Filamentous Smooth Muscle Myosin Is Regulated by Phosphorylation

Kathleen M. Trybus

Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254-9110

Abstract. The enzymatic activity of filamentous dephosphorylated smooth muscle myosin has been difficult to determine because the polymer disassembles to the folded conformation in the presence of MgATP. Monoclonal antirod antibodies were used here to "fix" dephosphorylated myosin in the filamentous state. The steady-state actin-activated ATPase of phosphorylated filaments was 30–100-fold higher than that of antibody-stabilized dephosphorylated filaments, suggesting that phosphorylation can activate ATPase activity independent of changes in assembly. The degree of regulation may exceed 100-fold, because steady-state measurements slightly overestimate the rate of product release from dephosphorylated filaments. Single-turnover experiments in the absence of actin showed that although dephosphorylated folded myosin released products at the low rate of 0.0005 s^{-1} (Cross, R. A., K. E. Cross, A. Sobieszek. 1986. *EMBO [Eur. Mol. Biol. Organ.] J.* 5:2637–2641) the rate of product release from dephosphorylated filaments was only 3–12-fold higher, depending on the ionic strength. The addition of actin did not increase this rate to any appreciable extent. Dephosphorylated filaments and dephosphorylated heavy meromyosin (Sellers, J. R. 1985. *J. Biol. Chem.* 260:15815–15819) thus have similar low rates of phosphate release both in the presence and absence of actin. These results show that light chain phosphorylation alone, without invoking other mechanisms, is an effective switch for regulating the activity of smooth muscle myosin filaments.

PHOSPHORYLATION of the 20-kD regulatory light chain by myosin light chain kinase is closely coupled to the initiation of contraction in smooth muscles (for review see Kamm and Stull, 1985). Consistent with this observation, biochemical studies with heavy meromyosin (HMM),[1] the soluble, regulated subfragment of myosin, showed that light chain phosphorylation increased the rate of product release by nearly 1,000-fold in the presence of actin, from 0.002 to 1.9 s^{-1} (Sellers, 1985). In vitro studies with whole myosin, however, suggested that the state of assembly of the myosin may be more important in determining activity than the state of phosphorylation of the regulatory light chain (Wagner and Vu, 1986, 1987).

Myosin can adopt two conformations in the presence of MgATP, depending on the solvent conditions and the state of phosphorylation of the myosin. In a typical solvent used for ATPase assays, namely, low ionic strength (50–75 mM KCl) and moderate concentrations of magnesium (2–4 mM), dephosphorylated myosin would mainly be in the assembly-incompetent folded conformation, with the myosin rod bent into thirds and the heads pointed down toward the tail (Trybus and Lowey, 1984). Phosphorylated myosin would be predominantly filamentous. When the actin-activated activity of these two species was compared, the degree of regulation by phosphorylation was high, and myosin appeared to be similar to HMM (Wagner and Vu, 1986, 1987).

To polymerize dephosphorylated myosin, high concentrations of magnesium (≥10 mM) are necessary. Unexpectedly, the actin-activated ATPase of these dephosphorylated filaments was only two- to threefold less than that of phosphorylated filaments (Wagner and Vu, 1986, 1987). This observation suggested that phosphorylation might increase activity primarily by altering the conformational state of the myosin, and not by directly affecting the rate of product release. Because filaments have been observed in relaxed smooth muscles (Somlyo et al., 1981; Gillis et al., 1988), their enzymatic activity must be "turned off" by some mechanism. If dephosphorylation does not inhibit the rate of ATP turnover from filaments, another regulatory system has to be invoked.

Here, antibodies were used instead of magnesium to stabilize dephosphorylated filaments. This permitted the enzymatic activity of dephosphorylated filaments to be examined under more physiological conditions, where myosin would normally disassemble. The results suggest that changes in the state of light chain phosphorylation alone can effectively regulate the activity of filamentous myosin.

Materials and Methods

Protein Preparation

Turkey gizzard myosin was prepared as described by Sellers et al. (1981). This myosin was ≥95% dephosphorylated as determined by glycerol/poly-

1. *Abbreviations used in this paper*: FTP, formycin triphosphate; HMM and LMM, heavy and light meromyosin, respectively.

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acrylamide gel electrophoresis (Perrie and Perry, 1970). Myosin was thiophosphorylated at low ionic strength as previously described (Trybus and Lowey, 1984). Smooth muscle myosin light chain kinase was isolated essentially according to Adelstein and Klee (1981), and calmodulin was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Turkey gizzard tropomyosin was isolated from an ethanol-acetone gizzard powder (Smillie, 1981). Actin was purified from chicken pectoralis acetone powder as described by Pardee and Spudich (1982). Monoclonal antibodies were purified from ascitic fluid on an Affi-Gel Protein A column (Bio-Rad Laboratories, Richmond, CA). Protein concentrations were determined at λ = 280 nm with the following extinction coefficients (1 mg/ml): myosin, 0.5; actin, 1.1; tropomyosin, 0.29; IgG, 1.45.

Filament Formation

Myosin in 10 mM KP_i, pH 7.5, 0.6 M KCl, 1 mM EGTA, 1 mM DTT, 1 mM NaN_3 was dialyzed into 20 mM imidazole, pH 7, 150 mM KCl, 3 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT. If the final assay conditions were 50 or 75 mM KCl, the filaments formed in 150 mM KCl were diluted with an appropriate amount of buffer without added salt. Antibody was either added to the filaments in 150 mM KCl, or after the filaments were diluted to the correct final salt concentration. The order of addition of antibody to filaments did not appear to affect the enzymatic activity.

Filament Pelleting Assay

The fraction of filamentous myosin was estimated from the amount of pelletable material after a 10-min spin at 23 psi (130,000 *g*) in an airfuge (Beckman Instruments, Inc., Fullerton, CA). Supernatant protein concentrations were determined by the Bradford method (1976) with a myosin standard curve.

Steady-state ATPases

Phosphate release was determined colorimetrically (Taussky and Shorr, 1953) after the reaction was stopped with SDS as described by White (1982). The myosin concentration was 0.25 mg/ml (1-μM sites), and the actin-tropomyosin (4:1 molar ratio) and antibody concentrations were as indicated in the figure legends and tables. The reaction was started by the addition of 2 mM MgATP, and the reaction stopped at three times during the initial 30% of the reaction. Rates were obtained from the average slope through these three time points.

Release of $\gamma^{32}P$ from Myosin

A rapid gel filtration method (Neal and Florini, 1973) was used to measure the release of bound phosphate from myosin. The Sephadex G-50 columns were prepared as described by Sellers (1985). 2 μM (γ^{32}P)MgATP (1 $\times$ 10^6 cpm/nmol) was added to 1-μM myosin sites (0.25 mg/ml); 10 s later 1 mM unlabeled MgATP was added. 100 μl was applied to the 1-ml column at various times, and the column immediately spun to separate free phosphate from myosin. The amount of phosphate bound to myosin, determined by counting an aliquot of the void volume, was plotted as a function of time. Reaction time is considered to be the time between addition of labeled MgATP and when the centrifuge was started; the length of the spin was 30 s. The moles phosphate per mole active site was calculated based on the concentration of myosin that was eluted from the column, which was typically 60-70% of the applied concentration.

Release of Formycin Triphosphate

2 μM formycin triphosphate (FTP) (Calbiochem-Behring Corp., San Diego, CA) was added to 1-μM myosin sites (0.25 mg/ml) until the fluorescence reached a maximum. Approximately 30 s later, 100 μM MgATP was added, and the decrease in FTP fluorescence followed as a function of time. The measurements were made with a fluorimeter (MPF44; Perkin-Elmer Corp., Norwalk, CT) thermostatted at 24°C; 4 mm square micro cells were used. The exciting wavelength was 313 nm (2-nm slit), and the emission monitored at 340 nm (2-nm slit).

Electron Microscopy

Filaments (25-50 μg/ml) were applied to a carbon-coated grid, negatively stained with 1% uranyl acetate, and examined with a Philips electron microscope (EM301; Philips Electronic Instruments, Inc., Mahwah, NJ) operated at 80 kV.

Results

Actin-activated ATPase Activity of Folded and Filamentous Gizzard Myosin

The actin-tropomyosin activated ATPase activity of myosin was determined under conditions where the dephosphorylated species was folded and phosphorylated myosin was filamentous (Fig. 1, circles). A $\geq$50-fold difference in the V_{max} of these two species (0.03 vs. 1.7 s^{-1}) was observed, suggesting that phosphorylation effectively regulates actin-activated ATPase activity when changes in conformational state occur.

The degree to which phosphorylation increases enzymatic activity when myosin is filamentous has been more difficult to establish, primarily because unusual solvent conditions (low pH or high concentrations of magnesium) must be used to prevent dephosphorylated myosin from disassembling. The activity of dephosphorylated filaments has been reported to approach to within threefold of the value obtained for phosphorylated filaments under some solvent conditions (Wagner and Vu, 1986, 1987). The loss of regulation was due to an increase in the rate obtained with the dephosphorylated species, a trend that was also observed here. In 10 mM $MgSO_4$ (50 mM KCl, pH 7), both dephosphorylated and phosphorylated myosin were filamentous, and the difference in V_{max} between the two species decreased to a factor of 11 (0.15 vs. 1.6 s^{-1}; Fig. 1, squares). Although these results suggest that phosphorylation has only small effects on activity when myosin is filamentous, it is possible that factors

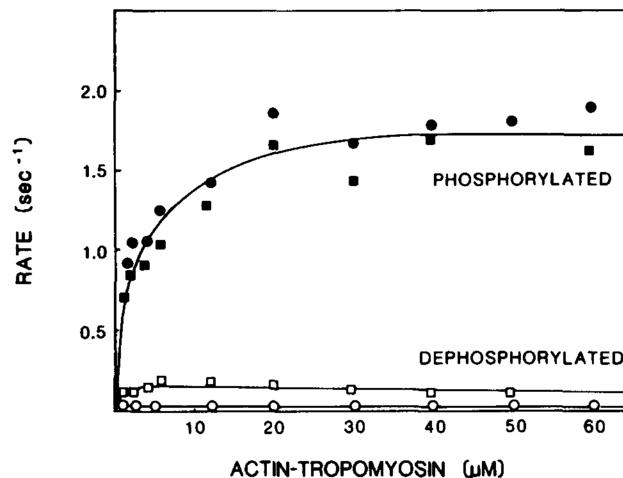

Figure 1. Actin-tropomyosin-activated ATPase activity of gizzard myosin in 3 and 10 mM $MgSO_4$. The ATPase activity of phosphorylated (●, ■) and dephosphorylated (○, □) myosin is plotted as a function of actin-tropomyosin (4:1 molar ratio) concentration. At both 3 (●) and 10 (■) mM $MgSO_4$, the V_{max} for phosphorylated myosin was 1.6-1.7 s^{-1}, and the K_m was 1-2 μM. For dephosphorylated myosin in 3 mM $MgSO_4$, the rate at 50 μM actin-tropomyosin was 0.03 s^{-1} (○). In 10 mM $MgSO_4$, the maximum rate obtained with dephosphorylated myosin increased to 0.17 s^{-1}, and the K_m was $\leq$1 μM (□). Pelleting assays estimated the percent soluble myosin under these conditions: at 3 mM $MgSO_4$, 76% of the dephosphorylated myosin and 14% of the phosphorylated myosin was soluble; at 10 mM $MgSO_4$, only 13% of the dephosphorylated myosin and 7% of the phosphorylated myosin was soluble. Conditions: 20 mM imidazole, pH 7, 50 mM KCl, 1 mM EGTA, 0.5 mM DTT, 37°C, 3 or 10 mM $MgSO_4$.

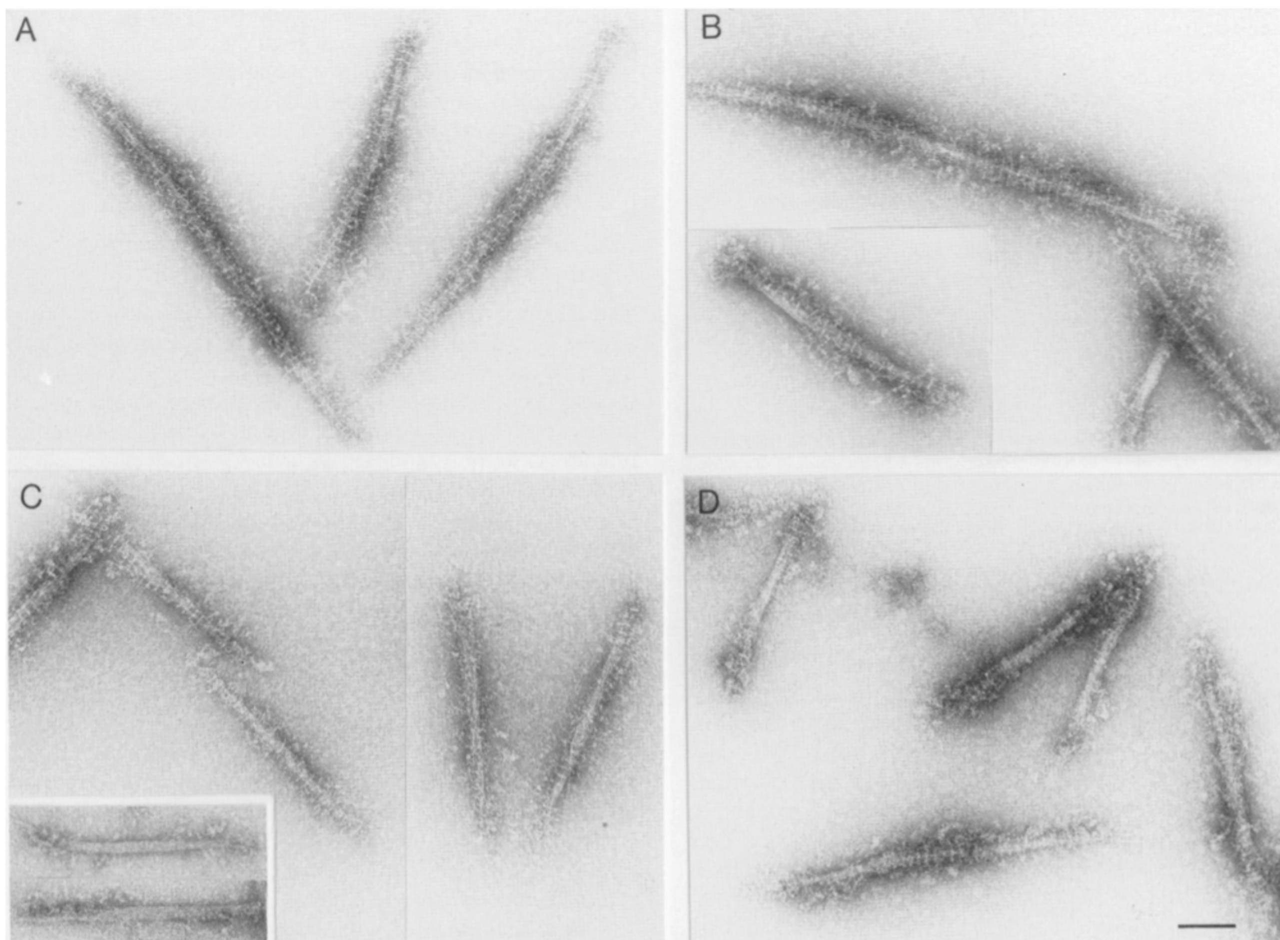

Figure 2. Antirod antibodies stabilize dephosphorylated filaments in the presence of nucleotide. Filaments were reacted with a 3:1 molar ratio of antibody/myosin. 1 mM MgATP was then added, and the filaments were negatively stained. Phosphorylated filaments were reacted with LMM.2 in *A* or with LMM.1 in *B*. Dephosphorylated filaments were reacted with LMM.2 in *C* or LMM.1 in *D*. Note the striations with a 15-nm periodicity. The inset in *C* shows filaments in the absence of antibody and nucleotide. Conditions: 20 mM imidazole, pH 7, 150 mM KCl, 3 mM $MgSO_4$, 1 mM EGTA, mM MgATP. Bar, 0.1 μm.

other than the change in conformation may have activated the dephosphorylated myosin.

Actin-activated ATPase Activity of Antibody-stabilized Filaments

The effect of conformation on activity was directly determined by comparing the rates of folded and dephosphorylated filamentous myosin in the same solvent. Antibodies with epitopes located in the central portion of the rod (S2.2, LMM.1, and LMM.2) were used to stabilize dephosphorylated filaments in the presence of MgATP (Trybus and Henry, 1989). Electron micrographs of the antibody–myosin complexes confirmed that dephosphorylated myosin remained assembled in the presence of nucleotide (Fig. 2, *C* and *D*). The antibody-stabilized filaments have a distinctive structure: periodic striations at 15-nm intervals are evident along the filament length. Filaments decorated with any of the three antibodies show this pattern, but it is most prominent in those filaments decorated with LMM.2 (Fig. 2, *A* and *C*). Similar striations are seen in the presence and absence of nucleotide, and with dephosphorylated (Fig. 2, *C* and *D*) and phosphorylated (Fig. 2, *A* and *B*) filaments. Striations were also seen with antibody-decorated filaments formed from rod, indicating that the striped pattern was caused by bound antibody, and was not due to ordering of the crossbridges.

The steady-state actin-activated ATPase activity of antibody-stabilized filaments was measured under conditions where dephosphorylated myosin normally assumes the folded conformation, and where phosphorylation therefore has a large effect on activity (50 mM KCl, 3 mM $MgSO_4$, as in Fig. 1). An actin–tropomyosin concentration of 30 μM was used so that maximal velocities would be measured. The antirod antibodies did not interfere with actomyosin interactions because the activity of phosphorylated filaments was unchanged in the presence of antibody (Table I). The rate of the antibody-stabilized dephosphorylated filaments, however, was as low as that obtained in the absence of antibody, suggesting that assembly into a filament does not activate the ATPase. Under these conditions, dephosphorylated myosin had a 30-fold lower rate than phosphorylated filaments (Table I).

The filaments formed at 50 mM KCl tended to aggregate side by side, although periodic striations were still evident on the antibody-decorated filaments. At the slightly higher salt concentration of 75 mM, the polymers showed less of a tendency to associate, and the actin-activated ATPase activ-

Table I. Effect of Antirod Antibodies on Filament Activity

Myosin	Antibody‖	−Actin	+Actin¶	Actin-activated rate	Percent soluble myosin
		s^{-1}	s^{-1}	s^{-1}	
Dephosphorylated*	None	0.017	0.063	0.046	76
	S2.2	0.020	0.059	0.039	0
	LMM.1	0.032	0.062	0.032	0
Dephosphorylated‡	none	0.087	0.195	0.108	13
Dephosphorylated§	none	0.140			94
Phosphorylated*	none	0.099	1.56	1.46	14
	S2.2	0.083	1.30	1.22	0
	LMM.1	0.088	1.65	1.56	0
Phosphorylated‡	none	0.178	1.49	1.32	7

* 20 mM imidazole, pH 7, 50 mM KCl, 3 mM $MgSO_4$, 1 mM EGTA, 0.2 mM DTT, 37°C.
‡ As in *, but 10 mM $MgSO_4$.
§ As in *, but 0.6 M KCl.
‖ 3:1 molar ratio of antibody/myosin. Antibody was added to filaments in 50 mM KCl.
¶ 30 μM skeletal muscle actin, 7.5 μM gizzard tropomyosin.

ity of dephosphorylated and phosphorylated filaments was also determined in this solvent. Pelleting assays confirmed that antibody remained bound to the filaments in the presence of actin.

In 75 mM KCl, the K_m was 2 μM, and maximal velocities could again be obtained. Similar to the results obtained in 50 mM KCl, the dephosphorylated filaments turned over MgATP at a rate 40–100-fold slower than the phosphorylated filaments (Table II). A large degree of regulation by phosphorylation was observed at both 24 and 37°C, at 1:1 and 3:1 molar ratios of antibody/myosin, and regardless of whether the antibody was added to filaments formed in 75 or 150 mM KCl. Slightly lower degrees of regulation were obtained with antibody S2.2 compared with the two anti-LMM antibodies, but the difference between phosphorylated and dephosphorylated myosin was still 40–50-fold.

The results obtained at both ionic strengths strongly suggest that phosphorylation can regulate the activity of filamentous myosin in solvents containing near physiological concentrations of magnesium. The reduced degree of regulation by phosphorylation observed at high magnesium concentrations does not appear to be a direct consequence of the assembly of the dephosphorylated myosin.

Release of Products from Folded and Filamentous Myosin in a Single Turnover

The effect of conformation on activity was further investigated by the use of single-turnover assays, in which only one ATP was added per active site. The rate of a very slowly cycling species can be overestimated in the steady state if there is even a small amount of myosin with a faster turnover rate for ATP (Wells and Bagshaw, 1985; Cross et al., 1986). To determine if myosin in a filament attains the same low rate of product release as the folded conformation, a rapid gel filtration method was used to measure the rate of release of radioactive phosphate in the absence of actin. As reported by Cross et al. (1986), the folded conformation in 150 mM KCl essentially traps the products of ATP hydrolysis, and phosphate is released at the very slow rate of 0.0005 s^{-1} (Fig. 3, filled circles; Table III). The rate of phosphate release from antibody-stabilized dephosphorylated filaments at this ionic strength was 0.006 s^{-1} (Fig. 3, open symbols). This value is an order of magnitude greater than that observed with the folded monomer, which appears to be essentially inactive, but it is still a very low rate of ATP turnover. Phosphorylation of the filaments further increased the rate of phosphate release by approximately a factor of four, resulting in a rate similar to that obtained with the extended monomer in high salt (Fig. 3, filled squares and triangles). Thus, at 150 mM salt, essentially three levels of activity can be distinguished.

To compare the rates obtained by single turnovers with the rates obtained for the steady-state ATPases, these experiments were repeated at lower ionic strength with the fluorescent nucleotide FTP. This analog has been shown to substi-

Table II. Actin-activated ATPase Rates of Antibody-stabilized Filaments

		Dephosphorylated myosin			Phosphorylated myosin			
°C	Antibody	−Actin	+Actin§	Actin-activated rate	−Actin	+Actin§	Actin-activated rate	Degree of regulation
		s^{-1}	s^{-1}	s^{-1}	s^{-1}	s^{-1}	s^{-1}	
37°C	none	0.009	0.020	0.011	0.106	1.68	1.57	143
	S2.2	0.020	0.047	0.027	0.099	1.29	1.19	44
	LMM.1	0.038	0.057	0.019	0.083	1.51	1.43	75
	LMM.2	0.033	0.037	0.004	0.095	1.54	1.45	≥100
	LMM.2*	0.027	0.042	0.015	0.116	1.42	1.30	87
	LMM.2‡	0.026	0.046	0.020	0.108	1.35	1.24	62
24°C	none	0.002	0.004	0.002	0.013	0.245	0.232	116
	S2.2	0.005	0.008	0.003	0.014	0.171	0.157	52
	LMM.1	0.007	0.007	–	0.021	0.261	0.240	≥100
	LMM.2	0.005	0.007	0.002	0.014	0.239	0.225	112

Conditions: 20 mM imidazole, pH 7, 75 mM KCl, 3 mM $MgSO_4$, 1 mM EGTA, 0.2 mM DTT. A threefold molar excess of antibody was added to filaments in 0.15 M KCl; the salt concentration was then lowered to 75 mM KCl.
* 1:1 molar ratio of antibody/myosin.
‡ A threefold molar excess of antibody was added to filaments in 75 mM KCl.
§ 20 μM skeletal muscle actin, 5 μM gizzard tropomyosin.

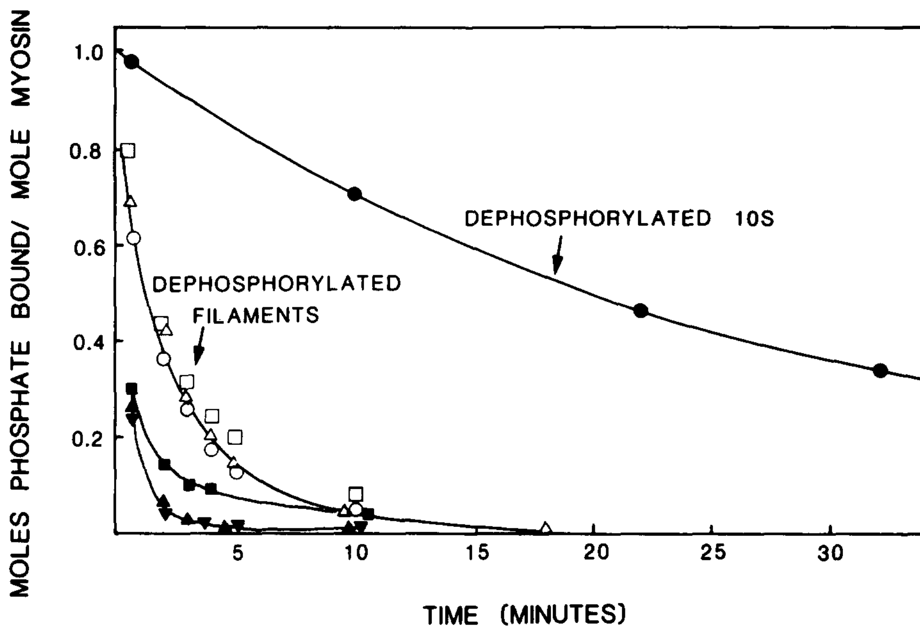

Figure 3. Release of $\gamma^{32}P$ in a single turnover. The release of radioactive phosphate as a function of time was followed by a rapid gel filtration method (see Materials and Methods). The folded monomer (●) released phosphate at a rate of 0.00053 s^{-1}. Antibody-stabilized dephosphorylated filaments (△, S2.2; ○, LMM.1; □, LMM.2) had a 10-fold higher rate of 0.006 s^{-1}. Phosphorylated filaments in the absence (■) or presence (▲, LMM.1) of antibody, or myosin in high salt (▼) was at least four times faster; 70% of the phosphate was released by the time the first point of this manual assay could be taken. Conditions: 20 mM imidazole, pH 7, 150 mM KCl, 3 mM $MgSO_4$, 1 mM EGTA, 0.5 mM DTT, 24°C.

tute for ATP in inducing the folded conformation (Cross et al., 1988). The fluorescence of the FTP approximately doubled upon binding to the active site; release of nucleotide was followed by the decrease in fluorescence with time. The rate of FDP release from the folded monomer at 150 mM KCl was 0.0006 s^{-1}, which agreed reasonably well with the rate of phosphate release determined by the column method.

At 50 mM KCl, dephosphorylated filaments released products at 0.0014 s^{-1} (Fig. 4 *B* and Table III), a rate ~fourfold lower than that obtained at 150 mM KCl. Dephosphorylated myosin not stabilized by antibody released products at essentially the same rate (0.0012 s^{-1}; Fig. 4 *A* and Table III), although this value probably reflects some contribution from filaments that did not disassemble. A decrease in the rate of product release with decreasing salt concentration has also been observed with HMM (Greene and Sellers, 1987). Phosphorylation caused a five- to sevenfold increase in the rate of FDP release (Fig. 4 *C*, Table III). These rates are within threefold of the values obtained in the steady state.

These results show that all myosins with an extended tail are not equivalent. At low ionic strength, dephosphorylated myosin in a filament releases products at a rate 10–20-fold lower than that from an extended monomer in high salt.

Upon addition of 5 μM actin-tropomyosin to the dephosphorylated dimers or the antibody-stabilized dephosphorylated filaments in 50 mM KCl, the rate increased less than twofold, to 0.0019 s^{-1} (Fig. 4, *A* and *B*; Table III). These values are within fourfold of the rates obtained in the steady state. The K_m for actin is 1 μM under these conditions, and thus these rates should be near V_{max}. It was not feasible to use higher actin concentrations, because of mixing problems and because the optical density at the exciting wavelength was too high. As expected, addition of actin-tropomyosin to the phosphorylated filaments resulted in a rate of FTP release that was too fast to measure by this method (Fig. 4 *C*).

By combining the data obtained in the steady state and by single turnovers, it can be seen that dephosphorylated filaments turn over ATP at the rate of 0.001–0.002 s^{-1} in low

Table III. Single-turnover Rates of Myosin Monomers and Filaments

Myosin	Antibody	ATP($\gamma^{32}P$)* 150 mM KCl	FTP‡ 50 mM KCl			ATP, steady-state 50 mM KCl
		−Actin	−Actin	+Actin§	Actin-activated rate	Actin-activated rate‖
		s^{-1}	s^{-1}	s^{-1}	s^{-1}	s^{-1}
dephosphorylated	none	0.0005	0.0012	0.0019	0.0007	0.0045
	LMM.1	0.006	0.0014	0.0019	0.0005	0.0051
Phosphorylated	none	≈0.02	0.006	fast		0.33
	LMM.1	≈0.02	0.006			

Conditions: 20 mM imidazole, pH 7, 50 or 150 mM KCl, 3 mM $MgSO_4$, 1 mM EGTA, 0.2 mM DTT, 24°C.
* Release of bound phosphate was followed by rapid gel filtration. Rates were estimated from semi-log plots.
‡ The fluorescent ATP analog, FTP, was used to follow nucleotide release. Rates were estimated from half-times of the reaction.
§ 5 μM skeletal muscle actin, 1.3 μM gizzard tropomyosin. Under these solvent conditions, the K_m of the steady-state actin-activated ATPase is ~1 μM actin-tropomyosin.
‖ 20 μM skeletal muscle actin, 5 μM gizzard tropomyosin.

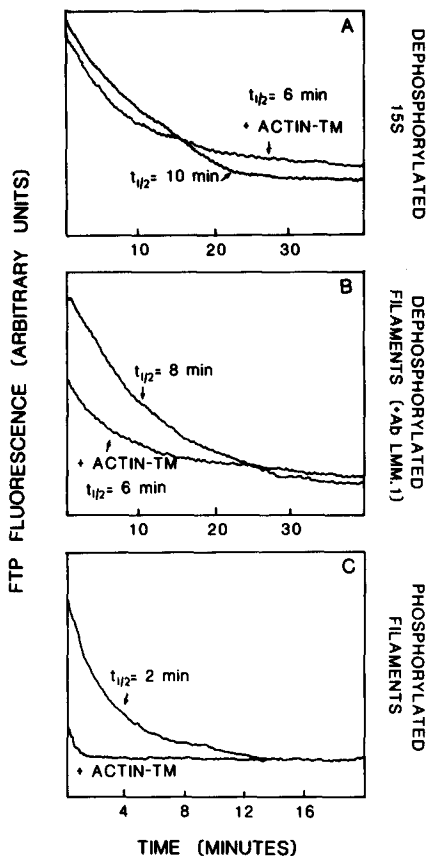

Figure 4. Release of FTP from filaments or folded myosin in the presence and absence of actin. 2 μM FTP was added to 1 μM myosin. When maximum fluorescence enhancement was observed, a 100-μM ATP chase was added. The decay in fluorescence was then followed as a function of time in the absence or presence of 5 μM actin-tropomyosin. (*A*) Folded dephosphorylated myosin. The rates are 0.0012 s^{-1} in the absence of actin, and 0.0019 s^{-1} in the presence of actin. (*B*) Dephosphorylated filaments stabilized with antibody LMM.1. The rates are 0.0014 s^{-1} in the absence of actin, and 0.002 s^{-1} in the presence of actin. (*C*) Phosphorylated filaments. The rate in the absence of actin was 0.006 s^{-1}, and too fast to measure in the presence of actin. Conditions: 20 mM imidazole, pH 7, 50 mM KCl, 3 mM $MgSO_4$, 1 mM EGTA, 0.5 mM DTT, 24°C. The data from these experiments are tabulated in Table III.

salt at 24°C, and that actin causes little activation of this rate. Phosphorylation of the myosin increases this rate to ~0.3 s^{-1} in the presence of actin, resulting in >100-fold increase in the rate of ATP turnover. Although these data do not exclude the possibility of a second regulatory system, phosphorylation by itself has a large effect on the activity of filamentous myosin.

Discussion

Light chain phosphorylation affects both the conformational state and the enzymatic activity of smooth muscle myosin, but it has not been established how closely coupled these two processes are. The question addressed here is whether phosphorylation of the regulatory light chain directly affects the rate of product release when the assembly state is held constant. More specifically, can phosphorylation regulate the activity of filamentous myosin, or is another regulatory system necessary to inhibit the activity of dephosphorylated filaments? The results show that the actin-activated activity of filamentous myosin can be increased to a large extent (~100-fold) by changes in light chain phosphorylation.

The idea that conformation determines activity first arose from the observation that folded myosin had a 10-fold lower steady state MgATPase than myosin with an extended tail, regardless of the state of light chain phosphorylation (Ikebe et al., 1983). This view was modified somewhat by the results of experiments in which single turnovers of MgATP were followed. Dephosphorylated folded myosin in 150 mM KCl "trapped" the products of ATP hydrolysis (0.0002 s^{-1}), whereas phosphorylated folded myosin had a 10-fold higher rate of product release (Cross et al., 1986, 1988). Phosphorylation could therefore modulate the rate of product release without changing conformation. Cross et al. (1988) also observed that the rate of nucleotide release increased to 0.02 s^{-1} for myosin with an extended tail, regardless of whether the myosin was monomeric or filamentous. This observation was unexpected because it suggested that dephosphorylated filaments were not "turned off" to the same extent as dephosphorylated HMM (Sellers, 1985).

Here the activity of antibody-stabilized dephosphorylated filaments was measured in a solvent where myosin normally disassembles. The results showed that all myosins with an extended tail did not have the same ATPase activity. The rate of product release from dephosphorylated filaments was low and similar to that of dephosphorylated HMM (0.001 s^{-1} at 50 mM KCl; 0.006 s^{-1} at 150 mM KCl; see Table III) (Sellers, 1985; Greene and Sellers, 1987). Monomeric myosin in high salt releases products at the faster rate of 0.02 s^{-1}. The similarity in rates between dephosphorylated filaments and HMM suggests that both species can undergo a common transition that results in the observed depression of product release. This transition could be related to the observed salt-induced movement of the heads of HMM from an upward to a downward orientation (Suzuki et al., 1985). Even though the heads of folded monomeric myosin bend down toward the tail (Onishi and Wakabayashi, 1982; Trybus and Lowey, 1984), the activity of this species is lower than that of filaments or HMM (0.0005 s^{-1}; see Table III). But in the case of folded myosin, interaction of the tail with the neck may stabilize the inhibited state more effectively than is possible in the filament or with HMM.

Phosphorylation increased the rate of product release from filamentous myosin approximately fivefold in the absence of actin (0.006 s^{-1} at 50 mM KCl; 0.02 s^{-1} at 150 mM KCl; see Table III), although this is much less than the enhancement seen in the presence of actin. Other techniques, such as limited proteolysis, have also detected differences between phosphorylated and dephosphorylated filaments, suggesting that a transition at the head/rod junction upon phosphorylation might play a role in determining enzymatic activity (Ikebe and Hartshorne, 1984).

Taken together, the results suggest that phosphate release in the absence of actin is affected both by phosphorylation and by changes in conformation. If conformation is kept constant, phosphorylation activates the rate of product release 5–10-fold; if the state of phosphorylation is kept constant, assembly into a filament causes up to a 10-fold activation of product release. The rates obtained in the absence of actin are important because these values are the basal levels from

which actin and phosphorylation accelerate product release. The more effectively the enzyme is "turned off," the greater will be the degree of regulation when this inhibition is removed.

Regulation of the rate of product release in the absence of actin was first described for the thick filament regulated scallop myosin. ADP release was inhibited 600-fold, and the rate of phosphate release decreased 50–100-fold upon removal of calcium (Jackson and Bagshaw, 1988). The lowest rate of phosphate release obtained for scallop myosin in the absence of calcium was similar to that obtained with smooth muscle myosin dephosphorylated filaments or HMM (0.002 s^{-1}).

The more physiologically relevant question is to what extent phosphorylation regulates the ATPase activity of myosin in the presence of actin. Wagner and Vu (1986, 1987) reported that under solvent conditions that favored assembly (i.e., high magnesium concentrations), the V_{max} of dephosphorylated smooth muscle myosin filaments was high, approximately half that obtained with phosphorylated filaments. A loss of regulation by phosphorylation was also observed here when 10 mM $MgCl_2$ was used to promote filament formation, but the highest values obtained were not as fast as those reported by Wagner and Vu (1987). It is not unreasonable that the solvent conditions necessary to prevent myosin from disassembling could also activate the dephosphorylated molecule. High concentrations of magnesium, for example, caused tension development in skinned gizzard fibers in the absence of phosphorylation (Ikebe et al., 1984). Other modifications, such as reaction of a thiol in the COOH-terminal 20-kD of the head region, can also mimic the effects of phosphorylation (Chandra et al., 1985).

The evidence presented here suggests that filament assembly per se does not cause a large increase in actin-activated activity. The steady-state actin-activated ATPase of antibody-stabilized dephosphorylated filaments in 3 mM $MgSO_4$ was 30–100-fold lower than that for phosphorylated filaments. Single-turnover experiments suggest that this ratio somewhat underestimates the degree of regulation by phosphorylation, which probably exceeds 100-fold. Filament assembly, however, is required for actin activation. When phosphorylated myosin was not totally assembed, the V_{max} decreased, indicating that folded phosphorylated myosin was not appreciably actin activated (Wagner and Vu, 1986, 1987). Binding of phosphorylated folded myosin to actin has not been measured, but dephosphorylated folded myosin binds 100-fold more weakly to actin than extended myosin (Ikebe and Hartshorne, 1986). The lack of actin activation of the folded form, whether phosphorylated or dephosphorylated, may therefore simply be due to its lack of interaction with actin. In contrast, the lack of actin activation of dephosphorylated HMM appears to be due to inhibition of the rate of phosphate release, and not a marked decrease in affinity for actin compared with the phosphorylated species (Sellers et al., 1982).

The in vitro results obtained here are consistent with the observation that phosphorylation is required for the initiation of contraction in smooth muscles (see review by Kamm and Stull, 1985). These results also suggest that even if no assembly-disassembly took place in vivo, phosphorylation by itself would be a good regulator of actomyosin ATPase activity. Another regulatory mechanism such as caldesmon does not appear to be essential. In some smooth muscles, myosin filaments can partially depolymerize and reform during cycles of contraction and relaxation (Godfraind-DeBecker and Gillis, 1988; Gillis et al., 1988). Both these studies and those of Somlyo et al. (1981) showed, however, that a high proportion of the myosin in relaxed smooth muscle cells exists in the form of filaments. If folded myosin forms to some extent in vivo, it would only confer additional regulation on an already efficient system.

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