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Biochemistry

Crosslinked myosin subfragment 1: A stable analogue of the subfragment-1·ATP complex*

(myosin conformational states/cooperativity/muscle contraction/troponin–tropomyosin)

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ABSTRACT **Myosin subfragment 1 (S-1) with its two reactive cysteine groups crosslinked by *N,N'-p*-phenylenedimaleimide (*p*PDM), is shown to be a stable analogue of S-1·ATP and S-1·ADP·P_i, the predominant complexes present during the steady-state hydrolysis of ATP by S-1. *p*PDM-S-1 binds to actin with about twice the affinity of S-1·ATP or S-1·ADP·P_i, whereas its affinity is 1/100th of that of S-1·5′-adenylyl imidodiphosphate and 1/1,000th of that of S-1·ADP. *p*PDM-S-1 is also similar to S-1·ATP and S-1·ADP·P_i in that its binding to actin is not inhibited by troponin–tropomyosin. In contrast, the binding of S-1, S-1·ADP, and S-1·5′-adenylyl imidodiphosphate to actin is markedly inhibited by troponin–tropomyosin in the absence of Ca^{2+} when actin is in large excess over S-1. This suggests that modifying S-1 with *p*PDM stabilizes a conformation which mimics that induced by the binding of ATP.**

Force generation in vertebrate skeletal muscle is thought to occur as a result of a cyclic interaction of myosin cross-bridges with actin (1). Various cross-bridge models of muscle contraction suggest that the globular head region of myosin undergoes a rotation or a conformational change while attached to actin, causing movement of the thin actin filaments past the thick myosin filaments (2–4). After this step of shortening, the myosin molecule detaches, and then reattaches to a different actin monomer to repeat the process. Because this cyclic process is coupled to the hydrolysis of ATP, it is thought that myosin must exist in two or more conformational states as ATP is hydrolyzed. In the absence of ATP (rigor) the cross-bridge seems to be bound to actin at a 45° angle (5, 6), whereas in the relaxed state this angle may be closer to 90° (5). Eisenberg and Greene have suggested that the 90° or relaxed conformation may be associated with states containing bound ATP or ADP·P_i, whereas the 45° or rigor conformation may be associated with states containing bound ADP or no nucleotide (7). Accordingly, it is of interest to be able to dissect and study the individual states. It is possible to study the structure of acto·S-1 (S-1, myosin subfragment 1) in the "45°" state, but it is difficult to study the structure of the "90°" ATP-bound state because of the rapidity with which ATP is hydrolyzed. Therefore, it would be useful to have a long-lived analogue of S-1·ATP that is similar to S-1·ATP and S-1·ADP·P_i in its interaction with actin. It was hoped that the binding of 5′-adenylyl imidodiphosphate (AMP-*P*[NH]*P*) would create such a state, but S-1·AMP-*P*[NH]*P* is apparently distinct from S-1·ATP because it has a different actin-binding affinity (8).

A candidate for an analogue of S-1·ATP is S-1 that has reacted with *N,N'-p*-phenylenedimaleimide (*p*PDM). Treatment of S-1 with *p*PDM in the presence of ADP results in crosslinking the two reactive sulfhydryl groups of S-1 (9), simultaneously trapping a molecule of ADP (10). Two observations suggest that *p*PDM-S-1 may be similar to S-1·ATP. The circular dichroism spectrum of *p*PDM-S-1 resembles that of S-1 in the presence of ATP and, like S-1 in the presence of ATP, *p*PDM-S-1 does not bind to actin at high ionic strength (11). Under the same high ionic strength conditions, S-1 having both sulfhydryl groups alkylated but not crosslinked is able to bind to actin. On the other hand, the intrinsic fluorescence of *p*PDM-S-1 resembles that of S-1·ADP rather than S-1·ATP (11, 12). To determine whether *p*PDM-S-1 is an analogue of S-1·ATP or S-1·ADP we have thoroughly studied the binding of *p*PDM-S-1 to actin in both the presence and the absence of troponin–tropomyosin. Our results suggest that *p*PDM-S-1 is an excellent analogue of S-1·ATP.

MATERIALS AND METHODS

Tropinin–tropomyosin was prepared according to Eisenberg and Kielley (13); all other proteins were prepared by procedures described elsewhere (14). S-1 was labeled with iodo[^{14}C]acetamide as described earlier (15). S-1 was modified with *p*PDM at 0°C as described by Wells and Yount (16). The modified S-1 contained 1.1–1.3 mol of *p*PDM per mol of S-1, and between 75% and 80% of the modified S-1 molecules contained trapped ADP. Because this *p*PDM-S-1 preparation had a relatively high actin-activated ATPase activity (4% of unmodified), the *p*PDM-S-1 was further purified by sedimenting with actin at a 1:5 mol ratio of actin to *p*PDM-S-1 in the absence of nucleotide at 45 mM ionic strength. The contaminant uncrosslinked S-1 sedimented with the F-actin, leaving purified *p*PDM-S-1 in the supernatant. This purified *p*PDM-S-1 differed from the original *p*PDM-S-1 preparation by having a much lower ATPase activity (0.2% of unmodified).

The binding of S-1 to actin–troponin–tropomyosin (regulated actin) in the presence of ATP was determined either by stopped-flow turbidity measurements (17) or by measuring the NH_4^+-ATPase activity of the free S-1 after sedimentation of acto·S-1 in a Beckman air-driven ultracentrifuge (18). Binding in the presence of AMP-*P*[NH]*P* was measured either by the NH_4^+-EDTA ATPase method or by measuring the iodo[^{14}C]-acetamide-labeled S-1 in the supernatant after centrifugation in an ultracentrifuge (19). The binding of *p*PDM-S-1 was measured by using [^{14}C]*p*PDM-labeled S-1 with the same centrifugation procedure that was used for iodo[^{14}C]acetamide S-1 binding.

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Abbreviations: S-1, myosin subfragment 1; *p*PDM, *N,N'-p*-phenylenedimaleimide; AMP-*P*[NH]*P*, 5′-adenylyl imidodiphosphate; (A-1)S-1, S-1 containing the 21,000-dalton alkali light chain 1; Ap_5A, P^1,P^5-di(adenosine-5′)pentaphosphate.

* Preliminary reports of this work have been presented at the 27th Annual Biophysical Society Meeting (28, 29), Feb. 13–16, 1983, San Diego, CA.

[γ-^{32}P]ATP and [2,8-^{3}H]ADP were from New England Nuclear; [1,4-^{14}C]maleic anhydride was from Amersham. [^{14}C]-*p*PDM was synthesized as described by Wells and Yount (16) and was purified by sublimation. *p*PDM from Aldrich was recrystallized from acetone and sublimated before use. AMP-*P*[NH]*P* and vanadate-free ATP were obtained from Sigma, and P^1,P^5-di(adenosine-5')pentaphosphate (Ap_5A) and ADP were purchased from P-L Biochemicals.

RESULTS

Binding of *p*PDM-S-1 to Unregulated Actin. The binding of *p*PDM-modified S-1 to actin was first examined at low ionic strength (μ = 18 mM) and 25°C, conditions that favor the binding of S-1 to actin. Various concentrations of actin were added to a fixed concentration of *p*PDM-S-1 and the results are shown in Fig. 1 as a double-reciprocal plot of fraction of *p*PDM-S-1 bound to actin vs. free actin concentration. From the abscissa intercept, the binding constant of *p*PDM-S-1 to actin is determined to be 4×10^4 M^{-1}. Even at this low ionic strength, the binding of *p*PDM-S-1 to actin is weak; i.e., the free actin concentration at which half of the *p*PDM-S-1 is bound is 25 μM. The ordinate intercept of Fig. 1 gives the fraction of *p*PDM-S-1 bound at infinite actin concentration as 0.88. The deviation of this value from the theoretical value of 1.0 suggests that ≈10% of the *p*PDM-S-1 may be denatured and unable to bind to actin. For comparison, Fig. 1 also shows earlier data (18) on the binding of S-1 to actin in the presence of ATP under the same conditions (broken line). The binding of *p*PDM-S-1 is only 2-fold stronger than the binding of S-1·ATP to actin (2.3×10^4 M^{-1}).

To determine the stoichiometry of binding of *p*PDM-S-1 to actin, this binding was examined under the same conditions by varying the concentration of *p*PDM-S-1 at a fixed concentration of actin. The Scatchard plot of these data, shown in Fig. 2, has an abscissa intercept of 1.1, indicating that 1 mol of *p*PDM-S-1 binds per mol of actin. In this experiment, the presence of denatured *p*PDM-S-1 does not affect the stoichiometry because S-1 is in great excess over actin. From the slope of the Scatchard plot, the binding constant of *p*PDM-S-1 to actin (3.7×10^4 M^{-1}) is in good agreement with the value determined in Fig. 1. Therefore, although *p*PDM-S-1 binds to actin weakly, the stoichiometry shows that there is specificity in its binding.

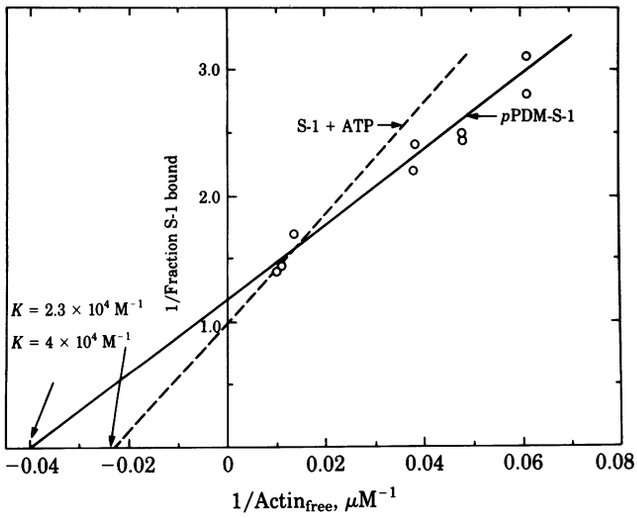

FIG. 1. Double-reciprocal plot of the fraction of *p*PDM-S-1 bound to actin as a function of free actin concentration. Binding was measured by using 9 μM [^{14}C]*p*PDM-S-1 in 5 mM KCl/2 mM $MgCl_2$/10 mM imidazole/1 mM dithiothreitol (μ = 18 mM) at 25°C, pH 7.0. The solid line gives a binding constant for *p*PDM-S-1 of 4.0×10^4 M^{-1}. The binding of unmodified S-1 in the presence of ATP is illustrated by the broken line, which gives a binding constant of 2.3×10^4 M^{-1}. In this experiment conditions were the same as for *p*PDM-S-1 except that 1 mM MgATP was present instead of 5 mM KCl.

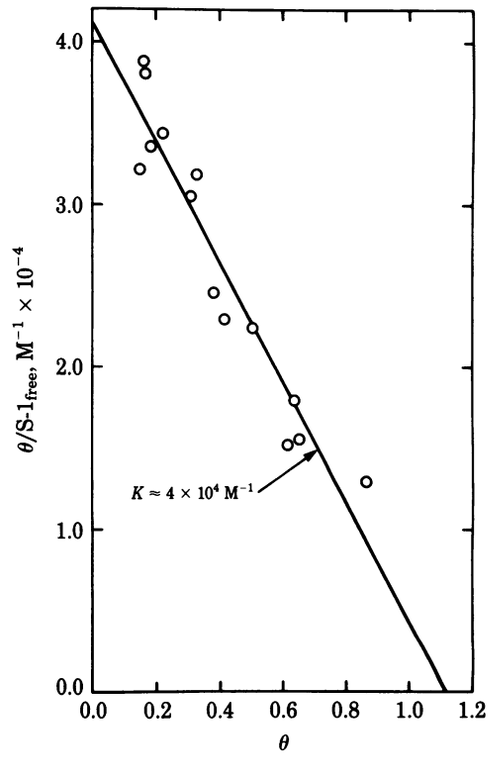

FIG. 2. Scatchard plot of the binding of *p*PDM-S-1 to actin. θ is defined as the number of moles of S-1 bound per mole of actin monomer. Actin was 26 μM and [^{14}C]*p*PDM-S-1 was varied between 4 and 88 μM; other conditions are the same as in Fig. 1. The binding constant is 3.7×10^4 M^{-1}.

The binding of *p*PDM-S-1 to actin was next examined over a range of ionic strengths, between 12 and 63 mM, to determine whether its similarity to the binding of S-1 in the presence of ATP is maintained. The data are plotted as *K* vs. $\mu^{1/2}$ on a semilogarithmic plot in Fig. 3. For comparison, data for the binding of S-1 to actin in the presence of ATP are also shown. Throughout the entire range of ionic strengths, *p*PDM-S-1 binds to actin with about twice the affinity of S-1·ATP or S-1·ADP·P_i. Therefore, *p*PDM-S-1 is similar to S-1·ATP over a large range of ionic strengths.

Binding of *p*PDM-S-1 to Regulated Actin. In addition to differences in affinity for pure actin, myosin–nucleotide complexes differ in their interaction with the troponin–tropomyosin–actin complex (regulated actin). This is illustrated in Fig. 4. In the presence of ATP there is no cooperativity in the binding of S-1 or the more tightly binding isozyme (A-1)S-1 and there is little Ca^{2+} sensitivity. In contrast, binding in the presence of AMP-*P*[NH]*P* shows marked cooperativity in the absence of Ca^{2+} and the binding is sensitive to Ca^{2+}. The binding in AMP-*P*[NH]*P* was done at high ionic strength to make the strength of binding similar to that in the presence of ATP. Therefore, the effect of troponin–tropomyosin can be used as a second criterion for the characterization of *p*PDM-S-1.

Fig. 5 shows the binding of *p*PDM-S-1 to regulated actin. It is clear that the binding of *p*PDM-S-1 to regulated actin resembles the binding of S-1 in the presence of ATP both in the lack of a Ca^{2+} effect and in the apparent absence of cooperativity. Both in the presence and in the absence of Ca^{2+}, the

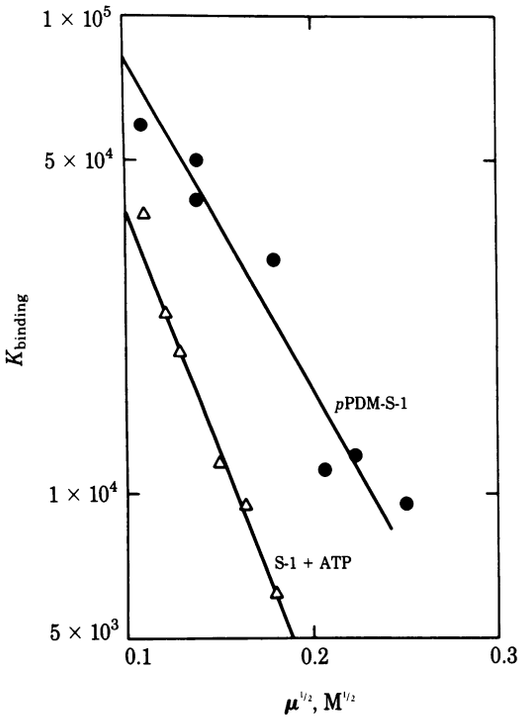

FIG. 3. Binding of S-1 to actin as a function of ionic strength. △, Binding of unmodified S-1 in the presence of 1 mM ATP; ●, binding of *p*PDM-S-1. The solutions contained 1.8 mM $MgCl_2$, 10 mM imidazole, 1 mM dithiothreitol, and a variable KCl concentration to produce the given ionic strength.

*p*PDM-S-1 data can be fitted by a theoretical curve for independent binding with $K = 8 \times 10^4\ M^{-1}$, about twice the value of the binding constant measured in the absence of troponin–tropomyosin. Although this fit is reasonable, we cannot rule out that the *p*PDM-S-1 binding may show a slight positive cooperativity. For example, in terms of the cooperative model of Hill *et al.* (20), if the *p*PDM-S-1 binds 2-fold stronger to regulated actin in the strong binding form than to regulated actin in the weak binding form, we could not experimentally detect the deviation from the curve for independent binding. In fact, a slight positive cooperativity in the binding could explain the 2-fold greater binding strength of *p*PDM-S-1 to regulated actin than to unregulated actin. Preliminary experiments utilizing a fluorescent probe to monitor tropomyosin movement also are consistent with slight positive cooperativity in the binding of *p*PDM-S-1 to regulated actin (21). It is interesting that troponin–tropomyosin does not cause the 2-fold strengthening of the binding of *p*PDM-S-1 to actin in the presence of ATP.

DISCUSSION

The data presented in this paper suggest that, in its interaction with actin, *p*PDM-S-1 closely resembles S-1·ATP and S-1·ADP·P_i, the steady-state species of S-1 that occur in the presence of ATP. Table 1 is a summary of binding constants of different S-1·nucleotide complexes to actin at 18 mM ionic strength, 25°C. Under these conditions, the binding constants of *p*PDM-S-1 and S-1·ATP or (S-1·ADP·P_i) to actin are $4 \times 10^4\ M^{-1}$ and $2 \times 10^4\ M^{-1}$, whereas S-1·AMP-*P*[NH]*P*, S-1·ADP, and S-1 alone bind several orders of magnitude more strongly. For example, S-1·AMP-*P*[NH]*P* binds about 100-fold stronger to actin than does *p*PDM-S-1. Thus, in its affinity for actin, *p*PDM-S-1 is very similar to S-1·ATP or S-1·ADP·P_i and very different from S-1 alone, S-1·ADP, or S-1·AMP-*P*[NH]*P*.

Another major similarity between *p*PDM-S-1 and S-1·ATP is the very slight effect of troponin–tropomyosin on their interaction with actin. This is in marked contrast to the behavior of S-1 (23), S-1·ADP (19), and S-1·AMP-*P*[NH]*P*, which bind to actin in a highly cooperative manner in the presence of troponin–tropomyosin. It is possible that the binding of *p*PDM-S-1 to actin does exhibit a small degree of cooperativity. In terms of the model of Hill *et al.* (20), if *p*PDM-S-1 (or for that matter S-1·ATP or S-1·ADP·P_i) binds only 2-fold stronger to regulated actin in the strong binding form than in the weak binding form, the binding curves would be experimentally indistinguishable

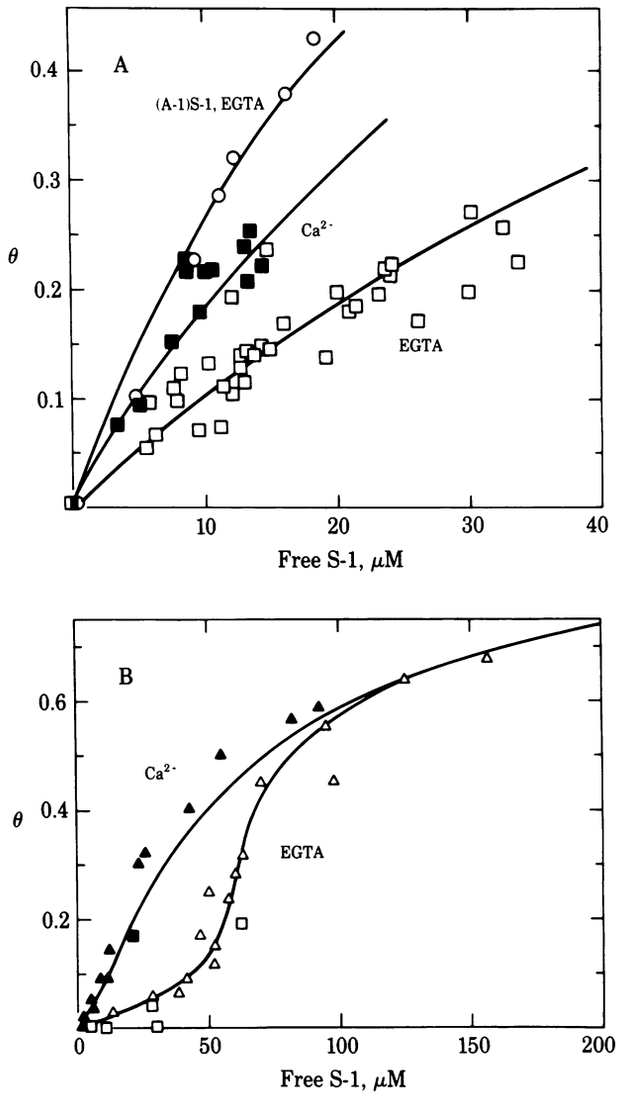

FIG. 4. Binding of S-1·nucleotide complexes to actin–troponin–tropomyosin. (*A*) Binding in the presence of ATP. Binding at very low S-1 concentrations (shown as single points near the origin) was measured by the Airfuge centrifugation technique and at higher S-1 concentrations by the stopped-flow turbidity technique. All experiments were done at 1 mM ATP/3 mM $MgCl_2$/10 mM imidazole/1 mM dithiothreitol and 1 mM EGTA (○, □) or 0.5 mM $CaCl_2$ (■), pH 7.0. In the absence of Ca^{2+} the actin concentration was generally 50 or 75 μM and the S-1 concentration was varied between 0.5 and 65 μM. With S-1 containing the 21,000-dalton light chain [(A-1)S-1] (○), the actin was maintained at 50 μM and S-1 was varied between 0.25 and 40 μM. (*B*) Binding of S-1 to actin–troponin–tropomyosin in the presence of AMP-*P*[NH]*P* at high ionic strength. Measurements were made using either the Airfuge technique (□, ■) or iodo[^{14}C]acetamide-labeled S-1 (△, ▲). The conditions were 4 mM AMP-*P*[NH]*P*, 5 mM $MgCl_2$, 10 mM imidazole at pH 7.0, 1 mM dithiothreitol, 150 mM KCl, 300 μM Ap_5A, and 1 mM EGTA (△, □) or 0.5 mM $CaCl_2$ (▲, ■), with 40 μM regulated actin and S-1 between 2 and 178 μM.

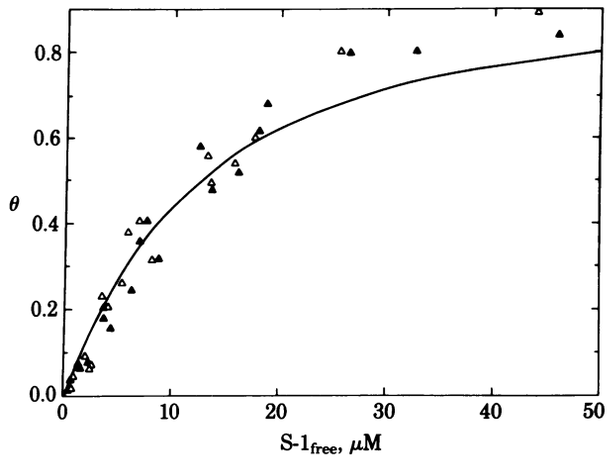

FIG. 5. Binding of *p*PDM-S-1 in the presence of troponin–tropomyosin. The conditions are those of Fig. 1 except for addition of 1 mM EGTA (△) or 0.5 mM $CaCl_2$ (▲) with 22 μM actin, 6 μM troponin–tropomyosin, and *p*PDM-S-1 between 2 and 60 μM. The solid line is the theoretical curve for independent binding of *p*PDM-S-1 to actin with $K = 8 \times 10^4$ M^{-1}.

from the curve for independent binding. Therefore further work is necessary to determine if there is a slight degree of cooperativity in the binding of *p*PDM-S-1 to regulated actin.

It having been established that crosslinking of S-1 by *p*PDM stabilizes a conformation which mimics that induced by the binding of ATP, the question arises as to the nature of this change. Our data indicate that the large difference in the effect of troponin–tropomyosin on the binding of S-1·AMP-*P*[NH]*P* and *p*PDM-S-1 to actin is not caused simply by the difference in their strength of binding to actin. At higher ionic strength, S-1·AMP-*P*[NH]*P* binds to actin with about the same affinity as S-1·ATP or *p*PDM-S-1, yet troponin–tropomyosin still has a strong cooperative effect on the binding of S-1·AMP-*P*[NH]*P* (Fig. 4). Thus, in addition to weakening the binding of S-1 to actin, modification of S-1 with *p*PDM causes some other change in the way that S-1 binds to actin.

Table 1. Binding constants of S-1 to actin at μ = 18 mM, 25°C

S-1 species	K, M^{-1}
*p*PDM-S-1	4×10^4
S-1·ATP	$2 \times 10^{4*}$
S-1·AMP-*P*[NH]*P*	$2 \times 10^{6\dagger}$
S-1·ADP	$3 \times 10^{7\ddagger}$
S-1 alone	$1 \times 10^{9\ddagger}$

*Refs. 17 and 18.
†Ref. 22.
‡Calculated on the basis that the binding of nucleotide to S-1 and acto·S-1 is insensitive to ionic strength.

A clue to this change may come from structural data, which suggest that tropomyosin can occupy two positions on actin (24–26). In one form, the weak form in the absence of Ca^{2+}, tropomyosin interferes with the binding of S-1 alone, S-1·ADP, and S-1·AMP-*P*[NH]*P*, to actin, but not with the binding of *p*PDM-S-1 (and S-1·ATP or S-1·ADP·P_i). It therefore appears that a substantial change in acto·S-1 structure may take place when S-1 binds ATP or is modified by *p*PDM, and this change allows the S-1·ATP and *p*PDM-S-1 to bind to actin without being affected by the position of tropomyosin. Fig. 6 schematically illustrates a possible model for the binding of S-1 in the different states to actin. In this model, tropomyosin has no effect on the binding of molecules in the 90° states (S-1·ATP and S-1·ADP·P_i) to F-actin. On the other hand, tropomyosin in the relaxing position simultaneously weakens the binding of molecules in the 45° states (S-1·ADP and S-1) and inhibits the change in S-1 angle associated with the release of P_i, thus inhibiting the ATPase cycle and preventing force development. The exact relationship between the ability of troponin–tropomyosin to inhibit S-1·ADP binding and prevent P_i release remains to be determined. This model predicts that cross-bridges containing bound ATP could be attached to actin in relaxed muscle, and recent evidence has

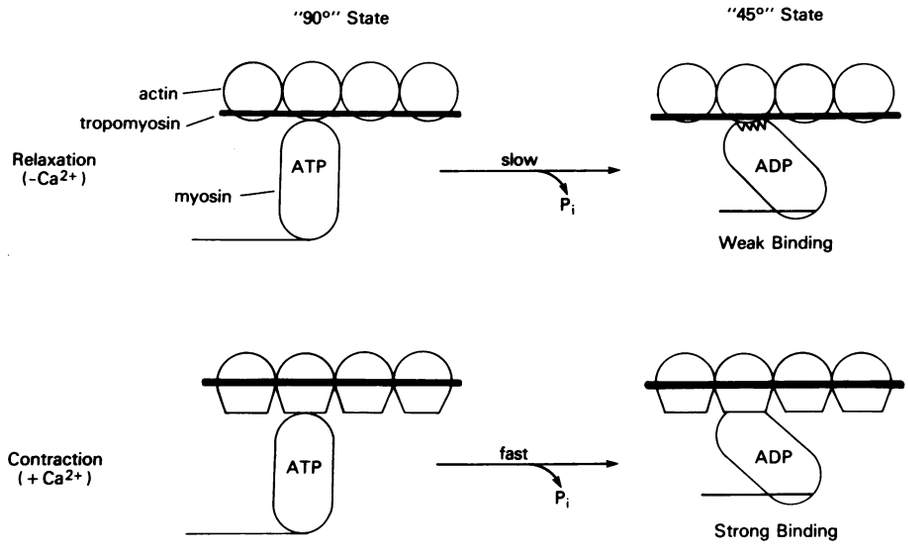

FIG. 6. Schematic illustration of the possible relationship between tropomyosin position and the chemical states of myosin heads. Actin is represented by open circles, tropomyosin is shown as solid rods, and myosin S-1 is shown as ovals containing either ATP or ADP. In the presence of Ca^{2+}, regulated actin is in the strong form. S-1·ATP binds weakly at a "90°" angle to the strong form of actin, whereas S-1·ADP binds strongly at a "45°" angle. The transition of S-1 from the weak binding 90° state to the strong binding 45° state occurs rapidly and is accompanied by rapid release of P_i. Rapid occurrence of this transition allows muscle contraction to occur. In the absence of Ca^{2+}, tropomyosin assumes a different position on the actin filament, forming the weak form of the regulated actin. Both S-1·ADP and S-1·ATP bind weakly to the weak form of regulated actin. In addition, the release of P_i and the associated change of S-1 from the 90° state to the 45° state is inhibited by tropomyosin and thus occurs very slowly. This causes muscle to relax.

shown that such attachment does indeed occur at very low ionic strength (27).

Until the structure of the acto·S-1·ATP complex is better understood, the structural changes that occur during the cross-bridge cycle will remain elusive. Studies on the stable *p*PDM-S-1-actin complex may be helpful in determining the structure of the acto·S-1·ATP complex and its role in muscle contraction and relaxation.

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