chain bands were cut from the stained gel and digested with H_2O_2, and the associated radioactivity was measured. The results from two independent myosin preparations are reported. With each of nine other preparations, qualitatively similar results were obtained; the 210,000- and 18,000-dalton subunits, but not the 16,000-dalton subunit, were phosphorylated as determined from densitometric scans of gel autoradiograms. Although this method is not as accurate as directly cutting out and assaying individual protein bands as was done for the two experiments shown in this table, in all nine preparations the phosphorylation level was <1.0 mol of phosphate per mol of subunit.

was phosphorylated with the partially purified *Dictyostelium* heavy chain kinase. A portion of the phosphorylated myosin was then incubated with bacterial alkaline phosphatase to remove most of the heavy chain phosphate. Because prolonged incubation with phosphatase at 22°C, chromatography, and associated manipulations may have altered the myosin, it was important to demonstrate that the effect was reversible and due solely to the presence of heavy chain phosphate. This was accomplished by rephosphorylating a portion ot the dephosphorylated myosin by using the heavy chain kinase. The integrity of the myosin and the distribution of phosphate are documented in Fig. 2. The endogenous level of unlabeled phosphate in the untreated myosin was not measured in this experiment, although, on the basis of previous *in vivo* labeling experiments, approximately 0.24 mol of phosphate per mol of heavy chain would be expected (see Table 1). The measured levels of *in vitro* phosphorylation (mol of [^{32}P]phosphate added per mol of heavy chain) were 0.34 for the first phosphorylation (or ≈0.6 mol of phosphate total per mol of heavy chain, assuming an endogenous phosphorylation level of ≈0.24), 0.06 for the dephosphorylated myosin, and 0.58 for the rephosphorylated myosin (Fig. 2). It is important to note that the total amounts of phosphate on the myosin heavy chain in the lanes in Fig. 2 that are labeled +P and +P,−P,+P are approximately the same. The higher level of incorporation of [^{32}P]phosphate into myosin that was pretreated with bacterial alkaline phosphatase (Fig. 2, lane +P,−P,+P) is a consequence of the endogenous unlabeled phosphate having been removed from the same site. The 18,000-dalton light chain was not phosphorylated.

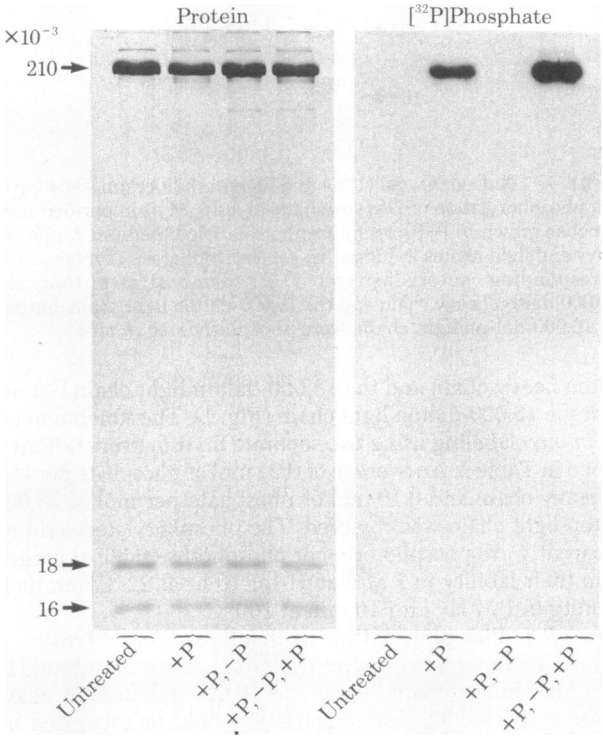

FIG. 2. NaDodSO$_4$ gel (12%) (*Left*) and its autoradiograph (*Right*), showing *in vitro* phosphorylation/dephosphorylation of purified *Dictyostelium* myosin. Myosin was purified from amoebae grown in nonradioactive medium (Untreated) and a portion of this myosin was phosphorylated *in vitro* by using the partially purified heavy chain kinase (+P). Some of this phosphorylated myosin was treated with bacterial alkaline phosphatase to remove phosphate (+P,−P) and a portion of the resulting myosin was then rephosphorylated (+P,−P,+P). Note that there was no proteolysis of myosin subunits and that only heavy chains were phosphorylated. Also, kinase and phosphatase were efficiently removed by Bio-Gel A-0.5 m column chromatography.

The ATPase activity of myosin assayed under high-salt conditions (0.6 M KCl/10 mM $CaCl_2$) was only slightly affected, with phosphorylation depressing the activity somewhat (Table 2). When assayed in low-salt conditions (actin-activation conditions), the myosin alone showed relatively small variations, with phosphorylation causing a slight increase in activity. However, when assayed in the presence of actin, substantial changes in ATPase activity were noted. Removal of heavy chain phosphate by bacterial alkaline phosphatase gave an 80% increase in ATPase specific activity (Fig. 3). The effect was reversible because adding back the phosphate decreased the ATPase specific activity.

Effect of Heavy Chain Phosphorylation on Self-Assembly. *Dictyostelium* myosin, like myosin from both muscle (25) and nonmuscle sources (1), has the ability to self-assemble into thick filaments under conditions of low ionic strength (15). To determine if heavy chain phosphorylation could alter this self-assembly, purified *Dictyostelium* myosin was phosphorylated with *Dictyostelium* heavy-chain kinase and subsequently dephosphorylated with bacterial alkaline phosphatase, as described above.

The untreated, phosphorylated, and dephosphorylated myosins were then assayed for their ability to form thick filaments at various KCl concentrations (Fig. 4*A*). In 50 mM KCl, the majority of the untreated myosin was sedimentable, due to assembly into thick filaments. As the salt concentration was increased, the myosin was increasingly solubilized, and at 150 mM KCl no assembly was detected. Phosphorylation of the

Table 2. Effect of phosphorylation on myosin ATPase activity

Preparation	ATPase specific activity, μmol P_i/min/mg myosin: High-salt, Ca^{2+}	Low-salt, Mg^{2+}
Untreated	0.69	0.013
+P	0.49	0.016
+P,−P	0.61	0.013
+P,−P,+P	0.45	0.017

The myosin preparations were those described in Fig. 2. The data shown are means of triplicate assays which differed by less than 10%. "High-salt, Ca^{2+}": assayed in 0.6 M KCl/10 mM $CaCl_2$/1.0 mM ATP/10 mM Tris·HCl, pH 8.0. "Low-salt, Mg^{2+}": assayed in 0.015 M KCl/5 mM $MgCl_2$/0.1 mM $CaCl_2$/1.0 mM ATP/25 mM 1,4-piperazinediethanesulfonic acid, pH 7.0.

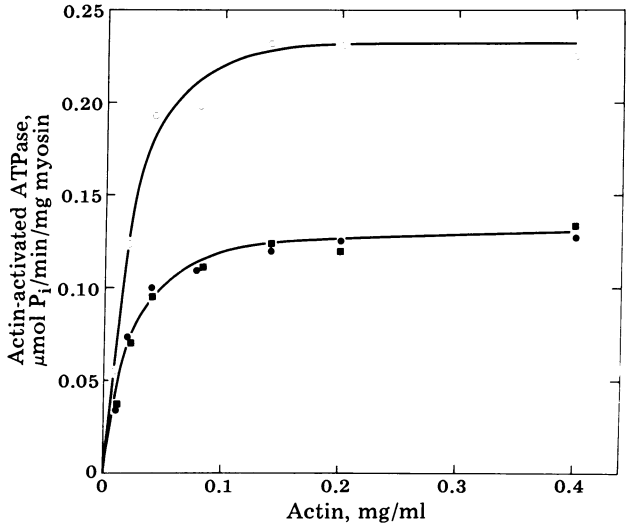

FIG. 3. Effect of phosphorylation on actin-activated myosin ATPase. The myosin preparations were those described in Fig. 2. The specific activity of myosin ATPase is plotted as a function of actin concentration. The values are means of duplicate assays which differed by less than 10%. Activity due to myosin alone has been subtracted, ○, +P, −P; ■, +P, −P, +P; ●, +P.

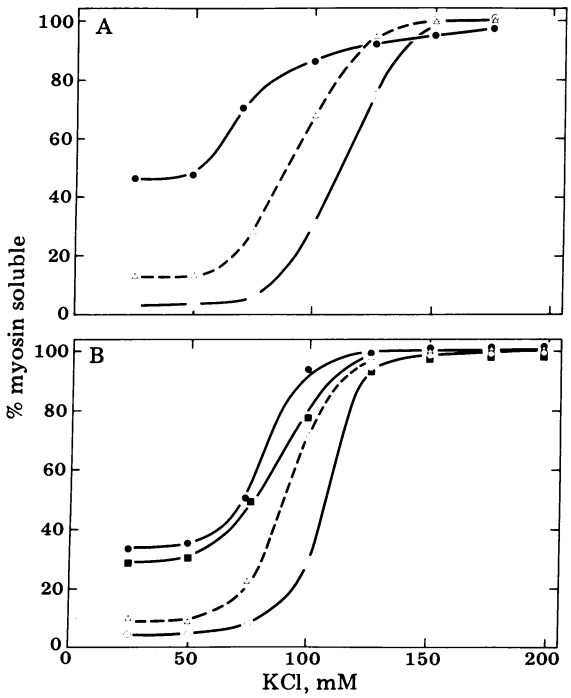

FIG. 4. Effect of phosphorylation on myosin self-assembly. (*A*) Myosin at 0.2 mg/ml was incubated in 0.1 mM EDTA/0.1 mM dithiothreitol/10 mM Tris·HCl, pH 7.5, at various KCl concentrations. Myosin that assembled into thick filaments was removed by centrifugation, and the remaining soluble myosin was measured by the Bradford reaction. In this experiment phosphorylated myosin (●) contained 0.58 mol of added [^{32}P]phosphate/mol heavy chain; dephosphorylated myosin (○) retained 0.12 mol of added [^{32}P]phosphate/mol heavy chain. △, Untreated. (*B*) A different myosin preparation was used and the dephosphorylated myosin (○) was rephosphorylated (■) to demonstrate reversibility of the effect on self-assembly. Myosin at 0.2 mg/ml was incubated in 0.1 mM dithiothreitol/0.02% sodium azide/10 mM Tris·HCl, pH 7.5, and assayed for assembly. In this experiment, the myosin preparations were those described in Fig. 2. △, Untreated; ●, +P.

heavy chain inhibited assembly. For example, at 75 mM KCl, only 25% of the phosphorylated myosin was assembled into filaments, compared to 70% of the untreated myosin. At other KCl concentrations the amount of phosphorylated myosin that assembled was also less than that found for the untreated material, such that the solubility curve was shifted to lower KCl concentrations. When this phosphorylated myosin was treated with bacterial alkaline phosphatase to remove the heavy chain phosphate (+P,−P), assembly was greatly enhanced; the solubility curve was shifted to higher KCl concentrations, showing even better assembly than the untreated myosin which had some phosphate on its heavy chain. Thus, there is a clear correlation showing that heavy chain phosphorylation inhibits self-assembly of *Dictyostelium* myosin.

To check for reversibility of this effect, another experiment with a different myosin preparation was carried out as above but, in addition, the dephosphorylated myosin was rephosphorylated (Fig. 4*B*). A similar result was obtained; the phosphorylated myosin solubility curve was shifted to lower KCl concentrations and the dephosphorylated myosin solubility curve was shifted to higher KCl concentrations. In addition, rephosphorylation of the dephosphorylated myosin (+P,−P,+P) shifted the solubility curve back to lower KCl concentrations, showing the effect to be completely reversible.

DISCUSSION

This study shows that a nonmuscle myosin has both heavy and light chains phosphorylated *in vivo* and that phosphorylation of the heavy chain inhibits the formation of myosin thick filaments. A recent report by Rahmsdorf *et al.* (26) suggested that myosin heavy chain of *Dictyostelium* was phosphorylated, although in their work phosphorylation occurred *in vitro* in cell extracts and myosin was not purified. The unusual single-headed *Acanthamoeba* myosin I has a 140,000-dalton subunit that can be phosphorylated *in vitro;* this phosphorylation is required for actin-activated ATPase activity (27).

The location of the phosphate group along the length of the heavy chain would be expected to determine which activity of the molecule is affected. For example, the tail portion of muscle myosin is a site of myosin–myosin interactions as shown by the observation that light meromyosin is capable of self-assembly (25). One would expect, therefore, that addition of phosphate groups to the tail portion of one *Dictyostelium* myosin molecule might affect its ability to interact with another molecule. Indeed, using chymotryptic cleavage we have recently found (unpublished data) that the *in vivo* and *in vitro* heavy chain phosphorylation site is present in the tail rather than in the head region of the myosin molecule.

Both Ca^{2+}-activated (Table 2) and actin-activated (Fig. 3) myosin ATPase activities were decreased by phosphorylation of the heavy chain. How might addition of phosphate to the myosin tail affect enzymatic activity in the head region of the molecule? In the case of actin-activated ATPase activity, the state of myosin assembly may directly influence its ability to interact with actin. However, the small decrease in ATPase activity of myosin alone (Table 2) was measured in 0.6 M KCl, a condition in which myosin is not aggregated. Phosphorylation of the heavy chain may be exerting an allosteric effect, and the phosphorylation site, although in the tail portion of the molecule, may be near the junction between head and tail, thus affecting assembly as well as enzymatic functions.

Striated muscle myosin thick filaments are readily visible in the electron microscope, are quite stable at physiological ionic strength (28), and are resistant to solubilization by relatively high salt concentrations (29). In smooth muscle it has been much harder to identify thick filaments, and visualization of these

structures depends on fixation conditions (30) and may also reflect the state of muscle activity (31). It has been difficult to identify myosin thick filaments ultrastructurally in nonmuscle cells although, when purified, most myosins form thick filaments at low ionic strength. It is assumed that such thick filaments are present at some time in the cell and interact with actin in a manner analogous to that in the striated muscle sarcomere (32).

Although thick filament assembly *in vitro* depends on the ionic strength of the solution, variations in salt concentration are unlikely to be an *in vivo* control mechanism. The intracellular KCl concentration for *Dictyostelium* amoebae has been estimated to be ≈50 mM by use of a K^+-selective electrode (J. D. Pardee, personal communication). Because changes in myosin solubility due to phosphorylation occur near this salt concentration (Fig. 4), heavy chain phosphorylation may be relevant to the control of assembly of the contractile apparatus in *Dictyostelium*.

Dictyostelium myosin also has one of its light chains phosphorylated *in vivo* (Fig. 1), similar to myosins from striated muscle (33), smooth muscle (34), and many nonmuscle sources (35–37). In those cases in which an effect has been observed, light chain phosphorylation exerted a positive control, increasing the myosin actin-activated ATPase activity. It has recently been demonstrated that light chain phosphorylation can also influence myosin self-assembly. Suzuki *et al.* (38) and Scholey *et al.* (39) have shown that phosphorylation of a light chain renders smooth muscle myosin and nonmuscle myosin resistant to solubilization by ATP.

This investigation was supported by a Cystic Fibrosis Foundation Fellowship and National Institutes of Health Training Grant CA09151-05 to E.R.K. and National Institutes of Health Grant GM-25240 to J.A.S.

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