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# Role of the 50-Kilodalton Tryptic Peptide of Myosin Subfragment 1 as a Communicating Apparatus between the Adenosinetriphosphatase and Actin Binding Sites

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ABSTRACT: Limited glutaraldehyde modification of tryptic myosin subfragment 1, which mainly consists of 26-, 50-, and 20-kilodalton (kDa) peptides, resulted in the selective cross-linking of the 20- and 50-kDa peptides. The cross-linking pattern was altered by nucleotides, depending on the base structure. Neither the reactive thiols on the 20-kDa peptide nor the reactive lysyl residue on the 26-kDa peptide was modified with the reagent, regardless of the presence or absence of nucleotide. Glutaraldehyde treatment of the protein resulted in marked increases in its $Mg^{2+}$-ATPase activity and affinity for actin. High ATPase activity and actin affinity were not produced if the treatment was conducted in the presence of ATP. These ATPase and actin binding properties of the protein derivatives are explained by assuming that glutaraldehyde "freezes" the existing interactions between the 20- and 50-kDa peptides in the activated and nonactivated conformational states, respectively. Taking into account the previous reports that the ATPase site resides between the 26- and 50-kDa peptides, and the 50-kDa peptide binds either ATP or actin, the present results suggest that the 50-kDa peptide acts as a communicating apparatus between the ATPase and actin binding sites of myosin. A simple model for the intersite communication is also proposed.

The interaction of actin with myosin heads and the actin-dependent activation of the $Mg^{2+}$-ATPase of the myosin molecule are crucial events of the mechanochemical transduction process in muscle and other motile systems. The

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globular head regions of the myosin molecule called subfragment 1 (S-1)[1] contain sites for nucleotide binding and hydrolysis (ATPase) and sites for actin binding (Mueller & Perry, 1962). A local change in the S-1 conformation due to nucleotide binding results in a change in actin affinity; binding of nucleotides to the ATPase site reduces the affinity of actin for the actin binding site and vice versa (Szent-Gyorgyi, 1947; Kiely & Martonosi, 1968). Thus, the communication between the ATPase and actin binding sites of S-1 is of great importance in understanding energy transduction in muscle contraction (Morales et al., 1982).

In recent years, significant gains in characterizing the topology of S-1 have been made possible by the application of limited proteolysis of S-1. As established by Balint et al. (1978), limited tryptic proteolysis of the heavy chain of S-1 produces mainly three peptide fragments of 26, 50, and 20 kDa which are aligned in this order within the heavy chain (Lu et al., 1978). Analysis by electrophoresis has revealed that these peptides remain associated under nondenaturing conditions (Hiratsuka, 1985a). A number of workers have referred to these peptides of S-1 as domains (Mornet et al., 1984; Muhlrad & Morales, 1984; Botts et al., 1984). Although the proteolysis of S-1 lowers the protein's affinity for actin (Botts et al., 1982), it does not have a significant effect on the ATPase properties of S-1 (Mornet et al., 1979). The tryptic S-1 has therefore become a valuable framework in which to assign specific groups and functionalities.

The actin binding sites in the rigor acto–S-1 complex have been characterized and are located at the 20- and 50-kDa peptides (Mornet et al., 1979; Yamamoto & Sekine, 1979; Sutoh, 1982). Furthermore, Muhlrad and Morales (1984) have separated and renatured the 20- and 50-kDa peptides of S-1 and find that the former has a high binding constant for actin ($10^{-6}$ M), whereas the latter shows only a weak affinity for actin. Functionally, the cleavage of the 20/50-kDa junction of S-1 does not have a significant effect on its ATPase properties (Mornet et al., 1979), but it does lower the protein's affinity for actin in the presence of ATP (Botts et al., 1982). However, whether this low affinity is due to a local effect restricted to changes solely in the actin binding site or whether it involves changes in the manner in which the 20- and 50-kDa peptides interact is not presently known.

Although the ATPase site has not been unequivocally characterized, it appears from photoaffinity labeling that the 26-kDa peptide is involved in the site (Szilagyi et al., 1979; Okamoto & Yount, 1985). In addition, evidence has accumulated for the involvement of the 50-kDa peptide in the ATPase site. The nucleotide binding to S-1 is sensed not only at the 26-kDa peptide but also at the 50-kDa peptide (Ajtai et al., 1982; Hozumi, 1983; Applegate & Reisler, 1984; Mocz et al., 1984; Labbe et al., 1984). Direct support for these results comes from recent studies of Mahmood and Yount (1984) and Hiratsuka (1985a). These authors have demonstrated that two types of ATP analogues are photochemically labeled mainly to the 50-kDa peptide in S-1, which had been implicated only in the binding of actin until recently. Therefore, taking into account the previous result that the 26- and 50-kDa peptides of S-1 are in contact with each other, at least partly (Hiratsuka, 1984c), we have proposed that the myosin ATPase site is located between the 26- and 50-kDa peptides of S-1 (Hiratsuka, 1985a). This suggestion is particularly interesting in view of an important role of the 50-kDa peptide as a communicating apparatus between the ATPase and actin binding sites. However, the manner in which the 50-kDa peptide is involved in the intersite communication remains obscure at present.

In order to examine this point further, we attempted to use chemical cross-linking reagents, which have been important tools in protein chemistry over the past 2 decades (Peters & Richards, 1977; Ji, 1983). If it were possible to "freeze" only the interactions between the 20- and 50-kDa peptides of S-1 with a cross-linking reagent, it would be possible to directly study the properties of the ATPase site in such a conformational state. Such studies could in turn be useful for the clarification not only of the involvement of the 20- and 50-kDa peptides in the ATPase reaction but also of the manner in which the ATPase and actin binding sites communicate with each other. In the present study, the interactions between the 20- and 50-kDa peptides of tryptic S-1 were selectively frozen with the protein cross-linking reagent glutaraldehyde (Habeeb & Hiramoto, 1968; Korn et al., 1972; Peters & Richards, 1977). It has been found that the freezing of the interactions alteres the ATPase and actin binding properties of tryptic S-1 and that the freezing pattern is altered by nucleotides. Taking into account the previous reports that the 50-kDa peptide is involved either in ATP binding or in actin binding, the present results suggest that the 50-kDa peptide of myosin S-1 acts as a communicating apparatus between the ATPase and actin binding sites. We have further endeavored to establish the manner in which the 26-, 50-, and 20-kDa peptides interact with each other.

## Materials and Methods

*Reagents.* Glutaraldehyde (SP, 25% in water) and TNBS (sodium salt) were purchased from Nakarai Chemical Co. IAEDANS was from Aldrich Chemical Co. $\alpha$-Chymotrypsin, diphenylcarbamyl chloride treated trypsin, and soybean trypsin inhibitor were from Sigma Chemical Co. ATP, ADP, and AMP were from Kohjin Co. GTP, GDP, UTP, ITP, and dATP were from Yamasa Shoyu Co. Trinitrophenyl derivatives of ADP, ATP, and GTP were prepared as described previously (Hiratsuka & Uchida, 1973; Hiratsuka, 1985b). Anthraniloyl and *N*-methylanthraniloyl derivatives of ATP and ADP were prepared as described previously (Hiratsuka, 1983). All other reagents were of reagent or biochemical research grade.

*Preparation of Proteins.* Rabbit skeletal myosin was prepared by the method of Perry (1955) with slight modification. F-Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt (1971). S-1 was prepared by chymotryptic digestion of myosin (Yagi & Otani, 1974) with modification as described by Weeds and Taylor (1975).

Tryptic S-1 was prepared as follows. Digestion of S-1 (7.5 mg/mL) was conducted with a 1:200 (w/w) ratio of diphenylcarbamyl chloride treated trypsin to S-1 in 50 mM KCl and 5 mM Mops (pH 7.0) at 25 °C. The reaction was stopped at 60 min by adding soybean trypsin inhibitor in an amount which was twice that of trypsin (w/w). Tryptic S-1 (5 mg/mL) was fluorescently labeled when necessary with a 10-fold molar excess of IAEDANS (Takashi, 1979) in buffer A (0.5 mM $MgCl_2$, 50 mM NaCl, and 40 mM borate, pH 8.5) at 25 °C for 30 min.

Denaturation of tryptic and undigested S-1's was carried out by incubating the protein (2 mg/mL) in buffer A with 2% SDS for 27 h at room temperature.

[1] Abbreviations: S-1, myosin subfragment 1; SDS, sodium dodecyl sulfate; NTP, nucleoside 5′-triphosphate; $PP_i$, pyrophosphate; $P_i$, orthophosphate; $V_i$, orthovanadate; TNBS, 2,4,6-trinitrobenzenesulfonate; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; kDa, kilodalton(s); Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

*Glutaraldehyde Treatment.* The original solution of glutaraldehyde was diluted with water to prepare the stock solution (0.5 M). The solution was stored at 0 °C. More dilute solutions in 2.5 mM $MgCl_2$, 0.25 M NaCl, and 0.2 M borate (pH 8.5) were prepared just prior to use.

Tryptic S-1 and undigested S-1 (1 mg/mL) were incubated with glutaraldehyde in buffer A in the presence and absence of 0.5 mM ligand at 25 °C. The glutaraldehyde concentration was 0.2 mM in all cases unless otherwise noted. The reaction was stopped by the addition of 1 M glycine/NaOH (pH 8.5) at a final concentration of 15 mM. The reaction mixture was then dialyzed overnight against 10 mM borate (pH 8.0) and 10 mM KCl for ATPase measurements and determinations of thiols and the reactive lysyl residue. All measurements were completed within 2 days after dialysis of samples.

When the protein samples were stored at 0 °C, no degradation of the cross-link was detected by SDS–polyacrylamide gel electrophoresis even after 16 days, consistent with the high stability of the glutaraldehyde-generated covalent bonds (Peters & Richards, 1977).

*ATPase Measurements.* The $K^+$- and $Ca^{2+}$-ATPase activities were measured at 25 °C in 1 mM ATP, 0.5 M KCl, and 50 mM Tris-HCl (pH 8.0) in the presence of 5 mM EDTA and $CaCl_2$, respectively. The $Mg^{2+}$-ATPase activity was measured at 25 °C in 1 mM ATP, 10 mM KCl, 1 mM $MgCl_2$, and 50 mM Tris-HCl (pH 8.0). The protein concentrations were 0.005 mg/mL ($K^+$- and $Ca^{2+}$-ATPases) and 0.05 mg/mL ($Mg^{2+}$-ATPase). The actin-activated ATPase was measured under the same conditions used for the $Mg^{2+}$-ATPase measurement, except for the presence of F-actin (0.08–0.4 mg/mL). $P_i$ liberated was determined by the method of Fiske and SubbaRow (1925).

*Turbidity Measurement.* The ability of tryptic and undigested S-1's to interact with actin was checked by monitoring turbidity at 400 nm (White & Taylor, 1976). The experiment was made at 24–26 °C in a final volume of 1 mL. The protein sample (0.8 mg/mL) was premixed with F-actin (0.4 mg/mL) in 50 mM Tris-HCl (pH 8.0), 10 mM KCl, and 1 mM $MgCl_2$. Turbidity was measured before and after the addition of 50 mM ATP (final concentration 1.2 mM).

*Determination of Thiols and of the Reactive Lysyl Residue.* The thiol content of unmodified and modified tryptic S-1 was measured by Ellman's titration (Ellman, 1959) in the presence of urea, as described previously (Uchida & Hiratsuka, 1971).

For the determination of the reactive lysyl residue, trinitrophenylation of the protein samples was carried out at 25 °C. TNBS (final concentration 0.1 mM) was added to a solution (1 mL) containing 0.45 mg of protein in 0.2 M Tris-HCl (pH 8.0) and 4 mM $MgCl_2$. The reaction was followed on the basis of the absorbance change at 345 nm. The number of trinitrophenylated amino groups was evaluated from the absorbance change ($\Delta\epsilon_{345}$ = 14 500) according to Okuyama and Satake (1960).

*SDS–Polyacrylamide Gel Electrophoresis.* Gel electrophoresis was carried out according to Weber and Osborn (1969) using 7.5% acrylamide gels. To follow the process of cross-linking of the protein, 50–100-$\mu$L aliquots of sample were withdrawn periodically and pipetted into equal volumes of a solution containing 0.1 M phosphate (pH 7.0), 2% SDS, 10% 2-mercaptoethanol, and 34% sucrose. Gels were stained with Coomassie Brilliant Blue. Phosphorylase *b* ($M_r$ 97 400), bovine serum albumin ($M_r$ 66 000), egg albumin ($M_r$ 45 000), chymotrypsinogen A ($M_r$ 25 700), soybean trypsin inhibitor ($M_r$ 20 100), and cytochrome *c* ($M_r$ 12 400) were used as molecular weight markers.

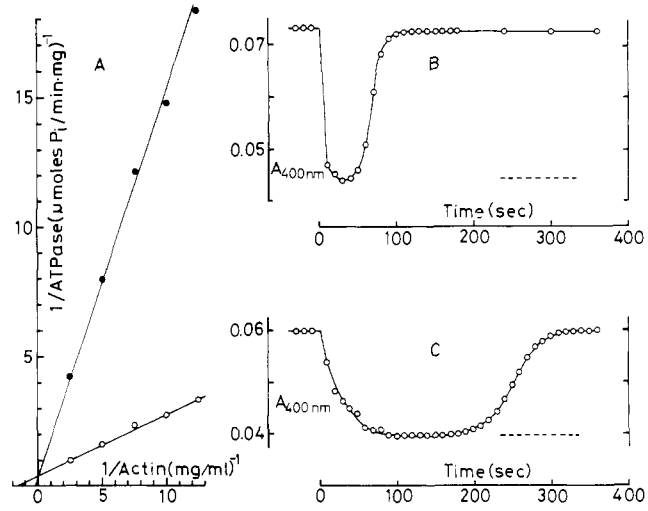


FIGURE 1: Comparison of the actin binding properties of tryptic and undigested S-1's. (A) Double-reciprocal plots of the actin-activated ATPase activity of tryptic (●) and undigested (○) S-1's; the ATPase activity was assayed at 25 °C in 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.05 mg/mL S-1, and 0.08–0.4 mg/mL F-actin. (B and C) Time courses of the change in turbidity of acto–S-1 (B) and acto–tryptic S-1 (C) upon the addition of ATP: the experiments were made at 24–26 °C in a final volume of 1 mL; the protein sample (0.8 mg/mL) was premixed with F-actin (0.4 mg/mL) in 50 mM Tris-HCl (pH 8.0), 10 mM KCl, and 1 mM $MgCl_2$; turbidity was measured at 400 nm (White & Taylor, 1976) before and after the addition of ATP (1.2 mM) at zero time; (---) the sum of turbidities of actin and S-1 measured individually.

For quantitative analysis of the formation of cross-linked products, the gels were scanned with a Yamato Ozumor 82 densitometer at 570 nm. Areas under peaks of the protein bands were measured.

*Concentration Determinations.* The concentration of S-1 was determined from the extinction coefficient ($A^{1\%}_{1cm}$) at 280 nm of 7.5 (Wagner & Weeds, 1977). Protein concentrations of glutaraldehyde–tryptic S-1 were determined by the biuret method (Gornall et al., 1949), standardized by using the $A^{1\%}_{1cm}$ of undigested S-1. S-1 and actin were assumed to have molecular weights of 120 000 (Weeds & Taylor, 1975) and 42 000 (Collins & Elzinga, 1975), respectively.

## Results

*ATPase and Actin Binding Properties of Tryptic S-1.* We first studied the ATPase and actin binding properties of our tryptic S-1. In agreement with the findings by Mornet et al. (1979) and Yamamoto and Sekine (1979), the various ATPase activities of S-1 alone were not affected by tryptic digestion. The specific activities of undigested S-1 were 1.3, 4.8, and 0.034 $\mu$mol of $P_i$/(min·mg) for $Ca^{2+}$-, $K^+$-, and $Mg^{2+}$-ATPase activities, respectively, while those of tryptic S-1 were 1.2, 4.5, and 0.035 $\mu$mol of $P_i$/(min·mg), respectively.

The actin-activated ATPases of undigested and tryptic S-1's were also studied by using a double-reciprocal plot of ATPase rate against actin concentration. As shown in Figure 1A, the linear regression analyses of the data for undigested and tryptic S-1's are consistent with the previous findings of Botts et al. (1982), who showed that the $V_{max}$ was unchanged but that the $K_m$ for actin was increased by tryptic cleavage. We obtained $K_m$ values of 12 and 82 $\mu$M for undigested S-1 and tryptic S-1, respectively. The $V_{max}$ was 2.4–2.5 $\mu$mol of $P_i$/(min·mg) for both S-1's.

The ability of S-1 to interact with actin can be followed directly by turbidimetry (White & Taylor, 1976). When ATP is added to acto–S-1, a rapid drop in turbidity occurs due to

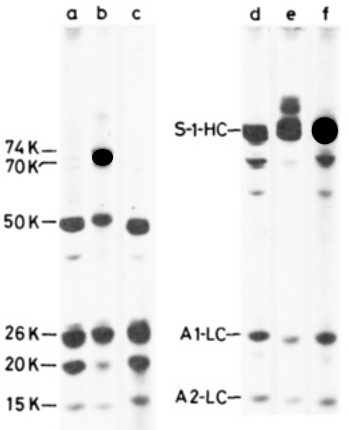


FIGURE 2: SDS gel (7.5%) electrophoresis of tryptic (a–c) and undigested (d–f) S-1's incubated with glutaraldehyde: (a and d) control; (b and e) glutaraldehyde–tryptic S-1 and –undigested S-1; (c and f) glutaraldehyde–tryptic S-1 and –undigested S-1, which were predenatured by SDS. The protein (1 mg/mL) was incubated with 0.2 mM glutaraldehyde in 0.5 mM $MgCl_2$, 50 mM NaCl, and 40 mM borate (pH 8.5) for 30 min at 25 °C. HC, heavy chain; A1, alkali 1; A2, alkali 2; LC, light chain.

dissociation of the protein complex, followed by a recovery as the ATP is exhausted and the rigor state is re-formed (Figure 1B). Although this recovery phase was markedly slowed in the case of tryptic S-1 (Figure 1C), it was able to interact with actin to a similar extent in the case of undigested S-1. These results thus support the findings of Mornet et al. (1979) and Botts et al. (1982). They reported that tryptic digestion of S-1 did not alter the extent of actin binding, as indicated by turbidity and fluorescence lifetime measurements, respectively.

*Reaction of Tryptic S-1 with Glutaraldehyde.* Digestion with trypsin of S-1 produces three main peptide fragments of 50, 26, and 20 kDa from the heavy chain together with a minor component of 15 kDa (lane a in Figure 2) (Balint et al., 1978; Mornet et al., 1979; Yamamoto & Sekine, 1979). Incubation of the tryptic S-1 with glutaraldehyde at a concentration of 0.2 mM caused the 20- and 50-kDa protein bands to diminish. On the other hand, new protein bands of the cross-linked products with apparent molecular weights of 70 000 (minor) and 74 000 (major) appeared on the SDS–polyacrylamide gels (lane b). However, most of the 26-kDa peptide remained unchanged. When the denatured tryptic S-1 was incubated with glutaraldehyde, none of the cross-linked products were formed (lane c). The glutaraldehyde treatment of undigested S-1 caused the protein bands of light chains to diminish, yielding the cross-linked products of the heavy and light chains (lane e). However, no cross-linked product of oligomers of the heavy chain was detected. It was again indicated that denaturation of the protein abolished the formation of the cross-linked products (lane f). These results suggest that we are looking only at intra-heavy-chain cross-linking and glutaraldehyde would therefore be potentially useful as a tool for selectively "freezing" the existing interactions between the 20- and 50-kDa peptides of tryptic S-1.

In order to check and confirm the results reported above, we examined the time course of the reaction of tryptic S-1 with glutaraldehyde. The experiment shown in Figure 3A indicates that concomitantly with the formation of the 70- and 74-kDa cross-linked products there is a progressive decrease in the amounts of the original 20- and 50-kDa peptides. At prolonged reaction times (longer than 60 min), a slight formation of the products with higher molecular weights was observed. No cross-linked product of the 20-, 26-, and 50-kDa peptides was detected below the 70- and 74-kDa protein bands.

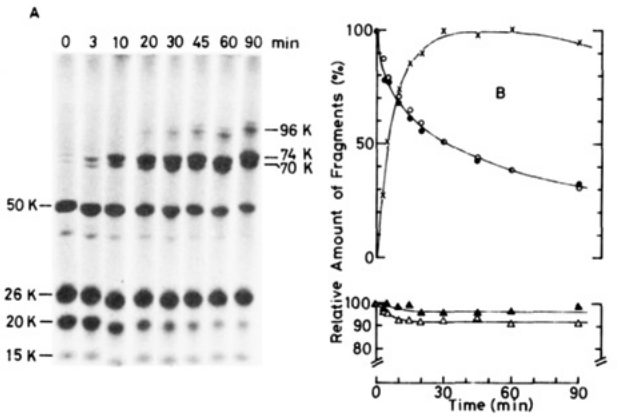


FIGURE 3: (A) SDS electrophoretic gels showing the time course of the reaction of tryptic S-1 with 0.2 mM glutaraldehyde. (B) Densitometric estimation of band intensities present in the SDS gel of tryptic S-1 during its reaction with 0.2 mM glutaraldehyde: (×) 70-kDa product + 74-kDa product; (○) 20-kDa peptide; (●) 50-kDa peptide; (▲) 15-kDa peptide; (△) 26-kDa peptide; percent is expressed as percent of the original band intensity (○, ●, △, and ▲) or of the maximal band intensity (×). Tryptic S-1 was incubated under the same conditions described in Figure 2 for the indicated times and then subjected to SDS gel (7.5%) electrophoresis.

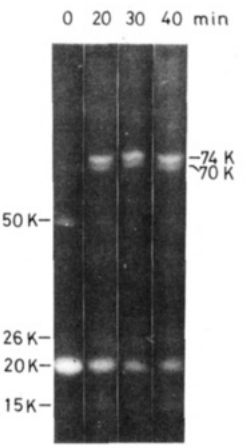


FIGURE 4: Identification of the 70- and 74-kDa cross-linked products. Prior to the reaction with glutaraldehyde, tryptic S-1 was fluorescently labeled with IAEDANS (see Materials and Methods). The labeled protein was incubated with glutaraldehyde under the same conditions described in Figure 2 for the indicated times and then subjected to SDS gel electrophoresis. Photographs of the gels were taken under UV illumination in the dark.

Quantitative analysis of the formation of the 70- and 74-kDa products was carried out by densitometric scanning of the gels (Figure 3B). The 70- and 74-kDa protein bands were counted together, since these bands could not be separated well. The data clearly indicate that the relative rates of disappearance of the 20- and 50-kDa peptides are nearly identical and that these peptides are converted into the 70- and 74-kDa products. This means that the 20- and 50-kDa peptides are cross-linked with a ratio of 1:1. After 90 min of reaction, 70% of the 20- and 50-kDa peptides was cross-linked, while most of the 26- and 15-kDa peptides (90–95%) was left un-cross-linked.

To confirm that either the 70-kDa product or the 74-kDa product indeed contains the 20- and 50-kDa peptides, we attempted to fluorescently label the 20-kDa peptide. The labeling was carried out with IAEDANS (Takashi, 1979). The fluorescently labeled tryptic S-1 was incubated with glutaraldehyde. As shown in Figure 4, the predominantly labeled band was the 20-kDa peptide. When the protein was incubated with glutaraldehyde, concomitantly with the decrease of fluorescence on the 20-kDa band there was a progressive increase of fluorescence on both the 70- and 74-kDa

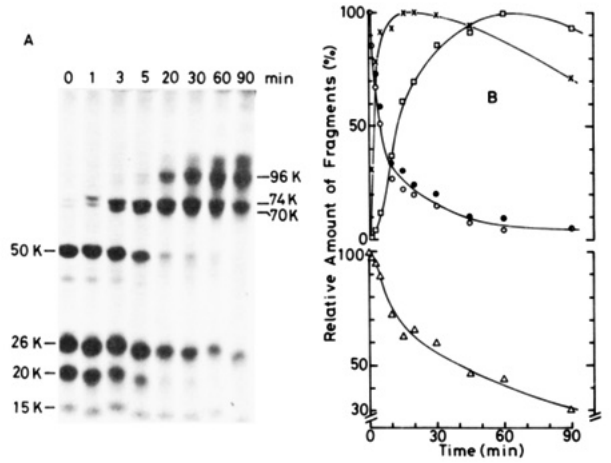


FIGURE 5: (A) SDS electrophoretic gels showing the time course of the reaction of tryptic S-1 with 0.5 mM glutaraldehyde. (B) Densitometric estimation of band intensities present in SDS gel of tryptic S-1 during its reaction with 0.5 mM glutaraldehyde: (□) 96-kDa cross-linked product; other symbols as in Figure 3; percent is expressed as percent of the original band intensity (○, ●, and △) or of the maximal band intensity (× and □). The experiments were made as described in Figure 3, except that the glutaraldehyde concentration was 0.5 mM.

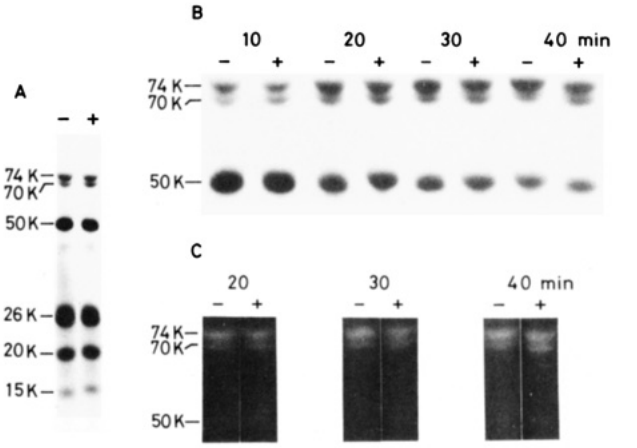


FIGURE 6: Comparison of the electrophoretic patterns of glutaraldehyde–tryptic S-1 prepared in the presence (+) and absence (−) of ATP: (A) protein banding patterns after incubation with glutaraldehyde for 20 min; (B) time courses of the formation of the 70- and 74-kDa cross-linked products; (C) location on gel of the cross-linked products containing the fluorescently labeled 20-kDa peptide (see Materials and Methods for the preparation of fluorescent tryptic S-1). The protein was incubated with glutaraldehyde for the indicated times as described in Figure 2 in the presence and absence of 0.5 mM ATP. Other conditions are described in Figure 4. The 26-, 20-, and 15-kDa peptides are not shown in (B) and (C).

bands. Therefore, we concluded that not only the major 74-kDa product but also the minor 70-kDa product consisted of the 20- and 50-kDa peptides.

Tryptic S-1 was also incubated under a higher concentration of glutaraldehyde (0.5 mM). Raising the reagent concentration accelerated the formation of the 70- and 74-kDa products (Figure 5A). However, under this condition, the 26-kDa peptide was also cross-linked. The data shown in Figure 5B indicate that the initial rapid disappearance of the 20- and 50-kDa peptides is followed by a second phase in which the 26-kDa peptide is converted into the 96-kDa product. Finally, 95% of the 20- and 50-kDa peptides and 70% of the 26-kDa peptide were cross-linked after 90 min of reaction. Therefore, we chose the glutaraldehyde concentration of 0.2 mM rather than 0.5 mM for the selective freezing of the interactions between the 20- and 50-kDa peptides.

*Effects of Nucleotides on the Reaction of Tryptic S-1 with Glutaraldehyde.* To determine whether ATP changes the formation of the cross-linked products of tryptic S-1 on glutaraldehyde treatment, the treatment was carried out in the presence of ATP. As shown in Figure 6A, the addition of 0.5 mM ATP to the incubation mixture changed the electrophoretic pattern of only the 70- and 74-kDa products; there was no significant difference in the amounts of the 20-, 26-, and 50-kDa peptides. We failed to estimate the individual amounts of the 70- and 74-kDa products densitometrically, since these protein bands could not be separated well as described above. However, the densitometric traces of the gels showed that there was no significant difference in the sum of amounts of the 70- and 74-kDa products between two samples prepared with and without ATP (not shown). To confirm this point further, visual inspection of the gels was made for these two protein samples as a function of time (Figure 6B). The effect exerted by ATP can be clearly seen in the increase of the amount of the 70-kDa product and in the decrease of the amount of the 74-kDa product, as compared to the corresponding gel of the sample prepared without ATP. It was also confirmed using the fluorescently labeled proteins that either the 70-kDa product or the 74-kDa product, which had been generated in the presence of ATP, indeed, contained the 20-kDa peptide as well as those generated in its absence (Figure 6C).

Effects of nucleotides other than ATP and the analogues on the formation of the 70- and 74-kDa products were also examined (not shown). Like ATP, ADP and $PP_i$ exerted effects on the formation of these products while AMP and $P_i$ did not. Among naturally occurring NTPs tested, only ATP and dATP were effective. A similar result was obtained with various ribose-modified NTP analogues (Hiratsuka & Uchida, 1973; Hiratsuka, 1983, 1984a, 1985b). The ATP analogues exerted their effects, but the GTP analogue did not. On the other hand, ADP–$V_i$ and ADP analogue–$V_i$ acted as effectors more efficiently than the corresponding NTP and NTP analogue, respectively. Similarly, $PP_i$–$V_i$ was superior to $PP_i$ in its effect. However, GDP–$V_i$ was again ineffective. These results clearly indicate that the effect of nucleotides on the formation of the 70- and 74-kDa cross-linked products is highly dependent on the base structure. Furthermore, the fact that the effect is limited to the adenine nucleotides, its analogues, and $PP_i$ suggests that various nucleotide bases other than adenine, especially guanine, prevent the 20- and 50-kDa peptides from being cross-linked in a manner similar to the case of ATP.

*Titration of Thiol Groups and the Reactive Lysyl Residue of Glutaraldehyde–Tryptic S-1.* S-1 contains two highly reactive thiols (Kielley & Bradley, 1956; Sekine & Yamaguchi, 1963) which are located on the 20-kDa peptide (Balint et al., 1978). Another well-defined residue of S-1 is one reactive lysyl residue, which is rapidly trinitrophenylated by TNBS (Kubo et al., 1960). This residue is located on the 26-kDa peptide (Mornet et al., 1980; Miyanishi & Tonomura, 1981; Hozumi & Muhlrad, 1981). Modification of these thiol and lysyl residues markedly affects the ATPase properties of S-1. Since glutaraldehyde reacts mostly with the free amino groups of proteins, but also to a lesser extent with thiol groups (Korn et al., 1972; Habeeb & Hiramoto, 1968), next we titrated free thiol groups and the reactive lysyl residue of the glutaraldehyde–tryptic S-1 with Ellman's reagent (Ellman, 1959) and TNBS, respectively.

As shown in Figure 7A, the total number of free thiol groups of tryptic S-1 determined after glutaraldehyde treatment was unchanged irrespective of the incubation time and conditions.

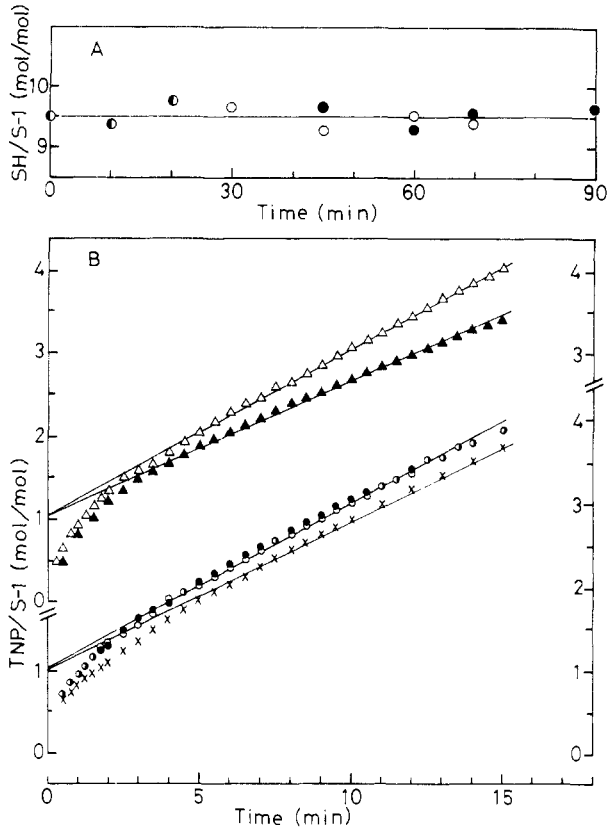


FIGURE 7: Determinations of thiol groups (A) and the reactive lysyl residue (B) of glutaraldehyde–tryptic S-1. Glutaraldehyde–tryptic S-1 was prepared in the presence and absence of 0.5 mM ATP, as described under Materials and Methods. (A) The total number of free thiol groups of the protein sample was determined by using Ellman's reagent (Ellman, 1959; Uchida & Hiratsuka, 1971); tryptic S-1 was incubated with glutaraldehyde for the indicated times in the presence (●) and absence (○) of ATP. (B) Tryptic S-1 was incubated with glutaraldehyde for 45 (○, ×) or 70 min (●) in the presence (×) and absence (○, ●) of 0.5 mM ATP: (▲) control undigested S-1; (△) control tryptic S-1; the experiments were made at 25 °C with the protein sample (0.45 mg/mL) in 0.2 M Tris-HCl (pH 8.0), 4 mM $MgCl_2$, and 0.1 mM TNBS; the reaction was followed on the basis of the absorbance change at 345 nm, and the number of trinitrophenylated amino groups was evaluated according to Okuyama and Satake (1960); TNP, 2,4,6-trinitrophenyl.

The mean value of 9.5 thiols/mol of tryptic S-1 we found agrees well with the value of 10 ± 0.8 mol reported for the undigested chymotryptic S-1 by Schaub et al. (1978). On the other hand, the time courses of trinitrophenylation of undigested and tryptic S-1's followed spectrophotometrically are biphasic, first fast and then slow (Figure 7B). In both cases, the existence of one reactive lysyl residue was clearly indicated by extrapolation of the slow reaction to zero time. Similar results were obtained with the glutaraldehyde–tryptic S-1. When the protein sample was prepared in the presence of ATP, the reactivity of the fast-reacting residue toward TNBS was diminished. In any event, the results reported in this paragraph show that neither the reactive thiols nor the reactive lysyl residue of tryptic S-1 is modified with glutaraldehyde under our experimental conditions. These results also support the previous report that the specificity of aldehydes toward the reactive lysyl residue of S-1 is less than that of TNBS (Takashi et al., 1982).

*ATPase Properties of Glutaraldehyde–Tryptic S-1.* The effect of glutaraldehyde treatment on the $Ca^{2+}$-, $K^+$-, and $Mg^{2+}$-ATPases of tryptic S-1 was also followed. Figure 8 shows the time courses of the changes in various ATPase activities when the protein is incubated with glutaraldehyde

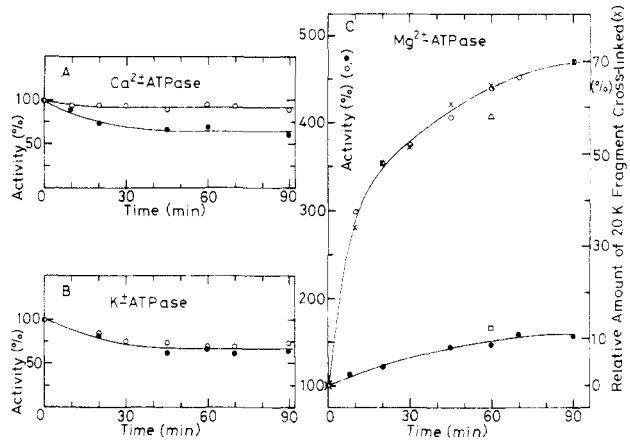


FIGURE 8: Effect of the reaction with glutaraldehyde on the $Ca^{2+}$- (A), $K^+$- (B), and $Mg^{2+}$-ATPase (C) activities of tryptic S-1. Glutaraldehyde–tryptic S-1 was prepared in the presence and absence of 0.5 mM ligand, as described under Materials and Methods: (○) no addition; (●) +ATP; (□) +$PP_i$; (△) +GTP. Control activities in micromoles of $P_i$ per minute per milligram: $Ca^{2+}$-ATPase, 1.2; $K^+$-ATPase, 4.5; $Mg^{2+}$-ATPase, 0.035. The relative amount of the cross-linked 20-kDa peptide (×) was obtained from the data shown in Figure 3B.

in the presence and absence of ATP. The $Ca^{2+}$- and $K^+$-ATPase activities of tryptic S-1 were decreased slightly with incubation time and reached a plateau after about 30–45 min; only about 10–30% loss of its activity was observed with all samples studied even after 90 min of incubation (Figure 8A,B).

In contrast to the above results, the $Mg^{2+}$-ATPase activity of tryptic S-1 was significantly enhanced by glutaraldehyde treatment (Figure 8C). When the protein was incubated with glutaraldehyde for 90 min in the absence of ligand, the activity was increased to 470% of the original activity. Furthermore, we found a good correlation between the magnitude of increase in the activity and the amount of cross-linked 20-kDa peptide. This suggests that the freezing of the interactions between the 20- and 50-kDa peptides causes the activation of the $Mg^{2+}$-ATPase of tryptic S-1. However, the high activity was scarcely produced if the glutaraldehyde treatment was conducted in the presence of ATP. $PP_i$ exerted this effect but GTP did not.

The $Mg^{2+}$-ATPase activity of glutaraldehyde–tryptic S-1 was also measured in the presence of actin. When the protein was incubated with glutaraldehyde in the absence of ligand, the actin-activated ATPase was enhanced slightly with incubation time. On the contrary, the ATPase was inhibited slightly in the case of the protein sample prepared in the presence of ATP (not shown).

In order to further clarify the effect of glutaraldehyde treatment on the actin-activated ATPase of tryptic S-1, we estimated the $K_m$ for actin and $V_{max}$ values of the protein samples, which had been incubated with glutaraldehyde for 60 min in the presence and absence of various ligands. These values were estimated from the modified double-reciplocal plot of ATPase rate against actin concentration (Barouch & Moos, 1971).

Table I summarizes the $V_{max}$ and $K_m$ values for various samples. The $V_{max}$ values were found to be nearly identical for all samples studied [0.32–0.36 μmol of $P_i$/(min·mg)]. However, they were only 13–15% of the value of undigested and tryptic S-1's [2.4–2.5 μmol of $P_i$/(min·mg)]. On the other hand, glutaraldehyde treatment resulted in a marked increase in affinity of tryptic S-1 for actin. The $K_m$ value of glutaraldehyde–tryptic S-1 (15 μM) was very close to that of undigested S-1 (12 μM). However, this high actin affinity was

Table I: Kinetic Constants $V_{max}$ and $K_m$ for Actin of Glutaraldehyde–Tryptic S-1 Prepared in the Presence and Absence of Various Ligands

| protein sample[a] | $V_{max}$[b] [μmol of $P_i$/(min·mg)] | $K_m$[b] (μM) |
|---|---|---|
| control S-1 | 2.4 | 12 |
| control tryptic S-1 | 2.5 | 82 |
| glutaraldehyde–tryptic S-1 | | |
| no addition | 0.34 | 15 |
| +ATP | 0.36 | 50 |
| +$PP_i$ | 0.34 | 42 |
| +GTP | 0.32 | 18 |

[a] Tryptic S-1 (1 mg/mL) was incubated with 0.2 mM glutaraldehyde in 0.5 mM $MgCl_2$, 50 mM NaCl, and 40 mM borate (pH 8.5) in the presence and absence of 0.5 mM ligand for 60 min at 25 °C. [b] The actin-activated ATPase activities were measured under the same conditions described in Figure 1A. The $V_{max}$ and $K_m$ values were obtained from the modified double-reciprocal plot (Barouch & Moos, 1971).

scarcely produced if the treatment was conducted in the presence of ATP or $PP_i$; the $K_m$ value was rather close to that of tryptic S-1. Again, GTP did not exert the effect in this case.

## Discussion

Glutaraldehyde is widely used as a cross-linking reagent for soluble and membrane-bound proteins (Peters & Richards, 1977; Ji, 1983). At neutral pH, aqueous glutaraldehyde solutions contain primarily the cyclic monohydrate and to a lesser extent the open-chain monomer and its hydrates (Korn et al., 1972). The active species is the unhydrated monomer; it reacts mostly with the free amino groups of proteins as a cross-linking reagent, yielding acid-stable cross-linked products (Habeeb & Hiramoto, 1968; Korn et al., 1972; Peters & Richards, 1977). Although the reaction of glutaraldehyde with protein is complex and not fully understood (Peters & Richards, 1977), under our experimental conditions, glutaraldehyde, because of low concentrations of reagent (0.2 mM) and protein (1 mg/mL), acted as a cross-linker for selectively freezing the interactions between the 20- and 50-kDa peptides within the heavy chain of tryptic S-1. Since glutaraldehyde solution contains an equilibrium mixture of the active and inactive species, there is a rather consistent relationship between the amount of glutaraldehyde added to the protein and the amount of lysyl residue consumed. This is close to 4 mol of glutaraldehyde per mole of lysyl residue for various proteins (Korn et al., 1972). If the same relationship holds also for tryptic S-1, only 3 mol of the cross-link is produced at the most per mole of the protein under our experimental conditions.

Treatment of tryptic S-1 with glutaraldehyde generated two types of cross-linked products. One type had an apparent molecular weight of 74 000 and the other 70 000. Both products were 1:1 complexes of the 20- and 50-kDa peptides. However, none of the cross-linked products of the 20- and 26-kDa peptides were detected under our experimental conditions. The reason appears to be that there is no suitable pair of lysyl residues between these peptides, which is susceptible to modification by glutaraldehyde. With the use of a photodynamic technique, the 20- and 26-kDa peptides were directly cross-linked (Hiratsuka, 1984c).

The origin of two cross-linked products is not well explained at present. However, the simplest and most attractive possibility is that the interacting site of the 20- and 50-kDa peptides exists in two conformational states and the site in one conformational state generates the 74-kDa product while that in the other conformational state generates the 70-kDa product. Upon addition of ATP, the formation of the 70-kDa product was accelerated while that of the 74-kDa product was suppressed. There was no significant difference in the sum of amounts of these products between two samples prepared in the presence and absence of nucleotide. This means that the precursors of the 70- and 74-kDa products are interconvertible upon the addition of nucleotide. After all, the results suggest that the interaction of ATP with the 26- and 50-kDa peptides (Szilagyi et al., 1979; Okamoto & Yount, 1985; Mahmood & Yount, 1984; Hiratuska, 1985a) induces conformational changes in the interacting sites of the actin binding peptides, the 20- and 50-kDa peptides (Mornet et al., 1979; Yamamoto & Sekine, 1979; Sutoh, 1982; Muhlrad & Morales, 1984).

The degree of conformational changes at the S-1 active site induced by various fluorescent nucleotides decreases in the order ADP = dADP > CDP >> UDP ≥ IDP > GDP (Hiratsuka, 1984a,b). This series represents roughly the order seen with corresponding natural NTPs for the ability to support superprecipitation of actomyosin (Maruyama & Gergely, 1962; Weber, 1969; Ghani & Watanabe, 1971; Hiratsuka, 1984a) and to contract and relax myofibrils (Weber, 1969). These results suggest that the ability of NTP to influence the actin binding site is highly dependent on the base structure of nucleotide and that the adenine moiety of the ATP molecule is essential for transmission of the conformational changes generated at the ATPase site to the actin binding site. Our present results are in agreement with these observations. ATP and dATP altered the cross-linking pattern of the 20- and 50-kDa peptides, but UTP, ITP, and GTP did not. Since $PP_i$ also exerted the effect, nucleotide bases other than adenine appear to prevent the NTP to interact with the protein in a manner similar to the case of ATP. This explanation is supported by the previous results that among nucleotide analogues having various base structures only the ADP and dADP analogues are uniquely buried in the active site of myosin ATPase (Hiratsuka, 1984a,b). Since actin binds either to the 20-kDa peptide or to the 50-kDa peptide (Mornet et al., 1979; Yamamoto & Sekine, 1979; Sutoh, 1982; Muhlrad & Morales, 1984) and the 50-kDa peptide contains the ATP binding site (Mahmood & Yount, 1984; Hiratsuka, 1985), it is logical to assume that the conformational changes generated by the adenine moiety of nucleotide bound at the ATPase site have to be transmitted to the actin binding site and the interacting sites of the 20- and 50-kDa peptides through the 50-kDa peptide.

Although glutaraldehyde was found to block neither the reactive thiols nor the reactive lysyl residue of tryptic S-1, the ATPase and actin binding properties of glutaraldehyde–tryptic S-1 were different in two protein samples prepared in the presence and absence of ligand. The high $Mg^{2+}$-ATPase activity and the low $K_m$ for actin of glutaraldehyde–tryptic S-1 prepared in the absence of ligand can be explained most simply by postulating that glutaraldehyde freezes the protein in the activated conformation. This explanation is supported by the fact that this activated conformation was scarcely produced in the presence of ATP. The protein sample prepared in the presence of ATP showed low $Mg^{2+}$-ATPase activity and a high $K_m$ for actin, as compared to the sample prepared in the absence of ligand. The reagent appears to freeze the protein in the nonactivated conformation under this condition. ATP and $PP_i$ were effective for the production of this nonactivated conformation but GTP was not. This pattern of effectiveness of nucleotide was consistent with that of the effectiveness of the formation of the 74- and 70-kDa cross-linked products, depending on the base structure of nucleotide. Thus, one can conclude that the activated conformation is produced by the cross-linking of the 20- and 50-kDa peptides

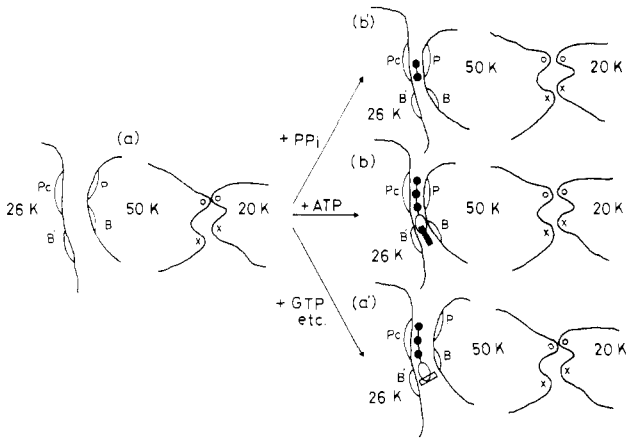


FIGURE 9: Proposed model for the communication between the ATPase and actin binding sites of tryptic S-1: (P) polyphosphate binding site; ($P_c$) catalytic site; (B and B′) base binding sites; (○ and ×) interacting sites between the 20- and 50-kDa peptides; (●) phosphate; (oval symbols) ribose; (■) adenine; (□) guanine. ATP and GTP are assumed to have the anti and syn conformations, respectively, when bound to protein (London & Schmidt, 1972; Gronenborn et al., 1984; Banerjee et al., 1985). The interaction between the 20- and 26-kDa peptides is not shown here. For details, see Discussion.

in which the 74-kDa product is predominantly generated, while the nonactivated conformation is produced by that in which either the 74-kDa product or the 70-kDa product is generated. It is likely that the conformational changes generated by the cross-linking of the 20- and 50-kDa peptides are transmitted to the ATPase site again through the 50-kDa peptide.

From the present studies, it is clear that the conformational changes at the ATPase site, which is located between the 26- and 50-kDa peptides, are transmitted to the interacting sites of the 50- and 20-kDa peptides, which contain actin binding sites, and vice versa. The overall data further provide support for the role of the 50-kDa peptide as a communicating apparatus between the ATPase and actin binding sites of S-1. If we accept this idea, our experiments can be easily interpreted in terms of a model shown in Figure 9. This model is developed on the assumptions that the nucleotide binding site resides between the 26- and 50-kDa peptides (Hiratsuka, 1985a). The model includes the provision that only the binding of ATP, dATP, and $PP_i$ induces a particular conformational change in the 26- and 50-kDa peptides, leading to closure of a nucleotide pocket (Hiratsuka, 1984a,b; present work), and the conformational transition is coupled to that at the interacting sites of the 20- and 50-kDa peptides. The ability of various nucleotides to affect the ATPase and actin binding properties is thus a consequence of a different property of the different conformations in the ATPase site and the interacting sites of the 20- and 50-kDa peptides. The interaction between the 20- and 26-kDa peptides, which has been previously suggested by us (Hiratsuka, 1984c), is not shown here.

The conformational state of the nucleotide binding site can be characterized as follows. The site is considered to be composed of four parts, designated P, $P_c$, B, and B′ (Figure 9a). Parts P and $P_c$ completely bind the triphosphate moiety of ATP; $P_c$ is the putative catalytic site for the triphosphate. Part B consists of a site capable of binding specifically to the intact adenine (Hiratsuka, 1984a,b, 1985a; present work) as well as having some affinity for the aromatic ring (Mahmood & Yount, 1984) by a hydrophobic ring interaction. Part B′ binds the various bases of nucleotide, including adenines having modified structures (Grammer et al., 1985). This part also has some affinity for the aromatic ring (Szilagyi et al., 1979; Okamoto & Yount, 1985).

In the absence of ligand, the ATPase site assumes an "open" conformational state (Figure 9a). Upon binding of ATP, the polyphosphate moiety must be first recognized because it has much greater affinity for myosin than the adenine moiety (Tonomura, 1972). Thus, parts $P_c$ and P surround the polyphosphate, causing the ATPase site to assume a "closed" conformational state (Figure 9b). This is not surprising because X-ray crystallographic studies have evidenced such a ligand-induced closure of the crevice of protein for hexokinase, phosphoglycerate kinase, dehydrogenases, and other various proteins (Steitz et al., 1982; Janin & Wodak, 1983). $PP_i$ can substitute for ATP (Figure 9b′). The interactions between adenine and parts B′ and B further stabilize the closed conformation. Especially the 6-amino group of adenine must play an important role in the stabilization of the conformation, as suggested previously (Hiratsuka, 1984a,b). However, the guanine base prevents the triphosphate portion of the nucleotide from interacting with part P, forcing the ATPase site to assume the open conformational state (Figure 9a′). Indeed, we have previously revealed that the ribose-modified analogues of GDP and IDP bound to the S-1 active site are less buried and thus more accessible to collision with acrylamide than those of ADP and dADP (Hiratsuka, 1984b). The reason may be that ATP tends to exist in the anti conformation when bound to protein while GTP and ITP tend to exist in the syn conformation (London & Schmidt, 1972; Gronenborn et al., 1984; Banerjee et al., 1985).

The conformational transition at the ATPase site, described above, is coupled to that at the interacting sites of the 20- and 50-kDa peptides. When the former site exists in the open state, the latter sites exist in the conformation in which the 74-kDa cross-linked product is predominantly generated (Figure 9a). On the other hand, when the former site exists in the closed state, the latter sites exist in that in which either the 74-kDa product or the 70-kDa product is generated (Figure 9b). Actin has a greater affinity for state a than for state b. Thus, binding of ATP produces state b, resulting in reduction in the affinity of actin for the actin binding site. Binding of actin stabilizes the open state of the ATPase site (Figure 9a), allowing a rapid release of the split products of ATP from myosin. This explanation is supported by the fact that myosin hydrolyzes GTP and ITP more rapidly than ATP and actin scarcely activates the myosin GTPase and ITPase (Stone & Prevost, 1973; Eccleston & Trentham, 1979); the ATPase site in the myosin–GTP and –ITP complexes (Figure 9a′) always assumes the conformational state rather similar to that observed with actomyosin (Figure 9a), regardless of the presence of actin, allowing rapid release of the split products from myosin.

In conclusion, our results suggest that the 50-kDa peptide acts as a communicating apparatus between the ATPase and actin binding sites of myosin S-1. The communication between two sites appears to be an example of a particularly long-range signal transmission within the myosin heavy chain. Of course, the present results do not exclude the possibility that both the ATPase and actin binding sites reside at or near the region where the 20-, 26-, and 50-kDa peptides are contiguous, as suggested previously (Hiratsuka, 1984c). However, the role of the 50-kDa peptide would be valid even in such a case, since this peptide can interact either with ATP or with actin (Mahmood & Yount, 1984; Hiratsuka, 1985a; Mornet et al., 1979; Yamamoto & Sekine, 1979; Sutoh, 1982; Muhlrad & Morales, 1984).

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**Registry No.** 5′-ATP, 56-65-5; $PP_i$, 14000-31-8; ATPase, 9000-83-3.

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