Stoichiometry of Labeling of Myosin's Proteolytic Fragments by a Purine Disulfide Analog of Adenosine Triphosphate†

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ABSTRACT: A site-specific analog of ATP, 6,6′-dithiobis-(inosinyl imidodiphosphate) (S_2P-PNP), inactivates the ATPase activities of myosin's proteolytic fragments, heavy meromyosin (HMM) and subfragment one (SF_1), by formation of mixed disulfides between the 6 position of the purine ring and certain key cysteines. The stoichiometry of the reaction was determined by quantitatively displacing the thiopurine nucleotides from the labeled enzymes with sodium [^{14}C]cyanide. The thiocyanatoenzyme formed regained 25% of the original activity showing that the cysteines modified were not essential for catalysis. The rate of uptake of label paralleled the rate of inactivation. HMM was completely inactivated when 4 mol of thiopurine nucleotide was bound. SF_1 made by a papain digestion of myosin incorporated 2 mol of thiopurine nucleotide when completely inactivated. Having adenylyl imidodiphosphate, a reversible competitive inhibitor of myosin's ATPase, present during the inactivation of HMM by S_2P-PNP demonstrated that only one cysteine per head needed to be blocked to inactivate the enzyme. Moreover, SF_1 made by a trypsin digest of HMM was completely inactivated when only 1.1 mol of the thiopurine nucleotide bound again indicating that blocking only a single cysteine per head was sufficient to cause inactivation. This sulfhydryl is thought to be at an ATP binding site distinct from the ATPase site. The properties of this second ATP binding site are consistent with it being an ATP regulatory site.

Little is known about the nature of either the ATPase or actin binding sites of myosin. Evidence is conflicting as to whether these two types of sites are the same or physically distinct (Barany and Barany, 1959; Kiely and Martonosi, 1968, 1969; Nanninga, 1964; Taylor, 1972; Weber and Murray, 1973). If they are distinct, they must be interacting as actin binding affects the ATPase activity of myosin (Eisenberg and Moos, 1968) and ATP under certain conditions prevents the binding of actin to myosin (Gergely, 1956; Takeuchi and Tonomura, 1971). It is also not known if the ATPase sites and the nucleotide binding sites which cause relaxation are the same. AMP-PNP,[1] a nonhydrolyzable competitive inhibitor of ATP hydrolysis, relaxes myofibrils (dos Remedios et al., 1972) and dissociates actomyosin (Yount et al., 1971b) suggesting simple binding of ATP and not its hydrolysis is the cause of relaxation.

Affinity labels are useful tools for investigating the nature of specific sites. Werber et al. (1974) have used Co(III)–ATP complexes to label myosin. These compounds form stable complexes with the enzyme but do not form covalent bonds to it. The only analog of ATP which is known to bind specifically and covalently to myosin is 6-thioinosine triphosphate (SH-TP) (Murphy and Morales, 1970; Tokiwa and Morales, 1971; Tokiwa, 1971; Stone, 1973).

The reactivity of SH-TP was greatly improved by making it nonhydrolyzable, i.e., replacing the β,γ-bridge oxygen with an "NH" group, and by oxidizing the free sulfhydryl to the disulfide (Yount et al., 1972). The structure of this analog, 6,6′-dithiobis(inosinyl imidodiphosphate) (S_2P-PNP), is shown in Figure 1. It irreversibly inactivates heavy meromyosin (HMM) in minutes while SH-TP normally requires 2–4 days (Murphy and Morales, 1970; Stone, 1973). The kinetics of the inactivation of HMM and subfragment one (SF_1) by S_2P-PNP have been described in detail (Yount et al., 1972). These kinetic findings indicate the presence of two interacting ATP binding sites per myosin head.

To understand more fully the action of S_2P-PNP, the stoichiometry of its binding was determined. S_2P-PNP is known to form a mixed disulfide between the 6 position of the purine ring and certain key cysteines of myosin. A potential problem with mixed disulfides of this type is the possibility of disulfide exchange. To reduce this possibility a reaction similar to that employed by Vanaman and Stark (1970) with Nbs_2-inactivated aspartate transcarbamylase was used. These authors found that cyanide stoichiometrically displaced the thionitrobenzoate moiety from the catalytic subunit to give a thiocyanato derivative of the enzyme. We have found an analogous reaction can be performed with the thiopurine nucleotide labeled HMM. The use of [^{14}C]cyanide allowed the stoichiometry of binding to be determined and a more stable derivative to be formed.

This paper describes the use of S_2P-PNP to determine the number of nucleotide binding sites on myosin's proteolytic fragments. Evidence will be given that in addition to the two well-established ATPase sites there are two other nucleotide binding sites on myosin. It will be shown that S_2P-PNP reacts specifically with four sulfhydryls per

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[1] Abbreviations used are: AMP-PNP, adenylyl imidodiphosphate; HMM, heavy meromyosin; SF_1, subfragment one; S_2P-PNP, 6,6′-dithiobis(inosinyl imidodiphosphate); Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); bicine, *N,N*-bis(2-hydroxyethyl)glycine; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; HMM-SP-PNP and SF_1-SP-PNP, the mixed disulfides between 6-thioinosinyl imidodiphosphate and heavy meromyosin and subfragment one, respectively; HMM-SCN and SF_1-SCN, the thiocyanato derivatives of heavy meromyosin and subfragment one, respectively; SCN-PNP, 6-thiocyanatoinosinylyl imidodiphosphate; SHP-PNP, 6-thioinosinyl imidodiphosphate; SH-TP, 6-thioinosine triphosphate; S_2TP, 6,6′-dithiobis(inosine triphosphate).

HMM and that two of these are at nucleotide binding sites distinct from the ATPase sites. Also a difference in the reactivity of SF_1 from a trypsin digestion of HMM and that from a papain digestion of myosin will be reported.

Experimental Section

Materials

S_2P-PNP was prepared by oxidation of SHP-PNP with sodium triiodide and characterized as described previously (Yount et al., 1972). AMP-PNP was prepared and characterized as described by Yount et al. (1971a). Both nucleotides were greater than 95% pure. [^{14}C]NaCN (60 Ci/mol) was purchased from Amersham/Searle and diluted 100-fold with carrier sodium cyanide. It was stored as a frozen solution (~20 m*M*). It was necessary to make up fresh cyanide solutions every 6–8 weeks as decomposition products (initially ammonium formate) gave increasing amounts of nonspecific labeling with time. Papain, trypsin, and soybean trypsin inhibitor were purchased from Worthington, Aquasol was from New England Nuclear, and ATP was from PL Biochemicals.

Myosin was prepared from rabbit skeletal muscle by the method of Kessler and Spicer (1952) except the actomyosin contamination was removed by two separate centrifugations of 0.3 *M* KCl solutions at 14,000*g* instead of by an ATP induced superprecipitation. HMM was prepared as described by Yount and Koshland (1963) except the myosin hydrolysis by trypsin was at pH 8.2 in 0.04 *M* $KHCO_3$. HMM was freeze-dried with an equal weight of sucrose and stored at −20°. SF_1 was prepared both by the digestion of HMM with trypsin (Nauss et al., 1969) and by the digestion of myosin with papain. HMM (15 mg/ml) was digested with trypsin (1 mg/ml) for 10 min at 25° in 0.10 *M* bicine (pH 7.6) and 10^{-5} *M* EDTA. The digestion was stopped by adding soybean trypsin inhibitor to 2 mg/ml. SF_1 was isolated on a G-200 column equilibrated with 10 m*M* bicine (pH 8.0) and 10^{-5} *M* EDTA at 4 °. The second protein peak, SF_1, was collected and concentrated to about 2 mg/ml by pressure filtration with an Amicon/Diaflo apparatus. Papain SF_1 was prepared as described by Margossian and Lowey (1973). Both types of SF_1 were stored at 4° for no longer than 1 week prior to their use.

Methods

ATPase Assay. The activity of HMM and SF_1 were generally measured using an NH_4^+ · EDTA stimulated ATPase assay. The assay conditions were 5.7 m*M* ATP, 0.51 *M* NH_4Cl, 0.034 *M* EDTA, 0.2 *M* KCl, and 0.057 *M* Tris (pH 8.0) at 25°. The enzyme concentration varied from 0.01 to 0.10 mg/ml depending on the relative activity. Samples were removed after 2 and 12 min and analyzed for inorganic phosphate using the method of Rockstein and Herron (1951). The optical density of the reduced phosphomolybdate complex was read at 700 nm using a Gilford 300-N rapid sampling spectrophotometer. Conditions for the Ca^{2+} · ATPase assay were 10 m*M* Ca^{2+}, 0.1 *M* KCl, 5 m*M* ATP, and 0.12 *M* Tris (pH 7.4) at 25°. The protein concentration used was approximately 2 mg/ml. After 2 and 12 min, 1-ml samples were removed and added to 1 ml of cold 10% trichloroacetic acid. After removal of protein by centrifugation, inorganic phosphate liberated in 10 min was determined as described above. The specific activities for the NH_4^+EDTA · ATPase (and Ca^{2+}· ATPase) averaged 2.3 (0.26), 2.8 (0.29), and 1.1 (0.13) mol of P_i per min per mmol of enzyme, respectively, for myosin, HMM, and SF_1.

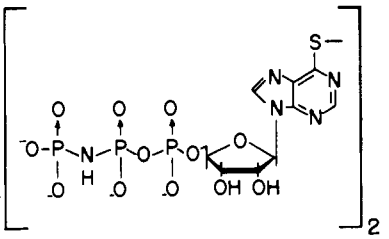

FIGURE 1: Structure of 6,6′-dithiobis(inosinyl imidodiphosphate) (S_2P-PNP).

Reactions with S_2P-PNP and NaCN. HMM and SF_1 were treated with NaCN prior to their inactivation with S_2P-PNP. Without this pretreatment the zero time sample incorporated from 1.5 to 2 mol of [^{14}C]cyanide/mole of HMM. Treating HMM with dithioerythritol also lowered this nonspecific labeling indicating that the cyanide was reacting with protein disulfide groups. The pretreatment with NaCN was overnight in 0.1 *M* KCl and 10 m*M* bicine (pH 8.0) at 0° with a 100- and 200-fold molar excess of cyanide (around 1 m*M*) for SF_1 and HMM, respectively. This pretreatment had no effect on either the Ca^{2+} or the EDTA · ATPase specific activities but did reduce the nonspecific labeling to 0.05–0.30 mol of cyanide/mol of enzyme, depending on the age of the cyanide solution. Excess cyanide was removed by two precipitations of the enzymes in 66% saturated $(NH_4)_2SO_4$ (pH 7.0).

The S_2P-PNP inactivations were done with a 10- and 20-fold molar excess of S_2P-PNP to SF_1 and HMM, respectively. Typically the S_2P-PNP concentration was around 0.1 m*M* and the enzyme concentrations were 4–5 μM for HMM and 8–10 μM for SF_1. The inactivation conditions were 10 m*M* bicine (pH 8), 50 m*M* KCl, and 1 m*M* $MgCl_2$, except where noted. Timed samples were removed over a 3-hr period and the inactivation was quenched by adding 2-ml aliquots to 19 ml of 74% saturated $(NH_4)_2SO_4$ (pH 7.0). The enzyme was pelleted by centrifugation at 23,000*g* (0–4°) for 10 min; the pellet was dissolved in 5 ml of cold water, and the enzyme was reprecipitated with 10 ml of saturated $(NH_4)_2SO_4$ (pH 7.0). After centrifugation as before, the supernatant was removed and the protein pellet dissolved in 3.0 ml of 10 m*M* bicine (pH 8.0) at 0°. Precipitation with $(NH_4)_2SO_4$ is an incomplete quench since some inactivation may occur during the precipitation. It is, however, a useful quench as it allows direct comparisons to be made between the extent of inactivation and the number of nucleotides bound; 1 ml of the protein solutions was used for ATPase assays. The other 2 ml was treated with Na^{14}CN at a 100- and 200-fold molar excess for SF_1 and HMM, respectively. After 2.5 hr at room temperature or 16 hr at 0°, the protein was precipitated by adding 13 ml of 77% saturated $(NH_4)_2SO_4$ and centrifuged at 23,500*g* (0–4°) for 10 min. The precipitate was dissolved in 2.0 ml of 5 m*M* TES (pH 7.0) and 30 m*M* KCl at 4°. The samples were dialyzed against this buffer at 4° for about 50 hr with four to five buffer changes to remove all noncovalently bound cyanide. Early experiments incorporated a "cold cyanide chase" (10 m*M* NaCN for 10 min prior to precipitation with $(NH_4)_2SO_4$) but subsequent experiments proved this to be unnecessary. The ^{14}C-labeled protein samples (0.5 ml) were dissolved in 5.0 ml of Aquasol and counted in minivials in a Beckman LS230 liquid scintillation counter.

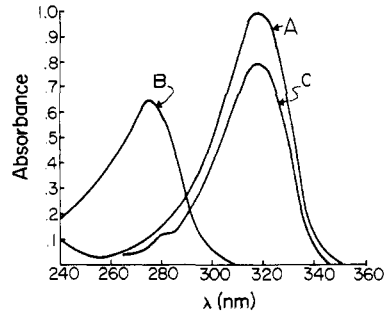

FIGURE 2: Absorption spectra of SHP-PNP, SCN-PNP, and the nucleotide released by the reaction of cyanide with HMM-SP-PNP. The spectra were taken in 66% saturated $(NH_4)_2SO_4$, pH 7.0. (A) SHP-PNP, (B) SCN-PNP, and (C) the supernatant after treatment of HMM-SP-PNP with NaCN and subsequent precipitation of the protein with $(NH_4)_2SO_4$. The conditions for the inactivation of HMM with S_2P-PNP and the displacement of the thiopurine nucleotide with cyanide are given in the Experimental Section.

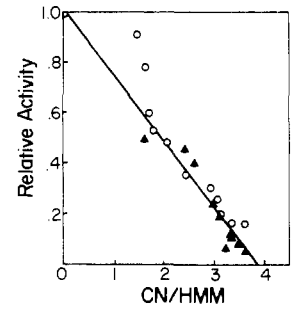

FIGURE 3: Observed ^{14}CN/HMM vs. relative ATPase activity. Conditions for the inactivation were: 10 m*M* bicine, pH 8.0, 50 m*M* KCl, 1 m*M* $MgCl_2$, 5 μM HMM, and 0.1 m*M* S_2P-PNP. Cyanide displacement was at room temperature for 2.5 hr in 10 m*M* bicine, pH 8.0; HMM was 5 μM and the [^{14}C]cyanide was 1.0 m*M*. (○) is for inactivations performed at 0° and (▲) is for inactivations performed at 25°.

The efficiency of counting was determined by the internal standard method.

A modified microbiuret was used for protein determinations using bovine serum albumin as a standard. The concentration of the serum albumin was determined using $\epsilon_{278}(1\%) = 6.6$ (Tanford and Roberts, 1952). To a 1.0-ml sample was added 4.0 ml of a 1:5 dilution of the standard biuret solution (Gornall et al., 1949). After 30 min the absorbance was read at 310 nm. The useful range of protein concentrations was 0.1–1.0 mg/ml. Although this method is more sensitive many common reagents (e.g., sucrose, Tris, and bicine buffers) affect the color yield and care must be taken to run appropriate controls in each case. The molecular weights used were 3.5×10^5 for HMM (Mueller, 1964) and 1.1×10^5 for SF_1 (Nauss et al., 1969).

Results

Cyanide Displacement of the Thiopurine Nucleotide. If the number of cyanides incorporated is to be equated with the number of nucleotides bound, the displacement of the thiopurine by cyanide must be selective and complete. To demonstrate the specificity of the displacement, the type of nucleotide released from thiopurine-labeled HMM by cyanide was determined. HMM was inactivated for 3 hr with S_2P-PNP under standard conditions. All noncovalently bound nucleotide was removed by two precipitations of HMM with $(NH_4)_2SO_4$. HMM-SP-PNP was treated with cyanide and then the protein was precipitated with $(NH_4)_2SO_4$. The absorption spectra of the supernatant was taken. The two possible products were SHP-PNP and SCN-PNP. The absorption spectra of these two compounds and the product of the cyanide treatment are shown in Figure 2. Within the limits of detection, no SCN-PNP was formed (less than 3%). The small peak at 280 nm is due to unprecipitated protein. As expected the cyanide cleaves the disulfide specifically to release the aryl group and to label the enzyme.

Not only must the cyanide displacement be selective, it must also displace all of the thiopurine nucleotide under the conditions used. To establish the reaction time necessary for complete displacement, HMM-SP-PNP was treated with a 200-fold excess of Na^{14}CN for various lengths of time at room temperature in 10 m*M* bicine (pH 8.0) and 50 m*M* KCl. The amount of cyanide incorporated leveled off after 1.5 hr. The thiopurine absorbs strongly at 320 nm when bound to the enzyme (Murphy and Morales, 1970). After

Table I: Effect of Additional S_2P-PNP on the Inactivation and Labeling of HMM.[a]

Time of Inactivation (min)	Control		Excess S_2P-PNP Reaction	
	cpm/mg of HMM	Relative ATPase Activity	cpm/mg of HMM	Relative ATPase Activity
120	11,900	0.15		
150	14,000	0.13	13,800	0.14
160			13,700	0.13
170			13,800	0.13
180	14,200	0.13	14,600	0.11

[a] Standard inactivation conditions (see text) were used. After 145 min, the reaction was divided into two fractions. The first fraction (control) was allowed to continue without further addition of S_2P-PNP. To the second fraction an additional 20-fold excess of S_2P-PNP was added to give a final concentration of 200 μM. Relative ATPase activity measurements and the level of ^{14}C incorporation were performed as described in the Experimental Section.

treatment with cyanide and removal of the liberated SHP-PNP this peak falls to less than 5% of its original value. The remaining absorbance could not be removed by treating either with additional NaCN or with β-mercaptoethanol. Thus, the cyanide displaces at least 95% of the purine analog within 1.5 hr at room temperature.

The thiocyanato derivative of HMM was found to be quite stable below pH 8.0 at 4°. The length of dialysis at pH 7.0 in 5 m*M* TES and 30 m*M* KCl at 4° had no significant effect on the amount of cyanide bound to HMM. Thus, extending the time of dialysis from 60 to 160 hr gave no change in the cyanide to enzyme ratio. The thiocyanato group exchanges very slowly if at all with free cyanide; 1.0 μM HMM-S^{14}CN was treated with 10 m*M* NaCN in 10 m*M* bicine (pH 8.0) at room temperature for various lengths of time up to 90 min. The enzyme was precipitated in 66% saturated $(NH_4)_2SO_4$ (pH 7.0) and dialyzed overnight in 5 m*M* TES and 30 m*M* KCl (pH 7.0) at 4°. Even after 90 min there was no loss of label. However, cyanide was readily liberated when HMM-S^{14}CN was treated with β-mercaptoethanol. After 2.5 hr in 0.4 *M* β-mercaptoethanol at 25° only 3% of the [^{14}C]cyanide remained bound to the HMM. These control experiments show that the number of cyanides bound to HMM are equal to the number of nucleotides bound by the enzyme.

Inactivation and Labeling of HMM by S_2P-PNP. HMM was inactivated at both 25 and 0° with a 20-fold excess of

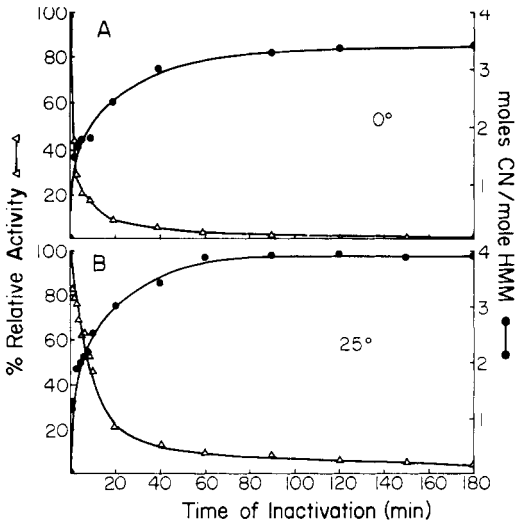

FIGURE 4: Labeling and inactivation of HMM by S_2P-PNP in the presence of 1.5 m*M* AMP-PNP. The conditions for the inactivations were the same as given in Figure 3 except 1.5 m*M* AMP-PNP was present during the inactivation and the $MgCl_2$ concentration was raised to 3 m*M*. (A) Inactivation at 0° and (B) at 25°; (△) the relative NH_4^+ EDTA·ATPase activity; (●) the observed ^{14}CN/HMM.

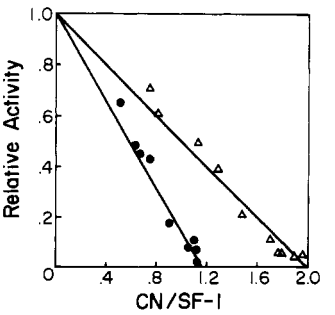

FIGURE 5: Observed $^{14}CN/SF_1$ vs. relative ATPase activity. Conditions for the inactivation were: 10 m*M* bicine (pH 8.0) at 0°, 50 m*M* KCl, 1 m*M* $MgCl_2$, 10 μM SF_1, and 0.1 m*M* S_2P-PNP. The cyanide displacement was at room temperature for 2.5 hr in 10 m*M* bicine (pH 8.0): SF_1 was 10 μM and the [^{14}C]cyanide was 1.0 m*M*. (●) Trypsin SF_1 and (△) papain SF_1.

S_2P-PNP. At either temperature the time course of inactivation and incorporation of cyanide proceeded in a parallel manner. The activity was based on NH_4^+ · EDTA stimulated ATPase but as reported previously (Yount et al., 1972) the Ca^{2+} activated ATPase gave essentially the same result. At both temperatures 3.6 ± 0.2 cyanides were bound after 3.0 hr of inactivation. When the enzyme was 50% active 2 mol of cyanide were bound per mol of HMM. A plot of the observed relative activity vs. the observed moles of cyanide per mole of HMM (Figure 3) gives a straight line with a ratio of 3.85 ^{14}CN/HMM for zero activity.

To demonstrate that the leveling off in incorporation of cyanide was due to loss of reactive sites and not to a depletion of S_2P-PNP, additional S_2P-PNP was added to inactivated HMM. The inactivation of HMM was allowed to proceed for 145 min with a 20-fold excess of S_2P-PNP at 0°. At this time the enzyme was 13% active. To half of the solution an additional 20-fold excess of S_2P-PNP was added. Samples were removed every 10 min for the next half hour. As shown in Table I this addition did not change either the subsequent rate of inactivation or the amount of nucleotide bound.

Effect of AMP-PNP on the Inactivation and Labeling of HMM by S_2P-PNP. AMP-PNP, a potent competitive inhibitor of HMM-ATPase, did not protect HMM from S_2P-PNP at 0° but rather enhanced the rate of inactivation. Since the rate of inactivation approached a limiting value as the S_2P-PNP concentration was increased even in the presence of AMP-PNP, it was postulated there were interacting ATP binding sites. It was hoped that by going to high AMP-PNP concentrations it would be possible to protect two of the sulfhydryls from reacting with S_2P-PNP. Figure 4A shows the effect of 1.5 m*M* AMP-PNP on the labeling pattern of HMM at 0°. Even at this high level of AMP-PNP the rate of inactivation was increased. The total number of cyanides incorporated after 3 hr went down to 3.2 in the presence of AMP-PNP from the value of 3.8 in its absence. The labeling pattern on the whole is significantly altered. Two thiopurine nucleotides appear to be incorporated very quickly, the remainder reacting more slowly; 50% inactivation corresponds to only 1.1 mol of thiopurine nucleotide incorporation indicating that blocking a single set of sulfhydryls is sufficient to inactivate HMM. Although little if any protection was afforded by 1.5 m*M* AMP-PNP at 0°, it was possible to distinguish two types of sulfhydryls.

At 25°, high concentrations of AMP-PNP do not enhance the rate of inactivation but rather slow it (Frye, O'Keefe and Yount, submitted for publication). As shown in Figure 4B, the labeling pattern for HMM inactivated by S_2P-PNP at 25° in the presence of 1.5 m*M* AMP-PNP is very similar to that in its absence. The enzyme was 4% active after incorporating 3.95 [^{14}C]cyanides. A little over 2 mol of nucleotide was bound to give 50% inactivation.

Effect of Pyrophosphate on the Inactivation and Labeling of HMM by S_2P-PNP. Kinetic investigations have shown that the rate of inactivation can be slowed by adding either pyrophosphate or tripolyphosphate (Frye, O'Keefe and Yount, submitted for publication). When these are added to the inactivation mixture even in large excess (up to 20 m*M* for tripolyphosphate), the labeling pattern is the same as that obtained after incorporation with S_2P-PNP alone, incorporating about 3.6 mol of cyanide after 3 hr and being 50% active after covalently binding two nucleotides.

Inactivation and Labeling of SF_1 by S_2P-PNP. Figure 5 shows the inactivation of SF_1 made both by trypsin digestion of HMM and papain digestion of myosin with a tenfold excess of S_2P-PNP at 0° over a three-hr period. The rate of uptake of label paralleled the rate of inactivation for both types of enzyme preparation. Surprisingly, trypsin SF_1 did not incorporate 2 mol of cyanide as expected from the HMM results, but rather only 1.1 ± 0.1 mol. When plotted as relative activity vs. CN/SF_1, the intercept for zero activity corresponded to 0.97 and 1.17 CN/SF_1 for two separate experiments. Figure 5 gives the data for the latter trial. In contrast, when SF_1 made by papain digestion was used the labeling results were quite different. As shown in Figure 5 when papain SF_1 is inactivated by S_2P-PNP at 0°, 2 mol of nucleotide are bound per mol of enzyme, with 1 mol of cyanide corresponding to 50% inactivation.

Location of [^{14}C]cyanide Label in HMM. The apparent loss of one set of sulfhydryls from trypsin SF_1 could be explained in two possible ways: either (i) the second sulfhydryl was in a portion of HMM removed during the digestion, or (ii) that in converting HMM to SF_1 the conformation around the second sulfhydryl is altered to such an extent that although still present in SF_1 it is much less reactive with S_2P-PNP. The following experiment was performed to distinguish between these two possibilities. HMM was labeled with S_2P-PNP under standard condi-

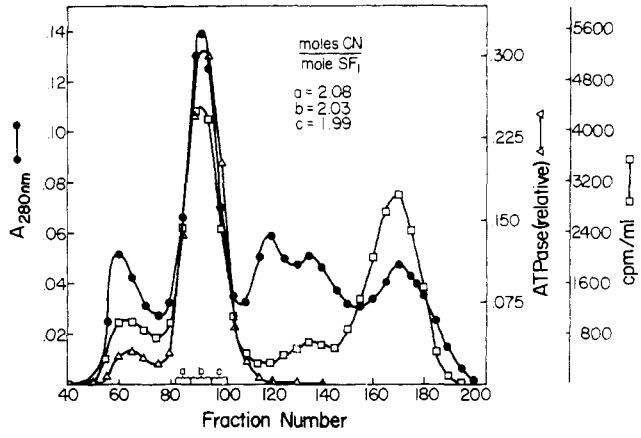

FIGURE 6: Sephadex G-200 separation of a trypsin digest of HMM-S^{14}CN. HMM-S^{14}CN (10 mg/ml) was digested with trypsin (0.66 mg/ml) for 8 min at 25° in 0.1 *M* bicine and 10^{-5} *M* EDTA (pH 7.6). The digestion was stopped by the addition of soybean trypsin inhibitor (1.3 mg/ml) and lowering the temperature to 0°. The G-200 column (5 × 80 cm) was equilibrated and the sample eluted with 10 m*M* Tris and 10^{-5} *M* EDTA (pH 7.0) at 4°. The flow rate was 1 ml/min. The fractions were 8 ml: (●) absorbance at 280 nm; (△) relative ATPase activity; (□) cpm/ml.

tions at 0°. To minimize denaturation, the [^{14}C]cyanide displacement was done at 4° for 18 hr instead of at room temperature for 2.5 hr. Control experiments showed that treatment in the cold for this length of time resulted in the same level of cyanide incorporation as did the room temperature treatment. All nonbound cyanide was removed by desalting on a G-25 column equilibrated in 30 m*M* KCl and 5 m*M* TES (pH 7.0) at 0°. The protein peak was pooled and concentrated to 10 mg/ml using an Amicon/Diaflo apparatus. The HMM-S^{14}CN was digested with trypsin as described in the Experimental Section at 25° for 10 min. The digestion was stopped by adding soybean trypsin inhibitor. The reaction mixture was placed on a G-200 column equilibrated with 10^{-5} *M* EDTA and 10 m*M* Tris (pH 7.0) at 4°. The elution profile (Figure 6) is the same as for an unlabeled sample. The first peak is mainly undigested HMM and a small amount of rods (Takeuchi and Tonomura, 1971). The second peak is SF_1, the third the trypsin–trypsin inhibitor complex, and the final two, proteolytic fragments. Radioactivity was in all peaks except the trypsin–trypsin inhibitor peak. The thiocyanato derivatives possess about 25% of the EDTA · ATPase activity of unmodified HMM or SF_1. The peak tubes containing HMM were pooled and concentrated. The ratio of cyanide to HMM was found to be 3.6. The leading edge, peak, and trailing edge of SF_1 region were each pooled separately and concentrated. They had a CN/SF_1 ratio of 2.08, 2.03, and 1.99, respectively, demonstrating the presence of both types of sulfhydryls in trypsin SF_1. This result indicates that the conformation around one of the reactive sulfhydryls in trypsin SF_1 is different than it is in HMM or papain SF_1.

Effect of AMP-PNP on Labeling Trypsin SF_1. When trypsin SF_1 was labeled with a tenfold excess of S_2P-PNP in the presence of 50 μM AMP-PNP at 0°, the total number of cyanides incorporated reaches 1.7 CN/SF_1 after 3 hr as opposed to only 1.1 without the addition of any AMP-PNP. When 1.5 m*M* AMP-PNP was present during the inactivation the total number of cyanides incorporated levels of ± 1.5 CN/SF_1. This observation is consistent with previous kinetic experiments which showed that AMP-PNP enhanced the rate of inactivation of both SF_1 and HMM. The incorporation of nucleotide was biphasc. The first nucleotide bound inactivated SF_1, the rest going on after the inactivation was complete.

Comparison of the Ca^{+2}- and EDTA · ATPase of SF_1 when Inactivated by S_2P-PNP. As noted previously (Yount et al., 1972) when HMM was inactivated by S_2P-PNP it showed a parallel loss in both the NH_4^+ · EDTA and Ca^{2+} · ATPases. Since trypsin SF_1 when inactivated by S_2P-PNP incorporated only one nucleotide, it was of interest to examine what effect this had on the Ca^{2+} activity. SF_1 was inactivated under standard conditions and timed samples precipitated with $(NH_4)_2SO_4$ as described in the Experimental Section. These precipitations were necessary to remove residual Mg^{2+} which would otherwise interfere with the Ca^{2+} · ATPase assays. As was found for HMM, the Ca^{2+}- and the EDTA · ATPases activities of SF_1 declined in a similar fashion. However, at all times the relative activity of the EDTA · ATPase was lower than that of the Ca^{2+} · ATPase. For instance, after 3 hr only 3% of the EDTA · ATPase activity remained while 12% of the Ca^{2+} · ATPase activity remained.

Discussion

The cleavage of organic disulfides by cyanide has been widely studied. Catsimpoolas and Wood (1966) have used cyanide to cleave cystine to yield cysteine and thiocyanoalanine. Under milder conditions, cyanide will stoichiometrically displace the thionitrobenzoate moiety from Nbs_2-labeled proteins. This reaction, which generates a thiocyanato derivative of the protein, has been used to study the role of sulfhydryl groups in aspartate transcarbamylase (Vanaman and Stark, 1970), isocitrate dehydrogenase (Chung et al., 1971), and aspartate aminotransferase (Birchmeier et al., 1973). In each case active enzyme was regenerated by reaction with cyanide demonstrating the modified sulfhydryl groups were not essential for catalysis.

By analogy it was thought cyanide would displace the thiopurine nucleotide moiety from S_2P-PNP labeled enzyme (eq 1 and 2). Several experiments established the

$$\boxed{\text{HMM}}\text{–SH} + \text{S}_2\text{P–PNP} \longrightarrow \boxed{\text{HMM}}\text{–S–SP–PNP} + \text{SHP–PNP} \quad (1)$$

$$\boxed{\text{HMM}}\text{–S–SP–PNP} + {}^{14}\text{CN}^- \longrightarrow \boxed{\text{HMM}}\text{–S}^{14}\text{CN} + {}^-\text{SP–PNP} \quad (2)$$

quantitative nature of eq 2. First, the displacement was complete. This was shown by the leveling off in the amount of cyanide incorporated during the displacement of the thiopurine nucleotide from HMM-SP-PNP and by the loss of the absorbance at 320 nm from HMM–SP-PNP as it was converted to HMM-SCN. Further evidence for the selective nature of the cleavage of HMM–S-SP-PNP was demonstrated by the absence (<3%) of formation of any SCN-PNP (Figure 2).

The cyanide displacement reaction has two immediate advantages. First, the thiocyanatoenzyme formed is more stable (Degani and Patchornik, 1974) and less subject to reaction with neighboring thiols than the parent disulfide derivative. Second, the use of radioactive cyanide removes the necessity to prepare radioactive derivatives of S_2P-PNP.

The use of radioactive cyanide to determine stoichiometry of labeling via formation of thiocyanato derivatives of proteins is limited by two potential side reactions. First, at alkaline pH these derivatives are known to undergo elimination reactions to form dehydroalanine and thiocyanate (Catsimpoolas and Wood, 1966). Under the same condi-

tions in denaturing solvents they may also undergo a cyclization reaction to form an iminothiazolidine ring with concomitant chain cleavage (Vanaman and Stark, 1970; Jacobson et al., 1973; Degani and Patchornik, 1974). Either reaction could lead to low values of cyanide incorporated per mole of enzyme. As a consequence, such conditions were avoided allowing the number of cyanides bound to be equated to the number of thiopurine nucleotides originally incorporated. Furthermore, the accompanying paper (Wagner and Yount, 1975) demonstrates that intact subunits of cyanide-labeled myosin could be isolated even though mildly alkaline denaturing conditions were used in their preparation.

Interestingly the reaction of cyanide with HMM-SP-PNP (or SF_1-SP-PNP) regenerated 25–30% of the original enzyme activity demonstrating that the cysteines modified are not essential for activity. This result is consistent with the cysteines modified being at the purine binding site rather than at the bond-breaking site. The number and location of the sites of reaction of S_2P-PNP with HMM and SF_1 are of marked importance since prior kinetic experiments (Yount et al., 1972) have given evidence for a second set of ATP binding sites on each head of myosin. This postulate was based on the observations that the rates of inactivation of HMM and SF_1 reached a limiting value as S_2P-PNP concentrations were increased. This type of kinetic behavior results when the rate-limiting irreversible inactivation step is preceded by a rapid, reversible binding of the inhibitor to a specific binding site on the enzyme (Kitz and Wilson, 1962; Fahrney and Gold, 1963). Since saturation effects of this type occurred even in the presence of substrate, interaction of S_2P-PNP at a second site was postulated. Moreover, these two sites must be coupled since binding of ATP to the active site enhances reaction at the second site and binding of an ATP-like molecule at the second site prevents ATP hydrolysis.

As shown in Figure 3, the incorporation of thiopurine nucleotide by HMM paralleled its inactivation by S_2P-PNP. HMM was ordinarily used in these labeling experiments instead of myosin as it was easier to manipulate. To quench various reactions, HMM and SF_1 could both be precipitated with $(NH_4)_2SO_4$ from relatively dilute solutions. These precipitations when performed with reasonable care did not affect the ATPase activities of either preparation. When myosin was treated similarly, on occasion, some of the precipitated protein would not redissolve requiring clarification before going on to further work. However, experiments with myosin have always given the same stoichiometry and labeling pattern as that obtained with HMM.

Surprisingly, complete inactivation required the blocking of four sulfhydryls per mole of enzyme. Our initial supposition was that S_2P-PNP was reacting at either the active site or a control site. These sites interacted in such a way that reaction of S_2P-PNP at either site inactivated the enzyme and in turn enhanced the rate of reaction at the alternate sites. This way complete inactivation always appeared to require two nucleotides per head. Further experiments, however (P. Wagner and R. Yount, manuscript in preparation), rule out reaction of S_2P-PNP at the active site. Briefly, binding experiments with completely blocked HMM-SP-PNP have shown that AMP-PNP binds to the two active sites with the same affinity and stoichiometry as to unmodified HMM. Experiments with SF_1-SP-PNP and SF_1 have given similar results. Thus S_2P-PNP appears not to react at the active site despite its profound effects on ATPase activity. This fact plus the difference in relative Ca^{2+}- and EDTA · ATPase values for trypsin SF_1 blocked with varying amounts of S_2P-PNP argue strongly against the thiopurine nucleotide being at the active site.[2]

For purposes of discussion it is useful to call the sites labeled by S_2P-PNP which inactivate the ATPase activity "regulatory sites".[3] Further support for this concept is the observation that neither HMM labeled with four thiopurine nucleotide groups nor trypsin SF_1 labeled with only one group will bind to F-actin (P. Wagner and R. Yount, manuscript in preparation). Thus the two key properties of myosin, ATP hydrolysis and actin binding, are lost on the binding of one or two -SP-PNP groups per head. Furthermore, myofibrils treated with S_2P-PNP or S_2TP are permanently relaxed (dos Remedios et al., 1972) and give polarization of fluorescence values identical with ATP-relaxed myofibrils. Studies with spin-labeled HMM (Tokiwa, 1971; Stone, 1973) also indicate that actin interaction is weakened after modification with thiopurine nucleotides. Thus a number of lines of evidence support the idea that S_2P-PNP binds covalently to a specific site with the properties of a regulatory site. Further discussion of this concept will be given in a future publication (Wagner and Yount, manuscript in preparation).

SF_1 arises from the head region of myosin and retains the ATPase and actin binding properties of myosin. The kinetics of its inactivation by S_2P-PNP indicated the presence of two interacting ATP binding sites per head (Yount et al., 1972). It was of interest to determine if both types of sulfhydryls modified in HMM by S_2P-PNP were also present in SF_1. As shown in Figures 5 and 6 both types of cysteines are located in SF_1. Neither the tail region nor the "hinge region" of HMM contribute to the ATP binding sites revealed by S_2P-PNP reactions. The ATP binding sites reported by Harrington and Himmelfarb (1972) to be in the rod region are therefore not the same as those reported here.

Surprisingly, the number of nucleotides bound by SF_1 was dependent upon the method of preparation (Figure 5). Complete inactivation of SF_1 made by a papain digestion of myosin required reaction with 2 mol of nucleotide. However, when SF_1 made by a trypsin digestion of HMM was used only 1.1 mol of nucleotide were incorporated for complete inactivation. Trypsin SF_1 still contained the second reactive sulfhydryl, since when HMM labeled with four cyanides was digested with trypsin, the isolated SF_1 had two cyanides bound (Figure 6). Further evidence for this supposition comes from studies of trypsin SF_1 inactivation by S_2P-PNP in the presence of AMP-PNP. In this case, the

[2] Blocking the single reactive cysteine in trypsin SF_1 with S_2P-PNP inhibited both the Ca^{2+}- and the EDTA · ATPases. However, the EDTA · ATPase was consistently lower than the Ca^{2+} · ATPase. After 90 min, only 4% of the EDTA · ATPase activity remained while 17% of the Ca^{2+} activity remained. If the thiopurine moiety bound to the active site and caused inactivation by physically blocking the binding of ATP, one would expect both the Ca^{2+}- and the EDTA · ATPases to be inactivated identically. If the inactivation resulted from the thiopurine nucleotide binding to a regulatory site, then the nonidentity of the two ATPases activities is easily explained.

[3] The term "ATP regulatory sites" has been introduced by Kominz (1971) to explain a variety of effects of ATP concentrations on actomyosin superprecipitation (Levy and Ryan, 1966; Stewart and Levy, 1970) and on myofibril contraction (Eisenberg and Moos, 1965; Kominz and Yoshioka, 1969). The evidence for such sites on myosin though is largely circumstantial and direct binding studies to reveal ATP binding sites weaker than the active sites have not been reported.

number of nucleotides bound increased to 1.5, if 1.5 m*M* AMP-PNP was present and to 1.7 if 50 μM AMP-PNP was used. The simplest explanation for the loss of reactivity at one of these sulfhydryls is that trypsin cleavage changes the conformation around one of these cysteines preventing its reaction with S_2P-PNP. The change in the enzyme's conformation induced by the trypsin treatment must be partially reversed by the binding of AMP-PNP to the ATPase site. The importance of these experiments is that they best illustrate that only a single sulfhydryl need be blocked to inactivate the enzyme. In addition, these results indicate a conformational difference in the two forms of SF_1. The use of both preparations as equivalent models for independent myosin heads may be unwarranted. This difference in reactivity argues for using papain rather than trypsin for preparing SF_1, as less structural alteration appears to occur.

Although two sulfhydryls per head react with S_2P-PNP, blocking only one of these is sufficient for complete inactivation. As shown in Figure 4A, when AMP-PNP was present during the inactivation of HMM by S_2P-PNP at 0°, two classes of sulfhydryls were revealed. One set reacted very quickly, the other more slowly. Blocking of the first set completely inactivated HMM, as 1.1 mol of nucleotide bound resulted in 50% inactivation. As mentioned above, the necessity of blocking only one type of cysteine was confirmed by the complete inactivation of trypsin SF_1 when only one thiopurine nucleotide was bound. The additional sulfhydryl labeled in trypsin SF_1 when AMP-PNP was added reacted after the enzyme had been inactivated by the modification of the first cysteine. The role, if any, of this second set of reactive sulfhydryls is unknown.

The inactivation of Ca^{2+}- and EDTA · ATPases by S_2P-PNP is quite different from the effect of other sulfhydryl reagents. Typically these other reagents first react with one set of sulfhydryls (SH_1) which when modified inactivate the EDTA · ATPase but stimulate the Ca^{2+} · ATPase. They then react with a second set of sulfhydryls (SH_2) which inactivate the Ca^{2+} · ATPase (see Reisler et al., 1974 for a summary of these effects). S_2P-PNP, however, never stimulates the Ca^{2+} · ATPase activity. Blocking a single sulfhydryl in trypsin SF_1 with the thiopurine nucleotide inactivated both the Ca^{2+}- and the EDTA · ATPases. Blocking either SH_1 or SH_2 alone inactivates the EDTA · ATPase while stimulating the Ca^{2+} · ATPase (Sekine and Kielley, 1964; Seidel, 1969). This difference in the effect of blocking a single cysteine with S_2P-PNP as compared to general sulfhydryl reagents suggests that the cysteines modified are themselves different. SH_1 and SH_2 have been widely studied and the sequence around them was determined (Yamashita et al., 1964, 1974). Both of these cysteines are on the heavy chains of myosin. As shown in the accompanying paper two of the cysteines modified by S_2P-PNP are located on the light chains (Wagner and Yount, 1975).

AMP-PNP appears to affect the inactivation of HMM by S_2P-PNP in two ways. At low concentrations it binds to the active site and in doing so enhances the reactivity of a sulfhydryl at a second binding site. At higher concentrations, AMP-PNP can also bind to the regulatory sites and thereby decrease the rate of inactivation. The relative importance of these two competing effects is a function of the temperature at which the inactivations are carried out. The rate of inactivation at 0° is enhanced by the addition of AMP-PNP. At this temperature 50% inactivation of HMM by 100 μM S_2P-PNP required approximately 15 min in the absence of AMP-PNP but less than 2 min in the presence of 1.5 m*M* AMP-PNP. At 25° high concentrations of AMP-PNP (or ATP) slow the rate of inactivation rather than enhancing it. At this temperature 1.5 m*M* AMP-PNP increased the time required for 100 μM S_2P-PNP to inactivate HMM 50% from 5 to 10 min (Figure 4B). This indicates a conformational difference in HMM at these two temperatures. This type of temperature dependent conformational change has been reported by other investigators looking at a wide variety of myosin's properties (Levy et al., 1959; Malik and Martonosi, 1972). This conformational change could modify either of the two effects of AMP-PNP. AMP-PNP binding to the active site at 25° might decrease the reactivity of the cysteines at the regulatory sites or the affinity of the regulatory sites for AMP-PNP might be greater at 25° than it is at 0°. Either of these effects alone or in concert could decrease the rate of the S_2P-PNP reaction.

Murphy and Morales (1970) and Tokiwa and Morales (1971) reported that SH-TP bound to only one site per head of myosin. The conclusion that this cysteine was at the active site was a logical one. However, in view of the work presented here the possibility that SH-TP also reacts at the so-called regulatory sites should be investigated. More recently Stone (1973) found that HMM which had been spin-labeled at SH_1 incorporated 4.6 mol of thiopurine nucleotide. It was assumed that two of the thiopurine groups were at the active sites and that the rest were bound nonspecifically. Again, it would seem that the results observed most likely reflect binding of the SH-TP moiety at regulatory sites. However, direct comparison of that work with the results presented here is difficult as there were significant differences in reaction conditions; SH-TP was used, the inactivation period was several days, and the HMM had already been modified with a spin-label.

In summary, our current model for the reaction of S_2P-PNP with myosin is that the ATPase sites are never labeled and that the inactivations result solely from S_2P-PNP binding to the so-called regulatory sites. Reaction of S_2P-PNP at this regulatory site inactivates the active site and prevents actin binding. AMP-PNP binds to the ATPase site and stimulates the reactivity of a sulfhydryl at the regulatory site. At higher concentrations AMP-PNP also binds to the regulatory sites and blocks S_2P-PNP reaction. The second set of two sulfhydryls labeled by S_2P-PNP in HMM are specific in the sense of their hyper-reactivity with S_2P-PNP. They may or may not be at a specific nucleotide binding site and therefore, nothing can be said at this time as to their function. In trypsin SF_1 the single cysteine labeled by S_2P-PNP is believed to be at the regulatory site. The additional sulfhydryl labeled by S_2P-PNP in trypsin SF_1 when AMP-PNP is added is presumed to be the same as that labeled in HMM and in papain SF_1. Experiments in progress are designed to establish the specific location and function of each set of reactive cysteines.

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