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Cooperative Turning on of Myosin Subfragment 1 Adenosinetriphosphatase Activity by the Troponin–Tropomyosin–Actin Complex

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ABSTRACT: In the field of muscle regulation, there is still controversy as to whether Ca^{2+}, alone, is able to shift muscle from the relaxed to the fully active state or whether cross-bridge binding also contributes to turning on muscle contraction. Our previous studies on the binding of myosin subfragment 1 (S-1) to the troponin–tropomyosin–actin complex (regulated actin) in the absence of ATP suggested that, even in Ca^{2+}, the binding of rigor cross-bridges is necessary to turn on regulated actin fully. In the present study, we demonstrate that this is also the case for the turning on of the acto·S-1 ATPase activity. By itself, Ca^{2+} does not fully turn on the acto·S-1 ATPase activity; at low actin concentration, there is almost a 10-fold increase in ATPase activity when the regulated actin is fully turned on by the binding of rigor cross-bridges in the presence of Ca^{2+}. This large increase in ATPase activity does not occur because the binding of S-1·ATP to actin is increased; the binding of S-1·ATP is almost the same to maximally turned-off and maximally turned-on regulated actin. The increase in ATPase activity occurs because of a marked increase in the rate of P_i release so that when the regulated actin is fully turned on, P_i release becomes so rapid that the rate-limiting step precedes the P_i release step. These results suggest that, while Ca^{2+}, alone, does not fully turn on the regulated actin filament in solution, the binding of rigor cross-bridges can turn it on fully. If force-producing cross-bridges play the same role in vivo as rigor cross-bridges in vitro, there may be a synergistic effect of Ca^{2+} and cross-bridge binding in turning on muscle contraction which could greatly sharpen the response of the muscle fiber to Ca^{2+}.

The protein complex troponin–tropomyosin is responsible for regulating the contraction and relaxation of skeletal muscle (Weber & Murray, 1973; Ebashi, 1980). Muscle contraction occurs only when Ca^{2+} binds to this complex. In solution, the troponin–tropomyosin complex causes about 95% inhibition of the acto–S-1[1] ATPase activity in the absence of Ca^{2+} (Ebashi & Kodama, 1966; Hartshorne & Mueller, 1967). Since this inhibition occurs without troponin–tropomyosin, significantly affecting the binding of S-1·ATP to actin, we proposed that troponin–tropomyosin inhibits the acto–S-1 ATPase activity by blocking the release of P_i (Chalovich et al., 1981, 1983), rather than the binding of S-1·ATP to actin as was suggested by the steric blocking model.

In addition to affecting the acto–S-1 ATPase activity, troponin–tropomyosin confers cooperativity on the binding of S-1·ADP to actin in the absence of ATP (Greene & Eisenberg, 1980b). In the absence of both ATP and Ca^{2+}, troponin–tropomyosin markedly inhibits the binding of S-1·ADP to actin at low levels of saturation of the actin with S-1·ADP. However, at saturation levels greater than 50%, the binding strength

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HMM, heavy meromyosin; NEM, *N*-ethylmaleimide; S-1, myosin subfragment 1; SH-1, the most reactive sulfhydryl group in S-1.

of S-1·ADP increases markedly. We have suggested that this cooperative effect of troponin–tropomyosin on the binding of S-1·ADP to actin may have a simple relationship to its effect on the acto·S-1 ATPase activity (Greene et al., 1987; Greene & Eisenberg, 1988). The troponin–tropomyosin–actin complex (regulated actin) can be thought of as existing in two states which have different affinities for S-1·ADP and different abilities to activate the S-1 ATPase activity. The turned-off state binds S-1·ADP weakly and is unable to activate the acto–S-1 ATPase activity, while the turned-on state binds S-1·ADP even more strongly than actin alone and also activates the acto–S-1 ATPase activity more than actin alone. On the basis of this model, the binding of S-1·ADP or Ca^{2+} to the troponin–tropomyosin–actin complex affects both the binding of S-1·ADP to actin and the ATPase activity by shifting the equilibrium between the two states of regulated actin from the turned-off state to the turned-on state. One of the major questions regarding this model is why it is physiologically useful for troponin–tropomyosin to affect both P_i release and S-1·ADP binding. Presumably, blocking P_i release without affecting S-1·ADP binding would be sufficient to cause skeletal muscle relaxation just as occurs in smooth muscle when myosin is dephosphorylated (Greene & Sellers, 1987).

Another major question regarding this model is the effect of Ca^{2+} on the state of the troponin–tropomyosin complex. X-ray diffraction studies have shown that the binding of Ca^{2+} to the troponin–tropomyosin complex causes a large shift in the position of the tropomyosin on the actin filament with the binding of rigor cross-bridges having almost no further effect (Haselgrove, 1972; Huxley et al., 1985). These studies have been interpreted to mean that the binding of Ca^{2+} alone is able to completely shift the regulated actin into the turned-on state. On the other hand, our studies on the cooperative binding of S-1·ADP to regulated actin in the absence of ATP show that there is significant cooperativity in the binding of S-1·ADP to regulated actin in the presence of Ca^{2+}. Therefore, even though Ca^{2+} activates the regulated acto·S-1 ATPase activity, binding studies indicate that 90% of the regulated actin is in the turned-off form in the presence of Ca^{2+} (Greene, 1982; Williams & Greene, 1983).

This same contradiction between biochemical and structural data has also occurred in studies of the tropomyosin–actin complex in the absence of troponin. Binding studies in the absence of ATP showed that S-1·ADP binds cooperatively to the tropomyosin–actin complex, with the cooperativity being indistinguishable from that observed with regulated actin in the presence of Ca^{2+} (Williams & Greene, 1983). In addition, numerous ATPase studies with tropomyosin have shown that it causes about 80% inhibition of the acto–S-1 ATPase activity. This inhibition is also cooperative in nature, with the ATPase activity markedly increasing when S-1 forms rigor bonds with the tropomyosin–actin complex (Bremel et al., 1972; Murray et al., 1980, 1981, 1982; Lehrer & Morris, 1982). Therefore, as with regulated actin, biochemical studies suggest that, in the absence of rigor bonds, a large fraction of the tropomyosin–actin units are in the turned-off form. This is in contrast to the structural studies which, on the basis of the three-dimensional image reconstruction of the tropomyosin–actin complex, suggest that, in the absence of troponin, tropomyosin is in the active position on the actin filament (Wakabayashi et al., 1975).

Although qualitative biochemical studies suggest that most of the tropomyosin–actin units are in the turned-off form for both the tropomyosin–actin complex and regulated actin in the presence of Ca^{2+}, no quantitative study has been done to determine if the ATPase and binding data are consistent with each other. Therefore, in the present study, we carried out a detailed investigation of the effect of both troponin–tropomyosin and tropomyosin on the acto–S-1 ATPase activity and on the steady-state binding of S-1 to actin in the presence of ATP. In these studies, we worked over the full range of cooperative behavior either by working at very low S-1 to actin ratios to turn off the regulated actin as much as possible or by using S-1 extensively modified with NEM (NEM·S-1) to turn it on fully (Pemrick & Weber, 1976; Nagashima & Asakura, 1982).

Our results confirm that all of the biochemical data can be explained by a simple two-state model for regulated actin. As we suggested previously (Chalovich et al., 1983; Greene et al., 1987), we find that there is no more than a 2-fold difference in the strength of binding of S-1·ATP to the turned-off and turned-on forms of regulated actin, which confirms our previous conclusion that, in the absence of Ca^{2+}, relaxation does not occur because the myosin cross-bridges bind weakly to the turned-off form of regulated actin but rather because P_i release becomes very slow (Chalovich et al., 1981; Chalovich & Eisenberg, 1982). In the presence of Ca^{2+}, our results suggest that, although the regulated actin is markedly shifted toward the turned-on form compared to the situation in the absence of Ca^{2+}, it is still about 90% in the turned-off form. We find that under this condition, K_{ATPase} (the apparent binding constant of S-1·ATP for actin obtained from the double-reciprocal plot of ATPase activity versus actin concentration) is nearly equal to $K_{binding}$ (the binding constant of S-1 to actin in the presence of ATP). This suggests that, under this condition, although P_i release has become must faster than it was in the absence of Ca^{2+}, it is still the rate-limiting step in the ATPase cycle. In contrast, in the fully turned-on system, K_{ATPase} becomes almost an order of magnitude larger than $K_{binding}$. It is this large difference between K_{ATPase} and $K_{binding}$, rather than an increase in V_{max}, which mainly accounts for the higher ATPase activity obtained with fully turned-on regulated actin compared to regulated actin in the presence of Ca^{2+}.

Materials and Methods

Proteins. Myosin was prepared from rabbit back and leg muscles according to the method of Kielley and Harrington (1960) and labeled on SH-1 by iodo[^{14}C]acetamide (Amersham) according to the method of Greene and Eisenberg (1980a). The extent of labeling was approximately 0.95 label per myosin head. Myosin subfragment 1 (S-1) was prepared by the method of Weeds and Taylor (1975) except that 1 mM DTT was included in our solutions. NEM·S-1 was prepared from the [^{14}C]acetamide-labeled S-1 or from unmodified S-1 by the method of Nagashima and Asakura (1982) using nonradioactive *N*-ethylmaleimide. In some of the experiments, NEM-S-1 was made by a modification of their procedure. Specifically, unmodified S-1 (17 μM) in 20 mM imidazole (pH 7.3) was first reacted with stoichiometric concentrations of NEM for 20 min at 25 °C, followed by a 10-fold excess of NEM for 80 min at 25 °C. The reaction mixture was then made 5 mM in DTT, dialyzed overnight, and clarified by centrifugation. S-1 was labeled with *N*-succinimidyl[2,3-^{3}H]propionate (Amersham) according to the method of Rosenfeld and Taylor (1984). Approximately 300 μM *N*-succinimidyl[2,3-^{3}H]propionate in toluene was spotted on a small piece of filter paper and air-dried. The filter paper was placed in a 5-mL Teflon beaker containing 3 mL of 70 μM S-1, stirred overnight at 5 °C, and then clarified by centrifugation. Rabbit skeletal muscle F-actin was prepared by the method of Spudich and Watt (1971) with an additional cen-

trifugation at 3.3 M KCl to separate α-actinin from actin. The F-actin was treated with Dowex 1-X8Cl to eliminate unbound nucleotide. Rabbit skeletal muscle tropomyosin and troponin–tropomyosin were prepared according to the method of Eisenberg and Kielley (1974). Regulated actin was prepared by mixing actin with a 100% excess (2 mol/7 mol of actin) of native tropomyosin and dialyzing overnight at 4 °C against 3 mM $MgCl_2$, 1 mM DTT, 4 mM imidazole (pH 7.0), and 0.1 mM EGTA.

In the experiment with fully turned-on regulated actin or tropomyosin–actin, we used an actual ratio of NEM·S-1 to actin of 2.67 to 5. Since only 75% of the NEM·S-1 can bind to actin in the presence of ATP (Williams et al., 1984), this gives a ratio of 2 NEM·S-1 to 5 actins; i.e., 40% of the actin was saturated with NEM·S-1 in this experiment. This ratio, which is sufficient to ensure that the regulated actin is fully turned on (Greene et al., 1987), was used as the basis of calculating the free actin concentration.

The protein concentrations were determined spectrophotometrically at 280 nm by using the following absorbances and molecular weights: myosin, $E^{1\%}_{280nm}$ = 0.56 cm^2/mg, M_r 480000; S-1, $E^{1\%}_{280nm}$ = 0.75 cm^2/mg, M_r 120000; actin, $E^{1\%}_{280nm}$ = 1.15 cm^2/mg, M_r 42000; tropomyosin, $E^{1\%}_{278nm}$ = 0.33 cm^2/mg, M_r 68000; troponin–tropomyosin, $E^{1\%}_{278nm}$ = 0.38 cm^2/mg, M_r 150000.

Actin-Activated ATPase Assays. Actin-activated S-1 ATPase activities were measured at 25 °C by measuring the rate of liberation of $[^{32}P]P_i$ from [γ-^{32}P]ATP (Chock & Eisenberg, 1979). Assays were carried out in 1.5 mL of solution under conditions described in the figure legends. A single assay usually consisted of five time points. In all figures, the rate for S-1 alone (and where appropriate, NEM·S-1) has been subtracted from the measured rates. Vanadate-free ATP was obtained from Sigma. [γ-^{32}P]ATP was obtained from New England Nuclear.

Binding Studies. The binding of S-1 to actin was studied by pelleting the acto·S-1 in a Beckman Airfuge (178000*g* for 20 min) or Beckman TL-100 tabletop ultracentrifuge (436000*g* for 8 min). The amount of S-1 in the supernatant was determined by three different methods. These were using unmodified S-1 and measuring the S-1 concentration by the NH_4^+/EDTA ATPase method (Chalovich & Eisenberg, 1982), using [2,3-^{3}H]propionyl-S-1, and using iodo[^{14}C]acetamide-modified S-1. The binding with the latter two preparations of S-1 was determined by direct liquid scintillation counting. Several different methods were used because we found that the large excess of NEM·S-1 used in these studies, even with its very low ATPase activity, tended to interfere with the use of NH_4^+/EDTA ATPase to measure the free S-1 in the supernatant.

Results

Quantitative analysis of the cooperative effect of tropomyosin and troponin–tropomyosin on the actin activated S-1 ATPase activity requires the complete characterization of both maximally turned-on and maximally turned-off regulated actin. Most of the previous studies on this cooperative effect have been carried out by reducing the ATP concentration or raising the S-1 concentration (Bremel & Weber, 1972; Bremel et al., 1972; Murray et al., 1980, 1981, 1982; Lehrer & Morris, 1982). However, both of these approaches present major problems in quantitatively analyzing the cooperativity which occurs in the presence of ATP. Lowering the ATP concentration not only causes formation of rigor bonds which turn on the regulated actin but also, of necessity, decreases the fraction of S-1 which is saturated with ATP. Since these two

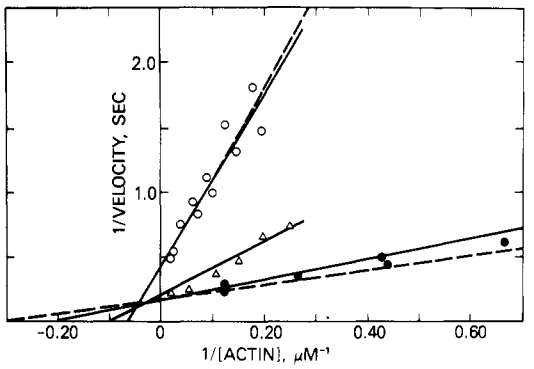

FIGURE 1: Double-reciprocal plots of actin-activated S-1 MgATPase activity versus free actin concentration. This experiment was done using unregulated actin (△), maximally turned-off regulated actin in Ca^{2+} (○), and fully turned-on regulated actin in Ca^{2+} (●). Conditions were 0.5 mM ATP, 2.5 mM $MgCl_2$, 7 mM imidazole (pH 7.0), and 0.5 mM $CaCl_2$ at 15 °C. In the experiment with maximally turned-off regulated actin (○), very low S-1 to actin ratios were used. The highest [S-1] used was 0.2 μM, which gave the same rates as 0.05 μM S-1, thereby ensuring the regulated actin was maximally turned off. In the experiment with fully turned-on regulated actin (●), 40% of the actin was saturated with NEM·S-1. The solid line through each set of data points was obtained by fitting the data directly to a hyperbola. The dashed lines are theoretical fits with the Hill et al. (1981) model using the rate constants given in Table II. All rates were corrected for the rate of S-1 alone (0.06 s^{-1}). In the absence of Ca^{2+} at 20 μM regulated actin, the acto·S-1 ATPase rate was 0.10 s^{-1}, showing that the regulated actin is highly responsive to Ca^{2+}.

effects are inseparable, it is not possible to understand the turning-on effect by itself. Turning on the system by raising the S-1 concentration is also difficult to analyze because the binding of S-1 to actin is so weak in the presence of ATP that a large excess of S-1 over actin has to be added to turn on the regulated actin even partially. This, in turn, makes the ATPase activity of the S-1 alone so high that it is difficult to measure the actin-activated ATPase activity.

We have previously shown that, both with tropomyosin alone and with the troponin–tropomyosin complex in the presence of Ca^{2+}, the addition of NEM·S-1 markedly increases the acto–S-1 ATPase activity (Williams et al., 1984; Greene et al., 1987). NEM·S-1 binds strongly to actin, even in the presence of ATP, and furthermore, it has no ATPase activity of its own (Williams et al., 1984). Therefore, like S-1 in the absence of ATP, NEM·S-1 probably forms rigor bonds with actin. Yet, because NEM·S-1 does this in the presence of ATP and has almost no ATPase activity of its own, it can be used to turn on the regulated actin fully without itself interfering with the measurement of the ATPase activity of the unmodified S-1 in the assay mixture. Therefore, in the present study, we used NEM·S-1 at a 2 to 5 molar ratio with actin (see Materials and Methods) to turn on the regulated actin fully; previous studies have shown that this molar ratio is more than sufficient to increase the ATPase activity to its maximal level (Williams et al., 1984; Greene et al., 1987). Maximal turning off, defined as the situation where added S-1 has no effect on the preexisting state of the regulated actin, was achieved by using very low ratios of S-1 to actin such that a 4-fold increase in S-1 concentration had no effect on the acto–S-1 ATPase activity.

We first examined the actin activation of the S-1 ATPase activity by regulated actin in the presence of Ca^{2+} at μ = 0.013 M and 15 °C. Figure 1 shows double-reciprocal plots of S-1 ATPase activity versus regulated actin concentration with maximally turned-off regulated actin, maximally turned-on regulated actin, and, for comparison, unregulated actin. The data show that, at low actin concentration, the ATPase activity

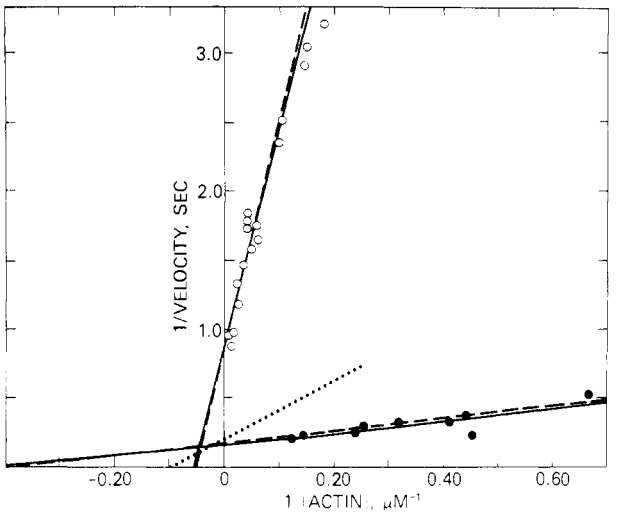

FIGURE 2: Double-reciprocal plots of actin-activated S1 MgATPase activity for maximally turned-off and fully turned-on tropomyosin–actin. Conditions are the same as in Figure 1. The highest [S-1] used was 0.4 μM, which gave the same rates as 0.1 μM S-1, thereby ensuring that the regulated actin was maximally turned off. The experiments with maximally turned-off (○) and fully turned-on tropomyosin–actin (●) were conducted as described in Figure 1. The dotted line is the plot obtained with unregulated actin (Figure 1). The solid line through each set of data points was obtained by directly fitting the data to a hyperbola, while the dashed theoretical lines were obtained by fitting the data to the model of Hill et al. (1981) using the rate constants given in Table II.

Table I: Comparison of V_{max}, K_{ATPase}, and $K_{binding}$[a]

preparation	V_{max} (s^{-1})	$K_{ATPase} \times 10^{-4}$ (M^{-1})	$K_{binding} \times 10^{-4}$ (M^{-1})
unregulated actin	5.7	8.8	4.0
regulated actin + Ca^{2+}	2.4	5.4	3.7
regulated actin + Ca^{2+} + NEM·S-1	6.0	22.0	3.7
tropomyosin–actin	1.2	4.9	3.7
tropomyosin–actin + NEM·S-1	6.1	37.0	3.7
regulated actin + Ca^{2+} + NEM·S-1[b]	8.3	3.1	0.4

[a] Conditions: 15 °C and μ = 0.013 M (see Figure 1). [b] Conditions: 15 °C and μ = 0.05 M obtained by using 39 mM KCl, 0.5 mM ATP, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 3 mM imidazole (pH 7.0), and 1 mM DTT.

of the turned-off regulated actin is about one-fourth that of the unregulated actin while the ATPase activity of the turned-on regulated actin is about twice that of the unregulated actin. Therefore, at low actin concentration, there is an 8-fold increase in the actin-activated ATPase activity when the regulated actin goes from maximally turned off to maximally turned on in the presence of Ca^{2+}. This 8-fold increase is caused by a 2-fold increase in V_{max} and a 4-fold increase in K_{ATPase}.

Figure 2 shows that under the same conditions, a similar phenomenon occurs with tropomyosin alone, which we have previously shown behaves just like troponin–tropomyosin in the presence of Ca^{2+} (Williams & Greene, 1983). There is about a 40-fold increase in the actin-activated ATPase activity when the actin-activated ATPase goes from maximally turned off to maximally turned on. This 40-fold increase is caused by a 5-fold increase in V_{max} and an 8-fold increase in K_{ATPase}. Comparing the turned-off complexes (Table I), there is a somewhat lower V_{max} observed with the turned-off tropomyosin–actin complex than with the turned-off regulated actin complex. In both cases, the turning-on process is accompanied by a marked increase in K_{ATPase}. In fact, since the presence of NEM·S-1 may cause as much as a 2-fold decrease in $K_{binding}$

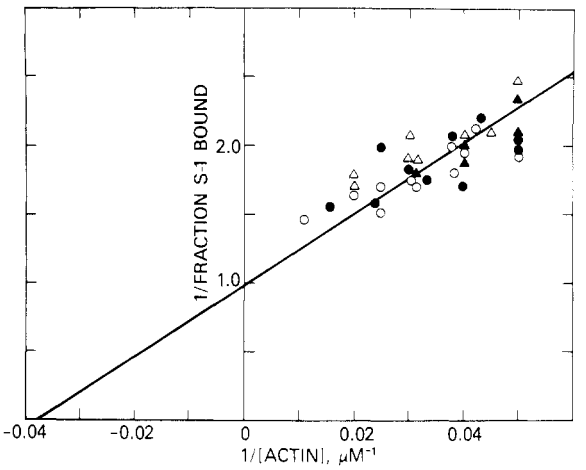

FIGURE 3: Binding of S-1 in the presence of ATP to turned-off and turned-on regulated actin plus Ca^{2+} or turned-off and turned-on tropomyosin–actin. The conditions were the same as in Figure 1. The binding of S-1·ATP and S-1·ADP·P_i to tropomyosin–actin was conducted either in the presence (●) or the absence of NEM·S-1 (○). We used the three different techniques described under Materials and Methods to measure free [S-1]. Since all three techniques gave similar results, we did not distinguish between the data. The binding of S-1·ATP and S-1·ADP·P_i to regulated actin with Ca^{2+} was conducted either in the presence (▲) or in the absence of NEM·S-1 (△) using the technique of measuring the free [S-1] with [^{14}C]acetamide-modified S-1. In the experiments with fully turned-on regulated actin plus Ca^{2+} or fully turned-on tropomyosin–actin, the actin was 40% saturated with NEM·S-1.

(see below), turning on the regulated actin or tropomyosin–actin complex may actually increase K_{ATPase} 16-fold rather than the 8-fold which we observe.

The large difference in K_{ATPase} which we observe between turned-off and turned-on regulated actin is somewhat surprising given our previous observations that there appears to be very little difference in the binding of S-1·ATP to the turned-off and turned-on forms of regulated actin (Chalovich et al., 1983; Greene et al., 1987). Therefore, we directly measured the steady-state binding of S-1 to both turned-on and turned-off regulated actin in the presence of ATP. As a control, we first investigated the effect of NEM·S-1 on the steady-state binding of S-1·ATP to unregulated actin. In this experiment, the three different methods described under Materials and Methods were used, and they all gave essentially the same result (data not shown). We found that S-1 bound about 2-fold more weakly to actin which was 40% saturated with bound NEM·S-1 than to control actin; i.e., $K_{binding}$ was 4×10^4 and 2×10^4 M^{-1} in the absence and presence of NEM·S-1, respectively. This result is in agreement with earlier studies of Nagahsima and Asakura (1982) which showed a small effect of bound NEM·S-1 on the actin-activated S-1 ATPase activity.

We next measured the binding of S-1·ATP to maximally turned-on and turned-off regulated actin in both the presence and absence of Ca^{2+} and with tropomyosin–actin. In the latter studies, we used the same three methods to measure the binding as we used with unregulated actin, and again, they all gave essentially the same results. In the binding studies with regulated actin in the presence and absence of Ca^{2+}, we only used one of these methods, measuring the amount of iodo[^{14}C]acetamide-modified S-1 bound to actin. Figure 3 shows that, in all cases, $K_{binding}$ was about 4×10^4 M^{-1}; the presence of troponin or turning on or turning off the actin had almost no effect. However, since we found that 40% saturation of the actin with NEM·S-1 weakens the binding of S-1·ATP to unregulated actin about 2-fold, these results are consistent

with the maximally turned-on state of regulated actin or tropomyosin–actin binding S-1·ATP about 2-fold more strongly than the maximally turned-off state of regulated actin. Therefore, these results are consistent with our previous finding that S-1·ATP binds about 2-fold stronger to the turned-on state than to the turned-off state of regulated actin (Greene et al., 1987).

Since turning on the regulated actin or tropomyosin–actin has a large effect on K_{ATPase}, but relatively little effect on the binding of S-1·ATP to actin ($K_{binding}$), it would appear that there is a large difference between K_{ATPase} and $K_{binding}$ with maximally turned-on regulated actin or tropomyosin–actin while there is relatively little difference with turned-off regulated actin or tropomyosin–actin. Table I shows that this is indeed the case. With the turned-on systems, K_{ATPase} is 6–10-fold larger than $K_{binding}$ while with the turned-off systems there is less than a 1.5-fold difference between K_{ATPase} and $K_{binding}$. Table I also shows that V_{max} increases with the turned-on systems compared to the turned-off systems although, with tropomyosin, this effect is not as prominent at 25 °C as at 15 °C (data not shown).

The value of K_{ATPase} is so large with the turned-on system that we were able to measure its value at 50 mM ionic strength. At this relatively high ionic strength, there is still about an 8-fold difference between K_{ATPase} and $K_{binding}$; K_{ATPase} has a value of 3×10^4 M^{-1}, while $K_{binding}$ has a value of about 4×10^3 M^{-1} (Table I). Given the weakness of the binding, this latter measurement cannot be considered accurate. It does, however, agree with the 10-fold weakening of the S-1 binding to actin which we have previously observed with a number of different nucleotides as the ionic strength is increased from 12 to 50 mM (Greene et al., 1983).

Discussion

In this study, by using NEM·S-1 to fully turn on regulated actin or tropomyosin–actin, we have been able to quantitatively analyze the properties of the fully turned-on system in the presence of ATP. The results show that much of the increase in ATPase activity which occurs when the system is fully turned on in the presence of Ca^{2+} is due to a marked increase in K_{ATPase} compared to $K_{binding}$; i.e., the actin-activated ATPase rate reaches its half-maximal value at almost an order of magnitude lower actin concentration than that required for half-maximal binding of S-1·ATP to actin. This is in contrast to the situation in the absence of NEM·S-1 where K_{ATPase} is nearly equal to $K_{binding}$ in the presence of Ca^{2+}. Both our work (Stein et al., 1979, 1984, 1985) and the work of Rosenfeld and Taylor (1984) have previously shown that a large difference between K_{ATPase} and $K_{binding}$ can only be explained by a kinetic model where P_i release is not rate limiting. The difference between K_{ATPase} and $K_{binding}$ observed with turned-on regulated actin and tropomyosin–actin is larger than the difference observed in the absence of troponin–tropomyosin (Chalovich et al., 1984). Furthermore, even at 50 mM ionic strength, we find almost an order of magnitude difference between K_{ATPase} and $K_{binding}$ in the fully turned-on system. This suggests that this phenomenon may be important physiologically. Just as the maximum actin-activated ATPase rate in vitro is reached with the S-1·ATP complex still mostly dissociated from actin, the rate of force development in muscle fibers may remain at its maximum level at physiologic ionic strength even though most of the weak binding cross-bridges are detached from actin. In fact, there is evidence that this does occur since the rate of force redevelopment in a skinned muscle fiber shows almost no dependence on ionic strength (Brenner & Eisenberg, 1986).

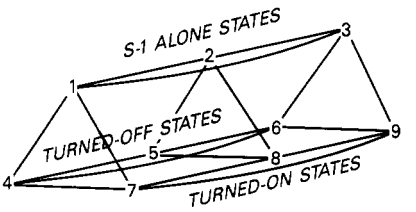

FIGURE 4: Kinetic model of Hill et al. (1981) for the regulated acto·S-1 ATPase activity and tropomyosin acto·S-1 ATPase activity. The numbering of the S-1 and acto·S-1 states is as follows: 1 = M·D·P_{i_I}; 2 = M·D·$P_{i_{II}}$; 3 = M·D; 4 = A·M·D·P_{i_I} in the turned-off form; 5 = A·M·D·$P_{i_{II}}$ in the turned-off form; 6 = A·M·D in the turned-off form; 7 = A·M·D·P_{i_I} in the turned-on form; 8 = A·M·D·$P_{i_{II}}$ in the turned-on form; and 9 = A·M·D in the turned-on form.

In contrast to the situation with fully turned-on regulated actin or tropomyosin–actin, when Ca^{2+} alone is added to regulated actin, there is a 2–4-fold decrease in V_{max}, and K_{ATPase} and $K_{binding}$ become nearly equal. This may indicate that, under this condition, the P_i release step becomes at least partially rate limiting in the ATPase cycle. In this respect, regulated acto·S-1 in the presence of Ca^{2+} may resemble the completely turned-off system in the absence of Ca^{2+}; in both cases, P_i release may be rate limiting although, of course, in the absence of Ca^{2+} the rate of P_i release is much slower so that the ATPase rate is completely turned off.

The marked difference in the kinetic properties of the regulated acto·S-1 system in Ca^{2+} with and without NEM·S-1 present strongly suggests that the binding of Ca^{2+} to regulated actin is not sufficient to turn on the regulated actin filament fully. Therefore, like our earlier binding studies, which showed cooperativity in the binding of S-1·ADP to regulated actin in Ca^{2+}, the present data strongly suggest that the binding of rigor S-1 to regulated actin has a major effect on the behavior of tropomyosin. This is in disagreement with X-ray diffraction studies on muscle fibers which showed that, even with thin filaments which were completely out of overlap, i.e., had no contact with myosin cross-bridges, the tropomyosin shifted its position almost completely when Ca^{2+} was bound (Haselgrove, 1972; Huxley et al., 1985). It is possible that the X-ray diffraction measurements are detecting only the conformational change caused by the actual binding of Ca^{2+} to the regulated actin and not the further conformational change which turns on the regulated actin filament.

The simplest explanation for the behavior of regulated actin and tropomyosin–actin in Ca^{2+} is that, in the absence of rigor bond formation, most of the actin units remain in the turned-off state. On this basis, a two-state model for the regulated actin filament is shown in Figure 4 (Hill et al., 1981). In this model, the regulated actin can exist in two states: a turned-off state, which is unable to activate the S-1 ATPase activity and which binds S-1·ADP relatively weakly, and a turned-on state, which markedly activates the P_i release step and binds S-1·ADP strongly. As we have described previously (Hill et al., 1980), the equilibrium constant between the two forms of regulated actin in the absence of S-1 is given by $L'Y$. In the presence of NEM·S-1, all of the regulated actin is in the turned-on form, and the model is nearly identical with the six-state model we have previously proposed to account for the acto–S-1 ATPase activity. As we discussed above, the presence of a rate-limiting step followed by rapid P_i release accounts for the marked difference observed between K_{ATPase} and $K_{binding}$ in the maximally turned-on system.

The opposite extreme, from the situation with NEM·S-1 bound, is the situation in the absence of Ca^{2+}. Here, $L'Y$ has a value of about 3000 so that essentially all of the actin is in the turned-off form (Greene & Eisenberg, 1980b). Since the

Table II: Rate Constants for the Model of Hill et al. (1981) (Figure 4)

rate constants[a]	rate	rate constants[a]	rate
$k_{12} = K_{45} = k_{78}$ (s^{-1})	6.5	$k_{14} = k_{25} = k_{36}$ (M^{-1} s^{-1})	$10^7(1 - p_2)$
$k_{21} = k_{54} = k_{87}$ (s^{-1})	20	$k_{71} = k_{82}$ (s^{-1})	125
$k_{23} = k_{56}$ (s^{-1})	0.1	k_{93} (s^{-1})	12.5
$k_{32} = k_{65}$ (M^{-1} s^{-1})	0.001	$k_{17} = k_{28} = k_{39}$ (M^{-1} s^{-1})	10^7p_2
k_{89} (s^{-1})	250	$k_{47} = k_{58}$ (s^{-1})	2α
k_{98} (M^{-1} s^{-1})	0.025	k_{69} (s^{-1})	20α
$k_{31} = k_{64}$ (s^{-1})	20	$k_{74} = k_{85}$ (s^{-1})	$\beta/2$
k_{97} (s^{-1})	600	k_{96} (s^{-1})	$\beta/20$
$k_{41} = k_{52} = k_{63}$ (s^{-1})	250		

[a] In this model, p_2 is the fraction of the tropomyosin–actin units in the turned-on form and is determined by the cooperativity values L' and Y [see eq 12; Hill et al. (1980) model]. The values of p_2 used were determined from the model of Hill et al. using values of $L' = 2.0$ and $Y = 20$ for regulated actin in the presence of Ca^{2+}, $L' = 3.15$ and $Y = 20$ for tropomyosin–actin, and $L' = 150$ and $Y = 20$ for regulated actin in the absence of Ca^{2+}, which we previously measured in the absence of Ca^{2+} (Greene & Eisenberg, 1980b, 1988; Williams & Greene, 1983). We used a value for α of 0.25, which from the equation $\beta = \alpha(1 - p_2)/p_2$ gives a value of $\beta = 742$. This latter value is comparable to the rate of the transition from the turned-on to the turned-off state of regulated actin as measured by Trybus and Taylor (1980). Increasing the values of α and β had no effect on the ability of the model to fit the experimental data. In fact, fitting the experimental data was even easier if the assumption was made that there was a rapid equilibrium between the turned-on and turned-off forms of regulated actin.

turned-off form of the regulated actin cannot activate P_i release, the overall rate of P_i release will depend on how easily the regulated actin can shift into the turned-on form. Since the shift of the regulated actin from the turned-off form to the turned-on form is highly unfavorable, in effect, the rate of P_i release will be reduced by a factor of about 3000 compared to the turned-on system. This will make the very slow rate of P_i release rate limiting in the absence of Ca^{2+}. Likewise, since S-1·ADP binds relatively weakly to the turned-off form, the binding of S-1·ADP will be markedly cooperative; at low ratios of S-1·ADP to actin, the binding will be weak, and only when enough S-1·ADP is bound to shift the tropomyosin into the turned-on form will the binding become strong.

Finally, in Ca^{2+} or with tropomyosin alone, the system will be intermediate between the fully turned-on and maximally turned-off systems. Earlier results suggest that here $L'Y$, the equilibrium constant between the turned-off and turned-on forms of regulated actin, has a value of about 50 (Williams & Greene, 1983). Thus, most of the regulated actin is in the turned-off form, but the equilibrium constant is considerably lower than in EGTA so it is much easier for the regulated actin to shift into the turned-on form. Thus, their is cooperative binding of S-1·ADP to actin, but it is considerably reduced compared to the situation in EGTA. In addition, since the overall rate of P_i release involves the transition from the turned-off form to the turned-on form as well as the P_i release step itself, the rate of P_i release becomes partially rate limiting in the cycle just as in the absence of Ca^{2+}. This accounts for the fact that in Ca^{2+} or with tropomyosin alone, K_{ATPase} and $K_{binding}$ become nearly equal and V_{max} is decreased, although to nowhere near as low a level as occurs in EGTA where $L'Y$ is about 3000 rather than 50.

The model we have presented for the action of tropomyosin and troponin–tropomyosin shows that blocking P_i release is not sufficient to cause relaxation. Unless the equilibrium constant between the turned-off and turned-on forms of regulated actin is markedly shifted toward the turned-off form, blocking the P_i release step in the turned-off form will have relatively little effect because the regulated actin will be able to shift easily into the turned-on form where P_i release is rapid. This qualitative explanation for the behavior of tropomyosin and troponin–tropomyosin in EGTA and Ca^{2+} can be made quantitative by giving values to the rate constants shown in Figure 4. Table II presents the values we have used in our quantitative model, and the dashed lines in Figues 1 and 2 show the fit we obtained with these values. The binding constants given in Table II fit the steady-state binding which we observed in Figure 3, and the values of L' and Y we have used are in good agreement with the values we used previously to explain the cooperative binding of S-1·ADP to regulated actin and tropomyosin–actin. It is clear that a model with only two states for the regulated actin can account for all of our biochemical data but that this is certainly not the only model. At least two states of the regulated actin are needed, but more states cannot be ruled out, nor is it possible to rule out that the turned-off and turned-on states differ in more than just the rate of P_i release and the strength of binding of S-1·ADP to actin. Furthermore, although we have fitted the data to the six-state model for reasons described elsewhere (Stein et al., 1985; Brenner & Eisenberg, 1986), the data could have also been fitted to the four-state model with the ATP hydrolysis step rate limiting (Rosenfeld & Taylor, 1984).

One major question which arises about this model of muscle regulation is why troponin–tropomyosin not only blocks the P_i release step but also weakens the binding of S-1·ADP. As we observed in smooth muscle (Greene & Sellers, 1987), effective muscle regulation can be obtained if S-1·ADP binds with about the same affinity to both the turned-on and turned-off forms of regulated actin, and the only difference between the two forms is their ability to activate the rate of P_i release. Therefore, there appears to be a dual effect of troponin–tropomyosin on the interaction of actin and myosin. On the one hand, tropomyosin blocks P_i release when it is in the turned-off form, and since, in the absence of Ca^{2+}, troponin completely shifts the tropomyosin into the turned-off form, this causes muscle relaxation. On the other hand, tropomyosin also weakens the binding of S-1·ADP to the turned-off form compared to the turned-on form, thus causing S-1·ADP to bind cooperatively to regulated actin in the presence of Ca^{2+}. Assuming that force-producing cross-bridges in vivo bind cooperatively like S-1·ADP in vitro, this cooperative binding may function to sharpen the response of the muscle fiber to Ca^{2+}. The work of Brandt and co-workers (Brandt et al., 1980, 1982, 1984) has shown the relationship between force development and Ca^{2+} concentration shows strong positive cooperativity. There is also now evidence in skinned muscle fibers that the binding of force-producing cross-bridges causes a fluorescence change in labeled troponin which is similar to the change caused by the binding of rigor cross-bridges (Zot et al., 1986; Guth et al., 1986, 1987; Schultie et al., 1987). In addition, partial removal of troponin from the regulated actin filament has been shown to decrease the cooperative response of the muscle fiber to Ca^{2+} as if a cooperative interaction between adjacent tropomyosin units were involved in this cooperative response (Brandt et al., 1987). Our results are therefore consistent with a model of muscle regulation in which Ca^{2+} and force-producing cross-bridges bind synergistically to the regulated actin filament and in this way cause a highly cooperative response of the muscle fiber to Ca^{2+}.

Registry No. ATPase, 9000-83-3; Ca, 7440-70-2.

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