Effect of Nucleotide on the Binding of *N,N'-p*-Phenylenedimaleimide-Modified S-1 to Unregulated and Regulated Actin

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ABSTRACT: In our previous study [Chalovich, J. M., Greene, L. E., & Eisenberg, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4909-4913], myosin subfragment 1 that was modified by having its two reactive thiol groups cross-linked by *N,N'-p*-phenylenedimaleimide (pPDM) was found to resemble the myosin subfragment 1-adenosine 5'-triphosphate (S-1·ATP) complex in its interaction with actin. In the present study, we examined the effect of actin on adenosine 5'-diphosphate (ADP) trapped at the active site of pPDM·S-1. Our results indicate first that, in the presence of actin, ADP is no longer trapped at the active site but exchanges rapidly with free nucleotide. Different pPDM·S-1·nucleotide complexes were then formed by exchanging nucleotide into the active site of pPDM·S-1 in the presence of actin. The binding of pPDM·S-1·ATP or pPDM·S-1·PP_i to actin is virtually identical with that of unmodified S-1 in the presence of ATP. Specifically, at μ = 18 mM, 25 °C, pPDM·S-1·ATP or pPDM·S-1·PP_i binds to unregulated actin with the same affinity as does S-1·ATP, and this binding does not appear to be affected by troponin-tropomyosin. On the other hand, pPDM·S-1·ADP and pPDM·S-1 with no bound nucleotide both show a small, but significant, difference between their binding to actin and the binding of S-1·ATP; pPDM·S-1 and pPDM·S-1·ADP both bind about 2- to 3-fold more strongly to unregulated actin than does S-1·ATP. In addition, troponin-tropomyosin confers slight cooperative strengthening and Ca^{2+} sensitivity on the binding of pPDM·S-1 and pPDM·S-1·ADP to actin. These results suggest that the nucleotide bound to the active site of pPDM·S-1 causes conformational changes in the protein which, in turn, subtly alter its interaction with actin; the stronger the binding of the S-1 to unregulated actin, the more cooperativity is observed in its binding to regulated actin.

The myosin cross-bridge is thought to oscillate between two major conformational states during the actomyosin-ATPase[1] cycle. In the conformation that occurs in the absence of ATP (strong-binding conformation), myosin binds very tightly to actin at about a 45° angle (Reedy et al., 1965; Moore et al., 1970); in the conformation that occurs in the presence of ATP (weak-binding conformation), myosin with bound ATP or ADP·P_i binds orders of magnitude more weakly to actin (Stein et al., 1979). The detailed structure of this latter conformation is not yet known, although it appears to be quite different from the conformation that occurs in the absence of ATP (Craig et al., 1985). These two conformations also differ in that troponin-tropomyosin markedly inhibits the binding of S-1 to actin when it is in the strong-binding conformation (Greene & Eisenberg, 1980) but has no significant effect when the S-1 is in the weak-binding conformation (Chalovich et al., 1981; Wagner & Giniger, 1981; Chalovich & Eisenberg, 1982). We previously found that a conformation of S-1 which resembles the weak-binding conformation can be stabilized by modifying S-1 with the bifunctional thiol reagent, *N,N'-p*-phenylenedimaleimide (Chalovich et al., 1983). pPDM-modified S-1 binds to unregulated actin with an affinity similar to that of S-1 in the presence of ATP. Furthermore, like the S-1·ATP and S-1·ADP·P_i complexes, the binding of pPDM·S-1 to regulated actin does not show marked cooperativity.

Reisler et al. (1974) originally reported that pPDM cross-links the critical thiol groups, SH_1 and SH_2, of S-1. This cross-linking reaction is greatly accelerated by MgADP (Burke & Reisler, 1977), which, in turn, is stoichiometrically trapped at the active site (Wells & Yount, 1979). The MgADP is considered to be trapped because it has a half-life of release of greater than 5 days at 0 °C when stored in the presence of nonradioactive nucleotide or EDTA (Wells et al., 1980). However, in the presence of actin, Wells and Yount[2] observed that the rate of release of the trapped ADP is greatly increased.

In our previous study on the interaction of pPDM·S-1 with actin (Chalovich et al., 1983), it was not determined whether ADP trapped at the active site of the pPDM·S-1 remains trapped in the presence of actin. In this paper we investigated this question by measuring the effect of actin on the release of ADP trapped at the active site of pPDM·S-1. Our results show that, in the presence of actin, nucleotide is no longer trapped at the active site of pPDM·S-1; it becomes rapidly exchangeable with free nucleotide although it remains tightly bound. This exchange reaction provided a convenient way to trap different nucleotides (ADP, ATP, or PP_i) at the active site of pPDM·S-1. Although these pPDM·S-1·nucleotide complexes all resemble the S-1·ATP and S-1·ADP·P_i com-

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[1] Abbreviations: S-1, myosin subfragment 1; acto-S-1, complex of actin and myosin subfragment 1; pPDM, *N,N'-p*-phenylenedimaleimide; pPDM·S-1, S-1 that has been modified with pPDM; AMP-PNP, 5'-adenylyl imidodiphosphate; regulated actin, troponin-tropomyosin-actin complex; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; ATPase, adenosinetriphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate.

[2] J. A. Wells and R. Yount, personal communication.

plexes in their weak binding to actin, they do show subtle differences in their binding to both unregulated and regulated actin: the stronger their binding to unregulated actin, the more cooperativity they show in their binding to regulated actin.

Materials and Methods

Myosin, actin, S-1, and troponin–tropomyosin were prepared as described previously (Stein et al., 1979). Concentrations were calculated spectrophotometrically at 280 nm with the following absorbances and molecular weights: myosin, $E^{0.1\%}$ = 0.56 cm^2/mg, M_r 480 000; S-1, $E_{280}{}^{0.1\%}$ 0.75 cm^2/mg, M_r 120 000; actin, $E^{0.1\%}$ = 1.15 cm^2/mg, M_r 42 000; troponin–tropomyosin, $E^{0.1\%}$ = 0.38 cm^2/mg, M_r 150 000.

S-1 was modified with pPDM at 0 °C in the presence of ADP as described by Wells & Yount (1982). The modified S-1 contained 1.1–1.3 mol of pPDM per mole of S-1, and about 75% of the modified S-1 molecules contained trapped ADP. The pPDM·S-1 was further purified by sedimenting with actin as described previously (Chalovich et al., 1983). The actin-activated ATPase of the purified pPDM·S-1 is 0.2% of the rate obtained with unmodified S-1. For many of the exchange studies (see Results), the modification of S-1 by pPDM was done in the presence of [^{3}H]ADP. For the binding studies, the S-1 was modified with [^{14}C]pPDM (specific activity of 8 × 10^{13} cpm/mol of S-1).

To remove most of the trapped ADP, pPDM·S-1 was treated twice with the anion exchange resin Dowex 1X-8 in the presence of actin. pPDM·S-1 (1.7 mg/mL) was mixed with actin (1.4 mg/mL) in 2 mM $MgCl_2$ and 4 mM imidazole (pH 7.0). A Dowex suspension (33% Dowex) was added as follows: 1 part of Dowex suspension to 4 parts of acto·pPDM·S-1 solution. The acto·pPDM·S-1 suspension was left with the Dowex at room temperature for 10 min with occassional stirring before being filtered through a sintered glass funnel. The Dowex treatment and filtration were repeated, and then the solution was made 0.4 M in KCl to dissociate the actin from the pPDM·S-1. The actin was separated from the pPDM·S-1 by centrifugation, and the pPDM·S-1 in the supernatant was concentrated with ammonium sulfate. After this treatment, only about 20% of the pPDM·S-1 molecules had bound ADP.

The exchange of nucleotide with the bound ADP of pPDM·S-1 was measured by Sephadex G-25 column chromatography (see below). Reactions were conducted at 25 °C in a 4-mL volume in the following solution: 10 mM imidazole (pH 7.0), 3 mM $MgCl_2$, 1 mM nucleotide, and 1 mM dithiothreitol. Varying concentrations of actin were mixed with 10 μM pPDM·S-1, and at the indicated time 0.9 mL of the reaction mixture was quenched with 0.1 mL of 4 M KCl to dissociate the actin from the pPDM·S-1. No significant exchange of nucleotide occurred after the KCl quench. The mixture was then centrifuged for 20 min at 180000g in a Beckman airfuge to remove the actin. The free nucleotide in the supernatant was separated from the pPDM·S-1·nucleotide complex by column chromatography at room temperature on a small Sephadex G-25 column (Pharmacia PD10) equilibrated with 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, and 200 mM KCl and eluted with the same buffer. Fractions of 0.5 mL were collected, and the radioactivity of the samples was determined.

The binding of [^{14}C]pPDM·S-1 to actin and actin–troponin–tropomyosin was measured with a Beckman airfuge (Chalovich et al., 1983). Solutions containing S-1 and actin and, in most cases, 1 mM nucleotide were stirred for several minutes at 25 °C. This permitted the added nucleotide to exchange with the trapped ADP of pPDM·S-1, resulting in about 80% of the pPDM·S-1 with bound ADP, PP_i, or ATP. The Dowex-treated pPDM·S-1 had about 20% of the molecules with bound ADP. Even with these inhomogeneities in the pPDM·S-1 populations, we found a significant effect of nucleotide on the binding of pPDM·S-1 to actin, and if anything, the observed differences would be greater if the populations were homogeneous. With regulated actin, binding experiments were concurrently performed in the presence and absence of Ca^{2+}. Aliquots of the solution taken before and after centrifugation and the supernatant after centrifugation were assayed for radioactivity in a Beckman LS-250 liquid scintillation counter to determine the total and free pPDM·S-1 concentrations. For independent binding isotherms, the data from each experiment, consisting of at least six points at different pPDM·S-1 concentrations, were fitted to the equation $K = \theta/[(1 - \theta)[\text{pPDM·S-1}]_{\text{free}}]$.

[^{14}C]ATP and [2,8-^{3}H]ADP were from New England Nuclear, $^{32}PP_i$ was from ICN, and [1,4-^{14}C]maleic anhydride was from Amersham. [^{14}C]pPDM was synthesized as described by Wells & Yount (1982) and was purified by sublimation. pPDM from Aldrich was recrystallized from acetone and sublimated before use.

Results

We first studied whether the trapped ADP on pPDM·S-1 was released in the presence of actin. The amount of ADP trapped on pPDM·S-1 was measured by a column method that separates the trapped ADP, found in the void volume, from the free ADP, which elutes later. This method can be used to measure the amount of ADP trapped at the active site of pPDM·S-1 since this trapped ADP is released very slowly after actin is removed by treatment with 0.4 M KCl and centrifugation (see Materials and Methods).

S-1 was reacted with pPDM in the presence of [^{3}H]ADP to make pPDM·S-1, containing trapped [^{3}H]ADP. This pPDM·S-1, at a concentration of 10 μM, was incubated with 25 μM actin at μ = 18 mM, 25 °C for 15 min, under conditions identical with those used in our previous binding study of pPDM·S-1 to actin. As shown in Figure 1 (open circles), this causes no significant release of the [^{3}H]ADP from pPDM·S-1. Since many of our previous binding experiments were conducted at 25 μM actin, these results show that ADP remained bound to pPDM·S-1 in those experiments. Very different results were obtained when the same concentration of pPDM·S-1, containing [^{3}H]ADP, was incubated with both 25 μM actin and 1 mM nonradioactive ADP. Under this condition, more than 95% of the [^{3}H]ADP is released (open squares). Figure 1 also shows that, in agreement with Wells et al. (1980), incubating pPDM·S-1 with 1 mM ADP in the absence of actin causes no significant release of ADP. Therefore, these results indicate that the release of the trapped ADP is greatly accelerated by actin. However, apparently because the binding constant of ADP to pPDM·S-1 is relatively strong in the presence of actin (>10^5 M^{-1}), the radioactive ADP remains bound to pPDM·S-1 in the presence of actin unless a high concentration of nonradioactive ADP is present so that exchange can take place.

The effect of actin on the rate of release of the trapped ADP was confirmed by mixing pPDM·S-1, containing trapped [^{3}H]ADP, with excess nonradioactive ADP and then quenching the reaction at varying times. As shown by the closed circles in Figure 2, the half-life of release of the trapped ADP at 25 °C is about 3 h in the absence of actin, whereas it is less than 1 min in the presence of 5 μM actin (closed squares). About 70–80% of the pPDM·S-1 has bound [^{3}H]-ADP initially, and within 1 min, all of this [^{3}H]ADP has

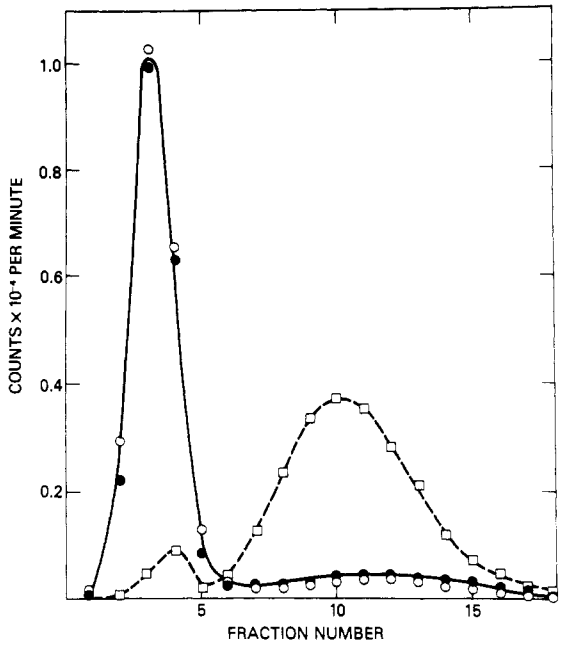

FIGURE 1: Release of [^{3}H]ADP from pPDM·S-1 as determined by column chromatography. The conditions were 10 mM imidazole (pH 7.0), 3 mM $MgCl_2$, and 1 mM dithiothreitol at 25 °C. pPDM·S-1 (10 μM), containing trapped [^{3}H]ADP, was incubated for 15 min at 25 °C with 25 μM actin (○) or 1 mM ADP (●) or with both 1 mM ADP and 25 μM actin (□). The reaction mixture was chromatographed on a PD-10 column to determine the distribution of free and bound ADP after quenching the reaction with KCl (see Materials and Methods).

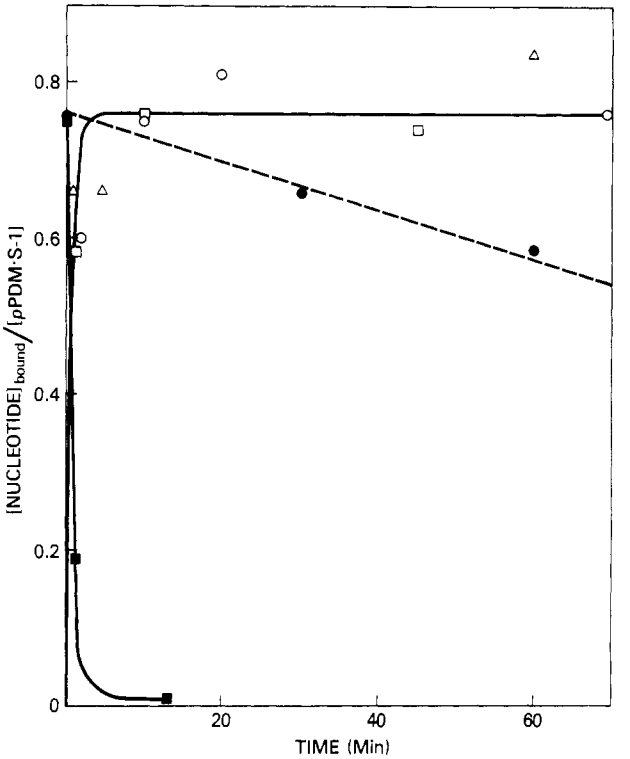

FIGURE 2: Rate of exchange of ADP bound to pPDM·S-1. Conditions are the same as in Figure 1. The rate of release of the bound [^{3}H]ADP of pPDM·S-1 was determined by incubating pPDM·S-1 (10 μM) with 1 mM ADP either in the absence of actin (●) or in the presence of 5 μM actin (■). The rate of binding of nucleotide to pPDM·S-1 was determined in the presence of 5 μM actin by incubating pPDM·S-1, containing bound nonradioactive ADP, with different nucleotides: 1 mM [^{3}H]ADP (○), 1 mM $^{32}PP_i$ (△), or 1 mM [^{14}C]ATP (□). The reactions were quenched at various times and then chromatographed on a PD-10 column. The dashed line is drawn through data obtained in the absence of actin and the solid lines are drawn through data obtained in the presence of actin.

exchanged with unlabeled ADP (Figure 2, open circles). These results confirm that, in the presence of actin, ADP remains bound to pPDM·S-1 but it is no longer trapped at the active site; it rapidly exchanges with free ADP in solution.

PP_i and ATP also rapidly exchange with the bound ADP of pPDM·S-1 in the presence of actin. As shown by the open symbols in Figure 2, ATP and PP_i, like ADP, completely exchange with the bound ADP of pPDM·S-1 in less than 1 min in the presence of actin. These results show that different pPDM·S-1·nucleotide complexes can readily be obtained by mixing pPDM·S-1 with excess nucleotide and actin. This enabled us to examine the effect of the bound nucleotide of pPDM·S-1 on the binding of pPDM·S-1 to unregulated actin. In addition, we used pPDM·S-1 that was treated twice with Dowex in the presence of actin to obtain pPDM·S-1 with almost no bound nucleotide (see Materials and Methods).

The binding of different pPDM·S-1·nucleotide complexes to unregulated actin was measured at μ = 18 mM, 25 °C, the same conditions used in our previous study on the binding of pPDM·S-1 to actin (Chalovich et al., 1983). Figure 3 shows that the nucleotide bound to pPDM·S-1 affects the strength of binding of pPDM·S-1 to unregulated actin. The Dowex-treated pPDM·S-1, containing almost no bound nucleotide, binds with the greatest affinity to actin (open circles), having an association constant of 5.3×10^4 M^{-1}. The presence of bound nucleotide (ADP, PP_i, or ATP) causes a slight reduction in the affinity of pPDM·S-1 for actin. This reduction is less in the presence of ADP ($K = 3.3 \times 10^4$ M^{-1}) than in the presence of PP_i or ATP ($K = 2.0 \times 10^4$ M^{-1}). From four different pPDM·S-1 preparations, the following binding constants were obtained for the acto·pPDM·S-1 association constants: in the absence of nucleotide, $K = 5.5 \pm 0.6 \times 10^4$ M^{-1}; in the presence of ADP, $K = 3.5 \pm 0.5 \times 10^4$ M^{-1}; in the presence of PP_i, $K = 2.7 \pm 0.5 \times 10^4$ M^{-1}; in the presence of ATP, $K = 2.3 \pm 0.4 \times 10^4$ M^{-1}. Therefore, there is a 2.5-fold

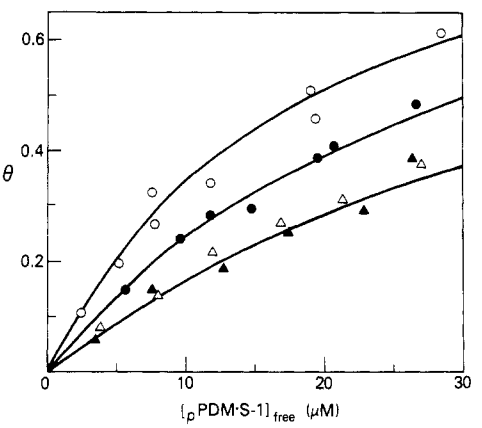

FIGURE 3: Binding of different pPDM·S-1·nucleotide complexes to actin at μ = 18 mM, 25 °C. In the absence of nucleotide (○), conditions were 5 mM KCl, 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, 1 mM DTT, and either 0.5 mM $CaCl_2$ or 1 mM EGTA with ion-exchange-treated S-1. In the presence of nucleotide, conditions were 1 mM nucleotide, 3 mM $MgCl_2$, 10 mM imidazole (pH 7.0), 1 mM DTT, and either 0.5 mM $CaCl_2$ or 1 mM EGTA with either ADP (●) or PP_i (△) or ATP (▲). Varying concentrations of [^{14}C]-pPDM·S-1 (2–50 μM) were added to 25 μM actin, and the amount of pPDM·S-1 bound to actin was determined after centrifugation. Data obtained in the presence and absence of Ca^{2+} were not significantly different and therefore are plotted together in this graph. θ is the number of moles of S-1 bound per mole of actin monomer. The theoretical curves are for independent binding of pPDM·S-1 to actin having an association constants of 5.3×10^4 M^{-1}, 3.3×10^4 M^{-1}, and 2.0×10^4 M^{-1} for data obtained in the absence of nucleotide, presence of ADP, and presence of ATP (or PP_i), respectively.

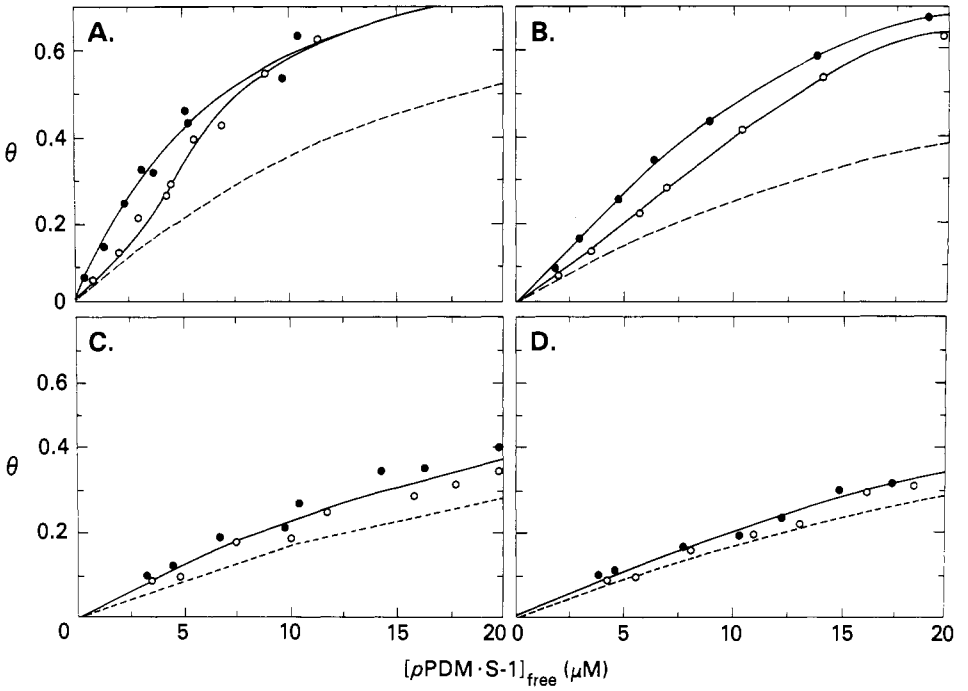

FIGURE 4: Binding of different pPDM·S-1·nucleotide complexes to regulated actin at μ = 18 mM, 25 °C. Binding studies were done in the presence of either 0.5 mM $CaCl_2$ (●) or 1 mM EGTA (○) under the same conditions as in Figure 3. Varying concentrations of [^{14}C]pPDM·S-1 were mixed with 20 μM regulated actin (ratio of 2:7 of troponin–tropomyosin to actin). Binding in the absence of nucleotide used ion-exchange-treated pPDM·S-1. Panels A–D show experiments carried out in the absence of nucleotide, presence of 1 mM ADP, presence of 1 mM PP_i, and presence of 1 mM ATP, respectively. The dashed line in each graph shows the binding isotherm obtained in Figure 3.

difference between the binding constant of pPDM·S-1 to actin in the absence of nucleotide and that obtained in the presence of ATP, while the binding constants of pPDM·S-1 to actin in the presence of ADP and perhaps in PP_i as well lie between these extremes. This difference in binding in the presence and absence of ATP was also observed if binding studies were done by varying the actin concentration while keeping the pPDM·S-1 concentration constant.

It is important to recognize that the effect of the bound nucleotide on the binding of pPDM·S-1 to actin is very small; with unmodified S-1, the binding of ATP at the active site causes the affinity of S-1 for actin to be reduced by more than 5 orders of magnitude (Stein et al., 1979; Chalovich et al., 1983). Therefore, regardless of the nucleotide bound to pPDM·S-1, the affinity of pPDM·S-1 for actin remains very similar to that of unmodified S-1·ATP and S-1·ADP·P_i (K = 2×10^4 M^{-1}), in agreement with our previous study (Chalovich et al., 1983).

Next, the binding of the different pPDM·S-1·nucleotide complexes to actin was measured in the presence of troponin–tropomyosin to determine whether nucleotide also affects the binding of pPDM·S-1 to regulated actin. This was done in both the presence and absence of Ca^{2+} under conditions identical with those used in Figure 3 with unregulated actin. As shown in Figure 4, the binding isotherms are altered by the nucleotide bound to pPDM·S-1. Comparison of the binding of the different pPDM·S-1·nucleotide complexes to regulated actin (solid lines) and unregulated actin (dashed lines) shows that troponin–tropomyosin has its greatest effect on the binding of pPDM·S-1 to actin in the absence of nucleotide (Figure 4A) and its smallest effect in the presence of ATP (Figure 4D). In the former case, troponin–tropomyosin appears to confer slight Ca^{2+} sensitivity on the binding and to strengthen it cooperatively about 3-fold; this cooperativity is shown by the binding of pPDM·S-1 to regulated actin becoming stronger as the concentration of pPDM·S-1 increases. At θ = 0.6, there was 30% more pPDM·S-1 bound to regulated actin than would have been the case in the absence of cooperativity, resulting in an association constant of 1.5 ± 0.2 × 10^5 M^{-1} for the binding of four different pPDM·S-1 preparations to regulated actin in this region of the binding isotherm. On the other hand, in the presence of ATP, troponin–tropomyosin does not significantly affect the binding in either the presence or absence of Ca^{2+}. Even up to 60% saturation of the actin with pPDM·S-1 (θ = 0.6), the binding of pPDM·S-1 to regulated actin in the presence of ATP showed no apparent cooperativity. From five different experiments, the binding constant of pPDM·S-1 to regulated actin in the presence of ATP was determined to be 2.4 ± 0.2 × 10^4 M^{-1}, which is only 5% higher than that obtained in the absence of troponin–tropomyosin. Therefore, when regulated actin is 60% saturated with pPDM·S-1, pPDM·S-1 with no bound nucleotide binds 7-fold more strongly to regulated actin than pPDM·S-1 in the presence of ATP.

The effects of ADP and PP_i on the binding of pPDM·S-1 to regulated actin appear to lie between the two extremes of no bound nucleotide and bound ATP. The binding obtained in the presence of ADP (Figure 4B) appears to closely resemble that obtained in the absence of nucleotide (Figure 4A), since it too shows Ca^{2+} sensitivity and slight cooperativity. When the sites on regulated actin are 60% occupied with pPDM·S-1·ADP, an association constant of 8.5 ± 0.8 × 10^4 M^{-1} for the binding of pPDM·S-1·ADP to regulated actin was determined from four experiments. Therefore, at this level of saturation, troponin–tropomyosin strengthens the affinity of pPDM·S-1·ADP to actin 2.5-fold. On the other hand, the binding of pPDM·S-1·PP_i to regulated actin (Figure 4C) closely resembles the binding of pPDM·S-1·ATP (Figure 4D) since it does not show any Ca^{2+} sensitivity or cooperativity. The binding constant of pPDM·S-1·PP_i to regulated actin was determined from four preparations to be 3.2 ± 0.2 × 10^4 M^{-1}, which is only about 20% higher than that obtained in the absence of troponin–tropomyosin.

These results show that the nucleotide bound to pPDM·S-1

affects the binding of pPDM·S-1 to regulated actin, but the effect is much less than that observed with unmodified S-1. Specifically, unmodified S-1 binds to regulated actin in a highly cooperative manner in the absence of nucleotide or in the presence of ADP, AMP-PNP, and PP_i (Greene, 1982; Williams & Greene, 1983) but binds with almost no cooperativity in the presence of ATP (Chalovich et al., 1981; Wagner & Giniger, 1981 Chalovich & Eisenberg, 1982). The binding of pPDM·S-1 t regulated actin also shows almost no cooperativity in the presence of ATP. However, in contrast to unmodified S-1, the binding of pPDM·S-1 to regulated actin shows only slight cooperativity in the absence of nucleotide or in the presence of ADP. This suggests that, irrespective of the nucleotide bound to pPDM·S-1, the conformation of pPDM-S-1 closely resembles that of unmodified S-1 in the presence of ATP.

Discussion

In this study, we obtained different nucleotide complexes of pPDM·S-1 by exchanging the bound ADP at the active site with other nucleotides in the presence of actin. Wells & Yount (1979) found that the rate of release of the bound ADP of pPDM·S-1 is very slow, which led them to suggest that the bound ADP is trapped at the active site by the cross-linking reaction. However, actin must change the microenvironment around the active site of pPDM·S-1 since, in the presence of actin, the rate of exchange of the so-called "trapped" ADP becomes quite rapid; that is, it is no longer trapped but is simply bound to the pPDM·S-1. Because this binding of nucleotide apparently remains strong even in the presence of actin ($K > 10^5$ M^{-1}), removal of the bound ADP in the presence of actin requires the addition of an anion-exchange resin and exchange of the bound ADP requires a large excess of another nucleotide.

It is not surprising that acto·pPDM·S-1 binds ADP with a binding constant greater t an 10^5 M^{-1}. Presumably, in the absence of actin, ADP binc , quites strongly to pPDM·S-1, at least as strongly as it does to unmodified S-1 ($K > 10^5$ M^{-1}) (Konrad & Goody, 1982). Since the binding of ADP to pPDM·S-1 has very little effect on the affinity of pPDM·S-1 for actin, by detailed balance, the binding of actin to pPDM·S-1 must have very little effect on the affinity of pPDM·S-1 for ADP. Therefore, when actin greatly increases the rate of dissociation of ADP from pPDM·S-1, it must simultaneously increase its rate of association as well. This differs from the acto·S-1 nucleotide interaction found with unmodified S-1, where, with ATP for example, actin greatly increases the rate of ATP dissociation from S-1 but has relatively little effect on the rate of ATP association (Sleep & Taylor, 1976). Therefore, in contrast to the situation with pPDM·S-1, actin greatly weakens the binding constant of ATP to unmodified S-1 and concomitantly ATP greatly weakens the binding of actin.

The ability of actin to accelerate the exchange of nucleotide at the active site of pPDM·S-1 without greatly affecting the binding of the pPDM·S-1 to actin allowed us to study the subtle effects of nucleotide on the binding of pPDM·S-1 to both unregulated and regulated actin. When pPDM·S-1 has almost no bound nucleotide or has bound ADP, its binding to unregulated actin is about 3-fold stronger than that of S-1·ATP, and correspondingly, its binding to regulated actin shows slight Ca^{2+} sensitivity and a further 3-fold strengthening with increasing pPDM·S-1 concentrations. The latter effect indicates cooperativity in the binding of pPDM·S-1 to regulated actin. On the other hand, in the presence of ATP or PP_i, the binding of pPDM·S-1 to unregulated actin is essentially the same as that of S-1·ATP, and correspondingly, the binding of pPDM·S-1 to regulated actin shows no apparent effect of troponin–tropomyosin on its binding in either the presence or absence of Ca^{2+}.

These results corroborate our earlier suggestion, based on studies with AMP-PNP (Greene, 1982), that troponin–tropomyosin can have a variable effect on the binding of S-1 to actin depending on the nucleotide bound at the active site. This can be explained by the model for the cooperative binding of S-1 to regulated actin proposed by Hill et al. (1980). In this model, each tropomyosin–troponin–actin unit along the regulated actin filament can exist in two forms, a weak S-1 binding form and a strong S-1 binding form. In the absence of Ca^{2+}, the equilibrium between these forms is shifted almost completely toward the weak S-1 binding form. The pronounced cooperativity in the binding of unmodified S-1 to regulated actin in the presence of ADP is explained by S-1 binding much more strongly to tropomyosin–actin units in the strong S-1 binding form than in the weak S-1 binding form. On the other hand, the absence of cooperativity in the binding of unmodified S-1 or pPDM·S-1 to regulated actin in the presence of ATP can be explained by there being less than a 2-fold difference in the binding strength of S-1·ATP or pPDM·S-1·ATP to tropomyosin–actin units in the weak and strong S-1 binding forms. On this basis, the slight cooperativity in the binding of pPDM·S-1 to regulated actin in either the absence of nucleotide or the presence of ADP would be explained by pPDM·S-1 binding about 3-fold more strongly to tropomyosin–actin units in the strong S-1 binding form than in the weak S-1 binding form. Hence, the greater the difference in the strength of binding of an S-1·nucleotide complex to the weak and strong S-1 binding forms of regulated actin, the greater the cooperativity observed in its binding to regulated actin.

Since the extent to which the fraction of tropomyosin–actin units is shifted into the strong S-1 binding form depends on the nucleotide bound to S-1, this suggests that the different acto·S-1·nucleotide complexes must have different structures. Therefore, rather than having S-1 exist in just two different conformations, a conformation that binds weakly to actin and a conformation that binds strongly to actin, there also appear to be intermediate conformations. We previously suggested that the weak- and strong-binding conformations of S-1 might also show large-scale structural differences when bound to actin; i.e., they might be equivalent to the so-called "90°" and "45°" cross-bridge states, respectively. Recent electron microscopy studies on S-1 cross-linked to actin provide some support for this idea (Craig et al., 1985). On this basis, the intermediate binding conformations, which can be artificially made by modifying S-1 with pPDM or by adding AMP-PNP to unmodified S-1, may differ structurally from both the 90° and 45° conformations, just as they differ in their affinity for actin and in the cooperative binding to regulated actin. The occurrence of these intermediate conformations may be related to the cross-bridge undergoing a continual rotation on the actin as it changes from the 90° to the 45° conformation during the cross-bridge cycle; this rotation may be linked to continual changes at the active site of myosin, and different stages of the rotation may be mimicked by S-1·AMP-PNP or the various pPDM·S-1 species examined in this paper. Therefore, rather than myosin existing in only two conformations (Shriver & Sykes, 1982), there may be a wide range of conformations.

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REFERENCES
Burke, M., & Reisler, E. (1977) *Biochemistry 16*, 5559–5563.
Chalovich, J. M., & Eisenberg, E. (1982) *J. Biol. Chem. 257*, 2432–2438.
Chalovich, J. M., Chock, P. B., & Eisenberg, E. (1981) *J. Biol. Chem. 256*, 575–578.
Chalovich, J. M., Greene, L. E., & Eisenberg, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4909–4913.
Craig, R., Greene, L. E., & Eisenberg, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3247–3251.
Eisenberg, E., & Greene, L. E. (1980) *Annu. Rev. Physiol. 42*, 293–309.
Eisenberg, E., & Hill, T. C. (1985) *Science (Washington, D.C.) 227*, 999–1006.
Greene, L. E. (1982) *J. Biol. Chem. 257*, 13993–13999.
Greene, L. E., & Eisenberg, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2616–2620.
Hill, T. L., Eisenberg, E., & Greene, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3186–3190.
Konrad, M., & Goody, R. (1982) *Eur. J. Biochem. 128*, 547–555.
Moore, P. B., Huxley, H. E., & DeRosier, D. J. (1970) *J. Mol. Biol. 50*, 279–295.
Reedy, M. K., Holmes, K. C., & Tregear, R. T. (1965) *Nature (London) 207*, 1276–1280.
Reisler, E., Burke, M., Himmelfarb, S., & Harrington, W. F. (1974) *Biochemistry 13*, 3837–3840.
Shriver, J., & Sykes, B. (1982) *Biochemistry 21*, 3022–3028.
Sleep, J. A., & Taylor, E. W. (1976) *Biochemistry 15*, 5818–5826.
Stein, L. A., Schwarz, R. P., Chock, P. B., & Eisenberg, E. (1979) *Biochemistry 18*, 3895–3909.
Wagner, P. D., & Giniger, E. (1981) *J. Biol. Chem. 256*, 12647–12650.
Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970.
Wells, J. A., & Yount, R. G. (1982) *Methods Enzymol. 85*, 93–116.
Wells, J. A., Sheldon, M., & Yount, R. G. (1980) *J. Biol. Chem. 255*, 1598–1602.
Williams, D. L., Jr., & Greene, L. E. (1983) *Biochemistry 22*, 2770–2774.

Calmodulin-Linked Equilibria in Smooth Muscle Myosin Light Chain Kinase†

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ABSTRACT: Competition experiments using 9-anthroylcholine, a fluorescent dye that undergoes calmodulin-dependent binding by smooth muscle myosin light chain kinase [Malencik, D. A., Anderson, S. R., Bohnert, J. L., & Shalitin, Y. S. (1982) *Biochemistry 21*, 4031], demonstrate a strongly stabilizing interaction between the adenosine 5′-triphosphate and myosin light chain binding sites operating within the enzyme–calmodulin complex but probably not in the free enzyme. The interactions in the latter case may be even slightly destabilizing. The fluorescence enhancement in solutions containing 5.0 μM each of the enzyme and calmodulin is directly proportional to the maximum possible concentration of bound calcium on the basis of four calcium binding sites. Evidently, all four calcium binding sites of calmodulin contribute about equally to the enhanced binding of 9-anthroylcholine by the enzyme. Fluorescence titrations on solutions containing 1.0 μM enzyme plus calmodulin yield a Hill coefficient of 1.2 and $K = 0.35 \pm 0.08$ μM calcium. Three proteolytic fragments of smooth muscle myosin light chain kinase, apparent products of endogenous proteolysis, were isolated and characterized. All three possess calmodulin-dependent catalytic activity. Their interactions with 9-anthroylcholine, in both the presence and absence of calmodulin, are similar to those of the native enzyme. However, the stabilities of their complexes with calmodulin vary. The corresponding dissociation constants range from 2.8 nM for the native enzyme and 8.5 nM for the 96K fragment to ~15 nM for the 68K and 90K fragments [0.20 N KCl, 50 mM 3-(*N*-morpholino)propanesulfonic acid, and 1 mM $CaCl_2$, pH 7.3, 25 °C]. A coupled fluorometric assay, modified from a spectrophotometric assay for adenosine cyclic 3′,5′-phosphate dependent protein kinase [Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R. (1982) *Biochemistry 21*, 5794], has provided the first continuous recordings of myosin light chain kinase phosphotransferase activity. The results show that smooth muscle myosin light chain kinase is a responsive enzyme, whose activity adjusts rapidly to changes in solution conditions.

Myosin light chain kinase is a calmodulin-dependent enzyme catalyzing phosphorylation of two of the regulatory light chains of myosin [cf. reviews by Stull (1980), Small & Sobieszek (1980), Hartshorne & Siemankowski (1981), and Perry et al. (1984)]. This reversible phosphorylation is apparently necessary for cross-bridging and contraction in smooth muscle as well as in the generalized motility systems of non-muscle cells. Light chain phosphorylation stimulates chicken gizzard actomyosin adenosinetriphosphatase activity (Sobieszek & Small, 1977) and assembly of gizzard myosin into bipolar filaments (Suzuki et al., 1978). Tension development correlates with myosin phosphorylation in both functionally skinned (Hoar et al., 1979) and intact smooth muscle fibers (deLanerolle & Stull, 1980). A role for light chain phosphorylation in the regulation of actomyosin adenosinetriphosphatase has also been shown for platelets (Adelstein & Conti, 1975), macrophages (Trotter & Adelstein, 1979), lymphocytes (Fechheimer & Cebra, 1982), the brain (Barylko

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