Activation of skeletal S-1 ATPase activity by actin-tropomyosin-troponin

Effect of Ca^{++} on the fluorescence transient

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ABSTRACT Regulation in striated muscles primarily involves the effect of changes in the free calcium concentration on the interaction of subfragment-1 (S-1) with the actin-tropomyosin-troponin complex (henceforth referred to as $[acto]_R$). At low concentrations of free Ca^{++} the rate of ATP hydrolysis by $(acto)_R$S-1 can be as much as 20-fold lower than that in the presence of high free Ca^{++}, even though the binding of S-1 to $(actin)_R$ in the presence of ATP is virtually independent of the calcium concentration. This implies that the mechanism of regulation involves a kinetic transition between actin-bound states, rather than the result of changes in actin binding.

In the current work, we have investigated the fluorescence transient that occurs with the binding and hydrolysis of ATP both at low and high free $[Ca^{++}]$. The magnitude of this transition at low free $[Ca^{++}]$ is higher than at high free $[Ca^{++}]$. At low free $[Ca^{++}]$, the rate of the fluorescence transient either stays constant or decreases slightly with increasing free actin concentrations, but at high free $[Ca^{++}]$ the rate increases slightly with increasing free actin concentration. The observed changes in rate are not great enough to be of regulatory importance. The results of the fluorescence transient experiments together with the binding studies performed at steady state also show that neither the binding of M·ATP or M·ADP·Pi to $(actin)_R$ is appreciably Ca^{++} sensitive. These data imply that an additional step (or steps) in the ATPase cycle, i.e., other than the burst transition, must be regulated by calcium.

INTRODUCTION

The contractile apparatus of muscle consists of actin and myosin filaments arranged in a highly ordered geometrical pattern in which the filament axes are parallel to the fiber axis, and each myosin filament is surrounded by six actin filaments. According to the cross-bridge theory of muscle contraction (1, 2), the globular heads of the myosin molecule extend from the myosin filament and interact with the actin filament, and muscle contraction is driven by the cyclical interaction of the actin and myosin filaments and the hydrolysis of ATP (3).

The regulation of contraction in striated muscle involves the protein tropomyosin, which is present in the groove formed by the actin filaments, and a triad of proteins called troponin which are bound to the tropomyosin. According to the steric blocking model of muscle contraction (4), at very low $[Ca^{++}]$, tropomyosin blocks the binding of the myosin head to the actin filament thus promoting relaxation. At higher $[Ca^{++}]$, the binding of calcium to specific sites on the troponin complex leads to a conformational change in the troponin-tropomyosin which alters the binding of the myosin head to actin. In opposition to the steric blocking model, Chalovich et al. (5) have demonstrated that myosin binds to the regulated thin filament (i.e., actin-tropomyosin-troponin) even at very low $[Ca^{++}]$ (5–7), and that the binding constant of the myosin head to actin is approximately constant independent of the calcium concentration (5, 6). Because the ATPase activity is significantly inhibited in the absence of calcium, Chalovich and Eisenberg (6) suggested that the rate-limitating transition involved in regulation would be a kinetic transition and not actin binding. They further suggested that the rate-limiting transition controlled by the regulatory proteins may be the release of Pi from the actomyosin-ADP-Pi complex (See Fig. 1, models). In agreement with this interpretation, Rosenfeld and Taylor (8) have shown that the rate of dissociation of various ligands from actoS-1 is regulated by calcium. However, Millar and Homsher (9) have failed to observe a direct effect of calcium on the phosphate release step. Thus changes in calcium concentration had no effect on the rate of tension decline after a rapid jump in the phosphate concentration (9). Walker et al. (11) have recently reported both a threefold calcium sensitivity of the rate of tension decline after a phosphate transient and a saturation of the rate of tension decline at high phosphate concentrations (10, 11). This raises the possibility that more than a single step associated with phosphate release may be regulated.

In the present work we have investigated the effect of troponin and tropomyosin on the rate and magnitude of the tryptophan fluorescence transient that occurs simul-

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taneously with the initial Pi burst[1] (12). These studies confirm earlier studies by Rosenfeld and Taylor (8) and show that the kinetic transition associated with the Pi burst is not greatly affected by Ca^{++} (8). In the presence of high [Ca^{++}] the burst rate increases slightly with increasing actin concentrations. However, we also find that at very low [Ca^{++}] the burst rate remains approximately constant independent of the actin concentration. These studies suggest that the burst rate in the nondissociating pathway (13) is rapid and not rate limiting. Taken together, the data imply that there is at least one additional transition between the hydrolysis step (i.e., initial Pi burst) and the product release step, and that it is one or more of these steps that is regulated by calcium.

METHODS AND MATERIALS

Proteins

The preparation of actin, myosin, S-1, and (A-1)S-1 was described earlier (14). All experiments were performed using the (A-1) isoform of S-1. The tropomyosin-troponin (T-T) complex was prepared from an ether powder of rabbit skeletal muscle as described previously (6). Protein concentrations were determined spectrophotometrically at 280 nm by using absorption coefficients of 750 cm^2/g for S-1, 1,150 cm^2/g for actin, and 380 cm^2/g (at 278 nm) for the T-T complex. The molecular weights used for S-1, actin, and T-T are 120 kD, 42 kD, and 150 kD, respectively.

Binding assays

The binding of (A-1)S-1 to actin, in the presence of ATP, was determined either by the instantaneous change in turbidity that occured upon rapidly mixing the proteins in a stopped-flow spectrophotometer (13) or by a cosedimentation with actin (6). In the sedimentation assay, tropomyosin was added to prevent nonspecific binding (6). To determine the extent of regulation, ATPase rates were measured at high and low [Ca^{++}] either by assaying the rate of Pi release (6), or in stopped flow studies by the time taken for the turbidity change to obtain its maximal value at ATP exhaustion when actin and S-1 bind completely (5).

Burst measurements

The burst magnitude was measured either using quench flow techniques described previously (13) or using stopped-flow techniques by comparing the fluorescence enhancement that occurs when actoS-1 binds to ATP with the decrease in fluorescence that occurs when S-1 + ATP is mixed with actin (see Appendix and reference 21). The rate of the fluorescence transient was measured using a stopped-flow device as described previously (13), and was determined from the rate of change of the fluorescence intensity. This fluorescence signal has been shown to be equal to the rate of the hydrolysis step (Pi burst) in the unregulated system (12, 15).

[1]The phosphate burst traditionally refers to the appearance of quenchable Pi before steady state is achieved. However, the tryptophan fluorescence enhancement observed when myosin is mixed with ATP occurs simultaneously with the Pi burst (12, 15), and it is generally assumed that the burst and fluorescence enhancement are two aspects of the same kinetic transition. In this paper, it is this transition that is referred to as the "burst." When using quench and fluorescence methods, the measured rates and magnitudes are not identical, but can be related (see Appendix).

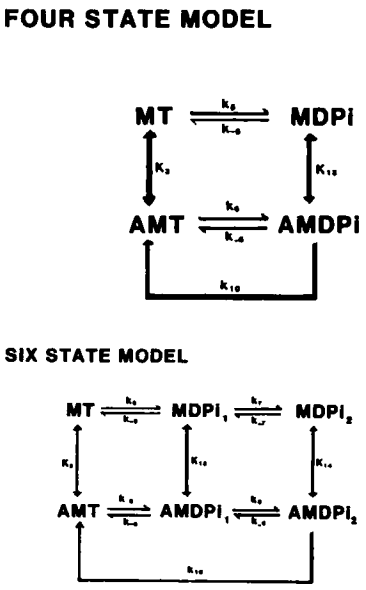


FIGURE 1 Four- and six-state models: in these models A = actin, M = Subfragment-1, T = ATP, D = ADP, Pi = inorganic phosphate. The dissociation constants K_3, K_{13}, K_{14} are assumed to represent rapid equilibrium processes.

RESULTS

The goal of this work is to determine whether the Ca^{++} concentration affects the rate of the Pi burst (or rapid hydrolysis step) of S-1 in the presence of actin-tropomyosin-troponin ($[actin]_R$) under conditions where the steady-state rate of ATP hydrolysis is Ca^{++} dependent. The burst of ATP hydrolysis can occur both when S-1 is free or when bound to actin (16). Therefore, to interpret the effect of regulatory proteins on the Pi burst, it is necessary to determine the fraction of S-1 that is bound to actin because only this portion can be affected by an actin based regulatory system. In Fig. 2 are shown double reciprocal plots of the fraction (A-1)S-1 bound to regulated actin vs. the free concentration of actin-tropomyosin-troponin, both at low free [Ca^{++}] and high free [Ca^{++}]. In the presence of high [Ca^{++}] the measured value of $K_{binding}$ is 27 μM, and at low [Ca^{++}] it is 44 μM, in agreement with earlier results (5, 6). The ATPase activity was 16 times higher at high free Ca^{++} that at low free Ca^{++} under these conditions (data not shown). Clearly then, calcium has little effect on the net

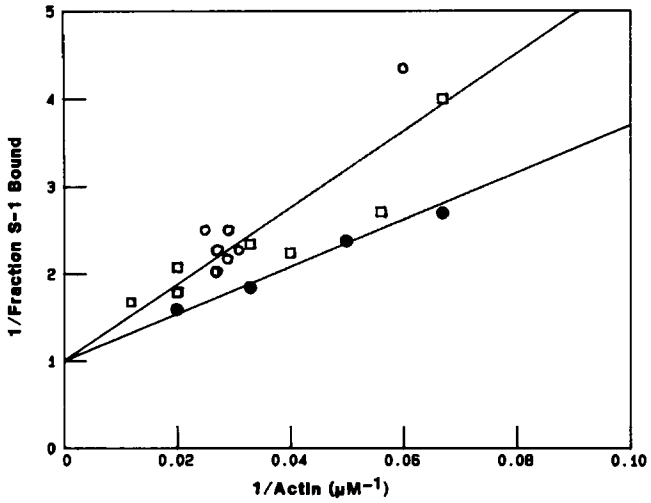


FIGURE 2 The binding of S-1 to actin-tropomyosin-troponin in the presence and absence of calcium. Closed circles, ●, are in the presence of calcium, performed using cosedimentation technique. □, ○, are in the absence of calcium. ○, stopped flow turbidity; □, cosedimentation. Conditions: 15°C, 8 mM Imidazole pH 7.0, 2.5 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, and either 0.67 mM $CaCl_2$ or EGTA. In stopped flow experiments S-1 concentration was 15–20 μM, in cosedimentation experiments the S-1 concentration was 0.1 μM. Ratio of actin/T-T was 4/1.

binding of actin-tropomyosin-troponin to the S-1-ATP and S-1-ADP-Pi complexes.

We next turn to measurements of the burst in the presence of tropomyosin-troponin. Under optimal low ionic strength conditions, and at very low concentrations of S-1, the maximum ATPase activity of S-1 is 22-fold higher in the presence of calcium than in the absence of calcium (6). However, the degree of inhibition decreases both in the presence of the high (A-1)S-1 concentrations that are necessary for chemical and fluorescence burst measurements, as well as the low ATP concentrations required for chemical burst measurements (Chalovich, unpublished data). This loss of inhibition may be due to strongly bound cross-bridges which activate the ATPase activity even in the absence of calcium (18, 19). The population of these strongly bound cross-bridges increases during the preparation of S-1 and separation into the component isozymes (A-1)S-1 and (A-2)S-1 (Chalovich, unpublished data). In the fluorescence burst studies described below, at least a ninefold difference between ATPase activities at high free Ca^{++} and those at low free Ca^{++} was obtained, whereas in the chemical quench studies a sevenfold difference was obtained.

To determine if there is a burst of ATP hydrolysis in the inhibited state, we compared the change in fluorescence intensity upon mixing (A-1)S-1 with [actin-tropomyosin-troponin + ATP] to the fluorescence intensity upon mixing [(A-1)S-1 + ATP] with actin-tropomyosin-troponin (20, 21; see Appendix). The experiments were performed under free regulated actin concentrations giving 44% binding of S-1 to actin in the steady-state. Fig. 3 shows the results of this experiment. In Fig. 3 *a*, (A-1)S-1 has been mixed with [actin-tropomyosin-troponin + ATP]. The fluorescence transient represents an 800 mvolt increase. Fig. 3 *b* shows that, in the converse experiment, a 150-mV fall in fluorescence occurs when (A-1)-S-1 + ATP is mixed with actin-tropomyosin-troponin.

As discussed in the Appendix, the magnitude of the

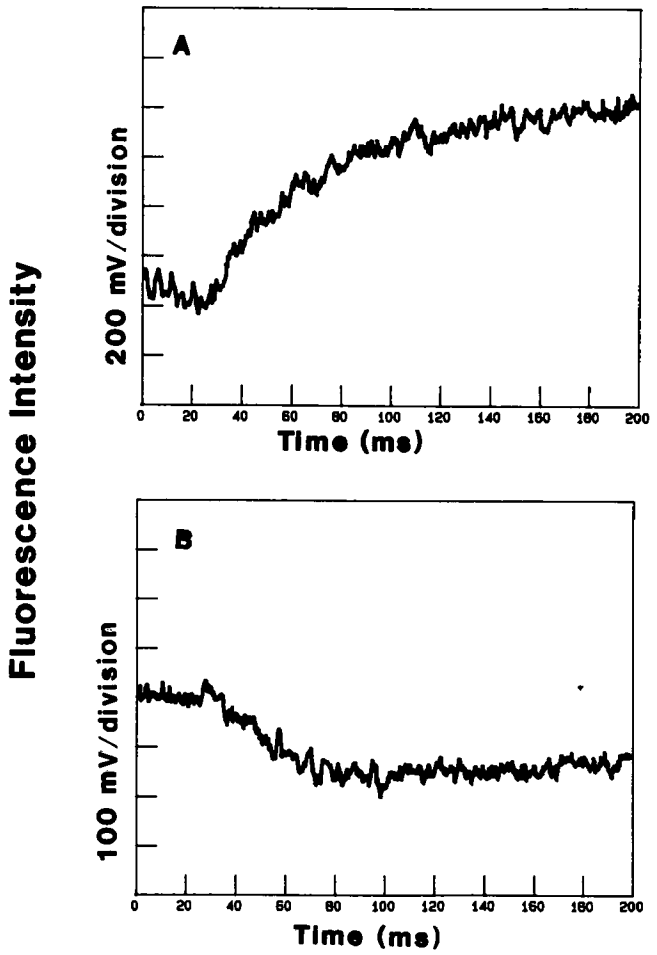


FIGURE 3 The magnitude of the Pi burst in the inhibited (zero calcium) state. Fig. 3 *A* shows the tryptophan fluorescence rise when S-1 is mixed with [actin + tropomyosin + troponin + ATP]. Fig. 3 *B* shows the fluorescence change observed when S-1 + ATP is mixed with actin + tropomyosin + troponin. Conditions: 15°C, 9 mM Imidazole pH 7.0, 2.5 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM EGTA, 0.5 mM DTT. Ratio of actin to Troponin-Tropomyosin was 4/1. Final protein concentrations were [S-1] = 15 μM, [actin] = 40 μM.

burst is given by:

$$(0.75)(800)/[800 - (-150)] = (0.75)(800/950) = 0.63,$$

where 0.75 is the magnitude of the burst in the absence of actin (see Table 1), and represents the fraction of the total S-1 in the myosin-products state. The above equation implies that under these conditions 63% of the S-1 is in the states (M**DPi + AM**DPi). Because 44% of the S-1 is bound to actin in this experiment, a significant population of the state AM**DPi exists in the inhibited state, and, therefore, K_{13} cannot be significantly weakened in the absence of calcium (see Discussion).

Table 1 shows that the burst magnitude at low free Ca^{++} is greater than that observed at high free Ca^{++}, and this is similar to the data observed in the absence of actin. This large magnitude is likely due to the slower steady-state rate which leads to an increase in the population of the myosin-products states.

For comparison with the fluorescence experiments, Fig. 4 shows the magnitude of the "apparent" Pi burst using quench flow techniques. The magnitude in the presence of actin was obtained at 52 μM free actin where 54% of the S-1 was bound to actin. Here the magnitude in the absence of regulated actin is 0.75 (i.e., corrected for by the irreversible binding magnitude 0.8 [13]) and 0.61 in the presence of regulated actin. The data show that the magnitude of the apparent Pi burst measured using chemical quench techniques is within 5% of the measured fluorescence magnitude. The chemical burst is an extrapolated value and, as described in the Appendix, this extrapolation results in an underestimate of the amount of actoS-1 in the myosin product states (22).

We next looked at the actin dependence of the burst rate using actin-tropomyosin-troponin both at high and low [Ca^{++}]. A typical trace is shown in Fig. 5 *a* and can be fit well by a single exponential. At high actin concentrations (>30 μM), however, a small, very fast second exponential (with rate ~450/s) was sometimes observed. This initial transient was believed to be an artifact related to the mixing of viscous solutions, and was ignored. Fig. 5 *b* shows the dependence of the observed fluorescence burst rate on the regulated actin concentration at high and low free [Ca^{++}]. The data show that the burst rate appears to increase slightly as a function of the regulated actin concentration at high free Ca^{++}, but tends to decrease slightly as a function of actin at low free Ca^{++}. The increase in the fluorescence enhancement rate as a function of increasing actin concentration was also seen with the unregulated system (20). This increase in rate was believed to represent the activation of the ATPase activity at low actin concentrations, and at higher actin concentrations the similarity of the burst rate constants in both the dissociated and actin bound pathways of the hydrolysis scheme (see Fig. 1 and reference 20). The finding that the burst rate in the absence of calcium falls only a few percent as a function of the regulated actin concentration implies that the rate of the burst in both the dissociated and nondissociated pathways are approximately equal under these conditions. The rate does not rise initially at low actin because there is little activation of the steady state ATPase activity under these conditions (see Appendix). It should be noted in Fig. 5 *b* that the rate of the fluorescence transient in the absence of actin appeared to depend on the calcium concentration. Statistical analysis of the data reveals that the rate of the fluorescence transient at zero actin was 37.3 s^{-1} ± 1.3 s^{-1} (n = 3) at very low

TABLE 1 Effect of tropomyosin-troponin on the burst magnitude of (A−1)S−1 determined using tryptophan fluorescence

Components	Fraction MT + AMT	Fraction MDPi + AMDPi	Fraction bound	Free actin
				μM
S−1*	0.25	0.75	—	0.0
S−1 + Actin‡ (Unregulated)	0.56	0.44	0.54	32.0
S−1 + Actin + TT‡ (+ Ca^{++})	0.56	0.44	0.54	32.0
S−1 + Actin + TT‡ (− Ca^{++})	0.37	0.63	0.44	34.0

The first line in the table using S−1 alone was determined using a combination of fluorescence and quench flow techniques as previously described (13), and these data are listed here for comparison only. Data in the presence of regulated or unregulated actin were performed under the conditions of Figs. 2–5. Conditions: 15°C, 9 mM Imidazole pH 7.0, 2.5 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM EGTA or $CaCl_2$, 1 mM DTT. Final protein concentrations: [S−1] = 15 μM, $[actin]_R$ = 40 μM. Ratio of actin to T−T was 4/1.

*Determined using both fluorescence and quench flow techniques (18).

‡Conditions: 15°C, 9 mM Imidazole pH 7.0, 2.5 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM EGTA or $CaCl_2$, 1 mM DTT: final protein concentrations were: S−1 = 15 μM, actin or actin-tropomyosin-troponin = 40 μM. The ratio of actin to tropoymyosin-troponin was 4/1.

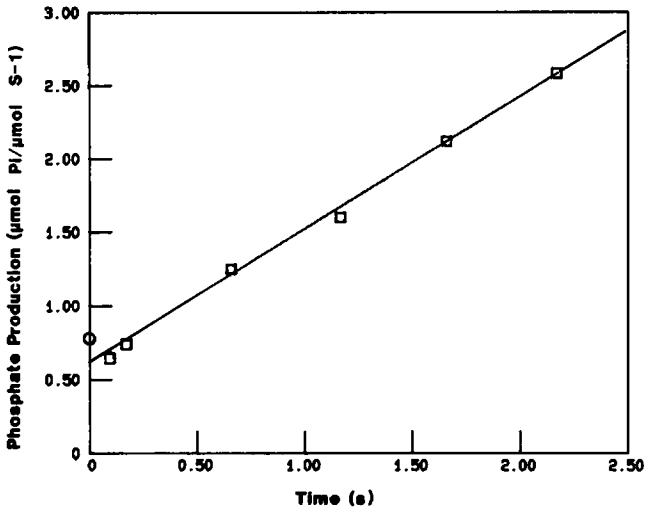


FIGURE 4 The magnitude of the Pi burst using quench flow techniques. ○, magnitude at zero actin; □, magnitude at 52 μM free actin, both corrected for by the irreversible binding magnitude. S-1 was mixed with actin-tropomyosin-troponin + ATP in the absence of calcium. Conditions: 150°C, 10 mM Imidazole, pH 7.0, 2.6 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM EGTA. Ratio actin/T-T was 4/1. Final [S-1] concentration was 15 μM.

calcium, and 41 $s^{-1} \pm 1\ s^{-1}$ ($n = 4$) at high [Ca^{++}]. These data suggest, possibly, a small effect of [Ca^{++}] on the burst rate of S-1 alone. However, assigning an average value of 39 s^{-1} to both transients is also well within the realm of possibility.

Hence, these data imply that the actual cleavage of the terminal phosphoanhydride linkage of ATP by S-1 is not significantly altered by the regulatory apparatus, and that the individual rate constants of the burst both in the dissociating as well as the nondissociating pathway appear to be very similar.

DISCUSSION

In agreement with several prior studies, we have confirmed that the inhibition of the actin activated ATPase activity of myosin S-1 occurs without a significant change in the binding constant of S-1 to actin (6). It was this original finding using $(actin)_R$ which led Chalovich and Eisenberg (6) to suggest that the step controlled by regulation was likely a kinetic transition between actin bound states. Since that time, it has also been demonstrated that while weak binding cross-bridges do remain bound in relaxed muscle, there is a decrease in the rate of cross-bridge cycling (23), a finding consistent with the notion that the mechanism of regulation involves control

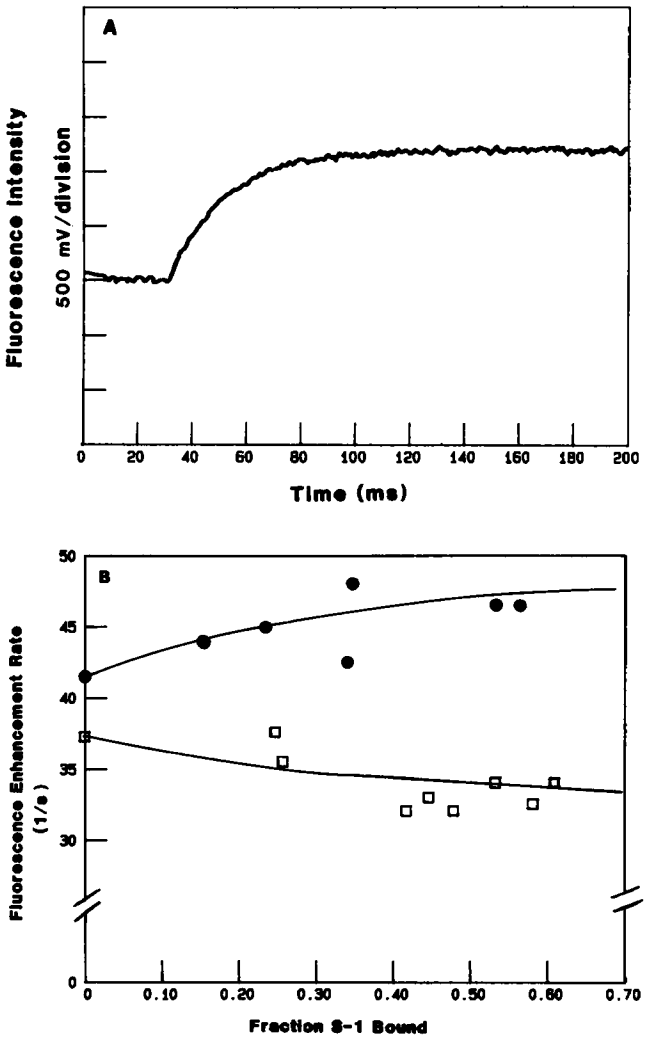


FIGURE 5 Effect of Calcium on the Rate of the Initial Pi burst. Fig. 5*A* shows a typical tryptophan fluorescence enhancement observed when S-1 is mixed with actin-tropomyosin-troponin + ATP in the presence of calcium. Fig. 5*B* shows the actin dependence of the fluorescence rate both in the presence and absence of Calcium. □, are in the absence of calcium, and ●, closed circles, are in the presence of Ca^{++}. Conditions: 15°C, 8 mM Imidazole pH 7.0, 2.5 mM $MgCl_2$, 0.5 mM ATP, 0.67 mM $CaCl_2$ or EGTA, 1 mM DTT, 15–20 μM S-1. The final actin concentration in 5*A* was 10 μM. Fraction bound was determined using 27 μM for the steady state binding constant in the presence of calcium and 44 μM for the binding constant in the absence of calcium. Ratio of actin to T-T was 4/1.

of a kinetic transition between actin-bound kinetic intermediates.

Although the actin binding that occurs during steady state ATP hydrolysis is weak, this binding is believed to be specific based on the following evidence: first the magnitude of the apparent dissociation constant, $K_{binding}$, has an approximately fixed relationship to the apparent Michaelis constant for the actin activated ATPase activ-

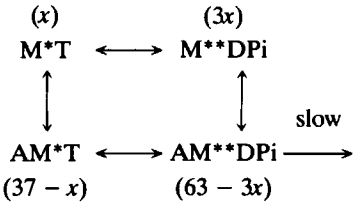
ity, K_{ATPase}, under constant ionic strength and temperature conditions (24). Second, approximately the same binding constant is determined using stopped-flow turbidity techniques at high S-1 concentrations or sedimentation techniques at very low S-1 concentrations (14). Third, using pPDM-labeled S-1 which resembles S-1-ATP, it has been shown that the binding saturates at a stoichiometry of 1:1 with actin both at low and high free [Ca^{++}] (25). Fourth, inhibition of this weak binding in solution is correlated with an inhibition of ATP hydrolysis (26). Fifth, Ca^{++}-independent weak binding has been observed in muscle fibers (27), and inhibition of this weak binding with caldesmon, without inhibition of the strong binding conformations, inhibits force production (28).

In contrast to the weak actin binding states, i.e., M-ATP and M-ADP-Pi discussed above, the binding of the M-ADP states to $(actin)_R$ are sensitive to the free Ca^{++} concentration (29). Because the release of phosphate from the weakly bound M-ADP-Pi state is a transition from a state whose actin binding shows minimal Ca^{++} dependency to a state whose actin binding demonstrates a strong Ca^{++} dependency, Chalovich and Eisenberg (6) suggested that Pi release is regulated by Ca^{++} (5, 6). Later, Rosenfeld and Taylor (8) showed that the release of phosphate is affected by Ca^{++} in agreement with Chalovich and Eisenberg (6).

Recent reports of the force transient of a single fiber after the rapid release of Pi from caged phosphate suggest that there is either no effect of calcium (9) or, at lower phosphate concentrations, a threefold effect of calcium on the rate of this transient (11), and based on these findings it was suggested that an additional isomerization exists before Pi release which may also be regulated (9, 10). Interestingly, an adequate quantitative fitting of much of the kinetic and biochemical data, from purified contractile proteins in solution, requires that an isomerization occur after the burst and before Pi release, although no direct evidence for this transition exists. The six-state model of ATP hydrolysis by actoS-1 includes an additional transition between the Pi burst and the release of Pi (22), but it is not clear if the two kinetically required transitions are identical. The existence of additional intermediate states would not be surprising since the binding of actin and nucleotides to S-1 occurs in multiple stages (31–33).

Rosenfeld and Taylor (8) did report earlier that alterations of [Ca^{++}] did not affect the rate of phosphate release (8). However, these authors did not show the burst rate as a function of the actin concentration (here plotted as burst rate vs. the fraction S-1 bound to actin), nor did they show the burst magnitude. We now show, in agreement with Rosenfeld and Taylor (8), that the rate of the fluorescence transient has little calcium sensitivity, and that this is true over a wide range of actin concentrations.

While direct binding studies have shown a lack of Ca^{++} effect on the binding of S-1 to $(actin)_R$ in the presence of ATP, they do not allow one to distinguish changes in the individual association constants. The present data of the burst magnitude allows changes in the individual equilibrium constants to be estimated. This can be seen as follows:



At very low calcium the ATPase activity is significantly reduced, and is much slower than the forward and backward rates of the burst in the actin free domain of the cycle. Hence it is reasonable to assume that M*T and M**DPi are approximately in equilibrium, and therefore M**DPi = 3M*T (13). Let M*T = x, then M**DPi = $3x$ and since the magnitude of the burst was 63% under these conditions, AM**T = $37 - x$ and AM**DPi = $63 - 3x$. Note, as expected, the sum of all four state probabilities is 100. Next we equate the sum of AM*T + AM**DPi to 44, the probability of being bound, and $x = 14\%$. Therefore 21% is in state AM**DPi and 23% is in the state AM*T, and significant binding occurs in both states. The data in Fig. 2 show that the binding constants of S-1 to "regulated" actin is 27 μM at high [Ca^{++}], and 44 μM at very low [Ca^{++}]. If a threefold difference between K_3 and K_{13} in the presence of calcium are assumed (21), then reasonable choices for these dissociation constants in the presence of calcium would be 18 and 54 μM, respectively. In the inhibited state Fig. 3 shows that, at 40 μM total actin, the sum of the states (M**DPi + AM**DPi) possesses 63% of the total S-1 present under conditions were 44% of the S-1 is bound to actin, and this has been shown above to imply that 21% is in the state AM**DPi making K_{13} = 68 μM and K_3 = 21 μM, showing that little change has occurred in these binding constants. Recent work from Taylors' laboratory (8) also suggests that the control of regulation must occur after the Pi burst. Their studies on the control of product plus substrate release rates using both ATP and 1-N^6-etheno-ATP as substrates imply that the burst rate in the nondissociating pathway is too large to be rate limiting for the cycle.

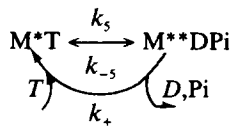
In summary, the tropomyosin-troponin complex does not inhibit the actin-activated ATP hydrolysis by inhibiting the burst of ATP hydrolysis. Furthermore, earlier studies have shown that the binding of myosin-ATP to

actin is not greatly affected by tropomyosin-troponin. Therefore, it is likely that either Pi release and/or a step preceeding it is regulated.

APPENDIX

A. Rate of the Pi Burst (20, 22)

The minimal model for the actin activated myosin ATPase activity is the four state model (see scheme 1) (13), and this model will be used as a starting point to discuss the presteady state kinetics of regulated actin. In the absence of actin the hydrolysis of ATP follows the following pathway:



Scheme 1

Here M = Subfragment-1, T = ATP, D = ADP, and Pi is inorganic phosphate. The star in the M*T species in the above scheme represents the fact that a tryptophan fluorescence enhancement occurs as a result of ATP binding. The two stars in the state M**DPi represents a further fluorescence enhancement. ATP binds to S-1 very rapidly and, therefore, the formation of M*T is immediate at saturating ATP concentrations (i.e., ATP > 0.5 mM). Following the formation of M*T, the system approaches a steady state and the fluorescence transient observed during this presteady state period is due to the formation of M**DPi. The rate constant for formation of M**DPi, which is usually refered to as the burst rate in the absence of actin, is given by (see Scheme 1)

$$R_B([A] = 0) = k_5 + k_{-5} + k_+.$$

In the absence of actin, k_+ is very slow, and it can therefore be ignored in the above equation.

In the presence of regulated actin the four state model is the minimal model capable of accounting for the data (Fig. 1 *a*). Data have been presented that suggests that in the presence of ATP, M*T, and M**DPi form rapid equilibrium complexes with actin (13). Because of this, the four state model can be written as if it consisted of only two states: (M**DPi + AM**DPi) and (M*T + AM*T). The fluorescence rate in this case (the burst rate), as a function of the actin concentration, is given by:

$$R_B([A]) = k'_5 + k'_{-5} + k'_6 + k'_{-6} + k'_{10},$$

where

$$k'_5 = k_5K_3/(K_3 + [A]);$$

$$k'_6 = k_6[A]/(K_3 + [A]); \; k'_{-6} = k_{-6}[A]/(K_{13} + [A]);$$

$$k'_{-5} = k_{-5}K_{13}/(K_{13} + [A]); \; k'_{10} = k_{10}[A]/(K_{13} + [A]).$$

As can be seen, this expression gives $k_5 + k_{-5}$ at zero actin and $k_6 + k_{10} + k_{-6}$ at infinite actin. Only the rate constants k_6, k_{-6}, and k_{10}, and the dissociation constants K_3 and K_{13} can be affected by the regulatory proteins in a simple way, and current evidence suggests that the steady-state binding is not significantly affected by the presence or absence of calcium (Fig. 2). Therefore it is reasonable to assume (also see Discussion of Fig. 3) that the binding constants K_3 and K_{13} do not change significantly, and that it is only necessary to focus on the kinetic transition rate constants k_6, k_{-6}, and k_{10}. Note that k_{10} can be viewed as equal to the rate of Pi release (or the rate of product release), and therefore the regulatory proteins can exert their influence in this model either by slowing the rate of the release of the products of hydrolysis or by slowing the rate of the ATP cleavage step.

B. Magnitude of the initial Pi Burst (13, 16, 22)

In the absence of actin the magnitude of the burst is defined as the fraction of active protein in the state M**DPi. The mathematical result is:

$$M_B([A] = 0) = k_5/(k_5 + k_{-5}).$$

In the presence of actin, the burst is defined as the fraction of active protein in the sum of the states M**DPi + AM**DPi, and in the four state model is given by:

$$M_B([A]) = (k'_5 + k'_6)/(R_B([A]).$$

Unfortunately, this is not a directly measurable quantity *using quench flow techniques,* because the steady-state rate interferes with the measurement. Instead, in the case of quench flow measurements, it is necessary to define a "reduced burst magnitude" or an "apparent burst magnitude" (8, 22) as the extrapolation of the phosphate production vs. time curve to $t = 0$. According to the four state model this is given by (Fig. 1 *a*):

$$M^R_B([A]) = (k'_5 + k'_6)/(R_B([A])\{1 - k'_{10}/[R_B([A])]\}$$

and since the steady state ATPase rate as a function of the actin concentration can be written as:

$$V_s([A]) = (k'_5 + k'_6)k'_{10}/(R_B([A])$$

it follows that (21):

$$M^R_B([A]) = M_B([A]) - Vs([A])/(R_B([A]).$$

C. Burst magnitude using fluorescence

The magnitude of the burst using fluorescence measurements has been described in detail before (21), and leads to an estimate of the actual fractional occupation of the states M**DPi + AM**DPi (i.e., the actual burst magnitude and not the reduced or apparent burst magnitude). Prior studies of the fluorescence burst comparing the mixing of ActoS-1 with ATP against the mixing of S-1 with ATP (15) showed that there was a discrepancy in the magnitudes that could be explained by a small increase in the tryptophan fluorescence of actoS-1 compared with S-1 alone. In the work described here either S-1 was mixed vs. $(Actin)_R$ + ATP, or S-1 + ATP was mixed vs. $(actin)_R$. In the latter mixing arrangement the state $(Acto)_RS$-1 without nucleotide bound is not found, and in the former while the theoretical possibility exists that some $(Acto)_RS$-1 will form transiently, the fact that the ATP concentration is saturating and 10–100-fold higher than the actin concentration makes it unlikely to appear in any significant concentration.

It will be shown below (21), that if F^1_M is the magnitude of the fluorescence enhancement signal when S-1 is mixed with Actin + ATP, and F^2_M is the magnitude measured when Actin is mixed with S-1 + ATP, then the burst magnitude is given by:

$$M^F_B([A]) = M_B([A] = 0)F^1_M/(F^1_M - F^2_M).$$

Note, that at $[A] = 0$, mixing buffer with S-1 + ATP will have no effect, and $F_M^2 = 0$; in this case:

$$M_B^F([A] = 0) = M_B([A] = 0).$$

Let the tryptophan fluorescence associated with the states in question be as follows:

$$M^*T = x,\ M^{**}DPi = y,\ AM^*T = nx,\ AM^{**}DPi = gy.$$

Also let the states AM*T and M*T always be in rapid equilibrium with dissociation constant K_3, with the same true of AM**DPi and M**DPi with K_{13}. The fraction of these two equilibria in the bound state is f_T and f_D, respectively given by:

$$f_T = [A]/(K_3 + [A]) \text{ and } f_D = [A]/(K_{13} + [A]).$$

At steady state in the absence of actin assume M**DPi/M*T = 3, and in the presence of actin, assume that f is the fraction of S-1 in the myosin-product states (=true burst). Also assume in the analysis that the final steady state achieved after mixing is the same with both mixing techniques, and M_t = total [S-1]. Finally, because the binding constants of actin of the various myosin-nucleotide states differ slightly, it is required that the free actin used be sufficiently high so that small transient changes in free actin that do occur do not significantly change the total free actin concentration.

In the first way of mixing we begin with all S-1 in the M-T states at $t = 0$ with fluorescence

$$(M^*T)x + (AM^*T)nx = M_t(1 - f_T)x + f_T M_t nx$$
$$= M_t[f_T nx + (1 - f_T)x].$$

After the transient all species are populated, and the steady-state fluorescence is:

$$= \{(1 - f)M_t[f_T nx + (1 - f_T)x] + (f)M_t[f_D gy + (1 - f_D)y]\}.$$

F_M^1 is the difference of these two expressions:

$$F_M^1 = \{(f)M_t[f_D gy + (1 - f_D)y]\} - \{(f)M_t[f_T nx + (1 - f_T)x]\}.$$

In the other mixing technique, before $t = 0$, 25% of the total protein is in the M*T state, and 75% is in the M**DPi state, and at $t = 0$ after mixing we assume this distribution between the myosin-ATP and myosin-product states is the same. Hence the initial fluorescence is given by:

$$0.25\,M_t[f_T nx + (1 - f_T)x] + 0.75\,M_t[f_D gy + (1 - f_D)y]$$

the final fluorescence is given above (i.e., steady state) and

$$F_M^2 = (0.75 - f)M_t[f_T nx + (1 - f_T)x]$$
$$- (0.75 - f)M_t[f_D gy + (1 - f_D)y].$$

Note that

$$F_M^2/F_M^1 = (f - 0.75)/f \quad \text{or} \quad f = 0.75F_M^1/(F_M^1 - F_M^2) \quad \text{(QED)}.$$

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