Phosphorylation of Thymus Myosin Increases Its Apparent Affinity for Actin but Not Its Maximum Adenosinetriphosphatase Rate†

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ABSTRACT: Vertebrate nonmuscle myosins contain two phosphorylatable light chains. The maximum rate, V_{max}, of the actin-activated adenosinetriphosphatase (ATPase) of unphosphorylated calf thymus myosin was found to be about 100 nmol/(min·mg), the same as that of thymus myosin with two phosphorylated light chains. However, the K_{app} (actin concentration required to achieve $^1/_2V_{max}$) of the unphosphorylated myosin was 15–20-fold greater than that of the phosphorylated myosin. When actin complexed with either skeletal muscle tropomyosin or calf thymus tropomyosin was used, the values for V_{max} were about the same as those obtained with F-actin. In the presence of skeletal muscle tropomyosin, the K_{app} of the unphosphorylated myosin was only 2–3-fold greater than that of the phosphorylated myosin, and in the presence of thymus tropomyosin, there was about a 5-fold difference in their K_{app} values. Thus, light chain phosphorylation regulates the actin-activated ATPase of thymus myosin not by increasing V_{max} but rather by decreasing the K_{app} of this myosin for actin. These rather small differences in K_{app} suggest that other proteins may be involved in the regulation of the actin-activated ATPase of thymus myosin. Regulated actin (actin plus skeletal muscle troponin–tropomyosin) was used to examine possible effects of thin-filament regulatory proteins. In the presence of calcium, phosphorylation caused only a slight increase in V_{max} and a 2-fold decrease in K_{app} of the regulated actin-activated ATPase of thymus myosin. In the absence of calcium, there was no significant activation of the unphosphorylated myosin by regulated actin, but at low ionic strengths, regulated actin activated the phosphorylated myosin almost as well in the absence of calcium as in its presence.

Vertebrate smooth muscle and nonmuscle myosins have similar properties and subunit compositions, being composed of two 200 000-dalton heavy chains and two pairs of light chains of M_r 20 000 and 17 000. Phosphorylation of the 20 000-dalton light chains, LC20,[1] causes a large increase in the actin-activated ATPase activities of these myosins [reviewed in Adelstein & Eisenberg (1980); Kendrick-Jones & Scholey (1981); Walsh & Hartshorne (1982)]. These light chains are phosphorylated by Ca^{2+}–calmodulin-dependent kinases that are specific for the myosin light chains (Sobieszek & Small, 1977; Dabrowska & Hartshorne, 1978; Hathaway & Adelstein, 1979; Adelstein & Klee, 1981; Bourguignon et al., 1982) and dephosphorylated by Ca^{2+}-insensitive phosphatases (Pato & Adelstein, 1980; Werth et al., 1982). Ca^{2+}-insensitive LC20 phosphorylation has also been observed in cell extracts (Trotter & Adelstein, 1979; Fechheimer & Cebra, 1982), but it is not known if this activity results from other types of kinases or from proteolysis of the Ca^{2+}–calmodulin-dependent kinases (Adelstein & Eisenberg, 1980). While phosphorylation of LC20 appears to be an important mechanism for regulating the actin-activated ATPases of vertebrate smooth and nonmuscle myosins, it does not preclude other regulatory systems (Hirata et al., 1980; Sobue et al., 1982; Chacko & Rosenfeld, 1982; Marston & Smith, 1984).

The phosphorylation of gizzard myosin has been studied in more detail than that of any other smooth muscle myosin. LC20 phosphorylation appears to be necessary for actin to activate the MgATPase of this myosin (Sobieszek & Small, 1977; Sherry et al., 1978; Sellers et al., 1981). The V_{max} of the actin-activated ATPase of unphosphorylated gizzard heavy meromyosin is only 4% of that of the phosphorylated heavy meromyosin (Sellers et al., 1982). Both heads of gizzard myosin must be phosphorylated for actin to activate the MgATPase of either head (Persechini & Hartshorne, 1981; Ikebe et al., 1982; Sellers et al., 1983). However, the mechanism of activation of mammalian nonmuscle and smooth muscle myosins appears to be quite different. Phosphorylation of one head of a nonmuscle myosin isolated from calf thymus (Wagner et al., 1985) stimulates the actin-activated ATPase of that head independent of the phosphorylation of the second head. Results with several mammalian smooth muscle myosins are consistent with the two heads of these myosins being activated independently (Chacko, 1981; Chacko & Rosenfeld, 1982).

We have examined the actin-activated ATPase of thymus myosin as a function of actin concentration by using both F-actin and F-actin complexed with skeletal muscle tropomyosin, skeletal muscle troponin–tropomyosin (regulated actin), or thymus tropomyosin. These results show that phosphorylation of thymus myosin has little effect on the maximum rate of the actin-activated ATPase, but rather it causes a 15–20-fold increase in the apparent affinity of this myosin for actin.

Materials and Methods

Skeletal muscle myosin was isolated from rabbit back and hind leg muscles, and actin and troponin–tropomyosin were

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ATPase, adenosinetriphosphatase; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; LC20, 20 000-dalton light chain of myosin; LC17, 17 000-dalton light chain of myosin; P_i, inorganic phosphate.

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prepared from the acetone powder of these muscles (Wagner & Weeds, 1977). Skeletal muscle tropomyosin was also isolated from this acetone powder (Smillie, 1982). Calf thymus tropomyosin was isolated by following the procedure of Cote & Smillie (1981) for the isolation of platelet tropomyosin. Thymus actin was isolated by following the procedure of Pardee & Bamburg (1979) for the isolation of brain actin. Regulated actin was prepared as described previously (Wagner & Stone, 1983), and tropomyosin–actin was prepared by mixing F-actin and tropomyosin at a 7:1.5 molar ratio. Column-purified turkey gizzard myosin was given to us by Dr. James Sellers. Turkey gizzard myosin light chain kinase was isolated as described by Adelstein & Klee (1981). Bovine brain calmodulin was given to us by Dr. Claude Klee. Calf thymus myosin was isolated by using a modification (Wagner et al., 1985) of the "ammonium sulfate" procedure of Scholey et al. (1982). The major modification was to load a 100-mL solution of 0.6 M KI, 10 mM $Na_2S_2O_3$, 0.1 mM EGTA, 10 mM $MgCl_2$, 10 mM ATP, 5 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 25 mM Tris, pH 7.5 at 4 °C, onto the Sepharose 4B (Pharmacia) column prior to loading the actomyosin. This brief exposure to KI improves the separation of myosin from actin, but it does not result in the loss of ATPase activity that occurs when thymus myosin is incubated in KI for several hours (Scholey et al., 1982; Wagner et al., 1985). This thymus myosin has about 20% phosphorylated LC20. These light chains were dephosphorylated by incubating the myosin, 7 mg/mL, with 1 ng/mL alkaline phosphatase (Sigma Type VII-N) for 16 h at 0 °C in 0.6 M NaCl, 1 mM $MgCl_2$, 0.2 mM DTT, and 30 mM Tris, pH 8.0. Alkaline phosphatase was removed by gel filtration (Wagner et al., 1985). Thymus and gizzard myosins were usually dialyzed into 150 mM KCl, 10 mM $MgSO_4$, 0.2 mM DTT, and 10 mM imidazole, pH 7.0. They were stored in this buffer at 0 °C for up to 1 week without any detectable change in ATPase activity.

Thymus and gizzard myosins, about 3 mg/mL, were phosphorylated by 10 nM gizzard myosin light chain kinase in 150 mM KCl, 10 mM $MgSO_4$, 0.2 mM DTT, 0.1 mM $CaCl_2$, 1 mM ATP, 0.5 μM calmodulin, and 10 mM imidazole, pH 7.0 at 25 °C. After 10 min, the light chains of these myosins were completely phosphorylated. Myosins with different levels of LC20 phosphorylation were obtained by removing aliquots from this reaction mixture after various lengths of time and stopping the phosphorylation by making the samples 2 mM in EGTA. The phosphorylated samples were kept at 0 °C and their ATPase activities determined within 1 h. The fraction of LC20 phosphorylated was determined by electrophoresis on urea/glycerol polyacrylamide gels (Perrie & Perry, 1970). On these gels, phosphorylated LC20 migrates more quickly than unphosphorylated LC20. The urea/glycerol gels were stained with Coomassie brilliant blue, and the fraction of LC20 phosphorylated was determined by use of a scanning gel densitometer. The results obtained by this method differed by less than 6% from those obtained by use of ^{32}P incorporation (Wagner et al., 1985).

Unless stated otherwise, the ATPase assays were performed in 50 mM KCl, 10 mM $MgSO_4$, 0.2 mM DTT, 1 mM EGTA, 2 mM [γ-^{32}P]ATP (1000 cpm/nmol), and 20 mM imidazole, pH 7.0 at 37 °C. The myosins were about 0.3 mg/mL, and the skeletal muscle actin was usually 14 μM. The ATPase reactions were started by adding 50 μL of myosin in 10 mM $MgSO_4$ and 150 mM KCl to 450 μL of reaction mixture. However, the same ATPase activities were obtained when the myosin and actin were first mixed in 0.5 M KCl and then diluted to low ionic strength. Aliquots were removed after 4, 9, 14, and 19 min, and the amount of inorganic phosphate was determined (Pollard & Korn, 1973). The ATPase assays were linear over this time period. In the absence of actin, the MgATPase activities of thymus myosin and gizzard myosin at 37 °C were respectively 10 and 30 nmol/(min·mg). These rates were subtracted from those observed in the presence of actin. Under comparable conditions, Heaslip & Chacko (1985) obtained an ATPase activity for gizzard myosin in the absence of actin of 21 nmol/(min·mg). At 25 °C, the ATPase activity of gizzard myosin was found to be 3 nmol/(min·mg), comparable to that reported by Persechini & Hartshorne (1981) and by Sellers et al. (1983).

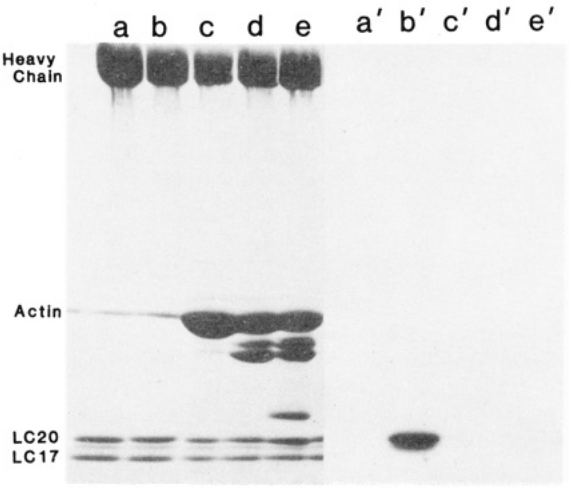

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of thymus myosin. (a–e) Coomassie brilliant blue stained gel; (a′–e′) autoradiogram of this gel. Thymus myosin, 0.9 mg/mL, was incubated at 37 °C for 10 min in 50 mM KCl, 10 mM $MgSO_4$, 0.2 mM DTT, 2 mM [γ-^{32}P]ATP, 20 mM imidazole, pH 7.0, and either (a and a′) 1 mM EGTA or (b and b′) 0.1 mM $CaCl_2$, 10 nM gizzard myosin light chain kinase, and 0.5 μM calmodulin, or (c and c′) 1 mM EGTA and 50 μM actin, or (d and d′) 1 mM EGTA and 15 μM skeletal muscle tropomyosin–actin, or (e and e′) 0.1 mM $CaCl_2$ and 15 μM regulated actin; 50 μg of myosin was applied to each well.

RESULTS

Effect of LC20 Phosphorylation on the Actin-Activated ATPase of Thymus Myosin. Electrophoresis on urea/glycerol gels showed that the unphosphorylated calf thymus and turkey gizzard myosins contained less than 5% phosphorylated LC20. These light chains can be fully phosphorylated by gizzard myosin light chain kinase in the presence of Ca^{2+} and calmodulin (Sellers et al., 1981; Wagner et al., 1985). When [γ-^{32}P]ATP was used, the fully phosphorylated myosins contained 1.9 ± 0.2 ^{32}P/myosin. Incubation of unphosphorylated thymus and gizzard myosins in MgATP for 10 min at 37 °C resulted in less than 4% increase in LC20 phosphorylation (Figure 1). Incubation of the phosphorylated myosins for 10 min at 37 °C did not result in any detectable dephosphorylation. The dependence of LC20 phosphorylation on the addition of both Ca^{2+} and myosin light chain kinase and the absence of phosphatase activity have been reported previously for both gizzard (Sellers et al., 1983) and thymus myosins (Wagner et al., 1985). Under the conditions used for the ATPase assays both the unphosphorylated and the phosphorylated myosins are filamentous (Wagner et al., 1985).

As reported previously (Scholey et al., 1982; Wagner et al., 1985), the actin-activated ATPase activity of unphosphorylated thymus myosin is about 15% of that of the fully phosphorylated myosin. However, when F-actin complexed with skeletal muscle tropomyosin or regulated actin in the presence of Ca^{2+}

Table I: ATPase Activities of Skeletal Muscle, Gizzard, and Thymus Myosins[a]

	MgATPase activities [nmol/(min·mg)]					
	F-actin		regulated actin		tropomyosin–actin	
myosin	EGTA	Ca^{2+}	EGTA	Ca^{2+}	EGTA	Ca^{2+}
skeletal muscle			20	440		
unphosphorylated gizzard[b]	9	13	6	15	15	38
phosphorylated gizzard[c]	86	83	25	255	239	239
unphosphorylated thymus[b]	13	12	8	50	57	60
phosphorylated thymus[c]	78	81	79	86	89	80

[a] The ATPase assays were performed at 37 °C in 50 mM KCl, 10 mM $MgSO_4$, 2 mM [γ-^{32}P]ATP, 0.2 mM DTT, 20 mM imidazole, pH 7.0, and either 0.1 mM $CaCl_2$ or 1 mM EGTA. The myosins were 0.30 mg/mL, and the skeletal muscle actin, regulated actin, and skeletal muscle tropomyosin actin were 14 μM. [b] Unphosphorylated gizzard and thymus myosins contained less than 5% phosphorylated LC20. [c] Phosphorylated gizzard and thymus myosins contained 100% phosphorylated LC20.

Table II: Actin, Tropomyosin–Actin, and Regulated Actin-Activated ATPases of Unphosphorylated and Phosphorylated Thymus Myosin[a]

	unphosphorylated myosin[b]		phosphorylated myosin[c]	
actin	V_{max} [nmol/(min·mg)]	K_{app} (μM)	V_{max} [nmol/(min·mg)]	K_{app} (μM)
skeletal muscle actin	125	65	114	5
skeletal muscle actin[d]	91[d]	77[d]	100[d]	4[d]
skeletal muscle tropomyosin + actin	114	12	118	4
thymus tropomyosin + skeletal muscle actin	90	20	100	3
thymus tropomyosin + thymus actin	103	9	108	3
skeletal muscle regulated actin	83	6	103	3

[a] The assays were performed at 37 °C in 10 mM $MgSO_4$, 50 mM KCl, 2 mM [γ-^{32}P]ATP, 0.2 mM DTT, and 20 mM imidazole, pH 7.0. The actin and tropomyosin–actin-activated ATPases were performed in 1 mM EGTA, and the regulated actin-activated ATPases were performed in 0.1 mM $CaCl_2$. [b] Unphosphorylated myosin had less than 5% phosphorylated LC20. [c] Phosphorylated myosin contained 100% phosphorylated LC20. [d] This thymus myosin was not treated with alkaline phosphatase. The unphosphorylated myosin had 15% phosphorylated LC20.

was used, the ATPase activities of the unphosphorylated thymus myosin were greater than 60% of those of the fully phosphorylated myosin (Table I). As only about 5% of the light chains were phosphorylated, the unphosphorylated thymus myosin appears to have significant ATPase activity. The thymus myosin used in the experiment given in Table I was purified by a brief incubation in 0.6 M KI (see Materials and Methods). Thymus myosin isolated without the use of KI (Scholey et al., 1982) and not treated with alkaline phosphatase had about 40% phosphorylated LC20. In 15 μM skeletal muscle tropomyosin–actin, the MgATPase of this myosin was 83 nmol/(min·mg) compared to 108 nmol/(min·mg) when fully phosphorylated. After treatment with alkaline phosphatase, the myosin contained 20% phosphorylated LC20, and in the presence of skeletal muscle tropomyosin, it had an actin-activated ATPase of 76 nmol/(min·mg).

Skeletal muscle tropomyosin increased the actin-activated ATPase activities of both unphosphorylated and phosphorylated gizzard myosin (Table I), but the ATPase activities of the unphosphorylated myosin were always much less than those of the phosphorylated myosin. A 2- to 3-fold stimulation of the actin-activated ATPase of phosphorylated gizzard myosin by gizzard tropomyosin has been reported by Sobieszek & Small (1977). Even larger stimulations by both gizzard and skeletal muscle tropomyosins have been reported (Yamaguichi et al., 1984; Heaslip & Chacko, 1985).

Thymus myosins with different levels of LC20 phosphorylation were prepared as described under Materials and Methods, and their actin-activated and skeletal muscle tropomyosin–actin-activated ATPase activities were determined. These ATPase assays were performed in 1 mM EGTA to prevent any additional phosphorylation (Wagner et al., 1985). There was nearly a linear correlation between the level of LC20 phosphorylation and the actin-activated ATPase (Figure 2), but when tropomyosin was present, the lower the level of phosphorylation the greater the ratio of ATPase activity to the fraction of LC20 phosphorylated.

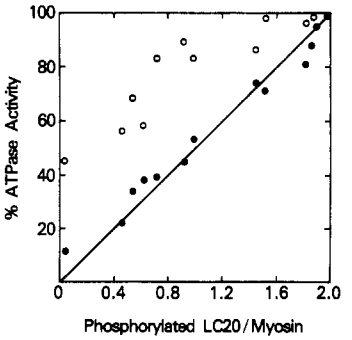

FIGURE 2: Actin-activated and tropomyosin–actin-activated ATPases of thymus myosins with different levels of phosphorylated LC20. The ATPase assays were performed at 37 °C in 50 mM KCl, 10 mM $MgSO_4$, 0.2 mM DTT, 2 mM [γ-^{32}P]ATP, 1 mM EGTA, and 20 mM imidazole, pH 7.0. The myosin was 0.3 mg/mL, the actin (●) was 14 μM, and the actin complexed with skeletal muscle tropomyosin (○) was 12 μM.

The MgATPase activities of unphosphorylated and fully phosphorylated thymus myosins were determined at various actin concentrations (Figure 3). The MgATPases of myosins have hyperbolic dependences on actin concentration. From plots of $1/V_{obsd}$ vs. 1/(actin), values for V_{max} and K_{app} (actin concentration required to achieve $^1/_2V_{max}$) can be determined. Data for both the unphosphorylated and the phosphorylated myosins extrapolated to the same V_{max}, about 120 nmol/(min·mg), but the K_{app} values were very different. The fully phosphorylated myosin had a K_{app} of 5 μM, while the unphosphorylated myosin had a K_{app} of 65 μM. Thymus myosin was usually treated with alkaline phosphatase to reduce the level of LC20 phosphorylation. As shown in Table II, the V_{max} and K_{app} values for the actin-activated ATPase of thymus myosin not treated with phosphatase (15% initial LC20 phosphorylation) were similar to those for the phosphatase-treated myosin. While skeletal muscle tropomyosin had little effect on the actin-activated ATPase of phosphorylated thymus myosin, the K_{app} of the unphosphorylated myosin was reduced

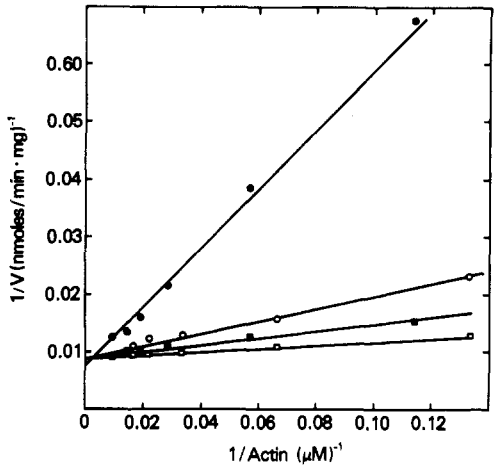

FIGURE 3: Actin-activated and skeletal muscle tropomyosin–actin-activated ATPases of thymus myosin. The conditions were as given in Figure 2, except the actin and tropomyosin–actin concentrations were varied. (●) Actin-activated ATPase of unphosphorylated myosin; (○) tropomyosin–actin-activated ATPase of unphosphorylated myosin; (■) actin-activated ATPase of phosphorylated myosin; (□) tropomyosin–actin-activated ATPase of phosphorylated myosin.

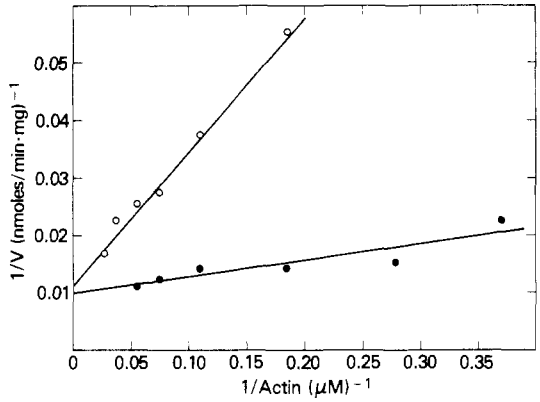

FIGURE 4: Actin-activated ATPase of thymus myosin in the presence of thymus tropomyosin. Conditions were as given in Figure 3, except the tropomyosin was from calf thymus. (○) Unphosphorylated myosin; (●) phosphorylated myosin.

to 12 μM (Figure 3). When skeletal muscle actin complexed with thymus tropomyosin was used, LC20 phosphorylation caused about a 7-fold decrease in K_{app} and only a slight increase in V_{max} (Figure 4). When thymus actin and thymus tropomyosin were used, there was only a 3-fold difference in K_{app} (Table II). The MgATPase activities of unphosphorylated and fully phosphorylated thymus myosin were also determined at different regulated actin concentrations in the presence of 0.1 mM $CaCl_2$. The V_{max} of the unphosphorylated myosin was about 80% of that of the fully phosphorylated myosin (Table II), and there was only about a 2-fold difference in their K_{app} values.

The actin-activated ATPases of thymus myosin in the presence of skeletal muscle tropomyosin were also determined under several other conditions to ensure that these results were not limited to one specific set of conditions. In 5 mM $MgSO_4$ instead of 10 mM $MgSO_4$, V_{max} and K_{app} were 75 nmol/(min·mg) and 7 μM for the unphosphorylated myosin and 92 nmol/(min·mg) and 4 μM for the phosphorylated myosin. In 10 mM $MgSO_4$ and 100 mM KCl, V_{max} and K_{app} were 85 nmol/(min·mg) and 10 μM for the unphosphorylated myosin and 87 nmol/(min·mg) and 4 μM for the phosphorylated myosin. While the ATPase assays for both unphosphorylated and phosphorylated thymus myosins were linear in 50 and 100 mM KCl, in 150 mM KCl the actin-activated ATPase assays of the unphosphorylated myosin were not linear; the rate of ATP hydrolysis increased with time, making it impossible to determine values for K_{app} and V_{max}. However, in 100 μM skeletal muscle tropomyosin–actin, the unphosphorylated myosin had an ATPase activity of about 40 nmol/(min·mg) (calculated from the amount of P_i released between 4 and 19 min after adding myosin to the assay). The actin-activated ATPase assays of the phosphorylated myosin were linear, and in the presence of skeletal muscle tropomyosin, the actin-activated ATPase of this myosin had a K_{app} of 6 μM and a V_{max} of 115 nmol/(min·mg).

The MgATPase activities of the unphosphorylated gizzard myosin did not increase as the actin or tropomyosin–actin concentrations were increased from 11 to 95 μM, and in the absence of calcium the rates for the unphosphorylated myosin were about 10% of those of the phosphorylated myosin. In 0.1 mM $CaCl_2$, the actin-activated ATPases of unphosphorylated gizzard myosin were about 15% of those of the fully phosphorylated (Table I), but these ATPase activities did not increase as the actin or tropomyosin–actin concentrations were increased.

The high ATPase activities of unphosphorylated thymus myosin do not result from phosphorylation during the ATPase assays. The unphosphorylated myosin was incubated in [γ-^{32}P]MgATP under the conditions used for the ATPase assays either in 1 mM EGTA with either actin or skeletal muscle tropomyosin–actin or in 0.1 mM $CaCl_2$ with regulated actin. After 10 min at 37 °C, the samples were electrophoresed on polyacrylamide gels in the presence of $NaDodSO_4$ (Figure 1). Autoradiograms of these gels showed than when myosin light chain kinase was not added, there was only about a 4% increase in the fraction of LC20 phosphorylated. The same result was obtained when thymus myosin was incubated with thymus tropomyosin and actin. There was also no obvious degradation of the myosin heavy chains or light chains during these incubations.

Interaction of Thymus Myosin with Regulated Actin. Regulated actin activated the MgATPase of skeletal muscle and phosphorylated gizzard myosins to a much greater extent in the presence of Ca^{2+} than in the presence of EGTA (Table I). However, regulated actin activated the MgATPase of phosphorylated thymus myosin almost as well in EGTA as in Ca^{2+} (Table I). Even at a ratio of 1 myosin to 272 actins, the regulated actin-activated ATPase of the phosphorylated thymus myosin in EGTA was 80% of that in Ca^{2+}. The regulated actin-activated ATPase activity of fully phosphorylated thymus myosin isolated without the use of KI was 79 nmol/(min·mg) in EGTA and 108 nmol/(min·mg) in Ca^{2+}. When phosphorylated thymus myosin was mixed with either skeletal muscle myosin or phosphorylated gizzard myosin, troponin–tropomyosin appeared to regulate the actin-activated ATPases of skeletal muscle and phosphorylated gizzard myosins but not that of the phosphorylated thymus myosin. While the regulated actin-activated ATPase of phosphorylated thymus myosin is almost Ca^{2+}-insensitive (Table I), that of the unphosphorylated myosin is Ca^{2+}-sensitive. Even in 60 μM regulated actin, the ATPase activity of unphosphorylated thymus myosin in EGTA was only 3 nmol/(min·mg).

The regulated actin-activated ATPase of phosphorylated thymus myosin was also examined at several different concentrations of KCl (Table III). Increasing the concentration of KCl from 50 to 150 mM had little effect on the regulated actin-activated ATPase activity in Ca^{2+}, but the ATPase activity in EGTA decreased as the concentration of KCl increased. In 150 mM KCl, the regulated actin-activated AT-

Table III: Regulated Actin-Activated ATPase of Phosphorylated Thymus Myosin[a]

KCl (mM)	MgATPase activity [nmol/(min·mg)]		
	EGTA	Ca^{2+}	EGTA/Ca^{2+}[b]
50	82	101	0.81
75	80	98	0.82
100	44	110	0.40
150	10	111	0.09

[a] The ATPase assays were performed at 37 °C in 10 mM $MgSO_4$, 2 mM [γ-^{32}P]ATP, 0.2 mM DTT, 20 mM imidazole, pH 7.0, either 0.1 mM $CaCl_2$ or 1 mM EGTA, and the KCl concentrations given above. [b] MgATPase activity in EGTA divided by MgATPase activity in $CaCl_2$.

Pase of phosphorylated thymus myosin had normal Ca^{2+} sensitivity. Even in 100 μM regulated actin (16 times the K_{app} in the presence of Ca^{2+}), the ATPase activity of phosphorylated thymus myosin in EGTA was only 10 nmol/(min·mg).

Discussion

Light chain phosphorylation regulates the actin-activated ATPase of thymus myosin not by increasing the maximum rate of ATP hydrolysis but rather by decreasing 15–20-fold the K_{app} of this myosin for actin. In the presence of skeletal muscle tropomyosin, phosphorylation causes only a 2–3-fold decrease in K_{app}, and in the presence of thymus tropomyosin, phosphorylation causes about a 5-fold decrease in K_{app}.

When 14 μM actin was used, the actin-activated ATPase of thymus myosin was found to increase nearly linearly with the level of LC20 phosphorylation (Figure 2). Under these conditions the K_{app} value for the actin-activated ATPase unphosphorylated thymus myosin is about 70 μM and that for the phosphorylated myosin is about 5 μM. Therefore, the MgATPase of the unphosphorylated myosin was only slightly stimulated, and that of the phosphorylated myosin was about 70% of V_{max}. As the level of LC20 phosphorylation was increased, the fraction of myosin having a K_{app} of 5 μM increased, resulting in a higher ATPase activity. The dependence of the tropomyosin–actin-activated ATPase on the level of LC20 phosphorylation appears very different from that of the actin-activated ATPase (Figure 2). However, these assays were performed in 12 μM tropomyosin–actin, which is close to the K_{app} of the tropomyosin–actin-activated ATPase of unphosphorylated thymus myosin. Thus, under these conditions the ATPase activity of the unphosphorylated myosin should be about $^1/_2V_{max}$.

While the actin-activated ATPase activities of phosphorylated thymus myosin in 150 mM KCl were comparable to those in 50 and 100 mM KCl, much higher concentrations of tropomyosin–actin were required to achieve significant activation of the unphosphorylated myosin in 150 mM KCl than were required in 50 and 100 mM KCl. Also in 150 mM KCl, the actin-activated ATPase assays of the unphosphorylated myosin were not linear, but rather they increased with time. In 150 mM KCl, MgATP depolymerizes filaments of unphosphorylated thymus myosin but not those of the phosphorylated myosin (Scholey et al., 1980). Monomeric myosin is expected to have a greater K_{app} than filamentous myosin. One possible explanation for the nonlinear ATPase assays is that actin promotes myosin filament formation. The physiological significance of the depolymerization of unphosphorylated thymus myosin is uncertain. At approximately physiological ionic strength, MgATP also depolymerizes unphosphorylated smooth muscle myosins (Suzuki et al., 1978; Scholey et al., 1980). However, in relaxed intact smooth muscles there are many myosin filaments even though the myosin is not phosphorylated (Somlyo et al., 1981).

The effect of LC20 phosphorylation on the actin-activated ATPase of thymus myosin is more like its effect on the actin-activated ATPase of rabbit skeletal muscle myosin than its effect on the actin-activated ATPase of gizzard myosin. Phosphorylation of skeletal muscle LC20 appears to cause a 2-fold decrease in the K_{app} of the actin-activated ATPase, but it does not affect V_{max} (Pemrick, 1980; Persechini & Stull, 1984). In contrast, phosphorylation of gizzard myosin LC20 appears to affect primarily V_{max} and not K_{app}. Under the conditions used for thymus myosin, the actin-activated and tropomyosin–actin-activated ATPases of unphosphorylated gizzard myosin did not increase with increasing actin concentration, and even in 100 μM tropomyosin–actin in the presence of EGTA, the rate of the unphosphorylated myosin was only 10% of that of the phosphorylated myosin. This result is consistent with those of Sellers et al. (1982), who found that LC20 phosphorylation causes a 25-fold increase in the V_{max} of the actin-activated ATPase of gizzard heavy meromyosin and only a 4-fold increase in affinity.

The actin-activated ATPases of a number of vertebrate nonmuscle myosins have been shown to be regulated by light chain phosphorylation (Adelstein & Eisenberg, 1980; Kendrick-Jones & Scholey, 1981). As usually only one or two relatively low actin concentrations were used, it is not known whether these phosphorylations increased V_{max} or decreased K_{app}. Trotter & Adelstein (1979) measured the MgATPase activity of macrophage myosin at several actin concentrations. The actin-activated ATPase of the unphosphorylated myosin was much less than that of the phosphorylated myosin, and it did not increase as the actin concentration was increased from 7 to 29 μM. However, the K_{app} for the phosphorylated myosin was about 14 μM. If the K_{app} of the actin-activated ATPase of the unphosphorylated myosin is 15–20-fold greater than that of the phosphorylated myosin, there would have been little activation of the unphosphorylated myosin even at the highest actin concentration used.

Is the difference in the K_{app} values of the actin-activated ATPases of phosphorylated and unphosphorylated thymus myosins large enough for this phosphorylation to regulate motile activity? A 5-fold difference in K_{app}, as is found when the unphosphorylated myosin is filamentous and when thymus tropomyosin is present, appears to be too small for this phosphorylation to be an effective regulatory system. If LC20 phosphorylation regulates myosin filament formation in vivo, phosphorylation could result in a large increase in ATPase activity. However, if the unphosphorylated myosin in vivo is filamentous, there must be some other way of inhibiting its interaction with actin. At low ionic strength and in the absence of calcium, skeletal muscle troponin inhibited the tropomyosin–actin-activated ATPase of unphosphorylated thymus myosin but not that of the phosphorylated myosin. While there is currently no convincing evidence for troponin in nonmuscle cells, these results do suggest how actin-binding proteins may be involved in regulating the actin-activated ATPases of nonmuscle myosins.

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