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The Covalent Modification of Myosin's Proteolytic Fragments by a Purine Disulfide Analog of Adenosine Triphosphate. Reaction at a Binding Site Other than the Active Site†

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ABSTRACT: A purine disulfide analog of ATP, 6,6′-dithiobis(inosinyl imidodiphosphate), forms mixed disulfide bonds between the 6 thiol group on the purine ring and certain key cysteines on myosin, heavy meromyosin, and subfragment one. The EDTA ATPase activities of myosin and heavy meromyosin were completely inactivated when 4 mol of thiopurine nucleotide was bound. When similarly inactivated, subfragment one, depending on its method of preparation, incorporated either 1 or 2 mol of thiopurine nucleotide. Modification of a single cysteine on subfragment one resulted in an inhibition of both the Ca^{2+} and the EDTA ATPase activities, but the latter always to a greater extent. Modification of two cysteines per head of heavy meromyosin had the same effect suggesting that the active sites were not blocked by the thiopurine nucleotides. Direct evidence for this suggestion was provided by equilibrium dialysis experiments. Heavy meromyosin and subfragment one bound 1.9 and 0.8 mol of [8-^{3}H]adenylyl imidodiphosphate per mol of enzyme, respectively, with an average dissociation constant of 5×10^{-7} *M*. Heavy meromyosin with four thiopurine nucleotides bound or subfragment one with two thiopurine nucleotides bound retained 65–80% of these tight adenylyl imidodiphosphate binding sites confirming the above suggestion. Thus previous work assuming reaction of thiopurine nucleotide analogs at the active site of myosin must be reevaluated. Ultracentrifugation studies showed that heavy meromyosin which had incorporated four thiopurine nucleotides did not bind to F-actin while subfragment one with one thiopurine nucleotide bound interacted only very weakly with F-actin. Thus reaction of 6,6′-dithiobis(inosinyl imidodiphosphate) at nucleotide binding sites other than the active sites reduces the rate of ATP hydrolysis and inhibits actin binding. It is suggested that these second sites may function as regulatory sites on myosin.

The role of ATP in contraction and relaxation of muscle remains an area of active investigation despite literally hundreds of papers on this subject. ATP appears to play a dual role in these reactions. In the presence of micromolar concentrations of Ca^{2+}, its hydrolysis is the immediate source of energy for movement. In the absence of Ca^{2+}, ATP acts to dissociate actin and myosin allowing myofibrils to relax. The ability to label ATP binding sites covalently with an ATP-like molecule should allow one to better define these two functions of ATP.

Recently we have reported on the stoichiometry and sub-

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unit location of the reaction of a purine disulfide analog of ATP, S_2P-PNP,[1] with myosin and its proteolytic fragments (Wagner and Yount, 1975a,b). Because the reaction of 1 mol of S_2P-PNP with 1 mol of subfragment one prepared by trypsin hydrolysis of HMM, (T)SF_1, inactivated both the Ca^{2+} and EDTA ATPase activities, the site of reaction was assumed to be the active site. However, careful measurement of the above two activities as a function of S_2P-PNP labeling showed that the EDTA ATPase activity was always lower than the Ca^{2+} ATPase activity. This suggested that S_2P-PNP was reacting not at the active site but at a different site which, when blocked, modified the rate of ATP cleavage. Accordingly, binding studies of [8-^{3}H]AMP-PNP, a known competitive inhibitor of ATP hydrolysis by myosin (Yount et al., 1971b), to both S_2P-PNP inactivated HMM and SF_1 were performed. Surprisingly, both HMM-SP-PNP and SF_1-SP-PNP bound AMP-PNP with the same affinity and with essentially the same stoichiometry as did the unmodified enzymes. Of additional interest is the observation reported here that HMM-SP-PNP and SF_1-SP-PNP no longer bind to actin. Thus, reaction of an ATP-like molecule at a site other than the active site markedly decreases both ATP hydrolysis and actin binding. The properties of this second site are consistent with it having a regulatory function.

Materials and Methods

AMP-PNP and S_2P-PNP were synthesized and characterized as described previously (Yount et al., 1971a, 1972). The purity of [8-^{3}H]AMP-PNP, purchased from ICN, was checked by ascending paper chromatography (Whatman 31 ET) using two different solvent systems: isobutyric acid–concentrated NH_4OH–H_2O, 66:1:33 and 0.1 *M* sodium phosphate (pH 6.8)–$(NH_4)_2SO_4$–1-propanol, 100:60:2. After development the chromatograms were air dried, cut into sections, and counted in 15 ml of Bray's solution (Bray, 1960). The [8-^{3}H]AMP-PNP (>95% pure) was diluted with unlabeled AMP-PNP (>96% pure) to give a specific activity of 8.9×10^{12} cpm/mol of nucleotide. Sodium [^{14}C]cyanide (Amersham/Searle) was used with the storage precautions given previously (Wagner and Yount, 1975a). Papain, trypsin, and soybean trypsin inhibitor were purchased from Worthington; ATP was from PL Biochemicals. All other reagents were of analytical grade.

Myosin and HMM were prepared as described previously (Wagner and Yount, 1975a). HMM used in the binding experiments with [8-^{3}H]AMP-PNP was further purified on a DEAE-cellulose column (Schliselfeld and Kaldor, 1973). (T)SF_1 used in the actin binding experiments was prepared from HMM (Nauss et al., 1969). (P)SF_1 used in the AMP-PNP binding studies was prepared by a papain digestion of myosin and purified by $(NH_4)_2SO_4$ fractionation (Margossian and Lowey, 1973). Actin (Regenstein and Szent-Gyorgyi, 1975) was extracted by gently stirring 1 g of acetone dried muscle powder in 25 ml of CO_2 free water for 10 min at 0°. The suspension was filtered using a Buchner funnel and the filtrate centrifuged at 80000*g* for 30 min at 4°. The supernatant containing G-actin was brought to 0.7 m*M* $MgCl_2$ and 1 m*M* Tes (pH 7.0) and allowed to stand for 2 hr at room temperature. The F-actin was subsequently collected by centrifugation at 80000*g* for 3 hr at 4° and stored as a pellet at 0–4° for up to 1 week. This preparation gave a single band on dodecyl sulfate acrylamide gels (Weber and Osborn, 1969). The molecular weights used were 3.5×10^5 for HMM (Mueller, 1964), 1.15×10^5 for SF_1 (Lowey et al., 1969), and 4.2×10^4 for G-actin (Elzinga et al., 1973).

The inactivation of HMM and SF_1 by S_2P-PNP has been described in detail (Yount et al., 1972; Wagner and Yount, 1975a). Conditions used for the inactivation were: 10 m*M* bicine (pH 8.0 at 0°), 50 m*M* KCl, 1 m*M* $MgCl_2$, 100 μM S_2P-PNP, and 5 μM HMM or 10 μM SF_1. After 3 hr at 0° less than 10% of the NH_4^+EDTA ATPase activity remained. Modification of four cysteines results in the complete inactivation of HMM under the above conditions. (T)SF_1 incorporates one thiopurine nucleotide for complete inactivation while (P)SF_1 requires reaction with two thiopurine nucleotides for complete inactivation (Wagner and Yount, 1975a).

As was shown previously cyanide stoichiometrically displaces the thiopurine nucleotides from HMM and SF_1 to give thiocyanato derivatives (Wagner and Yount, 1975a), referred to as HMM-$(SCN)_n$ and SF_1-$(SCN)_n$ where *n* equals the number of moles of thiocyanate per mole of enzyme. When the stoichiometry of the labeling with S_2P-PNP was determined by displacement of the thiopurine nucleotides with [^{14}C]cyanide, HMM and SF_1 were pretreated with unlabeled sodium cyanide. This pretreatment did not affect the ATPase activity but reduced the nonspecific labeling which results from the reaction of cyanide with protein disulfides.

The conditions for the cyanide displacement of the thiopurine nucleotides were: 10 m*M* bicine (pH 8.0 at 0°), 2 m*M* NaCN, and 10 μM HMM-SP-PNP and 20 μM SF_1-SP-PNP. After 16 hr HMM-SCN and SF_1-SCN were precipitated by addition of saturated $(NH_4)_2SO_4$ to 66% and collected by centrifugation at 20000*g* for 10 min. The protein pellets were dissolved in 1 m*M* $MgCl_2$, 0.3 *M* KCl, and 10 m*M* bicine (pH 7.4 at 0°) if they were to be used in binding studies with [8-^{3}H]AMP-PNP. If they were to be used in actin binding experiments the solution used was 1 m*M* $MgCl_2$, 0.1 *M* KCl, 5 m*M* potassium phosphate, and 10 m*M* bicine (pH 7.6). Excess cyanide and $(NH_4)_2SO_4$ were removed either by dialysis or by passage through a Sephadex G-25 column.

The binding of [8-^{3}H]AMP-PNP to HMM and SF_1 was determined by equilibrium dialysis using two sizes of Lucite cells designed after those of Myer and Schellman (1962). For most of the binding experiments each half-cell was 2.0 cm in diameter and 0.5 cm deep with a maximum volume of 1.5 ml. When a more rapid attainment of equilibrium was desired, smaller cells, 1.4 cm in diameter and 0.2 cm deep with a maximum volume of 0.3 ml, were used. Dialysis tubing was boiled three times in 1 m*M* EDTA for 10 min, stored in 0.1 m*M* EDTA, and rinsed with distilled water immediately prior to use. Tubing used with the small dialysis cells was stretched to increase its porosity (Craig and King, 1962). The dialysis buffer used was 1.0 m*M* $MgCl_2$, 0.3 *M* KCl, and 10 m*M* bicine (pH 7.4 at 4°). To one side of each cell was added either 1.2 or 0.25 ml of HMM (approximately 1 μM) or SF_1 (approximately 2 μM) and varying concentrations of [8-^{3}H]AMP-PNP (10^{-7} to 10^{-5} *M*).

[1] Abbreviations used are: S_2P-PNP, 6,6′-dithiobis(inosinyl imidodiphosphate); SH-TP, 6-thioinosine triphosphate; AMP-PNP, adenylyl imidodiphosphate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; bicine, *N,N*-bis(2-hydroxyethyl)glycine; (T)SF_1 and (P)SF_1, subfragment one prepared by trypsin digestion of heavy meromyosin and papain treatment of myosin, respectively; HMM, heavy meromyosin; HMM-$(SP\text{-}PNP)_n$ and $SF_1(SP\text{-}PNP)_n$, heavy meromyosin and subfragment one labeled with "*n*" moles of thiopurine nucleotide.

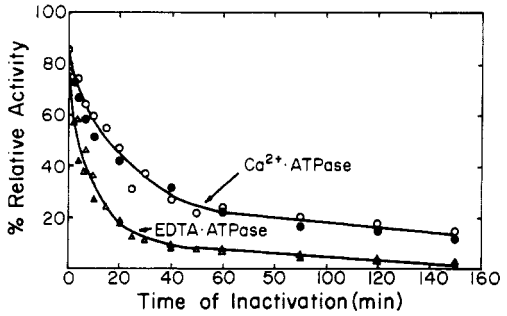

FIGURE 1: Comparison of the rates of inactivation of the Ca^{2+} and NH_4^+EDTA ATPase activities of HMM and (T)SF_1 by S_2P-PNP. Conditions for the inactivation of HMM were: 1.0 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* bicine (pH 8.0 at 0°), 5 μM HMM, and 100 μM S_2P-PNP. The conditions for the inactivation of (T)SF_1 were the same except the (T)SF_1 was 7 μM and the S_2P-PNP was 70 μM. At various times 2-ml samples were quenched by adding 19 ml of 74% saturated $(NH_4)_2SO_4$ and centrifuging at 23500*g* for 10 min. The resulting protein pellets were dissolved in 3 ml of 10 m*M* bicine (pH 8.0) and their Ca^{2+} and EDTA ATPase activities determined. (○) HMM Ca^{2+} ATPase activity; (●) SF_1 Ca^{2+} ATPase; (△) HMM NH_4^+EDTA ATPase activity; (▲) SF_1 NH_4^+EDTA ATPase activity.

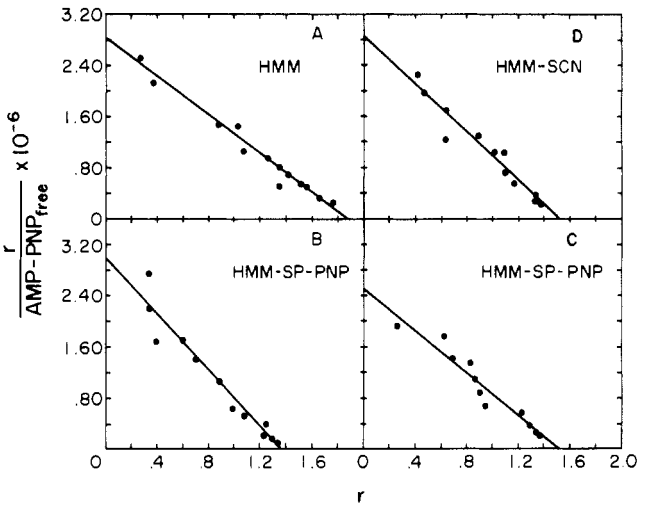

FIGURE 2: Scatchard plots for the binding of [8-^{3}H]AMP-PNP to HMM and its derivatives. Conditions for the equilibrium dialysis were: 10 m*M* bicine (pH 7.4 at 4°), 1.0 m*M* $MgCl_2$, 0.3 *M* KCl, 1.0 μM HMM, HMM-SP-PNP, or HMM-SCN and 10^{-7} to 10^{-5} *M* [8-^{3}H]AMP-PNP. Dialysis time: A, B, and D, 24 hr; C, 5 hr with the small cells. In experiment C, the HMM-SP-PNP was desalted using a Sephadex G-25 column equilibrated in the above buffer.

To the other side was added an equivalent concentration of [8-^{3}H]AMP-PNP in dialysis buffer. Dialysis was carried out for 5 hr (small cells) or 22 hr (large cells) at 4° on a rotary shaker. At equilibrium, the concentration of [8-^{3}H]AMP-PNP on both sides of the membrane was determined by counting 0.5 ml (or 0.1 ml) samples in 5 ml of Aquasol (New England Nuclear). Sufficient counts were taken to give less than 1.0% error. Efficiency of counting was determined by the internal standard technique. Protein concentrations (see below) were redetermined after dialysis.

Binding was analyzed by the following equation (Scatchard, 1949):

$$r/\text{AMP-PNP}_{\text{free}} = nK - rK$$

where $\text{AMP-PNP}_{\text{free}}$ is the concentration of unbound AMP-PNP, r is the number of moles of AMP-PNP bound per mole of enzyme, n is the total number of equivalent binding sites per protein molecule, and K is the apparent association constant. The data were analyzed with a least-squares program on a Wang 700 C programmable calculator to give the best straight line.

The extent of actin binding was determined by centrifugation in a preparative ultracentrifuge (Tawada, 1969; Takeuchi and Tonomura, 1971). HMM, SF_1, and their derivatives were dialyzed overnight at 4° against 1.0 m*M* $MgCl_2$, 0.1 *M* KCl, 5 m*M* potassium phosphate, and 10 m*M* bicine (pH 7.6 at 20°). The phosphate was included to keep F-actin polymerized (Eisenberg et al., 1972). The solutions were clarified by centrifugation at 120000*g* for 90 min at 4°, mixed with F-actin, and incubated for 30 min at room temperature. After centrifugation at 120000*g* for 90 min at 15–20°, the absorbance of the supernatants at 280 nm was determined. This absorbance, after correction for absorbance from centrifuged control solutions containing F-actin alone, was used as a measure of the enzyme which did not bind to actin. Small corrections (<5%) were necessary to correct for some HMM (SF_1) sedimentation in the absence of actin.

Ca^{2+} and NH_4^+ EDTA ATPase assays and protein concentrations (normally determined by a modified microbiuret) were performed as described previously (Wagner and Yount, 1975a). Since the microbiuret color is read at 310 nm, it was necessary when determining the concentrations of HMM-SP-PNP and SF_1-SP-PNP to subtract the thiopurine absorbance. The protein concentration determined this way was within 5% of the protein concentration determined using the standard biuret method at 550 nm (Gornall et al., 1949). In experiments using the small dialysis cells, protein concentrations were measured using a more sensitive fluorescamine fluorescent assay (Bohlen et al., 1973).

Results

Comparison of Ca^{2+} and EDTA ATPase Activities. Yount et al. (1972) reported that the rates of inactivation of HMM Ca^{2+} and EDTA ATPase activities by S_2P-PNP were identical. However, when (T)SF_1 was used, S_2P-PNP inactivated the EDTA ATPase activity to a greater extent than it did the Ca^{2+} ATPase activity (Wagner and Yount, 1975a). Since only a single cysteine is modified on (T)SF_1, it was thought that this difference in ATPase activities might not be seen when two cysteines per head were modified as found in HMM reactions. Figure 1 shows a comparison of the inactivation of (T)SF_1 and the inactivation of HMM in which four cysteines were modified. In both cases the Ca^{2+} and the EDTA ATPase activities were inhibited in a parallel manner but the latter at all times to a greater extent. These results suggested that S_2P-PNP might not be reacting at the active site but rather at a second bonding site which affects the ATPase activity.

Binding Studies of [8-^{3}H]AMP-PNP to HMM, HMM-SP-PNP, and HMM-SCN. To test if S_2P-PNP blocked the active sites of myosin, the binding of AMP-PNP to HMM, HMM-SP-PNP, and HMM-SCN was examined. AMP-PNP is a known potent competitive inhibitor of HMM ATPase (Yount et al., 1971b) and, therefore, can be used to determine the number of ATP binding sites and their association constants. Figure 2A shows a Scatchard plot for the binding of [8-^{3}H]AMP-PNP to HMM. HMM bound a total of 1.9 mol of AMP-PNP with an association constant of 1.5×10^6 M^{-1} (Table I) in good agreement with the values obtained by Schliselfeld (1974).

Figure 2B shows the results of one 22-hr equilibrium di-

Table I: Association Constants for HMM, SF_1, and Their Derivatives.[a]

	No. of Experiments	$K \times 10^{-6}$[d]	n[d]
HMM	2	1.50 ± 0.02	1.90 ± 0.03
HMM–SP-PNP[b]	2	2.30 ± 0.20	1.40 ± 0.05
HMM–SP-PNP[c]	1	1.65	1.52
HMM–SCN	2	1.68 ± 0.20	1.54 ± 0.02
SF_1	2	1.84 ± 0.30	0.82 ± 0.03
SF_1–SP-PNP	2	2.83 ± 0.50	0.53 ± 0.03
SF_1–SCN	3	2.40 ± 0.60	0.52 ± 0.03

[a] Buffer used was 10 mM Tris (pH 7.4) at 4°, 1 mM $MgCl_2$, and 0.30 M KCl. [b] 22-hr dialysis. [c] 5-hr dialysis. [d] Average ± average deviation.

alysis experiment for [8-^{3}H]AMP-PNP binding to HMM-SP-PNP. In this particular experiment 1 mol of HMM-$(SP\text{-}PNP)_{4.15}$ bound a maximum of 1.36 mol of AMP-PNP with an association constant of 2.2×10^6 M^{-1}. To verify that no thiopurine nucleotides had been lost during the dialysis, the remaining HMM-SP-PNP was pooled and treated with [^{14}C]cyanide. There was no significant loss of thiopurine nucleotides as 3.64 thiopurine nucleotides were still bound per mole of HMM. In addition, the EDTA ATPase activity remained 95% inhibited. The total number of [8-^{3}H]AMP-PNP bound by HMM-SP-PNP varied from 1.2 to 1.6 for five different HMM-SP-PNP preparations. However, the association constant was always within experimental error equal to that for unmodified HMM.

The length of time between stopping the S_2P-PNP inactivation and completion of the equilibrium dialysis experiments was approximately 48 hr. During this time the thiopurine nucleotides could have moved from the active sites by disulfide exchange allowing AMP-PNP to bind. For this reason smaller cells with shorter equilibrium times (<3 hr) were used. Figure 2C shows that even when the total time was reduced to 7 hr, HMM-SP-PNP still bound 1.52 mol of AMP-PNP with an association constant of 1.65×10^6 M^{-1}.

Figure 2D shows the results of AMP-PNP binding studies with HMM-SCN. The observed association constant was 1.88×10^6 M^{-1} and n was 1.52. HMM-SCN, like HMM-SP-PNP, appears to have lost part of the AMP-PNP binding sites. The most likely explanation for this decrease is the loss of some sites by denaturation.

Binding Studies of [8-^{3}H]AMP-PNP to SF_1. The results obtained with HMM were verified with experiments using (P)SF_1. (P)SF_1 was used since two cysteines per mole of this preparation are modified by S_2P-PNP. Thus, if either sulfhydryl modified was at the active site, no AMP-PNP should bind. [8-^{3}H]AMP-PNP binding to (P)SF_1 gave results analogous to those obtained with HMM (Figure 3 and Table I). Approximately 0.80 mol of AMP-PNP bound per mol of unmodified SF_1 with an association constant of 2.1×10^6 M^{-1}. (P)SF_1-$(SP\text{-}PNP)_{1.9}$ which retained less than 5% of the EDTA ATPase activity of unmodified (P)SF_1 bound 0.55 mol of AMP-PNP with an association constant of 2.3×10^6 M^{-1}. (P)SF_1–SCN gave results very similar to that obtained for (P)SF_1–SP-PNP: 0.51 AMP-PNP binding site with an association constant of 2.3×10^6 M^{-1}. Again, the 30% decrease in binding sites is attributed to denaturation of the modified (P)SF_1.

Actin Binding Studies. In the absence of added nucleotides HMM and SF_1 bind tightly to actin, $K > 10^6$ M^{-1} (Eisenberg et al., 1972; Margossian and Lowey, 1973). ATP and other nucleotides will dissociate these complexes (see Weber and Murray, 1973, for a review of these effects). Since S_2P-PNP is thought to react at nucleotide binding sites, the effect of this covalent modification on actin binding was examined. Preliminary experiments using a Model E analytical ultracentrifuge showed that only about 20% of HMM-SP-PNP bound to F-actin under conditions in which all of the HMM and most of the HMM-SCN were bound. To obtain a better understanding of this loss in actin binding, the F-actin concentration was varied. In the absence of scanning uv optics, the extent of actin binding was quantified using a preparative ultracentrifuge technique. As shown in Figure 4 over 80% of HMM bound to F-actin when the molar ratio of actin to HMM exceeded 3. HMM-SCN also binds to F-actin but with a weaker affinity as only 60% was bound at a molar ratio of actin to HMM-SCN of 7:1. However, at this ratio of actin to

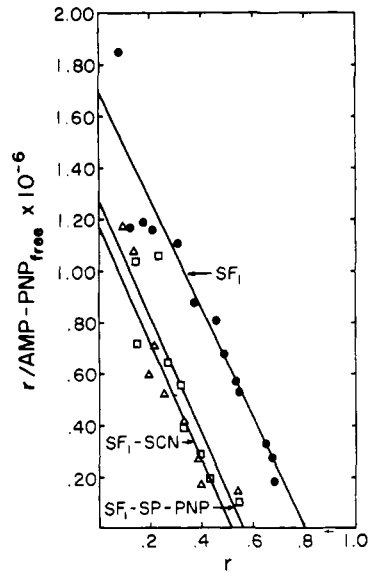

FIGURE 3: Scatchard plots for the binding of [8-^{3}H]AMP-PNP to (P)SF_1 and its derivatives. The conditions were the same as given in Figure 2 except (P)SF_1, (P)SF_1-SP-PNP, or (P)SF_1-SCN were approximately 2 μM. All dialysis were carried out for 24 hr. (●) (P)SF_1; (□) (P)SF_1-SP-PNP; and (△) (P)SF_1-SCN.

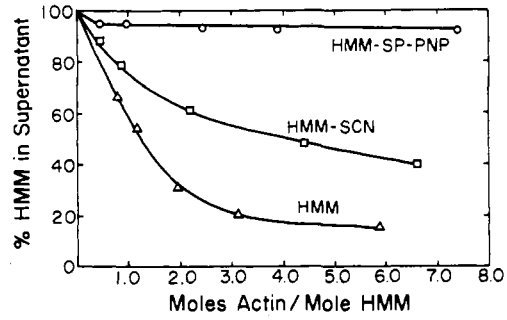

FIGURE 4: Binding of HMM and its derivatives to F-actin. Conditions were: 1.0 mM $MgCl_2$, 0.1 M KCl, 5 mM potassium phosphate, 10 mM bicine (pH 7.6 at 20°), approximately 1.0 mg/ml of HMM, HMM-SP-PNP, or HMM-SP-PNP, and 0.1–1.0 mg/ml of F-actin. The samples were centrifuged at 120000g for 90 min. The concentration of HMM remaining in the supernatant was determined as described under Materials and Methods. (△) HMM; (○) HMM-SP-PNP; and (□) HMM-SCN.

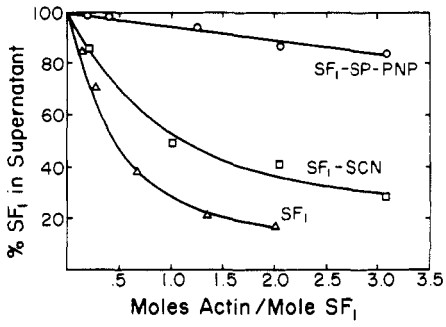

FIGURE 5: Binding of T(SF_1) and its derivatives to F-actin. The conditions were the same as described in Figure 4. (△) SF_1; (○) (T)SF_1-SP-PNP; and (□) SF_1-SCN.

HMM-SP-PNP, only 5% of the HMM-SP-PNP bound to the F-actin.

Since (T)SF_1 incorporates only one thiopurine nucleotide, it was possible to examine the effect of blocking a single cysteine per head on actin binding. As shown in Figure 5, SF_1 bound to F-actin, although somewhat more weakly than did HMM. This difference has been observed previously (Margossian and Lowey, 1973). Only 17% of the (T)SF_1-$(SP\text{-}PNP)_{1.01}$ bound to actin even at a molar ratio of actin to SF_1-SP-PNP of 3:1. SF_1-SCN bound to F-actin but as with HMM-SCN with a lower affinity than unmodified SF_1. These results indicated that S_2P-PNP reaction with a single cysteine per head at a nucleotide binding site other than the active site markedly reduces actin binding. In addition, the simple modification of the cysteine is not sufficient to give this effect, as SF_1-SCN and HMM-SCN both bind to F-actin although more weakly than unmodified HMM or SF_1

Discussion

Murphy and Morales (1970) showed that 6-thioinosine triphosphate (SH-TP) was hydrolyzed by myosin and induced shortening of myofibrils. From a transitory decrease in the absorbance of SH-TP when mixed with myosin, they calculated that there were two tight binding sites for the thiopurine nucleotide. They also found that high concentrations of SH-TP, 8 m*M*, after long periods of incubation, 48 hr at 0°, inactivated myosin. The inactive enzyme had 2.3 thiopurines covalently bound. Since SH-TP was a substrate for myosin, these covalent modifications were thought to be at the active sites.

To prevent hydrolysis to the diphosphate and thereby hopefully increase reactivity, the β,γ bridge oxygen of SH-TP was replaced with an imido group. Preliminary experiments with this analog, SHP-PNP, demonstrated that the inactivation required the disulfide, S_2P-PNP, rather than SHP-PNP itself (Yount et al., 1972). The mechanism of inactivation was a disulfide exchange between S_2P-PNP and key cysteine residues on myosin, HMM, or SF_1. The rate of inactivation of HMM reached a limiting value as the S_2P-PNP concentration was increased. This kinetic observation showed the analog formed a reversible complex with specific binding sites on HMM prior to a rate-limiting inactivation step. Particularly important was the observation that concentrations of ATP or AMP-PNP sufficient to block the active site increased the rate of inactivation. Even so, the rate of inactivation still reached a limiting value as the S_2P-PNP concentration was increased. Since similar results were obtained with SF_1, it was postulated that there were two interacting nucleotide binding sites per myosin head (Yount et al., 1972). It is possible that the binding sites observed kinetically for S_2P-PNP might not be ATP binding sites but rather hydrophobic pockets in which the two aromatic thiopurine rings bind. However, S_2MP, the thiopurine disulfide analog of AMP, has a K_I 15 times weaker than does S_2P-PNP (Yount et al., 1972). This indicates that the phosphate chain contributes to the affinity of the analog for its binding site.

The stoichiometry of the reaction of S_2P-PNP with HMM and SF_1 was determined by specifically displacing the thiopurine nucleotides with [^{14}C]cyanide (Wagner and Yount, 1975a). In the absence of other nucleotides the rate of uptake of thiopurine nucleotide paralleled the rate of inactivation; complete inactivation of the EDTA ATPase of HMM or myosin resulted from the modification of four cysteines. The addition of AMP-PNP enhanced the rate of reaction at two of the cysteines while the other two reacted at about the same rate as observed in the absence of AMP-PNP. Complete inactivation resulted from blocking only the two most reactive cysteines.

The necessity of blocking only a single cysteine per head was confirmed by experiments with SF_1. (T)SF_1 incorporated only one thiopurine nucleotide when inactivated by S_2P-PNP in the absence of other nucleotides, while (P)SF_1 incorporated two thiopurine nucleotides under identical conditions (Wagner and Yount, 1975a). The observations were consistent with S_2P-PNP reacting at both the active sites and a second set of binding sites. The single cysteine modified on (T)SF_1 was thought to be at the active site, while the two cysteines on HMM which were labeled rapidly in the presence of AMP-PNP would be at the second sites, as the active sites would be blocked by the AMP-PNP. Hence reaction of S_2P-PNP at either type of site would result in inactivation of the ATPase activity. However, a serious discrepancy was presented by a comparison of the Ca^{2+} and EDTA ATPase activities. When (T)SF_1 was inactivated with S_2P-PNP both ATPases were inhibited but the EDTA ATPase always to a greater extent (Wagner and Yount, 1975a). This finding was inconsistent with S_2P-PNP blocking the active site, since if that were the case, both activities should decrease to the same extent. Reaction of S_2P-PNP at a site other than the hydrolytic site could easily account for this discrepancy. Since only a single cysteine is modified on (T)SF_1, it is possible that this modification occurred at the second site rather than at the active site as originally thought. In HMM both the active sites and the second sites were thought to be blocked by S_2P-PNP. As shown in Figure 1, when HMM was inactivated by S_2P-PNP under conditions where four cysteines were modified, this discrepancy persisted; the EDTA ATPase was always inactivated to a greater extent than the Ca^{2+} ATPase. This strongly suggested that neither set of cysteines modified by S_2P-PNP were at the active sites.

To test the possibility that S_2P-PNP was not reacting at the active sites, binding studies with [8-^{3}H]AMP-PNP were performed. AMP-PNP binds reversibly to the active sites; it is a competitive inhibitor of ATP hydrolysis (Yount et al., 1971b) and gives an enhancement of protein fluoresence similar to that produced by ATP (Bagshaw et al., 1974). Binding studies with pyrophosphate (Tonomura and Morita, 1959; Nauss et al., 1969), ADP (Lowey and Luck, 1969), ATP under nonhydrolyzing conditions (Schliselfeld and Barany, 1968), and AMP-PNP (Schliselfeld, 1974) all give one tight binding site per head. The observed associa-

tion constants depend on the analog of ATP used, divalent cation, and temperature.

[8-^{3}H]AMP-PNP bound to HMM and (P)SF_1 under the conditions used in this work with an association constant[2] of 1.5–2.3 × 10^6 M^{-1}. HMM and SF_1 bound AMP-PNP with a molar ratio of approximately 1.9 and 0.80, respectively (Figures 2 and 3; Table I). These results are in good agreement with those obtained by Schliselfeld (1974) for HMM (K_a = 2.5 × 10^6 M^{-1}; n = 2.13). HMM-SP-PNP and (P)SF_1-SP-PNP retained 65–80% of the tight nucleotide binding sites. Since HMM-SCN and SF_1-SCN showed a similar reduction in binding capacity and since the affinity constants were unaffected by these modifications, it is believed that the loss in binding sites results from the presence of denatured protein.

There are three possible explanations as to why the reaction of HMM and SF_1 with S_2P-PNP does not prevent AMP-PNP binding. First, the thiopurine nucleotides might have been lost during dialysis. This is not the case as there were still 3.6 thiopurine nucleotides covalently bound to HMM-SP-PNP after completion of the binding experiment (Figure 2B). Alternatively S_2P-PNP might initially react at the active sites and then undergo disulfide exchange with other cysteines to unblock the active site. In experiments which required 7 hr from the end of the inactivation to completion of the equilibrium dialysis, HMM-SP-PNP bound the same number of AMP-PNP molecules as it did when 48 hr had elapsed (Figure 2B and C). If there was a slow movement of the thiopurine nucleotides out of the active sites, less AMP-PNP would have been able to bind after 7 hr than after 48 hr.

A rapid movement of the thiopurine nucleotides can also be shown to be unlikely. If S_2P-PNP were initially reacting at the active sites and then undergoing rapid disulfide exchange with other cysteines a second S_2P-PNP would have been able to react at the active site. However, prior experiments (Wagner and Yount, 1975a) showed that if HMM was inactivated with S_2P-PNP to give HMM(SP-PNP)$_4$ further addition of S_2P-PNP gave no additional labeling as would be expected if the above hypothesis were true. Since neither a fast nor a slow movement of the thiopurine nucleotides appears to occur, the most likely explanation is that S_2P-PNP does not react at the active sites but rather at some other type of binding sites.[3] It also seems likely that SH-TP (as S_2TP is believed to be the true inactivating species) does not react at the active sites as has been assumed by other workers (Murphy and Morales, 1970; Tokiwa, 1971; Tokiwa and Morales, 1971; Stone, 1973).

In addition to providing the energy for contraction, ATP is also needed for relaxation. In the absence of nucleotide, myosin, HMM, and SF_1 bind to actin with association constants of 10^5–10^6 M^{-1} at low ionic strength (Eisenberg et al., 1972; Margossian and Lowey, 1973). It is well known that ATP and some other polyphosphates will dissociate actomyosin (Weber and Murray, 1973). Since AMP-PNP dissociates actomyosin (Yount et al., 1971b), and relaxes myofibrils (dos Remedios, et al., 1972), the simple binding of nucleotide appears to be sufficient to cause relaxation. Although S_2P-PNP does not react at the active sites, it does react at specific binding sites. Binding of thiopurine nucleotides at these sites might affect actin interaction. As is shown in Figure 4, blocking four cysteines on HMM with S_2P-PNP prevented actin binding. The simple modification of the reactive cysteines cannot account for this effect of the covalently bound thiopurine nucleotides since their displacement with cyanide partially restored actin binding. Modification of 1.01 cysteines on (T)SF_1 drastically reduced the extent of actin binding (Figure 5). Although (T)SF_1-SP-PNP retained only 5% of the control EDTA ATPase activity, 17% bound to F-actin suggesting a weak interaction between (T)SF_1(-SP-PNP)$_1$ and F-actin.

The inability of HMM-SP-PNP to interact with F-actin is consistent with the work of other investigators. dos Remedios et al. (1972) found that S_2TP and S_2P-PNP permanently relaxed myofibers and caused the same fluorescence polarization changes as did ATP in the absence of Ca^{2+}. Tokiwa (1971) using spin-labeled myosin reported that the changes in the electron paramagnetic resonance spectrum induced by F-actin did not occur if the myosin was also modified by SH-TP. Stone (1973), performing similar experiments with spin-labeled HMM, concluded that modification by SH-TP allowed for only a weak interaction with actin.

In summary, S_2P-PNP does not react at the active sites of myosin but rather at a second type of binding sites. Thiopurine nucleotide binding at these sites profoundly affects two of the major properties of myosin, actin binding and ATP hydrolysis. We have suggested (Wagner and Yount, 1975a) that these sites may be regulatory sites, in which ATP binding has effects similar to what is seen after S_2P-PNP labeling. If so, the effects on the active site are unusual in that most allosteric effectors act to increase K_m rather than decrease V_m as seen here. It may be the main function of the site labeled is to regulate myosin-actin interaction rather than ATP cleavage since recent experiments have shown the effects of S_2P-PNP reaction on HMM Mg^{2+} ATPase are much less profound that they are on the Ca^{2+} or EDTA activated ATPase activity (P. Swetik, unpublished results).

Other workers (Eisenberg and Moos, 1965; Kominz and Yoshioka, 1969; Kominz, 1970; Stewart and Levy, 1970) have postulated two sites for ATP action based on studies of the ATP and Ca^{2+} concentration dependence of myofibriller contraction or actomyosin superprecipitation. It may be that S_2P-PNP is, in fact, reacting at one of these sites. It, however, remains to be shown by direct experimentation that ATP binds to this second set of sites and, if so, what in turn modulates its binding to myosin to allow actin interaction to occur.

[2] More recent measurements (P. Wagner and P. Swetik, unpublished results) using column purified [8-^{3}H]AMP-PNP have given association constants closer to 10^7 M^{-1}.

[3] The initial belief on finding four thiopurine nucleotides bound per HMM (Wagner and Yount, 1975a) was that two S_2P-PNP molecules reacted at the active sites and two at the so-called relaxing sites. However, the binding experiments reported here rule out reaction of S_2P-PNP at the active sites. This leaves open the question of the function of the second set of modified cysteines. These cysteines most likely are not at discrete binding sites, but rather are simply more reactive because of conformational changes induced by prior reaction of HMM with S_2P-PNP.

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Cross-Linking of Troponin with Dimethylimido Esters†

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ABSTRACT: The topology of troponin, the calcium binding regulatory protein in muscle, has been studied by cross-linking with different length dimethylimido esters. The results show that the three components of troponin are close to each other and that the troponin-I and -T are preferentially cross-linked being 0.6 nm or less apart. The largest cross-linked product is a complex which corresponds in molecular weight to the native troponin complex of 1 mol of each of the three components. Cross-linked troponin has lost the ability to make the actomyosin ATPase calcium sensitive although it does bind to actin-tropomyosin and tropomyosin, and it binds calcium normally. No effect of calcium on cross-linking could be detected.

Muscle contraction is regulated by the free calcium ion concentration (Heilbrun and Wiercinski, 1947). At low calcium concentrations (less than 10^{-8} *M*), the muscle is relaxed and the myosin cross-bridges on the thick filaments cannot interact with the actin containing thin filaments. At higher calcium ion concentrations (10^{-6} *M* or greater), actin-myosin interaction is allowed and contraction proceeds (Huxley, 1969). In vertebrate striated muscle, troponin, associated with actin and tropomyosin in the thin filaments, binds calcium and regulates contraction (Ebashi and Endo, 1968).

Troponin consists of three different proteins: troponin-T (37000 molecular weight in rabbit) which binds to tropomyosin, troponin-I (24000) which inhibits actin-myosin interaction in the presence and absence of calcium, and troponin-C (17800) which binds calcium (cf. Greaser and Gergely, 1971). The functional troponin which confers calcium-sensitive regulation on the actomyosin ATPase is composed of 1 mol of each of these components (Hartshorne and Driezen, 1973; Potter, 1974). The interactions among these proteins have been studied in many laboratories using a variety of chemical, physical, and structural techniques (see review by Weber and Murray, 1973).

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