Subunit Location of Sulfhydryl Groups of Myosin Labeled with a Purine Disulfide Analog of Adenosine Triphosphate†

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ABSTRACT: A purine disulfide analog of ATP, 6,6′-dithiobis(inosinyl imidodiphosphate), forms mixed disulfides with cysteine residues at what are believed to be ATP regulatory sites of myosin. Blocking these sites causes inactivation of the ATPase activity at the active sites. Two cysteine residues per head are specifically modified by this disulfide analog. The thiopurine nucleotides can be stoichiometrically displaced from myosin by [^{14}C]cyanide to give a more stable thiocyanato derivative of the enzyme. [^{14}C]Thiocyanatomyosin (3.7 ^{14}CN/myosin) was dissociated in 4 *M* urea and the individual subunits were isolated. The heavy chains each had 0.78 ^{14}CN bound per 200,000 molecular weight unit. The light chain with molecular weight of 20,700 had 1.00 ^{14}CN bound and the 16,500 molecular weight light chain had 0.65 ^{14}CN bound. The two 19,000 molecular weight light chains were not labeled. The two labeled light chains have only a single cysteine which is stoichiometrically modified. These two light chains show a high degree of homology and presumably perform identical functions in myosin. Their specific modification by the purine disulfide analog and their other known properties suggest that they contribute directly to the ATP regulatory sites and may, in fact, function as regulatory subunits.

The subunit structure of myosin from white skeletal muscle is now generally thought to consist of two heavy chains of molecular weight 200,000 (Gershman et al., 1969; Gazith et al., 1970) and four light chains. There are three different types of light chains with molecular weights of 20,700, 19,000, and 16,500 (Lowey and Risby, 1971; Sarkar et al., 1971; Weeds and Lowey, 1971; Frank and Weeds, 1974). These light chains will be referred to as LC_1, LC_2, and LC_3 in order of decreasing molecular weight.[1] The stoichiometry appears to be two LC_2 chains and a total of two LC_1 and/or LC_3 chains per myosin (Weeds and Lowey, 1971). There is still disagreement as to whether LC_1 and LC_3 are present in equal molar amounts or if there is more LC_1 than LC_3 (Lowey and Risby, 1971; Dreizen and Richards, 1972; Sarkar, 1972).

The exact contributions made by the heavy and light chains to the ATPase and actin binding properties of myosin have not yet been fully determined. LC_2 can be removed from myosin with Nbs_2[2] without affecting the ATPase activity (Weeds, 1969; Gazith et al., 1970). In addition, subfragment one (SF_1) made by a papain digestion of myosin is missing most of LC_2. Since SF_1 retains the ATPase and actin binding properties of myosin, LC_2 cannot be essential for either activity (Weeds and Lowey, 1971). LC_1 and LC_3, however, cannot be removed without losing both types of activity (Stracher, 1969; Gershman et al., 1969). It has been reported that the recombination of isolated inactive light and heavy chains partially regenerates ATPase activity (Stracher, 1961; Dreizen and Gershman, 1970; Dow and Stracher, 1971; Kim and Mommaerts, 1971). It cannot be determined, however, from these dissociation and recombination experiments if the light chains contribute directly to the nucleotide or actin binding sites.

An analog of ATP, 6,6′-dithiobis(inosinyl imidodiphosphate) (S_2P-PNP), inactivates heavy meromyosin (HMM) and SF_1 by forming mixed disulfide bonds with cysteines at ATP binding sites (Yount et al., 1972). As reported in the previous paper (Wagner and Yount, 1975), S_2P-PNP reacts specifically with two sulfhydryls per head of myosin. Myosin so modified is inactive as an ATPase and no longer binds actin but *does* bind adenylyl imidodiphosphate as tightly to the active site as does the native enzyme (Wagner and Yount, manuscript in preparation). These findings have led to the proposal of ATP regulatory sites which control myosin's ATPase activity and actin binding. One and possibly both sets of sulfhydryls are believed to be at the postulated regulatory sites.

This paper describes the subunit location of the cysteines which react with S_2P-PNP. Two of these cysteines are on the heavy chains and two are on the light chains, LC_1 and LC_3. Since the primary sequences of these two light chains are known and since they contain only a single cysteine (Frank and Weeds, 1974) the exact location of their modification is specified. It is suggested that the role of the light chains may be to regulate myosin's ATPase activity and interaction with actin by providing all or part of the proposed ATP regulatory sites.

Materials and Methods

Chemicals. S_2P-PNP was prepared by the oxidation of SHP-PNP with sodium triiodide as described previously (Yount et al., 1972). Sodium [^{14}C]cyanide (60 Ci/mol) was purchased from Amersham/Searle and diluted with carrier

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[1] The nomenclature used in this paper to designate the light chains is that of Sarkar et al. (1971). A number of alternate systems are currently in use. LC_1, LC_2, and LC_3 are equivalent respectively to alkali-1, DTNB (Nbs_2) and alkali-2 light chains (Weeds and Lowey, 1971); α, β, and γ (Dreizen and Richards, 1972); g_1, g_2, and g_3 (Hayashi, 1972).

[2] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); bicine, *N,N*-bis(2-hydroxyethyl)glycine; HMM, heavy meromyosin; SF_1, subfragment one; SDS, sodium dodecyl sulfate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; S_2P-PNP, 6,6′-dithiobis-(inosinyl imidodiphosphate); SH-TP, 6-thioinosine triphosphate.

sodium cyanide to give a specific activity of 1.42×10^{12} cpm/mol. Cyanide stock solutions were 50 mM and were stored at −20°. Cyanide in solution decomposes to give initially ammonium formate and subsequently unidentified brown products. The cyanide solutions were usable for 6–8 weeks before the level of nonspecific labeling from these decomposition products became prohibitive. Aquasol was purchased from New England Nuclear and papain from Worthington. DEAE-cellulose used throughout is Whatman DE 52. All other chemicals were of analytical grade.

Enzyme Preparations. Myosin was prepared from rabbit back and hind leg muscles as described previously (Wagner and Yount, 1975). It was further purified on a DEAE-cellulose column as described by Weeds and Lowey (1971) and was stored in 50% glycerol at −20°. SF_1 was made by a papain digestion of myosin and purified by $(NH_4)_2SO_4$ fractionation (Margossian and Lowey, 1973). After its precipitation with $(NH_4)_2SO_4$, SF_1 was dissolved in 20 mM bicine (pH 8.0) at 0° and stored for a maximum of 1 week at 4°.

Inactivation and Labeling of Myosin and SF_1. The inactivation of SF_1 by S_2P-PNP and the [^{14}C]cyanide displacement of the thiopurine nucleotide moiety were performed as described in the preceding paper (Wagner and Yount, 1975). Myosin was treated similarly except the KCl concentration was 0.6 M. Although most of the kinetic and labeling experiments have been performed with HMM or SF_1, experiments in which myosin was used gave the same results. Observations which are of particular importance to the work described in this paper are that S_2P-PNP modifies four cysteines for complete inactivation, that cyanide stoichiometrically displaces the thiopurine nucleotides to give thiocyanato myosin, and that the cyanide displacement regenerates 20–30% of control activity. As shown in the preceding paper (Wagner and Yount, 1975) treating HMM or SF_1 for 3 hr at 0° with S_2P-PNP results in greater than 90% inactivation. This length of time was used routinely for the experiments described in this paper.

Selective Dissociation of LC_2. After displacement of the thiopurine nucleotides from myosin with [^{14}C]cyanide and precipitation with $(NH_4)_2SO_4$, the myosin pellet was dissolved in 10 mM EDTA (pH 7.0) (0°), and 0.5 M KCl. After dialysis against this buffer to remove $(NH_4)_2SO_4$ and any free cyanide, the ^{14}C-labeled myosin was treated with Nbs_2 to selectively remove LC_2 (Weeds and Lowey, 1971). The thionitrobenzoate moieties, however, were not subsequently removed as the cyanide would also have been lost.

Dissociation and Fractionation of Light Chains. ^{14}C-labeled myosin both with and without prior treatment with Nbs_2 to remove LC_2 was dissociated in 4 M urea, 2.5 mM EDTA, and 25 mM bicine (pH 8.0) at room temperature. The light chains were fractionated on a DEAE-cellulose column as described by Weeds and Lowey (1971) except no dithiothreitol was added. The column was monitored at 230 nm and aliquots were taken from every second fraction for counting. The major peaks were pooled and concentrated by dialysis against sucrose (see below).

SDS Gel Electrophoresis. Procedures used for the SDS-acrylamide gel electrophoresis were those of Weber and Osborn (1969) with the following modifications. No reducing agents were added to the samples. Gels were prerun for 30 min to remove any residual ammonium persulfate. Protein samples were prepared by dialysis against 8 M urea, 0.1% SDS, and 0.01 M sodium phosphate (pH 7.0) for 4 hr at room temperature. The gels were fixed overnight in 10% trichloroacetic acid and 50% methanol prior to

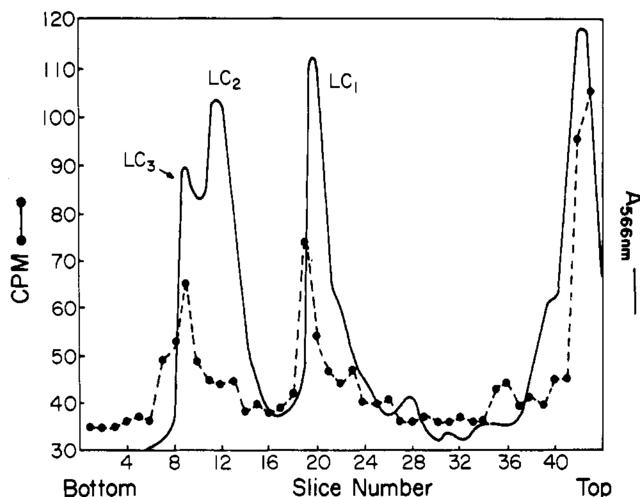


FIGURE 1: Analysis of an SDS-acrylamide gel electrophoresis of [^{14}C]cyanide-labeled myosin. The myosin had 3.6 ^{14}CN bound. The gel was 10% acrylamide and run in 0.1% SDS as described under Materials and Methods. (—) Absorbance at 566 nm; (●—●) cpm/2-mm slice.

staining with Coomassie Blue. After destaining in 7.5% acetic acid and 5% methanol, the gels were scanned at 566 nm with a Gilford gel scanning attachment and sliced with a Mickle gel slicer (Brinkmann). Two 1-mm sections were digested with 0.2 ml of 30% H_2O_2 at 70° in sealed miniscintillation vials. Aquasol (5 ml) was added and the digested gels were counted in a Beckman LS230 scintillation counter.

General Techniques. ATPase assays and protein determinations were performed as described previously (Wagner and Yount, 1975). Dilute protein solutions were sometimes concentrated by placing them in dialysis tubing and covering the tubing with sucrose (Schwarz/Mann, Enzyme Grade). Prior to protein determination, the sucrose was removed by dialysis against 2 mM TES (pH 7.0) (0°) and 30 mM KCl. The amount of cyanide bound was determined by dissolving a 0.5-ml sample in 5.0 ml of Aquasol and counting in minivials as described above. The efficiency of counting was determined by the internal standard method. The molecular weights used were 4.7×10^5 for myosin (Gershman et al., 1969) and 1.15×10^5 for SF_1 (Lowey et al., 1969).

Results

Myosin when completely inactivated by S_2P-PNP incorporated approximately four thiopurine nucleotides per mole of enzyme as determined by reaction with [^{14}C]cyanide (Wagner and Yount, 1975). Figure 1 shows a scan of an SDS-acrylamide gel electrophoretogram of such cyanide-labeled myosin. A typical light chain pattern for white skeletal muscle myosin was obtained. A significant amount of [^{14}C]cyanide was found in the heavy chains. There were also two peaks of radioactivity coinciding with light chains LC_1 and LC_3.

SF_1 made by a papain digestion of myosin incorporated 1.9 mol of thiopurine nucleotide on being inactivated with S_2P-PNP (Wagner and Yount, 1975). Figure 2 is an analysis of an SDS gel electrophoretic separation of cyanide-labeled papain SF_1. Again both the heavy and light chains have radioactivity associated with them. There were three peaks of radioactivity in the light chain region. Two of these peaks coincided with the protein peaks believed to be LC_1

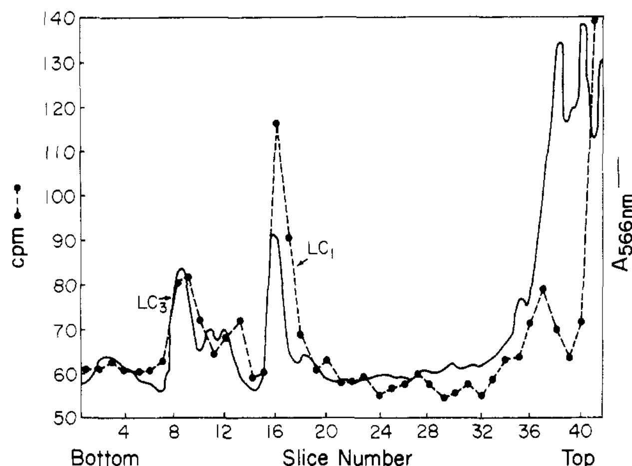


FIGURE 2: Analysis of SDS-acrylamide gel electrophoresis of [^{14}C]cyanide-labeled papain SF_1. SF_1 had 1.9 ^{14}CN bound. The gel was 10% acrylamide and run in 0.1% SDS as described under Materials and Methods. (—) Absorbance at 566 nm; (●—●) cpm/2-mm slice.

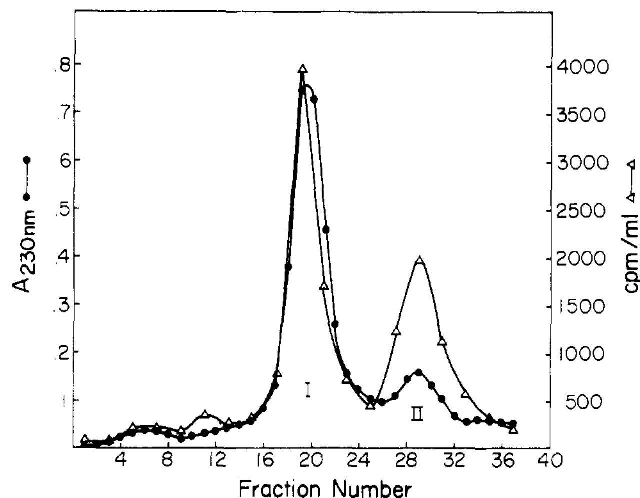


FIGURE 3: Chromatographic separation of the total light chain fraction from [^{14}C]cyanide-labeled myosin. The DEAE-cellulose column (1.2 × 30 cm) was equilibrated with 0.05 *M* potassium phosphate (pH 6.0) at 4°. The light chains were eluted with a linear gradient of 0.05- to 0.35 *M* potassium phosphate (pH 6.0) at 4°. The flow rate was 40 ml/hr and 4-ml fractions were collected. The fractions were monitored by reading their optical density at 230 nm (●); samples were taken from every second fraction and counted as described under Materials and Methods, (△) cpm/ml.

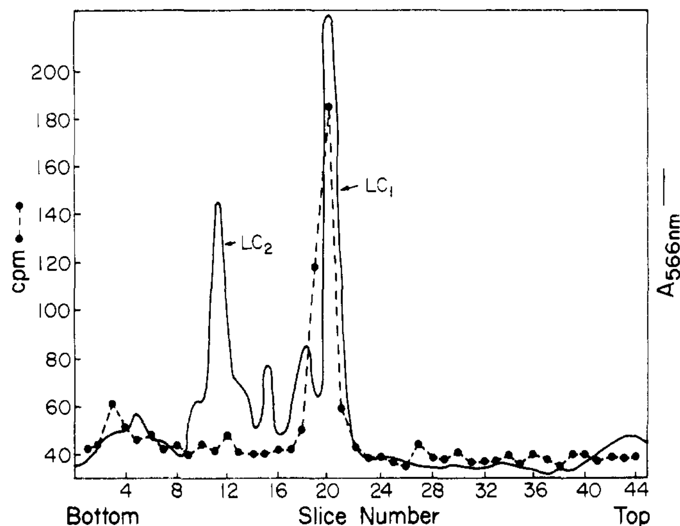


FIGURE 4: Analysis of SDS-acrylamide gel electrophoresis of peak I from Figure 3. Conditions are the same as described in Figure 1. (—) Absorbance at 566 nm; (●—●) cpm/2-mm slice.

Table I: Cyanide to Protein Ratios for Various Myosin Subunits.

Subunit	Mol Wt	cpm/mg[a]	Molar Ratio[b]
Myosin	470,000	11,200	3.71
Heavy chain	200,000	5,530	0.78
LC_1 (fraction A)	20,700	68,400	1.00
LC_2 (released by NbS_2)	19,000	8,070	0.11[c]
LC_3 (fraction C)	16,500	55,870	0.65

[a] Protein concentrations were determined by microbiuret and the cpm by liquid scintillation as described under Materials and Methods. [b] Specific activity = 1.42×10^{12} cpm/mol of ^{14}CN. [c] Contaminated with LC_1 and LC_3.

and LC_3. The third and smallest peak of radioactivity did not align with any protein peak. Thus, gels of labeled myosin and SF_1 both indicate that LC_1 and LC_3 have cyanide bound to them.

It was not possible, however, from the above experiments to determine the cyanide to subunit stoichiometry. To do this and to establish unequivocally the identity of the subunits it was necessary to isolate larger quantities of labeled light chains. Cyanide-labeled myosin (approximately four ^{14}CN/mol of myosin) was prepared as described under Materials and Methods and dissociated in 4 *M* urea. The light chains were isolated and concentrated as described under Materials and Methods. The fractionation of the light chains on a DEAE-cellulose column is shown in Figure 3. Only two major protein peaks were obtained. Both of them contained significant amounts of [^{14}C]cyanide. The two protein peaks were pooled individually and concentrated, and the CN/protein content was determined. The first peak (peak I) had 0.45 [^{14}C]cyanide per light chain using an average molecular weight of 20,000. An SDS-acrylamide gel electrophoresis of this fraction (Figure 4) showed that there were two major proteins present. The mobilities of these bands corresponded to LC_1 and LC_2. There were also present two polypeptides of intermediate mobility. About 80% of the counts were in the peak which corresponded to LC_1 and less than 5% in LC_2.

The second major peak from the DEAE-cellulose column (peak II) had a low cyanide content, 0.20 cyanide/mol of light chain using an average molecular weight of 17,000. As determined by SDS-acrylamide gel electrophoresis (not shown) LC_2, LC_3, and some higher molecular weight proteins were present in this peak. LC_3 was the predominant species and was the only labeled peptide present.

To obtain a better value for the stoichiometry of labeling of LC_1 and LC_3, it was necessary to isolate them free of major contaminants, principally LC_2. LC_2 can be selectively removed by treating myosin with Nbs_2 (Weeds, 1969; Gazith et al., 1970). ^{14}C-labeled myosin (3.71 CN/myosin) was treated with Nbs_2 as described under Materials and Methods. The supernatant obtained after precipitation of the Nbs_2-treated myosin was concentrated on a DEAE-cellulose column as described by Weeds and Lowey (1971). This fraction had only 0.11 [^{14}C]cyanide/mol of light chain using an average molecular weight of 19,000 (Table I). SDS-acrylamide gel electrophoresis showed it to be a mixture of polypeptides, with the major band being LC_2. There were also present small amounts of LC_1, LC_3, and a few

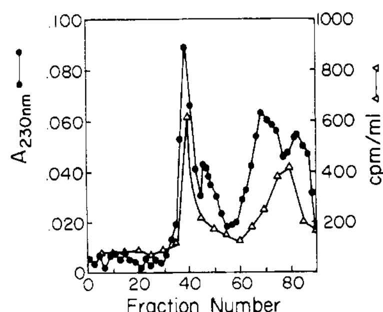


FIGURE 5: Chromatographic separation of the light chain fraction isolated from [^{14}C]cyanide-labeled myosin treated with Nbs_2. The DEAE-cellulose column (1.2 × 30 cm) was equilibrated with 0.05 *M* potassium phosphate (pH 6.0) at 4°. The light chains were eluted with a linear gradient of 0.05-0.35 *M* potassium phosphate (pH 6.0) at 4°. The flow rate was 40 ml/hr and 6.5-ml fraction were collected. The fractions were monitored by reading their optical density at 230 nm (●); samples were taken from every second fraction and counted, (△) cpm/ml. Fraction A, tubes 33-42; B, tubes 47-53; C, tubes 63-75; and D, tubes 81-87.

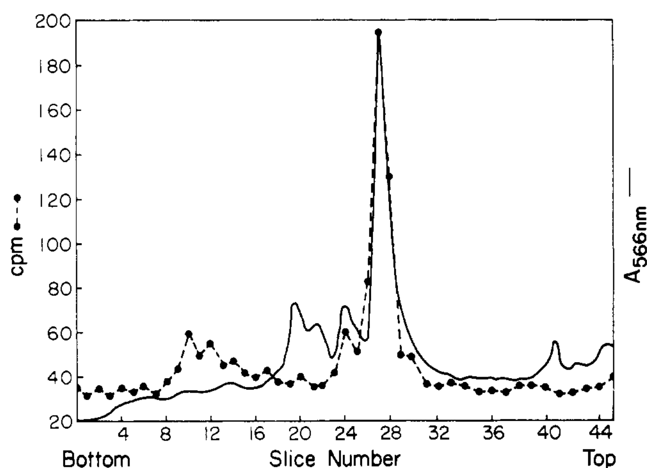


FIGURE 6: Analysis of SDS-acrylamide gel electrophoresis of fraction A (Figure 5). The gel was 12.5% acrylamide and run in 0.1% SDS as described under Materials and Methods. (——) Absorbance at 566 nm; (●—●) cpm/2-mm slice.

minor higher molecular weight bands.

After most of LC_2 had been removed by treatment with Nbs_2, the remaining myosin was dissociated in 4 *M* urea as described before. The precipitate from this dissociation, mostly heavy chains, had a [^{14}C]cyanide to protein ratio of 0.78 using a molecular weight of 200,000 (Table I). The light chain fraction was treated as described under Materials and Methods and fractionated on a DEAE-cellulose column (Figure 5). Four protein peaks and two radioactive peaks were observed. One of the radioactive peaks matched the first 230-nm absorbance peak. The other was located between the last two absorbance peaks. The four protein peaks were collected and designated A, B, C, and D in the order of their elution. The individual fractions were concentrated in dialysis bags covered with sucrose. The sucrose was removed by dialysis against 2 m*M* TES (pH 7.0) (0°) and 30 m*M* KCl.

An analysis of an SDS-acrylamide gel electrophoretogram of fraction A is shown in Figure 6. There is one major protein band and three minor contaminants. The mobility of the major band matched that of LC_1. All of the radioactivity except for a small peak in the region of very low molecular weight is in LC_1. Fraction A had 1.00 mol of

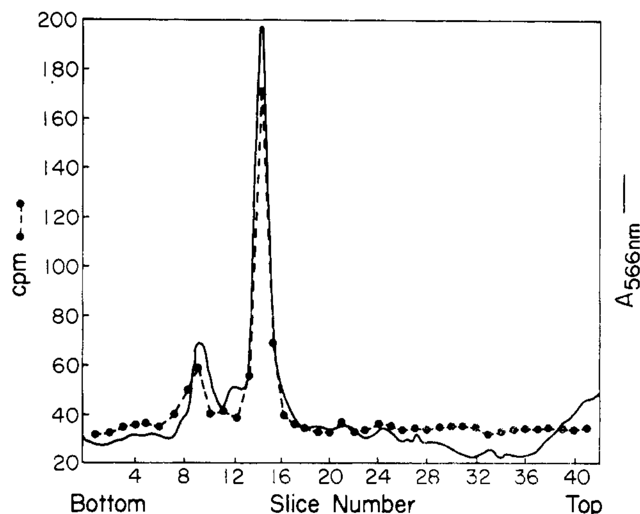


FIGURE 7: Analysis of SDS-acrylamide gel electrophoresis of fraction C (Figure 5). The gel was 12.5% acrylamide and run in 0.1% SDS as described under Materials and Methods. (——) Absorbance at 566 nm; (●—●) cpm/2-mm slice.

Table II: Amino Acid Composition of Light Chains.[a]

	(Residues/100 residues)			
	Fraction A	LC_1	Fraction C	LC_3
Lys	9.9	10.3	7.1	7.4
His	0.8	1.0	0.9	1.3
Arg	2.4	2.1	2.9	2.6
Asp	10.7	11.0	11.5	12.7
Thr	4.1	4.0	4.6	4.7
Ser	4.3	4.2	5.3	5.3
Glu	16.2	16.1	16.0	16.8
Pro	7.2	6.9	(5.6)[b]	2.5
Gly	7.7	6.6	9.5	8.3
Ala	10.9	11.3	7.3	8.3
Val	5.9	5.7	5.4	6.1
Met	2.8	3.3	5.0	3.7
Ile	4.6	4.8	5.2	4.6
Leu	7.6	7.3	8.3	8.3
Tyr	1.5	1.6	1.6	2.0
Phe	4.2	4.4	4.0	5.4

[a] The amino acid composition for a 24-hr hydrolysate of fractions A and C from the DEAE-cellulose column shown in Figure 5 are compared to those determined by other investigators for LC_1 and LC_3. The data shown for LC_1 and LC_3 are the average amino composition taken from Weeds and Lowey (1971) and Hayashi et al. (1973). [b] Subject to a relatively large error as the peak from the analyzer was very small.

[^{14}C]cyanide/mol of light chain using a molecular weight of 20,700 (Table I). An amino acid analysis of fraction A (Table II) corresponded well to the previously published values of LC_1 (Weeds and Lowey, 1971; Hayashi et al., 1973).

The second peak of the DEAE-cellulose column fraction B had 0.31 [^{14}C]cyanide/mol of light chain using an average molecular weight of 20,000. SDS-acrylamide gel electrophoresis of this fraction revealed a mixture of LC_1 and LC_2 with traces of unidentified polypeptides.

An analysis of an SDS-acrylamide gel electrophoretogram of fraction C is shown in Figure 7. It contained one major and one minor polypeptide. Both peaks contained [^{14}C]cyanide. The mobility of the major band matched that of LC_3. Fraction C had 0.65 cyanide/light chain using a molecular weight of 16,500 (Table I). The amino acid com-

position of this fraction is given in Table II. It corresponds well to the previously published amino acid compositions of LC_3 (Weeds and Lowey, 1971; Hayashi et al., 1973).

The final peak, fraction D, had 0.33 cyanide/mol light chain using a molecular weight of 16,500. It contained a single polypeptide with a mobility slightly greater than that of LC_3 when electrophoresed on SDS–acrylamide gels. This peptide is thought to arise from cleavage of LC_3.

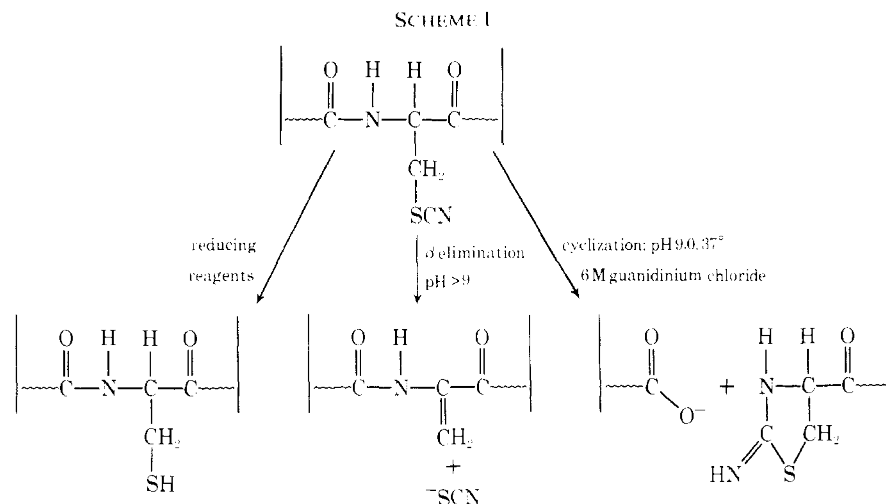
Discussion

S_2P-PNP was designed as a site specific analog of ATP to label covalently the nucleotide binding sites of myosin (Yount et al., 1972). It inactivates myosin via the formation of mixed disulfide bonds between the sulfur at the 6 position of the purine ring and certain key cysteines of myosin. It is of interest to determine the subunit location of these cysteines. However, a potential problem in using mixed disulfides of this type is the possibility of transfer of the thiopurine nucleotide to other cysteines by disulfide exchange. This would be particularly true under dissociating conditions. For this reason a more stable derivative was sought.

The thiopurine nucleotide moiety can be quantitatively displaced from myosin with cyanide to give thiocyanatomyosin (Wagner and Yount, 1975). The thiocyanato moiety is more stable than the mixed disulfide (Degani and Patchornik, 1974) and, therefore, should be less likely to undergo randomization. Furthermore, the specific cysteines modified by S_2P-PNP can readily be identified and quantified if [^{14}C]cyanide is used in the displacement. As demonstrated in the previous paper (Wagner and Yount, 1975), the number of [^{14}C]cyanides incorporated is equal to the number of thiopurine nucleotides originally bound.

Although the thiocyanato derivative is more stable than the disulfide, there are several important limitations in its use as shown in Scheme I. The thiocyanato moiety can be readily removed by reducing reagents (Chung et al., 1971; Wagner and Yount, 1975). For this reason the labeling, isolation, and identification procedures must be carried out in the strict absence of reducing agents. At alkaline pH, the thiocyanoalanyl residues can undergo β elimination to give dehydroalanine and free thiocyanate (Catsimpoolas and Woods, 1966). An additional problem arises from the ability of thiocyanoalanyl residues to cyclize with their own amino groups. This cyclization results in cleavage of the peptide bond with the peptide containing the C-terminal residue having an iminothiazolidine ring at its N-terminus (Catsimpoolas and Wood, 1966; Vanaman and Stark, 1970). Jacobson and coworkers (1973) and Degani and Patchornik (1974) have investigated this cyclization reaction in detail. Chain cleavage is most rapid and complete under denaturing conditions between pH 8 and 9 at 37°. Hence in this work the pH was maintained close to neutrality and the temperature kept below 25° to give reasonable yields of intact ^{14}C-labeled subunits.

SCHEME I



That some cleavage of [^{14}C]cyanide-labeled proteins has occurred is evidenced by the presence of extra protein bands in SDS–acrylamide gels of myosin and SF_1 (Figures 1 and 2). Although it is not possible to state unequivocally the origin of these polypeptides a logical guess can be made for some of them. The single cysteines of LC_1 and LC_3 are located 14 amino acids from the C-terminal end (Frank and Weeds, 1974). When these cysteines are labeled with cyanide, the potential cleavage products from LC_1 and LC_3 would be a ^{14}C-labeled peptide with a molecular weight of approximately 1400 and unlabeled polypeptides with molecular weights of about 19,000 and 15,000, respectively. The small unlabeled bands often seen migrating directly in front of LC_1 and LC_3 may represent these latter two polypeptides (Figures 4 and 7). The small peak of radioactivity of high mobility and low staining ability that often appears toward the bottom of the gels (Figures 2, 4, 6, and 7) may be the 1400 molecular weight fragment. However, the relatively small amounts of these unusual polypeptides indicated that the amount of cleavage occurring during the 4-hr dialysis at room temperature in 8 *M* urea, 0.1% SDS, and 0.01 *M* sodium phosphate (pH 7.0) was low. This low level of cleavage suggested that even the dissociation of myosin in 4 *M* urea at pH 8.0 might give reasonable yields of intact light chains.

Attempts to isolate the individual light chains on a DEAE-cellulose column failed repeatedly, as LC_2 was present in significant quantities in all protein fractions. Problems with resolving light chains on DEAE-cellulose columns have been reported previously (Weeds and Lowey, 1971; Lowey and Holt, 1972). Undoubtedly, the lack of dithiothreitol in our experiments made the resolution even more difficult. The elution of the total light chain fraction from the DEAE-cellulose column (Figure 3) gave only two protein peaks rather than the three expected if the light chains were completely resolved. Peak I contained a mixture of LC_1 and LC_2 as well as a number of other polypeptides (Figure 4). Since most of the radioactivity was associ-

ated with LC_1 and since LC_1 made up roughly one-half of the total protein, the 0.45 [^{14}C]cyanide/mol of light chain for the entire peak I indicated that LC_1 was stoichimetrically labeled. It is clear that LC_2 was not labeled by S_2P-PNP even though it is known to contain two cysteines (Weeds and Lowey, 1971). Similar conclusions can be drawn from the SDS-acrylamide gels of [^{14}C]cyanide-labeled myosin and SF_1 (Figures 1 and 2).

To obtain LC_1 and LC_3 pure, it was necessary to remove LC_2 by treating the cyanide-labeled myosin with Nbs_2. The light chain fraction liberated by Nbs_2 was principally LC_2 and contained only 0.11 mol of cyanide/mol of light chain giving further evidence of it not being labeled by S_2P-PNP. The radioactivity of this fraction is attributed to contamination with LC_1 and LC_3.

Removal of LC_2 allowed the remaining light chains to be separated satisfactorily by column chromatography. The first peak, fraction A, eluted from the DEAE column (Figure 5) gave a single major band on SDS-acrylamide gels (Figure 6). The mobility of this band matched that of LC_1 and contained most of the [^{14}C]cyanide. Fraction A had 1.00 cyanide/mol using a molecular weight of 20,700. Its amino acid composition (Table II) agreed remarkably well with published values of LC_1, despite the fact that only enough labeled protein was available to do a single time hydrolysis. Since LC_1 contains only one cysteine, the labeling was stoichiometric. The second peak, fraction B, was a mixture of LC_1 and the LC_2 which had not been removed by the Nbs_2 treatment. Fraction C contained primarily LC_3 as shown by its electrophoretic mobility on SDS-acrylamide gels (Figure 7), and its amino acid composition (Table II). This fraction has 0.65 [^{14}C]cyanide/mol of LC_3 using 16,500 as the molecular weight. Since LC_3 has but a single cysteine, it too within experimental error was stoichiometrically labeled by S_2P-PNP. The final fraction, D, was a polypeptide with an electrophoretic mobility of SDS-acrylamide gels slightly greater than that of LC_3. It is thought to be one of the products of the cleavage of LC_3 by cyanide. The similarity of this fraction's amino acid composition to that of LC_3 (data not shown) makes this explanation reasonable. The radioactivity present (0.33 cyanide/light chain) may be due to the presence of some of the ^{14}C-labeled 1400 molecular weight fragment. In addition, elution of this ^{14}C-labeled fragment between peaks C and D may cause the radioactivity not to follow to the protein exactly.

These results clearly show the specificity of S_2P-PNP reaction with myosin, at least as far as the light chains are concerned. Since there are a total of 2 mol of LC_1 and LC_3, the light chains can account for only half of the thiopurine nucleotides incorporated. The other two reactive cysteines must be on the heavy chains. This is substantiated by the observation that myosin after depletion of light chains by treatment with Nbs_2 and 4 M urea had 0.78 mol of ^{14}CN per 2×10^5 g. Determination of the specific location of the cyanides on the heavy chains will require peptide mapping. Cleavage of the cyanide-labeled heavy chains caused by the formation of iminothiazolidine should allow the reactive cysteine to be placed near the C-terminal end (small radioactive peptide would result) or near the N-terminal end (large radioactive peptide would result). Present evidence (Young et al., 1972; Starr and Offer, 1973) indicates that the head region of myosin contains the N-terminal end of the heavy chains.

It is conceivable that LC_1 and LC_3 were not the true sites of action of S_2P-PNP, but that the cyanide migrated to these residues when the protein was denatured. This seems unlikely since neither of the cysteines on LC_2 were labeled while the single cysteines on LC_1 and LC_3 were essentially stoichiometrically modified. Furthermore, the heavy chains which contain approximately 17 cysteines per chain were labeled by a single cyanide. Therefore, if the cyanide on LC_1 and LC_3 resulted from migration, it would have been a specific migration, an unlikely situation under the denaturing conditions used.

The sequences around the single cysteines in LC_1 and LC_3 are identical (Weeds and Lowey, 1971; Frank and Weeds, 1974). These two light chains are homologous and undoubtedly arose from the same parent gene. LC_1 has 41 additional amino acids on its N-terminus which accounts for the 4200 difference of molecular weights of LC_1 and LC_3. Five out of the first eight amino acids at the N-terminus of LC_3 are different from the corresponding region on LC_1. The rest of the sequences are identical (Frank and Weeds, 1974). The extensive homology and selective labeling of LC_1 and LC_3 by S_2P-PNP indicate that these two light chains have the same function in myosin. It is of interest to note that the sequence around the cysteine in LC_1 and LC_3 is also found in one of the two light chains from bovine cardiac myosin (Weeds and Frank, 1972). Efforts are underway to see if it, too, is labeled by S_2P-PNP.

Murphy and Morales (1970) reported 6-thioinosine triphosphate modified two cysteines per myosin to give complete inactivation. When the labeled myosin was dissociated by raising the pH to 11, the thiopurine moiety was found in the light chain fraction. Later reports (Murphy, 1971), however, indicated that the label was on the heavy chains and that the earlier results were artifacts arising from disulfide exchange at the alkaline pH used to isolate the light chains. The exact location of the cysteines on the heavy chains labeled by 6-thioinosine triphosphate remains to be determined.

The specific reaction of S_2P-PNP with the cysteines in LC_1 and LC_3 is a unique property of this ATP analog. General sulfhydryl reagents, i.e., N-ethylmaleimide, iodoacetamide, and Nbs_2, react most rapidly with two sets of sulfhydryls, generally referred to as SH_1 and SH_2 (Reisler et al., 1974, and references therein). Both SH_1 and SH_2 are known to be on the heavy chains (Trotta et al., 1968; Hayashi, 1972). The sequences around SH_1 and SH_2 are different than those around the cysteines of LC_1 and LC_3 (Yamashita et al., 1964, 1974; Frank and Weeds, 1974).

The function of myosin's light chains remains an area of intense interest. Myosins from various muscle types with different ATPase activities have different light chain compositions (Lowey and Risby, 1971; Sarkar et al., 1971). Removal of the light chains inactivates myosin (Gershman et al., 1969; Stracher, 1969). Although not widely confirmed by other workers, it has been reported that the recombination of the dissociated light and heavy chains partially restores the ATPase activity (Stracher, 1969; Dreizen and Gershman, 1970). Likewise, the mixing of light chains from fast muscle with the heavy chains of slow muscle results in an ATPase activity of intermediate value (Kim and Mommaerts, 1971; Dreizen and Richards, 1972). Cross-reinnervation of cat muscles causes slow twitch muscles to become fast twitch and vice versa. Myosin's ATPase and light chain composition also change in a consistent manner (Weeds et al., 1974). All of these studies indicate that the light chains are involved in controling the ATPase activity of myosin. The stoichiometric labeling of LC_1 and LC_3 by S_2P-PNP is

suggestive that they contribute directly to the postulated "ATP regulatory sites" of myosin. The evidence for such "ATP regulatory sites" is discussed in the preceding paper (Wagner and Yount, 1975). Definitive proof as to whether these sites involve only the light chains, the heavy chains, or possibly both awaits further experimentation.

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