

Regulation of actomyosin ATPase activity by troponin–tropomyosin: Effect of the binding of the myosin subfragment 1 (S-1)·ATP complex

(muscle regulation/cooperativity/regulated actin)

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ABSTRACT In our model of regulation, the observed lack of cooperativity in the binding of myosin subfragment 1 (S-1) with bound ATP to the troponin–tropomyosin–actin complex (regulated actin) is explained by S-1·ATP having about the same affinity for the conformation of the regulated actin that activates the myosin ATPase activity (turned-on form) and the conformation that does not activate the myosin ATPase activity (turned-off form). This predicts that, in the absence of Ca^{2+} , S-1·ATP should not turn on the regulated actin filament. In the present study, we tested this prediction by using either unmodified S-1 or S-1 chemically modified with *N,N'*-*p*-phenylenedimaleimide (*p*PDM·S-1) so that functionally it acts like S-1·ATP, although it does not hydrolyze ATP. We found that, in the absence of Ca^{2+} , neither S-1·ATP nor *p*PDM·S-1·ATP significantly turns on the ATPase activity of the regulated complex of actin and S-1 (acto·S-1). In contrast, in the presence of Ca^{2+} , *p*PDM·S-1·ATP binding almost completely turns on the regulated acto·S-1 ATPase activity. These results can be explained by our original cooperativity model, with *p*PDM·S-1·ATP binding only ≈ 2 -fold more strongly to the turned-on form than to the turned-off form of regulated actin. However, our results are not consistent with our alternative model, which predicts that if *p*PDM·S-1·ATP binds to actin in the absence of Ca^{2+} but does not turn on the ATPase activity, then it should also not turn on the ATPase activity in the presence of Ca^{2+} .

Skeletal muscle contraction is regulated by Ca^{2+} , which binds to the regulatory complex troponin–tropomyosin (1, 2). In the presence of ATP at low free Ca^{2+} concentration, muscle fibers are relaxed and the associated actin-activated ATPase activity is inhibited. However, a low concentration of Ca^{2+} is not in itself sufficient to produce relaxation; ATP is also necessary because rigor cross-bridges on the thin filament can activate muscle contraction and prevent relaxation even in the absence of Ca^{2+} (3, 4). This activation by rigor cross-bridges, which occurs at low ATP concentration, was intensively studied *in vitro* by Weber and coworkers (5–9). They found that rigor bridges [actin and myosin subfragment 1 complex (acto·S-1) or acto·S-1·ADP] cooperatively turn on the ATPase activity of regulated acto·S-1 both in the presence and absence of Ca^{2+} . This turning on, which is optimized by high ratios of S-1 to actin and by low ATP concentration, occurs with the troponin–tropomyosin–actin complex (regulated actin) in the presence and absence of Ca^{2+} and with tropomyosin–actin, but not with pure actin. Based on these observations, Weber and coworkers proposed that rigor cross-bridges cooperatively turn on tropomyosin–actin units consisting of one tropomyosin molecule and seven actin monomers (6).

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Rigor bridges not only cooperatively turn on the ATPase activity but, in a related phenomenon, they have also been found to bind cooperatively to regulated actin. S-1 and S-1·ADP bind more weakly to regulated actin at low levels of saturation of the actin with S-1 than at high levels of saturation (10, 11). This cooperativity has been observed in both the presence and absence of Ca^{2+} , but it is much more pronounced in the absence of Ca^{2+} . The original model of Hill *et al.* (12) accounted for this cooperativity by suggesting that regulated actin can exist in two forms. In the turned-off form (formerly referred to as weak-binding form), the binding of S-1 and S-1·ADP is inhibited and there is very little activation of the S-1 ATPase activity, while in the turned-on form (formerly referred to as strong-binding form), the binding of S-1 and S-1·ADP is even stronger than to unregulated actin, and there is marked actin activation of the S-1·ATPase activity. On this basis, the turning on of the ATPase activity by rigor bridges occurs because they bind with much greater affinity to the turned-on form than to the turned-off form of regulated actin, thereby shifting the regulated actin units into the turned-on form. Moreover, even in the presence of Ca^{2+} , it is necessary to have bound rigor bridges to fully turn on the regulated actin filament, since Ca^{2+} itself only partially shifts the regulated actin into the turned-on form.

In contrast to rigor cross-bridges, cross-bridges with bound ATP (or bound $\text{ADP}\cdot\text{P}_i$) do not bind with any apparent cooperativity to regulated actin either in the presence or absence of Ca^{2+} (13–15). On the basis of the model of Hill *et al.* (12), this indicates that S-1·ATP binds with, at most, 2-fold greater affinity to the turned-on than to the turned-off form of regulated actin. This, in turn, leads to the prediction that, in contrast to rigor bridges, S-1·ATP should not significantly turn on the regulated acto·S-1·ATPase activity in the absence of Ca^{2+} . However, Lehrer and Morris (16) reported that, in the absence of Ca^{2+} , S-1·ATP does appear to turn on the regulated acto·S-1 ATPase activity.

In the presence of Ca^{2+} , our model (12) gives two different predictions depending on the relative affinity of S-1·ATP for the turned-on and turned-off forms of regulated actin. If S-1·ATP binds equally well to the two forms, then it will not turn on the regulated acto·S-1·ATPase activity. However, if S-1·ATP binds slightly stronger to the turned-on form than to the turned-off form of regulated actin, then it will turn on the regulated acto·S-1 ATPase activity. In fact, workers in several laboratories have reported such turning on in the presence of Ca^{2+} (6, 9, 16), but it has never been clear whether it is due to the S-1·ATP itself or to trace contami-

Abbreviations: S-1, myosin subfragment 1; acto·S-1, complex of actin and myosin subfragment 1; *p*PDM, *N,N'*-*p*-phenylenedimaleimide; *p*PDM·S-1, S-1 that has been modified with *p*PDM; regulated actin, troponin–tropomyosin–actin complex; MalNEt, *N*-ethylmaleimide; MalNEt·S-1, S-1 that has been modified extensively with MalNEt.

nation of the S-1 with a denatured fraction that forms ATP-resistant rigor cross-bridges with actin.

In the present study, we have investigated whether the binding of S-1·ATP significantly turns on the regulated acto-S-1 ATPase activity and the tropomyosin-acto-S-1 ATPase activity. In many of our experiments, we used S-1 chemically modified with *N,N'*-*p*-phenylenedimaleimide (*p*PDM), which crosslinks the SH-1 and SH-2 groups, making the modified S-1 (*p*PDM·S-1) behave functionally like S-1·ATP (17). We find that, under conditions in which S-1·ATP or *p*PDM·S-1·ATP bind extensively to regulated actin, they do not significantly turn on the regulated acto-S-1 ATPase activity in the absence of Ca²⁺. However, *p*PDM·S-1·ATP significantly turns on the regulated acto-S-1 ATPase activity in the presence of Ca²⁺, apparently because it binds ≈2-fold stronger to the turned-on form of regulated actin than to the turned-off form. These data are consistent with our original model of regulation in which regulated actin is always in equilibrium between the turned-off and turned-on forms, but they rule out our alternative model of regulation (18) in which regulated actin can exist in a continuum of forms but only one of these forms can occur under a given condition.

MATERIALS AND METHODS

Troponin-tropomyosin and tropomyosin were prepared according to Eisenberg and Kielley (19); all other proteins were prepared by procedures described elsewhere (20). S-1 that has been extensively modified with *N*-ethylmaleimide (MalNET·S-1) was prepared as described by Williams *et al.* (21). Binding studies showed that in the presence of ATP, ≈80% of the MalNET·S-1 binds to actin. The MalNET·S-1 concentration was always corrected for this nonbinding population. S-1 was modified with either radioactive or nonradioactive *p*PDM·S-1 at 0°C as described by Wells and Yount (22). [¹⁴C]*p*PDM was synthesized as described by Wells and Yount; *p*PDM was sublimated before use (17). The modified S-1 contained ≈1.2 mol of *p*PDM per mol of S-1, and between 75% and 80% of the modified S-1 molecules contained trapped ADP. The *p*PDM·S-1 was further purified by sedimenting with F-actin at a 1:5 (mol/mol) ratio of actin to *p*PDM·S-1 in the absence of nucleotide at 45 mM ionic strength (17). The contaminant uncrosslinked S-1 sediments with the F-actin, leaving purified *p*PDM·S-1 in the supernatant. This was routinely done twice and, in some experiments, as many as four times.

RESULTS

Our previous results showed that *p*PDM·S-1 binds with about the same affinity to regulated actin as S-1·ATP (17). We also showed that, like S-1 in the presence of ATP, *p*PDM·S-1 does not bind cooperatively to regulated actin in the presence of ATP. This is in contrast to *p*PDM·S-1 in the absence of ATP, which does show slight cooperative binding to regulated actin (23). Since troponin-tropomyosin does not confer cooperativity on the binding of *p*PDM·S-1·ATP to actin, *p*PDM·S-1·ATP appears to bind with about equal affinity to the turned-off and turned-on forms of regulated actin. This leads to the prediction that *p*PDM·S-1 should not significantly turn on the ATPase activity of regulated acto-S-1 in the absence of Ca²⁺. The turning on of the regulated actin filament by *p*PDM·S-1 was measured by adding a low concentration of unmodified S-1 and then measuring the degree to which its ATPase activity was activated by the regulated actin filament at various levels of saturation with *p*PDM·S-1, while always maintaining a constant free actin concentration of 10 μM.

Of course, to determine the relative amount of turning on caused by *p*PDM·S-1, it was necessary to determine the ATPase rate when the regulated actin was fully turned on.

This was done by measuring, under the identical conditions used with *p*PDM·S-1, the extent to which MalNET·S-1 binding turns on regulated actin (21). Similar to *p*PDM·S-1, MalNET·S-1 itself has negligible ATPase activity, but in contrast to *p*PDM·S-1, it is permanently in the rigor state even in the presence of ATP. In the case of MalNET·S-1, the concentration of actin was maintained at 10 μM, calculated on the basis that 80% of the MalNET·S-1 binds to actin (see *Materials and Methods*), while with *p*PDM·S-1, the concentration of free actin was calculated from the known binding constant of *p*PDM·S-1 to regulated actin in the presence of ATP (23).

The extent to which *p*PDM·S-1 and MalNET·S-1 turned on the regulated acto-S-1 ATPase activity in the absence of Ca²⁺ is shown in Fig. 1. The S-1·ATPase activity is plotted as a function of actin sites saturated with either MalNET·S-1 (open circles) or *p*PDM·S-1 (closed circles); the plots are obviously quite different. With increasing saturation of the actin filament with MalNET·S-1, the ATPase activity of regulated acto-S-1 increased to a maximum rate of ≈16.0 s⁻¹, which occurred when the regulated actin filament was ≈50% saturated with MalNET·S-1. This is consistent with the binding data of S-1·ADP to regulated actin, which showed that all of the tropomyosin-actin units are in the turned-on form at a S-1·ADP to actin ratio of ≈0.5 (10, 11). Compared to the results obtained with MalNET·S-1, *p*PDM·S-1 does not

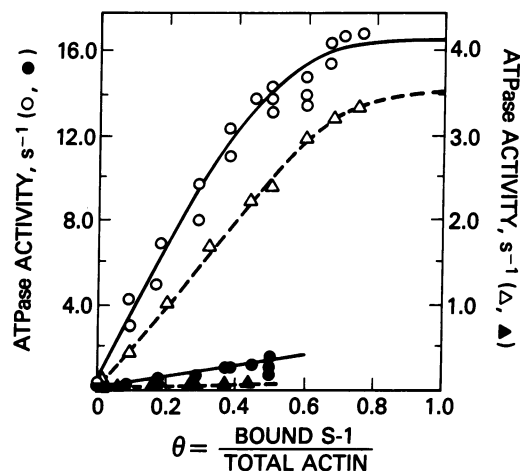


FIG. 1. Turning on of the regulated acto-S-1 ATPase activity in the absence of Ca²⁺. The ATPase activity of regulated acto-S-1 was measured at various saturations of the actin filament with *p*PDM·S-1 (●), S-1·ATP (Δ), or MalNET·S-1 (○, Δ). In all cases, the rate (ordinate axis) is expressed per total unmodified S-1 concentration. θ is the number of moles of S-1 bound per mole of actin monomer. In the experiment comparing the extent to which *p*PDM·S-1 (●) turns on the regulated acto-S-1 ATPase activity relative to that obtained with MalNET·S-1 (○), conditions were as follows: 3.5 mM KCl/3 mM imidazole, pH 7.0/3 mM MgCl₂/1 mM ATP/1 mM dithiothreitol/1 mM EGTA (μ, 18 mM) at 25°C. Various concentrations of either *p*PDM·S-1 (6–50 μM) or MalNET·S-1 (0.5–30 μM) were added to regulated actin (10–40 μM), while always maintaining a constant free actin concentration of 10 μM. The extent of turning on was determined by measuring the actin-activated ATPase of unmodified S-1 on the pH Stat, using trace amounts of unmodified S-1 (0.1–2 μM). In the experiment comparing the extent to which S-1·ATP (Δ) turns on the regulated acto-S-1 ATPase activity relative to MalNET·S-1 (Δ), conditions were 7 mM imidazole, pH 7.0/2.5 mM MgCl₂/0.5 mM ATP/1 mM dithiothreitol/1 mM EGTA at 15°C. Various concentrations of either S-1 (2–27 μM) or MalNET·S-1 (1–30 μM) were added to regulated actin while always maintaining a constant free actin concentration of 10 μM. In computing θ for the S-1·ATP data, the value used for the association constant of S-1·ATP to regulated actin under these conditions was $4 \times 10^{-4} \text{ M}^{-1}$ (unpublished data). Actin-activated ATPase activities of S-1 were corrected for the rate of S-1 alone.

significantly turn on the regulated acto-S-1 ATPase activity. For example, at 50% saturation of the actin filament with *p*PDM-S-1, the ATPase activity of regulated acto-S-1 is <10% of the maximal turned-on rate. Moreover, the slight turning on of the regulated acto-S-1 ATPase activity by *p*PDM-S-1 that we did observe may have been due to trace contamination of *p*PDM-S-1 with rigor-type cross-bridges. It would take only a very small amount of this contaminant to account either partially or totally for the observed turning on. On the other hand, since we repeatedly obtained this low level of turning on, even after repeating the purification of *p*PDM-S-1 several times (see *Materials and Methods*), this slight turning on may have been due to *p*PDM-S-1 itself binding 2-fold more strongly to the turned-on than to the turned-off form of regulated actin. In fact, our results obtained below in Ca^{2+} provide evidence that this may well have been the case. In any event, our results do show that, in agreement with the prediction of our model (12), *p*PDM-S-1 does not significantly turn on the regulated acto-S-1 ATPase activity in the absence of Ca^{2+} .

We next examined whether S-1·ATP itself is able to turn on the regulated actin filament. We could not perform this experiment at 25°C where we studied the effect of *p*PDM-S-1 because, as we increased the S-1 concentration, the ATPase activity became too high to measure. Therefore, we performed the experiment at 15°C. As in our experiments with *p*PDM-S-1, the concentration of free actin was calculated from the binding constant of S-1·ATP to regulated actin (see Fig. 1 legend). Fig. 1 shows that, just like *p*PDM-S-1, S-1·ATP (closed triangles) does not significantly turn on the actin-activated ATPase activity. Even when the actin is almost 50% saturated with S-1·ATP, the ATPase rate is <5% of the fully turned-on actin-activated ATPase rate measured with MalNEt-S-1 (open triangles). Therefore, these data, in combination with the results obtained with *p*PDM-S-1, strongly suggest that S-1·ATP cannot turn on the regulated actin-activated ATPase activity in the absence of Ca^{2+} .

The next question that arises is whether S-1·ATP can turn on the regulated acto-S-1 ATPase activity in the presence of Ca^{2+} . As discussed in the Introduction, the prediction of our model as to whether S-1·ATP should turn on the regulated acto-S-1 ATPase activity in the presence of Ca^{2+} depends on the relative affinity of S-1·ATP to the turned-on and turned-off forms of regulated actin. We could not investigate this question using S-1·ATP itself because the ATPase activity becomes much too high to measure, even at a low temperature. Therefore, we investigated this question using *p*PDM-S-1. The experiments were done under the same conditions we used in Fig. 1 in the *p*PDM-S-1 experiments, and again we used MalNEt-S-1 to measure the maximal turned-on rate. As shown in Fig. 2A, the maximal turned-on rate of $\approx 20 \text{ s}^{-1}$ was achieved when the actin filament was only $\approx 20\%$ saturated with MalNEt-S-1, which is a much lower percentage than that needed in the absence of Ca^{2+} . This is consistent with the cooperativity observed in the binding of S-1·ADP to regulated actin in the presence of Ca^{2+} ; in these experiments, all of the tropomyosin-actin units were turned on when the ratio of S-1·ADP to actin was 0.20 (10, 11).

We next measured the extent to which *p*PDM-S-1 turned on the regulated acto-S-1 ATPase activity in the presence of Ca^{2+} . As shown in Fig. 2A, when *p*PDM-S-1 was bound to the regulated actin filament, it significantly turned on the regulated acto-S-1 ATPase activity in the presence of Ca^{2+} , in contrast to the results obtained in the absence of Ca^{2+} . For instance, when the actin filament was 40% saturated with *p*PDM-S-1, the ATPase activity of regulated acto-S-1 was $\approx 70\%$ of the maximal turned-on rate. This extent of turning on indicates that *p*PDM-S-1 binds ≈ 2 -fold more strongly to the turned-on form than to the turned-off form of regulated actin.

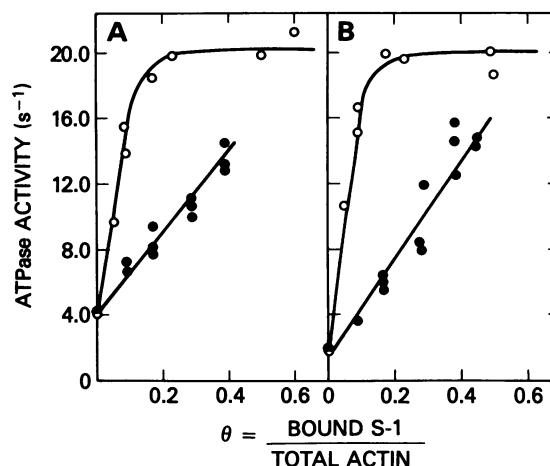


FIG. 2. Turning on of the regulated acto-S-1 ATPase activity in the presence of Ca^{2+} (A) or tropomyosin-acto-S-1 ATPase activity (B). The actin-activated ATPase activity of S-1 was measured at various saturations of the actin filament with either *p*PDM-S-1 (●) or MalNEt-S-1 (○). This experiment was performed in the same manner as that described in Fig. 1 for the turning on of regulated acto-S-1 by *p*PLA-S-1 in the absence of CaCl_2 , except 0.5 mM CaCl_2 was added. All rates were expressed per total unmodified S-1 concentration.

The turning on that we observe cannot simply be caused by trace contamination of rigor-type cross-bridges in our *p*PDM-S-1 preparation. If rigor-type cross-bridges were completely responsible for the turning on of the regulated acto-S-1 ATPase activity in the presence of Ca^{2+} , we should have observed much more turning on by *p*PDM-S-1 in the absence of Ca^{2+} . For example, when the actin is 50% saturated with *p*PDM-S-1, the extent of turning on of the regulated acto-S-1 ATPase activity in the presence of Ca^{2+} is equivalent to having 8% of the actin sites containing rigor-type cross-bridges. However, this level of contamination would have produced an ATPase activity of regulated acto-S-1 in the absence of Ca^{2+} 2- to 3-fold higher than we actually measured at 50% saturation of the actin filament with *p*PDM-S-1. At most, rigor bridges could be causing about one-half of the turning on we observe in the presence of Ca^{2+} . If this is the case, the remainder of the turning on observed would be due to *p*PDM-S-1 binding ≈ 1.5 -fold more strongly to the turned-on form than to the turned-off form of regulated actin, rather than 2-fold more strongly, as would be necessary if the *p*PDM-S-1 were causing all of the turning on.

We also examined the extent to which *p*PDM-S-1 and MalNEt-S-1 turn on the tropomyosin-acto-S-1 ATPase activity. We previously found in studying the binding of S-1 to actin that tropomyosin alone acts almost exactly like troponin-tropomyosin in the presence of Ca^{2+} (24). Consistent with these binding studies, the effect of *p*PDM-S-1 on tropomyosin-actin was very similar to its effect on regulated actin in the presence of Ca^{2+} (Fig. 2B). As with regulated actin in the presence of Ca^{2+} , *p*PDM-S-1 significantly turned on the tropomyosin-acto-S-1 ATPase activity, with the rate being $\approx 70\%$ of the maximal turned-on rate when the actin filament was 40% saturated with *p*PDM-S-1. Therefore, the results with both tropomyosin-actin and regulated actin in the presence of Ca^{2+} suggest that *p*PDM-S-1 significantly turns on the actin filament in a cooperative manner. This in turn indicates that *p*PDM-S-1 binds slightly more strongly (at most, 2-fold) to the turned-on form of regulated actin compared to the turned-off form. This accounts for the turning on of the regulated acto-S-1 ATPase activity in the presence of Ca^{2+} and the lack of turning on in the absence of Ca^{2+} . Furthermore, with only this slightly greater affinity of *p*PDM-S-1 for the turned-on form, the binding curve of

*p*PDM·S-1 to regulated actin would not appear cooperative but instead would be indistinguishable from the curve for independent binding, in agreement with our binding data (23).

Since all of our results with *p*PDM·S-1 were consistent with our model of muscle regulation, we reinvestigated the observation of Lehrer and Morris (16) that S-1·ATP turns on regulated actin both in the presence and absence of Ca^{2+} at relatively high ionic strength (50 mM). Based on our present results, we would not expect to observe significant turning on of the regulated actin at 50 mM ionic strength, either in the absence or presence of Ca^{2+} , because at this ionic strength very little S-1 binds to actin in the presence of ATP. However, Lehrer and Morris did not use MalNEt·S-1 in their studies, so they were unable to compare their results with the maximal turned-on ATPase rate. Therefore, we have repeated the experiments of Lehrer and Morris, working at the same ionic strength, pH, temperature, and protein concentrations that they used (see figure 3 of their paper).

Fig. 3 shows the ATPase activity as a function of S-1 concentration measured at 5 μM actin concentration. With unregulated actin, the actin-activated ATPase rate was, of course, independent of the S-1 concentration; at this ionic strength, so little S-1 binds to actin that from the lowest to the highest S-1 concentration used, the free actin concentration

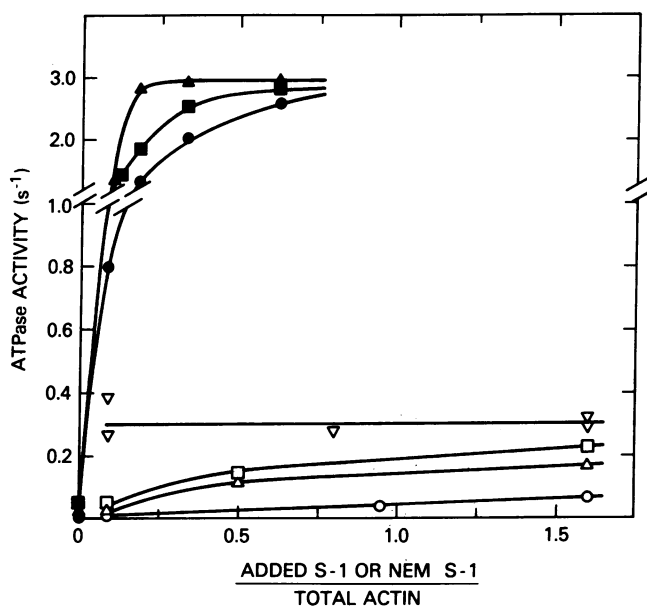


FIG. 3. Turning on of the acto-S-1 ATPase activity by S-1 at high ATP concentrations and by MalNEt·S-1 (NEM S-1). Conditions were as follows: 22.5 mM NaCl/15 mM Tris·HCl, pH 7.9/1 mM ATP/5 mM MgCl_2 /1 mM dithiothreitol/0.1 mM CaCl_2 or 0.5 mM EGTA (μ , 50 mM) at 23°C. Actin-activated ATPase activity of S-1 was measured by the release of P_i from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. This was done with unregulated actin (∇), tropomyosin actin (Δ , \blacktriangle), regulated actin in the presence of Ca^{2+} (\square , \blacksquare), and regulated actin in the absence of Ca^{2+} (\circ , \bullet). In measuring the turning on of the ATPase activity by S-1 (open symbols), various concentrations of S-1 (0.4–8.0 μM) were added to 5 μM actin. In measuring the turning on of the ATPase activity by MalNEt·S-1 (closed symbols), various concentrations of MalNEt·S-1 (0.5–8.0 μM) were added to actin, which was held at a constant free actin concentration of 5 μM . The ATPase activity was then measured with 1 μM unmodified S-1. The abscissa axis for the MalNEt·S-1 data is identical to θ , the same abscissa used in Figs. 1 and 2, since essentially all of the added MalNEt·S-1 is bound to actin. With unmodified S-1, S-1·ATP and S-1·ADP· P_i bind so weakly at this ionic strength that <2% of the added S-1 is bound to actin. Therefore, the abscissa axis for the unmodified S-1 data is essentially equal to the ratio of the free S-1 concentration to the total actin concentration. The activities were corrected for the rate of S-1 alone (0.03 s^{-1}). All rates were expressed per total unmodified S-1 concentration.

would not decrease by >2%. In contrast to the results of Lehrer and Morris (16), the rate of the regulated acto-S-1 ATPase activity in the absence of Ca^{2+} was very low, showing only negligible actin activation or turning on. However, in the presence of Ca^{2+} , the regulated acto-S-1 ATPase activity did increase as a function of S-1 concentration, just as did the tropomyosin–acto-S-1 ATPase activity. These results are very similar to those obtained by Lehrer and Morris, although at 5 μM actin we obtained only about one-half the ATPase activity that they reported. However, we also obtained similar results at 10 μM actin, where all of the ATPase rates (including those measured in the presence of MalNEt·S-1) were about twice as high.

We next used MalNEt·S-1 to obtain the maximal turned-on rate under identical conditions. As observed at lower ionic strength, the value of the maximal turned-on rate was about the same with regulated actin in the presence and absence of Ca^{2+} and with tropomyosin–actin. Comparing the ATPase activity of regulated acto-S-1 and tropomyosin acto-S-1 to this maximal turned-on rate, we find that the extent of turning on is, in fact, a small percentage of the maximal turned-on rate. Furthermore, the extent of turning on is consistent with our *p*PDM·S-1 results (Fig. 2), if we assume that S-1·ATP and S-1·ADP· P_i bind to the turned-off form of regulated actin with an association constant of $3 \times 10^{-3} \text{ M}^{-1}$ (15) and with a 2-fold stronger affinity to the turned-on form. Therefore, our results at 50 mM ionic strength support our conclusion that both *p*PDM·S-1 and S-1·ATP bind, at most, 2-fold stronger to the turned-on form of regulated actin compared to the turned-off form.

DISCUSSION

One of the major questions in the regulation of muscle contraction is whether the myosin cross-bridge with bound ATP binds significantly more strongly to the turned-on form of regulated actin than to the turned-off form. If it does, then in the absence of Ca^{2+} , like the rigor cross-bridge, it should show cooperative binding to the regulated actin and, in addition, it should shift the regulated actin to the turned-on form, thereby increasing the actin-activated ATPase activity. In this study we found that when *p*PDM·S-1, an analogue of S-1·ATP, is bound to actin, it significantly turns on the regulated acto-S-1 ATPase activity in the presence but not in the absence of Ca^{2+} . We previously observed that, in the presence of ATP, *p*PDM·S-1 does not bind with apparent cooperativity to regulated actin either in the presence or absence of Ca^{2+} , although we did observe slight cooperativity in the binding in the absence of ATP (23). The data in the presence of ATP are consistent with *p*PDM·S-1·ATP binding ≈ 2 -fold stronger to the turned-on form of regulated actin than to the turned-off form. Such a slight difference in affinity would not produce enough cooperativity to be experimentally distinguishable from independent binding, while at the same time, based on the model of Hill *et al.* (12), it is enough of a difference to account for the turning on in the presence of Ca^{2+} but not in the absence of Ca^{2+} . This is because Ca^{2+} itself causes an increase in the fraction of tropomyosin–actin units in the turned-on form, as shown by the observation that less MalNEt·S-1 has to be bound to the actin filament to maximally turn on the regulated acto-S-1 ATPase in the presence of Ca^{2+} than in the absence of Ca^{2+} . When *p*PDM·S-1 binds to regulated actin in Ca^{2+} , it pushes the remaining tropomyosin–actin units to the turned-on form and thereby fully turns on the regulated acto-S-1 ATPase activity.

Our observations that at 25°C *p*PDM·S-1·ATP does not bind cooperatively to the regulated actin filament and does not turn on the actin-activated ATPase activity in the absence of Ca^{2+} are consistent with our observations that at 15°C S-1·ATP itself is unable to turn on the regulated actin in the

absence of Ca^{2+} and also does not bind cooperatively to regulated actin (13–15). It is also consistent with direct binding studies, which show that $p\text{PDM}\cdot\text{S}\cdot\text{1}\cdot\text{ATP}$ and $\text{S}\cdot\text{1}\cdot\text{ATP}$ bind only ≈ 2 -fold stronger to turned-on regulated actin than to turned-off regulated actin (unpublished data). Finally, our results are consistent with the observation that, in relaxed skinned muscle fibers, extensive binding of myosin cross-bridges to actin can occur at very low ionic strength without turning on force development (25).

Further evidence that $\text{S}\cdot\text{1}\cdot\text{ATP}$ cannot turn on the regulated acto-S-1 ATPase activity in the absence of Ca^{2+} comes from our studies at 50 mM ionic strength where Lehrer and Morris (16) have reported such turning on does occur. In contrast, our results show that, at this ionic strength where there is no significant binding of $\text{S}\cdot\text{1}\cdot\text{ATP}$ to actin, $\text{S}\cdot\text{1}\cdot\text{ATP}$ does not significantly turn on the regulated actin either in the presence or absence of Ca^{2+} . In fact, our data in the presence of Ca^{2+} agree with the data of Lehrer and Morris. However, since they did not compare their measurements with the ATPase activity that occurs when the regulated actin is fully turned-on, they may not have recognized what a small amount of turning-on they were actually observing under this condition. It is likely that the reason no significant turning-on occurs in the presence of Ca^{2+} at 50 mM ionic strength is because so little binding occurs at this relatively high ionic strength. We did not attempt to determine whether $\text{S}\cdot\text{1}\cdot\text{ATP}$ turned on the regulated actin in the presence of Ca^{2+} at very low ionic strength, where significant binding occurs, but Weber (9) has performed experiments of this type with the tropomyosin-actin complex and in agreement with our results with $p\text{PDM}\cdot\text{S}\cdot\text{1}$, saw turning on under this condition. We would expect that this is due to $\text{S}\cdot\text{1}\cdot\text{ATP}$ binding 2 times more strongly to the turned-on form of regulated actin than to the turned-off form, just as we have observed with $p\text{PDM}\cdot\text{S}\cdot\text{1}$.

Although all of the data we have presented in this paper fit with our original model of cooperativity, the results do not fit with our alternative model of cooperativity (18). Unlike our original model, in which tropomyosin exists in only two positions on the actin filament, in our alternative model, tropomyosin can exist in a continuum of positions on the actin filament; the cooperativity observed in the binding of a given S-1 state then depends on how much it must displace the tropomyosin from its original turned-off position in order to bind. However, the alternative model has less flexibility than the original model in that, under a given condition, tropomyosin can only exist in a single position rather than in an equilibrium between two positions. Moreover, a given S-1 state can only bind to regulated actin in one position rather than in two positions, the turned-off position and the turned-on position, as in the original model. This, in turn, means that a given S-1 state must bind to actin in the same position in both the presence and absence of Ca^{2+} . Therefore, if a S-1 state pushes the tropomyosin into the turned-on position when it binds to actin in the presence of Ca^{2+} , it will, likewise, have to push the tropomyosin into the turned-on position if it binds in the absence of Ca^{2+} .

In trying to fit our data to the alternative model, the key observations that must be explained are that $p\text{PDM}\cdot\text{S}\cdot\text{1}$ turns on the acto-S-1 ATPase activity in the presence of Ca^{2+} , but

not in the absence of Ca^{2+} , although in both cases it binds to actin. Since $p\text{PDM}\cdot\text{S}\cdot\text{1}$ almost completely turns on the ATPase activity in the presence of Ca^{2+} , it must be pushing almost all of the tropomyosin into the turned-on position. Therefore, the alternative model predicts that, in the absence of Ca^{2+} , $p\text{PDM}\cdot\text{S}\cdot\text{1}$ should only be able to bind if here too it pushes all of the tropomyosin into the turned-on position. However, this is contrary to our data, which show that even though $p\text{PDM}\cdot\text{S}\cdot\text{1}$ clearly binds to the regulated actin in the absence of Ca^{2+} , it could not be pushing the tropomyosin into the turned-on position because it does not turn on the acto-S-1 ATPase in the absence of Ca^{2+} . Therefore, because $p\text{PDM}\cdot\text{S}\cdot\text{1}$ turns on the regulated acto-S-1 ATPase activity in the presence but not in the absence of Ca^{2+} , although it binds in both cases, our data can only be explained by our original model of cooperativity.

- Weber, A. & Murray, J. M. (1973) *Physiol. Rev.* **53**, 612–673.
- Ebashi, S. (1980) *Proc. R. Soc. London Ser. B* **207**, 259–286.
- White, D. C. S. (1970) *J. Physiol. (London)* **208**, 583–605.
- Reuben, J. P., Brandt, P. W., Berman, M. & Grundfest, H. (1971) *J. Gen. Physiol.* **57**, 385–407.
- Bremel, R. D. & Weber, A. (1972) *Nature (London) New Biol.* **238**, 97–101.
- Bremel, R. D., Murray, J. M. & Weber, A. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 267–275.
- Murray, J. M., Knox, M. K., Trueblood, C. E. & Weber, A. (1980) *FEBS Lett.* **114**, 169–173.
- Murray, J. M., Weber, A. & Knox, M. K. (1981) *Biochemistry* **20**, 641–649.
- Murray, J. M., Knox, M. K., Trueblood, C. E. & Weber, A. (1982) *Biochemistry* **21**, 906–915.
- Greene, L. E. & Eisenberg, E. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2616–2620.
- Greene, L. E. (1982) *J. Biol. Chem.* **257**, 13993–13999.
- Hill, T. L., Eisenberg, E. & Greene, L. E. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3186–3190.
- Chalovich, J. M., Chock, P. B. & Eisenberg, E. (1981) *J. Biol. Chem.* **256**, 575–578.
- Wagner, P. D. & Giniger, E. (1981) *J. Biol. Chem.* **256**, 12647–12650.
- Chalovich, J. M. & Eisenberg, E. (1982) *J. Biol. Chem.* **257**, 2432–2437.
- Lehrer, S. S. & Morris, E. P. (1982) *J. Biol. Chem.* **257**, 8073–8080.
- Chalovich, J. M., Greene, L. E. & Eisenberg, E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4909–4913.
- Hill, T. L., Eisenberg, E. & Greene, L. E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 60–64.
- Eisenberg, E. & Kielley, W. W. (1971) *J. Biol. Chem.* **249**, 4742–4748.
- Stein, L. A., Schwarz, R. P., Jr., Chock, P. B. & Eisenberg, E. (1979) *Biochemistry* **18**, 3895–3909.
- Williams, D. L., Greene, L. E. & Eisenberg, E. (1984) *Biochemistry* **23**, 4150–4155.
- Wells, J. A. & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4966–4970.
- Greene, L. E., Chalovich, J. M. & Eisenberg, E. (1986) *Biochemistry* **25**, 704–709.
- Williams, D. L. & Greene, L. E. (1983) *Biochemistry* **22**, 2770–2774.
- Brenner, B., Schoenberg, M., Chalovich, J., Greene, L. & Eisenberg, E. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7288–7291.