Phosphorylation Kinetics of Skeletal Muscle Myosin and the Effect of Phosphorylation on Actomyosin Adenosinetriphosphatase Activity†

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ABSTRACT: Purified rabbit skeletal muscle myosin is phosphorylated on one type of light-chain subunit (P-light chain) by calmodulin-dependent myosin light chain kinase and dephosphorylated by phosphoprotein phosphatase C. Analyses of the time courses of both phosphorylation and dephosphorylation of skeletal muscle myosin indicated that both reactions, involving at least 90% of the P-light chain, were kinetically homogeneous. These results suggest that phosphorylation and dephosphorylation of rabbit skeletal muscle myosin heads are simple random processes in contrast to the sequential phosphorylation mechanism proposed for myosin from gizzard smooth muscle. We also examined the effect of phosphorylation of rabbit skeletal muscle myosin on the actin-activated ATPase activity. We observed an apparent 2-fold decrease in the K_m for actin, from about 6 μM to about 2.5 μM, with no significant effect on the V_{max} (1.8 s^{-1}) in response to P-light-chain phosphorylation. There was no significant effect of phosphorylation on the ATPase activity of myosin alone (0.045 s^{-1}). ATPase activation could be fully reversed by addition of phosphatase catalytic subunit. The relationship between the extents of P-light-chain phosphorylation and ATPase activation (at 3.5 μM actin and 0.6 μM myosin) was essentially linear. Thus, in contrast to results obtained with myosin from gizzard smooth muscle, these results suggest that cooperative interactions between the myosin heads do not play an important role in the activation process in skeletal muscle. Since the effect of P-light-chain phosphorylation is upon the K_m for actin, it would appear to be associated with a significant activation of ATPase activity only at appropriate concentrations of actin and salt. Myosin preparations stored for longer than 5 days at 0 °C have been found to have no significant response to P-light-chain phosphorylation, and the effect of aging was an enhancement of ATPase activity in the absence of phosphorylation.

It is now generally accepted that in vertebrate smooth and scallop muscles the primary calcium-dependent regulatory switch is located on the myosin molecule itself and is characterized by a dependency upon the conformation of the light-chain subunits [cf. Kendrick-Jones & Scholey (1981)]. While it was initially thought that calcium regulation in vertebrate skeletal muscle was solely thin filament linked (Lehman & Szent-Gyorgyi, 1975), some subsequent studies suggest that this is not the case and that there is a coexisting thick filament linked system. The precise nature of this putative system has not been clarified. Some studies suggest it involves calcium binding directly to the regulatory light chain, in analogy with the scallop regulatory system (Lehman, 1978; Pulliam et al., 1983). Alternatively, calcium- and calmodulin-dependent phosphorylation of the myosin light chains (Yazawa & Yagi, 1978; Perry, 1979; Blumenthal & Stull, 1980) has been suggested to play a regulatory role (Pemrick, 1980) in analogy with the vertebrate smooth muscle. However, in vitro studies by others (Perry, 1979) have failed to support a regulatory role for light-chain phosphorylation in vertebrate skeletal muscle. The calcium-binding properties of skeletal muscle myosin have been studied, and it has failed to demonstrate Ca^{2+}-specific binding capability (Bagshaw, 1980) although Lehman's studies clearly implicate such a process (Lehman, 1978). Finally, it has been shown that the skeletal muscle P-light chain,[1] while structurally similar to the scallop regulatory subunit, cannot functionally replace it (Sellers et al., 1980; Scholey et al., 1981). In contrast, vertebrate smooth muscle regulatory light chains can functionally replace the scallop subunits, creating a phosphorylation-dependent hybrid (Kendrick-Jones et al., 1981).

Although much published biochemical evidence would appear to rule out a thick filament linked regulatory system in vertebrate skeletal muscle, some more recent studies of living muscle have renewed interest in this possibility. Measurements of isometric twitch potentiation (posttetanic and staircase potentiation) and P-light-chain phosphorylation in rat skeletal muscles suggest a correlation between these two events (Manning & Stull, 1979, 1982; Klug et al., 1982). In addition, Crow & Kushmerick (1982a) demonstrated that a reduced energy cost for tension maintenance may be associated with phosphorylation of the light chain. These authors also suggested, on the basis of measurements of unloaded shortening velocity, that there was a reduction in the cross-bridge cycling rate in response to phosphorylation (Crow & Kushmerick, 1982b). Direct measurements of ATPase activity in permeabilized skeletal muscle fibers are in agreement with this idea (Cooke et al., 1982). However, Butler et al. (1983) recently have shown that light-chain phosphorylation in mouse skeletal muscle is not linked in an obligatory manner with a reduced cross-bridge cycling rate.

We have recently reevaluated the effect of light-chain phosphorylation by using a reconstituted system of purified rabbit skeletal muscle myosin, actin, and myosin light-chain kinase. In some experiments, purified phosphatase C was also included. Myosin was purified by a novel procedure and was stored and assayed as synthetic myosin filaments. This preparation has been thoroughly characterized elsewhere and is notable for its well-defined structural homogeneity (Persechini & Rowe, 1984). We have been able to show, using this system under specific conditions, a clear activation of the

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MLCK, myosin light-chain kinase; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; P-light chain, the phosphorylatable 18.5-kDa light chain of rabbit skeletal muscle myosin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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actomyosin ATPase in response to light-chain phosphorylation. In addition, we have examined the steady-state kinetics of myosin phosphorylation. We have found, in contrast with results obtained for smooth muscle myosin (Persechini & Hartshorne, 1983), that phosphorylation of skeletal muscle myosin appears to be described by a single kinetic process and is, therefore, not sequential.

Materials and Methods

Reagents. ATP (grade I), SDS, acrylamide, methylenebis(acrylamide), and DTT were obtained from Sigma Chemical Co. (St. Louis, MO). Triton X-100 was obtained from Kodak (Rochester, NY). Other reagents were Malinckrodt (Paris, KY) or Fisher (Fairlawn, NJ) analytical grade. Institutional deionized water was further purified by treatment with a Milli-Q purification system (Millipore Corp., Bedford, MA). [γ-^{32}P]ATP was synthesized by the method of Walseth & Johnson (1979).

Proteins. Synthetic myosin filaments were prepared from freshly excised, minced rabbit latissimus dorsi muscles. All volumes are relative to the initial mass of minced tissue, and all operations were carried out at 2–4 °C. Minced tissue was homogenized for 8 s by using a Waring commercial blender on the "hi" setting in 4 volumes of a solution containing 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM KCl, 2 mM EGTA, 0.2 mM DTT, and 3% (v/v) Triton X-100, final pH 7.6 (buffer A). The myofibrillar suspension was sedimented at 10000*g* for 10 min, and the supernatant fraction was discarded. This procedure was repeated 4 times for a total of five washes in buffer A. The homogenization time was reduced to 5 s in all washes subsequent to the initial one. Prior to the extraction of myosin, the myofibrils were washed 3 times as described above in a solution which contained 10 mM Tris-HCl, 1 mM $MgCl_2$, 150 mM KCl, 2 mM EGTA, and 0.2 mM DTT, final pH 7.6 (buffer B). After the final wash in buffer B, the myofibrillar pellet was suspended by a 3-s homogenization at the "lo" blender setting in 1 volume of a solution containing 0.2 M sodium phosphate, 1 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT, final pH 7.2. This viscous mixture was then immediately subjected to centrifugation at 22000*g* for 30 min. The supernatant solution was filtered through glass wool, and the ATP concentration was raised 0.5 mM by addition of a 100 mM stock solution (pH 7.5). This was followed by centrifugation at 100000*g* for 4 h. The clear supernatant solution was removed, with care being taken not to disturb the actomyosin gel at the bottom of the tubes, and diluted 10-fold in 1 mM $MgCl_2$ for precipitation of myosin. The myosin precipitate was collected by centrifugation at 10000*g* for 15 min. Myosin was then dissolved in 0.3 M KCl, 10 mM Tris-HCl, and 0.2 mM DTT, final pH 8.0 (buffer C). Clarification at 48000*g* for 15 min yielded a clear, viscous solution. Solubilized myosin was precipitated by quickly stirring into an equal volume of ice-cold water. The resultant crude synthetic myosin filaments were exhaustively dialyzed (overnight) against a solution containing 10 mM Tris-HCl, 150 mM KCl, 1 mM $MgCl_2$, and 0.2 mM DTT, final pH 8.0 (buffer D). Any residual actin contamination was then eliminated by centrifugation at 100000*g* for 1.5 h. For this step, a swinging-bucket rotor was found to be most effective. The final supernatant solution, a suspension of purified synthetic myosin filaments, was usually about 3 mg/mL, and the total preparative procedure was completed within 2 days. The yield was approximately 4 mg of myosin/g of muscle. Preparations were assessed for purity by polyacrylamide gel electrophoresis in the presence of SDS and generally were found to be in excess of 95% pure with the major contaminant being C-protein (3%). Purified filaments were not found to contain a significant level of light-chain phosphate, as determined by isoelectric focusing (Silver & Stull, 1982). A thorough physical characterization of filaments prepared in this way will be presented elsewhere (Persechini & Rowe, 1984); they have been found to be unusually monodisperse with respect to both filament length and width.

Rabbit skeletal muscle myosin light-chain kinase was purified by a procedure similar to that described by Yazawa & Yagi (1978). Calmodulin was purified from frozen bovine brain as described by Dedman et al. (1975). Actin was purified from rabbit skeletal muscle as described by Driska & Hartshorne (1975). The catalytic subunit of phosphoprotein phosphatase (phosphatase C) was purified from bovine cardiac muscle as described by Chou et al. (1977). Concentrations of proteins were determined by either the microbiuret assay (Itzhaki & Gill, 1964) or the dye binding assay (Spector, 1978).

Measurement of Light-Chain Phosphorylation and ATPase Activity. Unless otherwise specified, all assays were carried out at 25 °C in 25 mM Tris-HCl, 3.5 mM $MgCl_2$, 75 μM $CaCl_2$, and 1 mM [γ-^{32}P]ATP ($\sim$10 cpm/pmol), pH 7.5. Other conditions are given in relevant figure legends. Phosphorylation was measured by blotting 100-μL aliquots of reaction mixture on to squares of Whatman 3MM filter paper, followed by protein precipitation and washing in trichloroacetic acid as described by Corbin & Reimann (1975). Washed bound precipitate was assayed for ^{32}P by aqueous Cherenkov counting. Analysis of phosphorylation and dephosphorylation time courses was carried out as described elsewhere (Orsi & Tipton, 1979; Persechini & Hartshorne, 1983). ATP hydrolysis was assayed as described elsewhere (Persechini & Hartshorne, 1981). In experiments where actomyosin ATPase activity was correlated with the extent of phosphorylation, myosin was incubated, in the absence of actin, with MLCK to allow light-chain phosphorylation. After 5 min, 10 mM EGTA (pH 7.2) was added to give a final concentration in the assay of 200 μM EGTA. Aliquots were then removed at 7 and 9 min and assayed for ^{32}P incorporation. We have never observed a significant difference between measurements of phosphate incorporation at 7 and 9 min, and additional experiments (data not shown) indicate that after EGTA addition phosphorylation is stable for a minimum of 1 h. The actin-activated ATPase reaction was initiated 10 min after the addition of ATP by addition of a solution of F-actin to produce the desired final assay conditions. Zero time in the ATPase time course is, therefore, 10 min after ATP additon. Because of this experimental protocol, P-light-chain phosphorylation was carried out under slightly different conditions from those stated for accompanying ATPase measurements due to compensation for the volume increase with the actin addition. In the most extreme case encountered at the highest actin concentration, there was a 30% dilution (i.e., KCl concentration in the phosphorylation reaction was 86 mM). However, in most assays, the dilution was 10% or less.

Polyacrylamide Gel Electrophoresis and Isoelectric Focusing. Discontinuous gel electrophoresis in the presence of SDS with a 7.5-20% polyacrylamide gradient was carried out as described by Laemmli (1970). Isoelectric focusing of myosin light chains was carried out as described by Silver & Stull (1982).

Results

Kinetics of Phosphorylation and Dephosphorylation. The time course of phosphorylation of rabbit skeletal muscle myosin by MLCK was analyzed as shown in Figure 1A. The

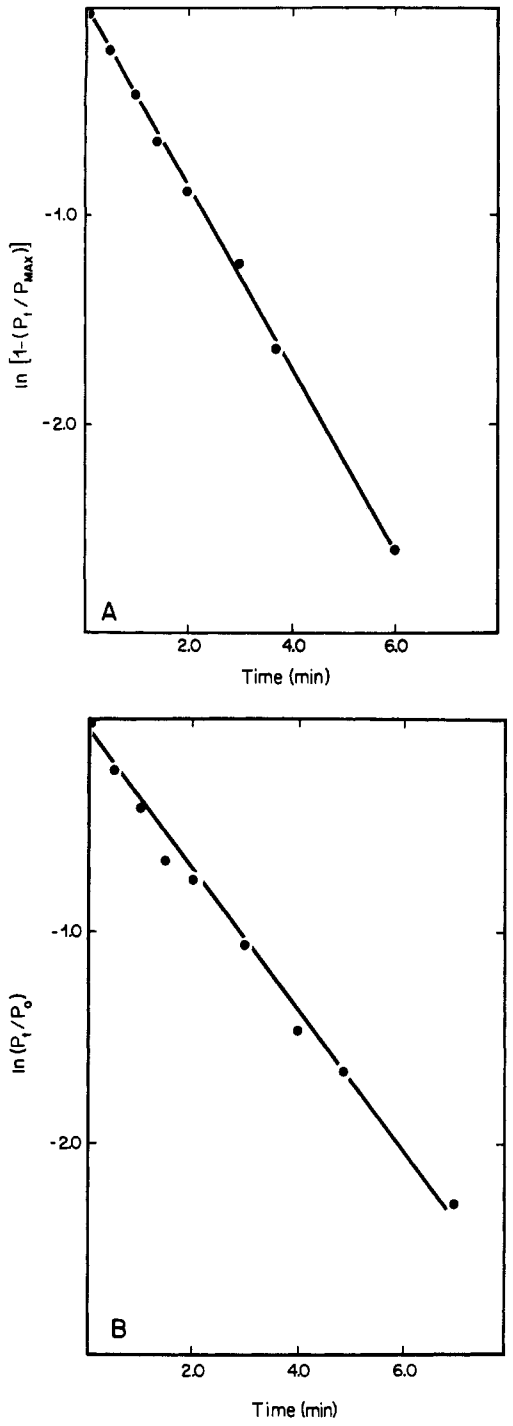

FIGURE 1: (A) Time course of rabbit skeletal muscle myosin phosphorylation by purified MLCK. Reaction was initiated by addition of ATP at $t = 0$; P_t = moles of ^{32}P per mole of P-light chain measured at time t; P_{MAX} = maximum phosphorylation of 0.92 mol of ^{32}P/mol of P-light chain. Reaction conditions included 2.5 nM MLCK, 60 nM calmodulin, and 0.95 μM myosin. (B) Time course of rabbit skeletal muscle myosin dephosphorylation by phosphoprotein phosphatase C; P_0 = extent of phosphorylation at the time of addition of phosphatase (0.96 mol of ^{32}P/mol of P-light chain). Myosin was prephosphorylated by incubation for 15 min under standard conditions with MLCK, followed by addition of EGTA to 200 μM. Phosphatase was then added to the reaction mixture at $t = 0$. Reaction conditions included 3 nM MLCK catalytic subunit, 9 μg/mL phosphoprotein phosphatase C, 0.95 μM myosin, and 60 mM KCl. Volume changes on addition of EGTA and phosphoprotein phosphatase C were less than 5%.

phosphorylation was, under these conditions, a pseudo-first-order process. This indicates that the phosphorylation of skeletal muscle myosin is not sequential and that all bound

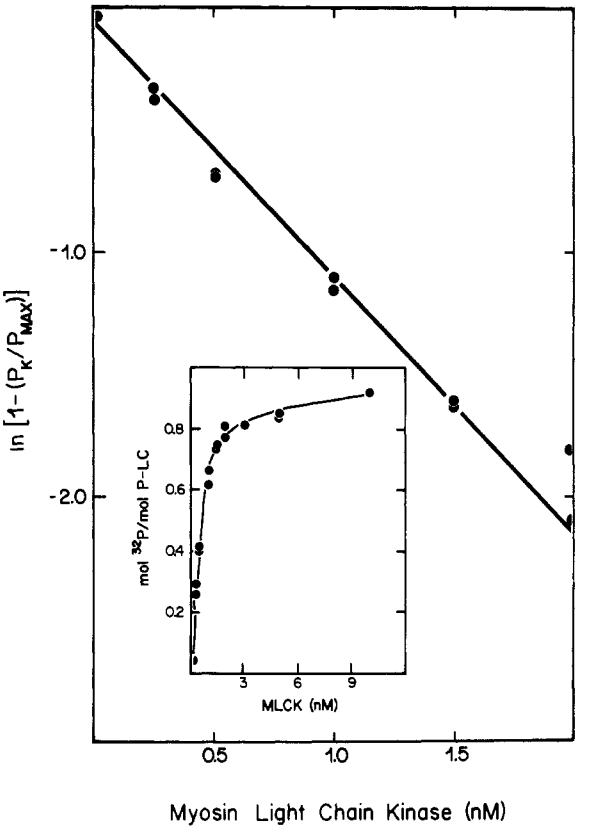

FIGURE 2: P-light-chain phosphorylation with increasing concentrations of MLCK. Phosphorylation was measured 10 min after incubation with the indicated MLCK concentrations. Assays were initiated by addition of ATP. P_K = phosphorylation measured at 10 min at a given MLCK concentration; P_{MAX} = maximum phosphorylation attainable at saturating [MLCK] (0.92 mol of ^{32}P/mol of P-light chain). Inset: Complete saturation profile. Reaction conditions included 75 nM calmodulin, 0.95 μM myosin, and 60 mM KCl.

light-chain substrate is equivalent. Equivalence of the light chains is also supported by experiments which demonstrated that the dephosphorylation time course from 0.96 to 0.09 mol/mol of P-light chain was described by a single logarithmic line (Figure 1B). Thus, dephosphorylation of skeletal muscle myosin by phosphoprotein phosphatase C is also a pseudo-first-order process.

Another approach for analysis of the kinetic parameters was to determine the dependence of P-light-chain phosphorylation measured at a fixed time (10 min) on the concentration of MLCK in the assay mixture (Figure 2). There was a simple first-order dependence on the MLCK concentration, as would be expected from the time course analysis, up to a level of ^{32}P incorporation equivalent to 90% of the available sites. An examination of Figure 2 also illustrates the negligible contaminating MLCK activity typical of the myosin preparations.

Effect of Light-Chain Phosphorylation on ATPase Activity. Using our assay conditions, we were able to obtain measurements of ATPase activity linear with time over the 6-min interval examined. Typical time courses are shown in Figure 3. Actin was added at zero time, and the intercept on the y axis represents ATP cleavage by myosin in the absence of actin [10 nmol (mg of myosin)$^{-1}$ min^{-1} or 0.045 s^{-1}]. Under most conditions, actomyosin ATP hydrolysis has been reported to be very nonlinear (Strzelecka-Golaszewska et al., 1979), and at lower ionic strengths (35 mM KCl), we also observed this to be the case (data not shown). For the purpose of these studies, a linear time course was obtained at a higher ionic strength (60 mM KCl). As shown in Figure 3, there was a substantial increase in the rate of ATP hydrolysis with P-

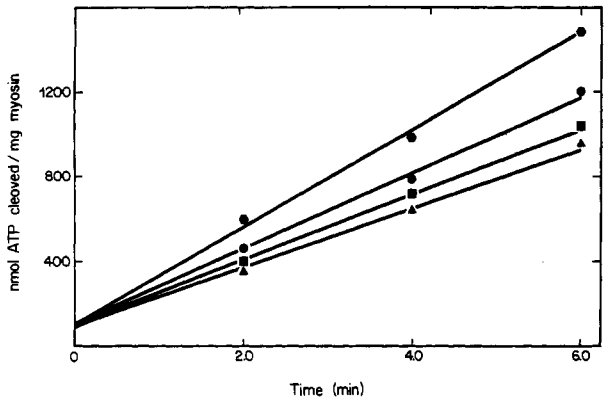

FIGURE 3: Time courses of actomyosin ATP hydrolysis at different levels of P-light-chain phosphorylation. Experiments carried out as described under Materials and Methods to attain stable levels of 0.04 (▲), 0.15 (■), 0.55 (●), and 0.82 (●) mol of ^{32}P/mol of P-light chain prior to actin addition. Final reaction conditions included 60 mM KCl, 60 nM calmodulin, 3.45 μM actin, 0.57 μM myosin, and MLCK concentrations at 0.0 (▲), 0.25 (■), 1.5 (●), and 8.0 (●) nM.

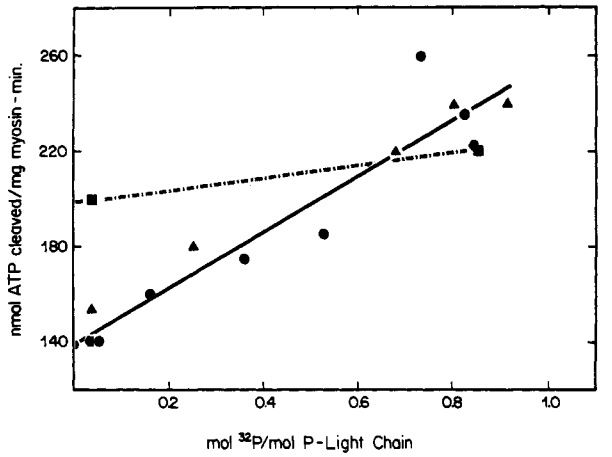

FIGURE 4: Relationship between actomyosin ATPase activity and the extent of P-light-chain phosphorylation. Data from three different myosin preparations are shown (▲, ●, ●). Data from one preparation stored for 7 days at 0 °C are also included (■). Final reaction conditions included 60 mM KCl, 60 nM calmodulin, 3.45 μM actin, 0.57 μM myosin, and MLCK concentrations from 0 to 8.0 nM.

light-chain phosphorylation. The MgATPase activity of myosin alone was unaffected (data not shown). The relationship between the extent of P-light-chain phosphorylation and the increase in actomyosin ATPase activity is shown in Figure 4. There was a linear correlation ($r = 0.95$) with a maximum activation of ~75% above the nonphosphorylated actomyosin ATPase activity. Maintenance of a response to phosphorylation was observed in preparations stored for no more than 5 days on ice. Aging increased actomyosin ATPase activity of the nonphosphorylated species (Figure 4). Comparison of aged and fresh preparations by SDS–polyacrylamide gel electrophoresis (not shown) revealed no obvious differences, such as would be expected if proteolysis were involved in the aging process.

The activation of actomyosin ATPase activity by phosphorylation of the P-light chain was itself not dependent upon a particular ionic strength in a range from 30 to 60 mM KCl, as shown in Figure 5. The rates of ATPase activity shown in Figure 5 are nominal and are based on measurements 10 min after addition of actin. It can be seen that the apparent magnitude (percentage increase) of the activation decreased with decreasing ionic strength.

The effect of P-light-chain phosphorylation on actomyosin ATPase activity was fully reversible, as shown in Figure 6.

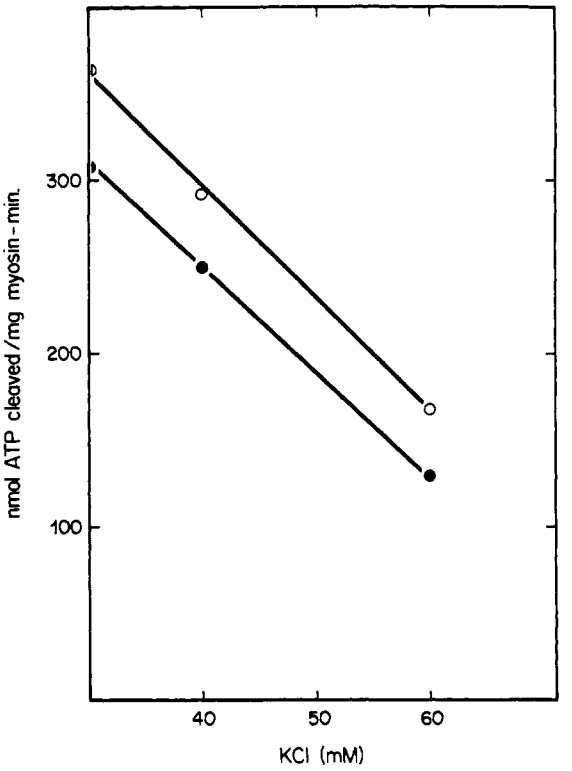

FIGURE 5: Effect of ionic strength on actin-activated ATPase activity of phosphorylated and nonphosphorylated myosin. Measurements of ATP hydrolysis were made at 10 min after addition of actin. Myosin was incubated with either 0.0 (●) or 3.0 (○) nM MLCK to give stable levels of 0.04 and 0.80 mol of ^{32}P/mol of P-light chain, respectively. Reaction conditions included 60 nM calmodulin, 3.45 μM actin, and 0.57 μM myosin.

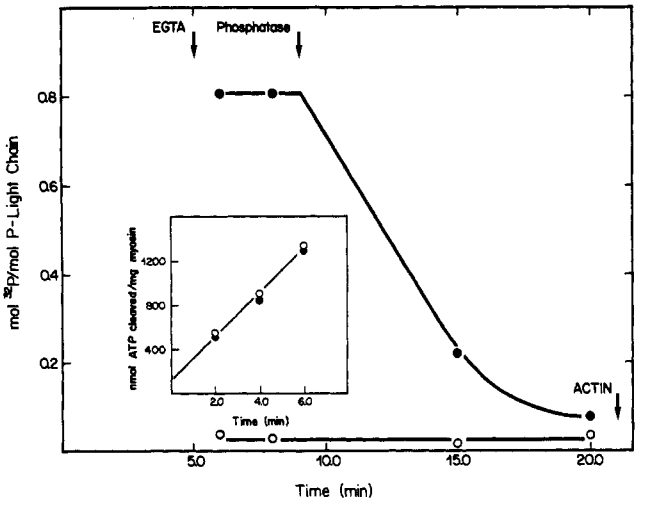

FIGURE 6: Reversal of phosphorylation-dependent activation by phosphoprotein phosphatase C. The phosphorylation reaction was initiated in either the presence (●) or the absence (○) of 3.5 nM MLCK by addition of ATP. Additions included 200 μM EGTA, 10 μg/mL phosphoprotein phosphatase C, and 4.6 μM actin at the times indicated in the figure. Total dilution by these additions was less than 10% of the final volume. Inset: Time courses of ATP hydrolysis, initiated by the addition of actin as indicated in the figure. Final reaction conditions included 60 mM KCl, 60 nM calmodulin, and 0.57 μM myosin. The nonzero intercept of the ATPase time course represents hydrolysis which occurred prior to actin addition.

Incubation of phosphorylated myosin with purified phosphatase C resulted in hydrolysis of phosphate from the light chain with the loss of activation of actomyosin ATPase activity. The rate of hydrolysis for dephosphorylated myosin was 195 nmol min^{-1} (mg of myosin)$^{-1}$ (see inset of Figure 6). Under comparable conditions, the rate of phosphorylated myosin was 290 nmol

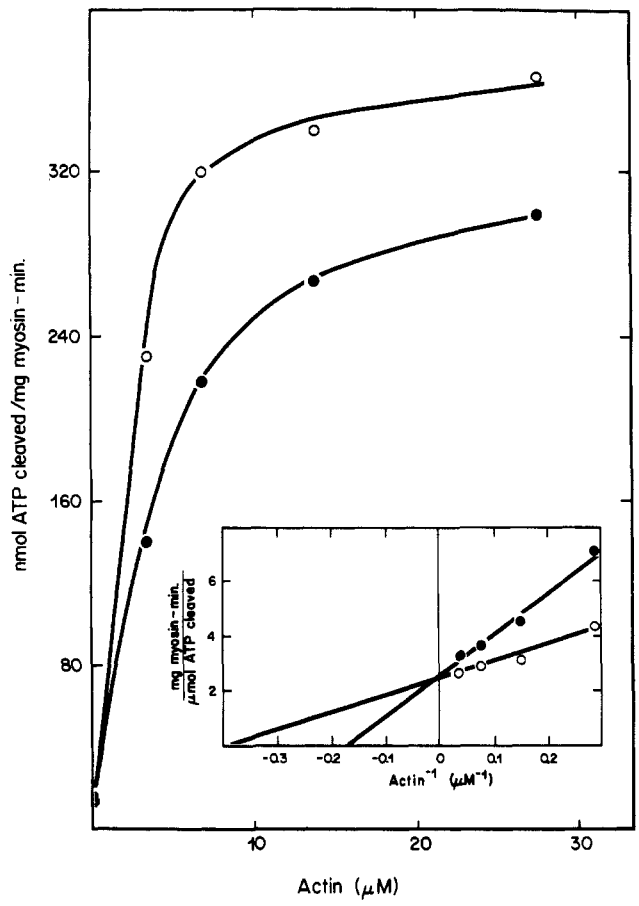

FIGURE 7: Actin dependence of phosphorylated and nonphosphorylated myosin ATPase activities. Myosin phosphorylation and subsequent measurements of actin-activated ATPase activities were carried out as described under Materials and Methods in the presence (○) or absence (●) of 3.5 nM MLCK. In the presence of MLCK, myosin was phosphorylated to a level of 0.92 mol of ^{32}P/mol of P-light chain. Inset: Data plotted in the double-reciprocal form. Final reaction conditions included 60 mM KCl, 60 nM calmodulin, and 0.57 μM myosin.

min^{-1} mg^{-1} (see Figure 7). The reversibility of the activation by phosphatase reduces the possibility of activation being associated with some change other than P-light-chain phosphorylation.

The dependencies of phosphorylated and nonphosphorylated myosin ATPase activities on the actin concentration are presented in Figure 7. These data are plotted in the double-reciprocal form in the inset to the figure. The data suggest that the effect of phosphorylation is to reduce the K_m value for actin from 6 to 2.5 μM. The V_{max} value is not affected significantly by phosphorylation and is approximately 400 nmol mg^{-1} min^{-1} (1.8 s^{-1}). We have found no evidence for an effect of calcium on the actin-activated ATPase activity of either phosphorylated or nonphosphorylated myosin (data not shown), in contrast with some other reports (Lehman, 1978; Pulliam et al., 1983). It has been observed that seemingly insignificant levels of regulated actin contamination (<0.1% by weight) are associated with a residual calcium-sensitive response, even with a large excess of unregulated actin present (A. Persechini and A. J. Rowe, unpublished results). Care must be taken to ensure that preparations are free of such contamination.

Discussion

Kinetics of Myosin Phosphorylation. The kinetic studies of myosin phosphorylation were carried out at low substrate concentrations (~1 μM myosin) so that reaction time courses might be expected to be pseudo first order. It is clear that the rates of both phosphorylation and dephosphorylation, as well as the relationship between phosphorylation and enzyme concentration, are indicative of a single pseudo–first-order process accounting for at least 90% of the available sites. This is in contrast to the situation with smooth muscle myosin (Persechini & Hartshorne, 1983). In the latter system, studies carried out under similar conditions are consistent with the nonequivalence of light-chain substrate bound to the two heads, resulting in a sequential phosphorylation mechanism. This rather striking difference between these two myosin types may be related to the ability of smooth muscle myosin to exist in a "bent-monomer" or "10s" conformation (Suzuki et al., 1982; Trybus et al., 1982). Perhaps in such a conformation, one or another of the phosphorylation sites on the two light chains is sterically blocked, thereby creating a sequential mechanism. Obviously, such a simple cause and effect relationship is rather speculative at this point, and further study will be required to test its validity. However, it can be concluded from these studies that sequential myosin light-chain phosphorylation does not appear to be a general phenomenon.

Activation of Actomyosin ATPase by Light-Chain Phosphorylation. It was first reported by Pemrick (1980) that phosphorylation of the P-light chain appeared to modulate the actin-activated ATPase activity of skeletal muscle myosin. She reported a decrease in the K_m value for actin with no significant effect on V_{max}. While there are qualitative similarities between her results and our findings, there are also significant differences. First, Pemrick reported a nonlinear relationship between P-light-chain phosphorylation and activation. Our results, however, indicate that the overall correlation is linear. The reason for this discrepancy is not clear, but it may be related to the lower ionic strength used in Pemrick's studies. Furthermore, the physical state of the myosin is not well-defined in those studies. It is not clear that the myosin aggregate is equivalent at all levels of phosphorylation. This is particularly critical in view of the fact that "fractional" phosphorylation was achieved by mixing soluble, fully phosphorylated, and nonphosphorylated myosins together followed by precipitation (Pemrick, 1980). Therefore, nominal rates of hydrolysis may not be comparable at all levels of phosphorylation. A correlation may be more easily characterized with a system in which the nature of the myosin aggregate and the linearity of the ATPase reaction have been established. Second, there is a difference in the magnitude of the change in K_m. Pemrick reports a 4-fold decrease from 1.3 to 0.35 μM in going from 0 to 0.82 mol of ^{32}P/mol of P-light chain. We have observed about a 2-fold decrease, from 6 to 2.5 μM. Some of this difference may be due to differing ionic conditions in the assays. However, Pemrick's analysis is confused by the fact that over most of the range of actin concentrations examined there was a molar excess of myosin heads.

One possible interpretation of the decrease in the K_m value for actin with P-light-chain phosphorylation is that phosphorylation simply increases the apparent affinity of myosin for actin. However, it may be difficult to interpret kinetic data obtained with filamentous myosin and actin because of steric influences (Sleep et al., 1980). Also, recent reports suggest that the mechanism of actomyosin ATP hydrolysis may be heterogeneous, possibly involving "nondissociating" (high actin affinity) and "dissociating" (low actin affinity) pathways. Cheung & Reisler (1983) and Pope et al. (1981) have reported that there appear to be at least two classes of actin-activated myosin ATPase binding affinities with K_m values differing by roughly a factor of 30. The low-affinity activity appears

similar to heavy meromyosin (HMM) or subfragment 1 (SF-1) (Cheung & Reisler, 1983). Shukla et al. (1983) have reported, on the basis of isotope exchange studies, a similar division in the hydrolytic pathway. In our studies, we appear to be measuring only the high-affinity activity as our values for K_m and V_{max} (using either phosphorylated or nonphosphorylated myosin) are in this range (Pope et al., 1981). Because of these complications, it is difficult to assign a single mechanistic interpretation to our results. For example, a change in pathway distribution might be consistent with our results. Shukla et al. (1980) have found that proteolysis or loss of P-light chain affects the isotope exchange pattern obtained in response to actin activation. While the mechanistic basis is obscure at this time, it seems clear that in vitro P-light-chain phosphorylation has a stimulatory effect on the purified actin-activated myosin ATPase and that, unlike other myosin-linked systems (Persechini & Hartshorne, 1981; Chantler et al., 1981), there is no evidence of significant cooperativity between the skeletal muscle myosin heads in this process.

Studies of the actin dependence of smooth muscle myosin ATPase activity suggest that in this system both K_m values for actin and V_{max} values are affected by P-light-chain phosphorylation (Sellers et al., 1982). The K_m value is decreased by a factor of 4 while the V_{max} value is increased by a factor of 25. This change in the K_m value is similar to that observed with skeletal muscle myosin in response to P-light-chain phosphorylation, suggesting that similar mechanisms may be involved. However, the dramatic effect of phosphorylation on V_{max} seen with the smooth muscle system appears to be absent in skeletal muscle.

It is well established that in skeletal muscle the primary regulatory switch is on the thin filament; however, it would also appear that P-light-chain phosphorylation can additionally modulate the activity of the system. The physiological importance of P-light-chain phosphorylation has been suggested by a number of studies. However, the precise nature of the in vivo response and its possible relationship to the in vitro effect we have observed are issues which are currently unresolved. A proposed model may accommodate a correlation between P-light-chain phosphorylation and one or more of the following: (1) isometric twitch potentiation (Manning & Stull, 1982; Klug et al., 1982); (2) a reduced energy cost for tension maintenance (Crow & Kushmerick, 1982a); (3) a reduced cross-bridge cycling rate immediately after a prolonged isometric tetanus (Crow & Kushmerick, 1982b; Butler et al., 1983) or in isometrically contracting permeabilized muscle fibers (Cooke et al., 1982); (4) a reduction in the apparent K_m value for actin (Pemrick, 1980; this study). Further study of the effects of P-light-chain phosphorylation in vitro may provide information helpful in illuminating this complex situation.

It is not clear why other investigators have failed to observe the in vitro manifestations of light-chain phosphorylation, but there appear to be at least two possibilities: First, with decreasing ionic strength, measurements of nominal ATPase rates show a reduced effect of P-light-chain phosphorylation, due probably to a much reduced K_m value for actin (Pemrick, 1980; Pope et al., 1981). Therefore, if even moderate actin concentrations are used, the effect might not be observed at low ionic strength. Second, we have found that the extent of activation by P-light-chain phosphorylation is dependent upon the age of the myosin preparation. Storage on ice will stabilize the effect, although by day 5 it is often reduced or absent. It is possible that myosin prepared by a more standard methodology is less stable, resulting in loss of the effect before assays can be carried out. At present, we have not defined the factors in the preparation and storage of myosin which are critical for maintenance of the phosphorylation effect, and this is an area of high priority in future research.

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Registry No. ATPase, 9000-83-3.

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Comparison of Effects of Smooth and Skeletal Muscle Tropomyosins on Interactions of Actin and Myosin Subfragment 1†

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ABSTRACT: The ATPase activity of acto-myosin subfragment 1 (S-1) was measured in the presence of smooth and skeletal muscle tropomyosins over a wide range of ionic strengths (20–120 mM). In contrast to the 60% inhibitory effect caused by skeletal muscle tropomyosin at all ionic strengths, the effect of smooth muscle tropomyosin was found to be dependent on ionic strength. At low ionic strength (20 mM), smooth muscle tropomyosin inhibits the ATPase activity by 60%, while at high ionic strength (120 mM), it potentiates the ATPase activity 3-fold. All of these ATPase activities were measured at very low ratios of S-1 to actin, under conditions at which a 4-fold increase in S-1 concentration did not change the specific activity of the tropomyosin–acto·S-1 ATPase. Therefore, the potentiation of the ATPase activity by smooth muscle tropomyosin at high ionic strength cannot be explained by bound S-1 heads cooperatively turning on the tropomyosin–actin complex. To determine whether the fully potentiated rates are different in the presence of smooth muscle and skeletal muscle tropomyosins, S-1 which was extensively modified by *N*-ethylmaleimide was added to the ATPase assay to attain high ratios of S-1 to actin. The results showed that, under all conditions, the fully potentiated rates are the same for both tropomyosins. Therefore, the difference in the effect of smooth muscle and skeletal muscle tropomyosins on the acto·S-1 ATPase activity at low ratios of S-1 to actin appears to be due to a greater fraction of the tropomyosin–actin complex being turned on in the absence of S-1 with smooth muscle tropomyosin than with skeletal muscle tropomyosin. This interpretation was supported by the equilibrium binding studies of S-1 to actin at 120 mM ionic strength in the presence of the ATP analogue adenyl-5′-yl imidodiphosphate. These studies indicate that in the absence of S-1, a greater fraction of the tropomyosin–actin complex is in the strong S-1-binding form with smooth muscle tropomyosin than with skeletal muscle tropomyosin. Hence, our data provide evidence that the fraction of tropomyosin–actin complex in the strong S-1-binding form correlates with the effect of tropomyosin on the actin-activated ATPase rate.

Skeletal muscle contraction is controlled by troponin–tropomyosin, a complex of proteins which lies along the actin filament (Ebashi et al., 1969). In the absence of Ca^{2+}, troponin–tropomyosin markedly inhibits the actomyosin ATPase activity, thus causing muscle relaxation. Although Ca^{2+} sensitivity is conferred on the actomyosin system only by the complete troponin–tropomyosin complex, tropomyosin alone has interesting effects on the actomyosin ATPase activity (Bremel et al., 1972; Weber & Murray, 1973; Eaton et al., 1975; Murray et al., 1975, 1980a,b; Lehrer & Morris, 1982). The effect of tropomyosin itself may be physiologically relevant since in certain contractile systems, such as smooth muscle, tropomyosin is bound to the actin filaments but no troponin is present. Furthermore, as with other complex allosteric systems, studying a simpler version of the system can often be informative.

We previously showed that the effect of skeletal muscle tropomyosin on the binding of myosin subfragment 1 (S-1)[1] to actin in the absence of ATP is very much like that of troponin–tropomyosin in the presence of Ca^{2+} (Williams & Greene, 1983). Tropomyosin strengthens the binding of S-1 to actin in a cooperative manner; that is, at low ratios of S-1 to actin, tropomyosin has little effect on the binding, while at high ratios of S-1 to actin, tropomyosin increases the strength of binding 3-fold. This cooperative effect of tropomyosin is explained by the model of Hill et al. (1980) which assumes

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[1] Abbreviations: AMP-PNP, adenyl-5′-yl imidodiphosphate; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; NEM-S-1, myosin subfragment 1 extensively modified with NEM; S-1, myosin subfragment 1.