generation (Harrington, 1979).

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Nucleotide-Induced States of Myosin Subfragment 1 Cross-Linked to Actin†

Anh M. Duong and Emil Reisler*

Department of Chemistry and Biochemistry and Molecular Biology Institute, University of California, Los Angeles, California 90024

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ABSTRACT: Actomyosin interactions and the properties of weakly bound states in carbodiimide-cross-linked complexes of actin and myosin subfragment 1 (S-1) were probed in tryptic digestion, fluorescence, and thiol modification experiments. Limited proteolysis showed that the 50/20K junction on S-1 was protected in cross-linked acto–S-1 from trypsin even under high-salt conditions in the presence of MgADP, MgAMPPNP, and $MgPP_i$ (μ = 0.5 M). The same junction was exposed to trypsin by MgATP and MgATPγS but mainly on S-1 cross-linked via its 50K fragment to actin. *p*-Phenylenedimaleimide-bridged S-1, when cross-linked to actin, yielded similar tryptic cleavage patterns to those of cross-linked S-1 in the presence of MgATP. By using *p*-nitrophenylenemaleimide, it was found that the essential thiols of cross-linked S-1 were exposed to labeling in the presence of MgATP and MgATPγS in a state-specific manner. In contrast to this, the reactive thiols were protected from modification in the presence of MgADP, MgAMPPNP, and $MgPP_i$ at μ = 0.5 M. These modifications were compared with similar reactions on isolated S-1. Experiments with pyrene-actin cross-linked to S-1 showed enhancement of fluorescence intensity upon additions of MgATP and MgATPγS, indicating the release of the pyrene probe on actin from the sphere of S-1 influence. The results of this study contrast the "open" structure of weakly bound actomyosin states to the "tight" conformation of rigor complexes.

Muscle contraction is believed to involve cyclic interaction between myosin crossbridges and actin filaments. Such interaction results in the sliding of the two filaments past each other and the generation of force (Huxley & Niedergerke, 1954; Huxley & Hanson, 1954). The widely accepted mechanism of force generation describes two states of myosin crossbridges (Huxley, 1969; Huxley & Simmons, 1971; Huxley & Kress, 1985). Initially, myosin heads would bind to actin at a 90° angle and then rotate to a 45° angle during the power

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stroke to generate force. Recent biochemical work has indeed suggested the existence of at least two alternating actin binding states, the strongly and weakly attached myosin crossbridges (Eisenberg & Hill, 1985). In the strongly bound state, myosin heads are inferred to retain the same properties and perhaps an orientation similar to that of rigor crossbridges (Cooke, 1986). The transition between the strong and weak binding states is believed to involve structural changes in actomyosin complexes (Trayer & Trayer, 1988).

Presently, experimental data characterizing the weakly bound state remain inadequate. However, interestingly, recent investigations have suggested the existence of a weakly bound state in the cross-linked actin–subfragment 1 (acto–S-1)[1] (XLAS-1) system in the presence of MgATP. Electron microscopy studies show that the cross-linked S-1 assumes a wide range of angles with respect to actin filaments in the presence of MgATP (Craig et al., 1985; Applegate & Flicker, 1987). This result is supported by EPR work showing great rotational mobility of the cross-linked S-1 under the same conditions (Svensson & Thomas, 1986). Since the ATPase activity of the XLAS-1 is near V_{max} (Mornet et al., 1981), it has been suggested that the "disorganized" cross-linked S-1 is "attached" most of the time to actin in a manner representative of an active state (Craig et al., 1985). In addition to this, the ability of the troponin–tropomyosin complex to inhibit the ATPase activity of XLAS-1 implied a weakly bound state for the cross-linked S-1 (King & Greene, 1987). However, the alternative possibility that XLAS-1 and ATP might be actually yielding "tethered" acto–S-1 complexes could not be excluded.

In the present work, tryptic digestions, maleimide modifications, and fluorescence measurements were employed to study the properties of cross-linked acto–S-1 complexes in the presence of nucleotides. Tryptic digestion data indicated that the 50/20K junction of cross-linked S-1 was cleaved in the presence of MgATP and MgATPγS but not in the presence of MgADP or MgAMPPNP (at μ = 0.5 M). Thiol modifications showed that the reactive cysteines on S-1, i.e., SH_1 and SH_2, were exposed to reagent when the cross-linked acto–S-1 bound MgATP or MgATPγS. The reactive thiols could not be unmasked by MgADP, MgAMPPNP, or $MgPP_i$ even under high ionic strength condition. Fluorescence measurements of pyrene-actin cross-linked to S-1 showed unmasking of pyrene probes by ATP or ATPγS, but not ADP. These results are discussed in terms of the conformations of the strongly and weakly bound actomyosin states.

Materials and Methods

Reagents. Trypsin, soybean trypsin inhibitor, subtilisin, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), ADP, ATP, AMPPNP, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). ATPγS was obtained from Boehringer Mannheim. *p*-Nitrophenylmaleimide (pNPM) and *N,N'-p*-phenylenedimaleimide (pPDM) were from Aldrich Chemical Co. (Milwaukee, WI). *N*-(1-Pyrenyl)iodoacetamide was a product of Molecular Probes (Eugene, OR). All other reagents used were of analytical grade.

Proteins. Actin was prepared from rabbit muscle according to the methods of Spudich and Watt (1971). S-1 was prepared by chymotryptic digestion of myosin as previously described (Weeds & Pope, 1977). Protein concentrations were determined at 280 nm by using $A^{1\%} = 7.5$ cm^{-1} for S-1 and 11 cm^{-1} for actin. The three forms of tryptic S-1 (25/50/20K, 25/70K, and 75/20K) were prepared as described previously (Chen et al., 1987).

Cross-Linking of Acto–S-1 Complexes. S-1 and actin (1 mg/mL each) were allowed to form a complex by incubation at 22 °C for 30 min in 0.1 M NaCl/10 mM imidazole, pH 7.0. EDC was subsequently added to a final concentration of 1 mg/mL to start the cross-linking reaction (Sutoh, 1983). The reaction was allowed to proceed for 2 h and was stopped by adding 10 mM β-mercaptoethanol. S-1 which had been inactivated with 4× molar excess of pNPM (pH 8.0) was cross-linked to actin under identical conditions.

The cross-linked acto–S-1 complexes were centrifuged at 40000 rpm (4 °C) in the presence of 10 mM MgATP to remove the un-cross-linked S-1. The supernatant contained only S-1. The pellets containing cross-linked acto–S-1 were homogenized and dialyzed in 0.1 M NaCl/25 mM Tris, pH 8.0, or in 0.1 M NaCl/10 imidazole, pH 7.0. The concentration of S-1 in the isolated pellet was estimated by measuring the concentration of S-1 recovered in the supernatant.

Tryptic Digestions of Cross-Linked Acto–S-1. All digestions were carried out in a buffer composed of 10 mM imidazole/2 mM $MgCl_2$, pH 7.0, with the NaCl concentration adjusted to the appropriate ionic strength conditions. To avoid depletion of ATP due to a rapid hydrolysis of MgATP by the cross-linked acto–S-1 complex, the digestions were performed at 4 °C. The weight ratio of trypsin to cross-linked S-1 was 1:2.5. The cross-linked acto–S-1 was digested in the absence of nucleotides and in the presence of 10 mM MgATP or 2 mM MgATPγS, with an ionic strength of 0.16 M. S-1 which had been inactivated with pNPM and subsequently cross-linked to actin was also digested at μ = 0.16 M and in the presence of 2 mM MgATP. Cross-linked acto–S-1 which had been modified with 7 μM pPDM in the presence of MgATP (Applegate & Flicker, 1987) was digested in a buffer of 0.16 M ionic strength, at 4 °C, and with a trypsin to S-1 weight ratio of 1:2.5. Tryptic digestions in the presence of 3 mM MgADP, 3 mM MgAMPPNP, or 4 mM $MgPP_i$ were carried out at μ = 0.5 M and at the same trypsin to S-1 ratio and temperature as above. At given time intervals, aliquots were removed from the proteolytic reactions, and soybean trypsin inhibitor (3× trypsin concentration) was added to stop the digestions. Catalase was added to all samples as an internal standard. The samples were denatured and run on SDS–polyacrylamide gel electrophoresis.

Subtilisin Digestions of Cross-Linked Acto–S-1. Cross-linked acto–S-1 was digested with subtilisin at a weight ratio of 1:10 (subtilisin:S-1) in the presence or absence of 2 mM MgATPγS and at 22 °C. The ionic strength was maintained at 0.16 M as mentioned before. At given time intervals, aliquots were removed, and the reactions were stopped by PMSF (3× enzyme concentration). The samples were denatured and run along with catalase (used as an internal standard) on SDS–polyacrylamide gel electrophoresis.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out according to the procedure of Laemmli (1970) using 7.5% (w/w) polyacrylamide gels.

Densitometric Measurements and Digestion Rates. The optical densities of Coomassie Blue stained protein bands and

[1] Abbreviations: S-1, myosin subfragment 1; pNPM S-1, S-1 modified with *p*-nitrophenylenemaleimide; pPDM S-1, S-1 with the SH_1 and SH_2 groups bridged by *p*-phenylenedimaleimide; XLAS-1, acto-S-1 cross-linked with carbodiimide (EDC); pyrene-actin, actin labeled at Cys-374 with *N*-(1-pyrenyl)iodoacetamide; AMPPNP, adenyl-5'-yl imidodiphosphate; ATPγS, adenosine 5'-*O*-(3-thiotriphosphate); ATPase, adenosinetriphosphatase; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide; pNPM, *p*-nitrophenylenemaleimide; pPDM; *N,N'-p*-phenylenedimaleimide; SDS, sodium dodecyl sulfate.

the respective mass distributions were determined with a Biomed soft laser scanning densitometer Model SL-504-X (Fullerton, CA) interfaced with an Apple II-e personal computer. To account for gel loading variations, the intensities of all protein bands in each lane were normalized to the intensity of the catalase band in the same lane. The differences in dye absorption were corrected by dividing the normalized band intensities by the molecular weights of the respective protein species. The digestion rates at the 50/20K junction of cross-linked S-1 were obtained by monitoring the combined intensities of the 95K–actin and 70K–actin bands as previously described (Duong & Reisler, 1987a,b).

Maleimide Modification of Cross-Linked Acto–S-1. All reactions were carried out at 4 °C and in a buffer containing 25 mM Tris, pH 8.0, and the NaCl concentration adjusted to the appropriate ionic strength. Cross-linked acto–S-1 was modified at an ionic strength of 0.16 or 0.5 M by using an 8× molar ratio of pNPM or pPDM to cross-linked S-1. At an ionic strength of 0.16 M, the cross-linked acto–S-1 was modified in a rigor complex and in the presence of 10 mM MgATP, 3 mM MgATPγS, or 3 mM MgADP. Under an ionic strength condition of 0.5 M, the reactions were performed in the presence of 3 mM MgADP and 3 mM MgAMPPNP. At given time intervals, aliquots were removed, and the reaction was terminated with 1 mM DTT.

pNPM Modification of S-1. Modification of S-1 by pNPM was carried out in 30 mM KCl/25 mM Tris, and at pH 8.0. The molar ratios of pNPM to S-1 ranged between 0 and 4:1, and the reactions took place at 4 or 22 °C. At indicated times, the reactions were terminated by adding 1 mM DTT to the modification system.

ATPase Assay. The MgATPase activity of cross-linked acto–S-1 was measured as previously described (Applegate & Flicker, 1987). Mg^{2+}-ATPase, Ca^{2+}-ATPase, and EDTA K^+-ATPase activities of S-1 were assayed as reported by Kielley and Bradley (1956).

Pyrene Labeling of Actin. Actin was labeled with *N*-(1-pyrenyl)iodoacetamide by using the methods of Cooper et al. (1983). Briefly, actin was polymerized in 0.1 M NaCl/2 mM $MgCl_2$ and subsequently reacted with a 1.5× molar excess of pyrene for 17 h. The labeled F-actin was pelleted, homogenized, depolymerized, and passed through two Sephadex G-50 Penefsky columns to remove excess pyrene. The labeling efficiency was greater than 70% as determined by the methods of Kouyama and Mihashi (1981). Pyrene-actin was cross-linked to S-1 with EDC and processed as described above. To reduce the concentration of un-cross-linked actin in the final product, the sample of cross-linked pyrene–acto–S-1 was recycled as described by Greene (1984). In this way, the amount of un-cross-linked actin in the sample was decreased from ~80% to ~50% of total actin.

Fluorescence Measurements. Fluorescence measurements of cross-linked pyrene–acto–S-1 were performed in the Spex Fluorolog spectrophotometer as previously described (Miller et al., 1988). The excitation and emission wavelengths were set at 368 and 407 nm, respectively. In this work, approximately 0.10 μM pyrene-actin cross-linked to S-1 was used to measure the change of fluorescence upon the addition of 3.0 mM MgADP, 0.3 mM MgATP, 1.2 mM MgATP, and 0.3 mM MgATPγS. The buffer was 0.1 M KCl/25 mM Tris, pH 8.0 (22 °C).

Results

Cross-Linking of Tryptic S-1 to Actin. In order to identify the products of tryptic digestions of cross-linked acto–S-1 by their electrophoretic mobilities, three forms of tryptically cleaved S-1 (25/50/20K, 25/70K, and 75/20K) were cross-linked to actin with EDC and run on SDS gels. The standard preparations of tryptic S-1 (25/50/20K), i.e., S-1 cleaved at both the 25/50K and 50/20K junctions, yield well-documented products upon EDC cross-linking to actin (Mornet et al., 1981; Sutoh, 1983). These products are represented by the 50K–actin and 20K–actin bands on SDS gels (Figure 1, lane 2). The cross-linking of the 25/70K S-1 to actin generated mostly the 70K–actin band (Figure 1, lane 4). Minute amounts of 50K–actin and 20K–actin were also formed due to minor cleavage at the 50/20K junction. Finally, the cross-linking of the 75/20K S-1 to actin yields the 75K–actin and 20K–actin bands (Figure 1, lane 6). The 95K–actin band present in this lane results from the cross-linking of actin to residual intact S-1. It should be noted that in agreement with previous observations (Kassab et al., 1981; Sutoh, 1983), the 75K–actin band migrates anomolously, at a lower mobility than the 70K–actin band (Figure 1, lanes 4 and 6).

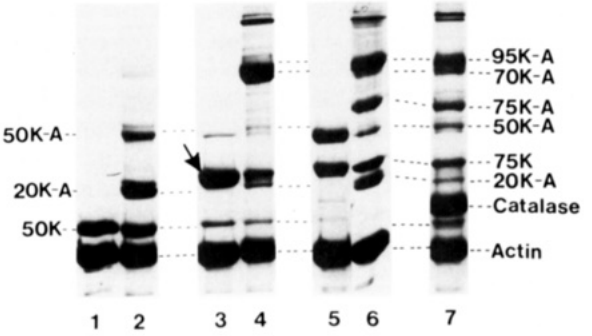

FIGURE 1: Representative electrophoretic patterns of tryptic S-1 cross-linked to actin and the tryptic digestion of cross-linked acto–S-1 (XLAS-1). S-1 was cleaved with trypsin (see Materials and Methods) to yield the 25/50/20K S-1 (lane 1), 25/70K S-1 (lane 3), and 75/20K S-1 (lane 5). The 20K and 25K bands are not displayed on this gel. The S-1 samples in lanes 1, 3, and 5 were cross-linked to actin with EDC to yield the products shown in lanes 2, 4, and 6, respectively. Lane 7 represents tryptic digestion in the presence of MgATP of pNPM–S-1 cross-linked to actin. 95K–A, 70K–A, 75K–A, 50K–A, and 20K–A correspond to actin cross-linked to the 95K, 70K, 75K, 50K, and 20K fragments of S-1, respectively. The arrow in lane 3 identifies the 70K fragment.

Lane 7 in Figure 1 shows the generation of the 70K–actin, 75K–actin, 50K–actin, and 20K–actin products from the digestion in the presence of MgATP of pNPM S-1 cross-linked to actin.

Effects of Nucleotides on Tryptic Cleavage of Cross-Linked Acto–S-1. The binding of actin to S-1 is known to inhibit tryptic cleavage at the 50/20K junction in myosin (Szilagyi et al., 1979; Mornet et al., 1981; Yamamato & Sekine, 1979). In analogy to the un-cross-linked acto–S-1 system, tryptic digestion of XLAS-1 in the absence of nucleotides occurs slowly, yielding mainly the 70K–actin product (Figure 2). This indicates that the cleavage occurs predominantly at the 50/25K junction of the cross-linked S-1 while the 50/20K junction is protected by actin. In the presence of MgADP, MgAMPPNP, or $MgPP_i$, XLAS-1 was digested with trypsin under high-salt conditions (μ = 0.5 M). Under these conditions, the un-cross-linked acto–S-1 dissociates completely (Greene, 1982), and the tryptic cleavage at the 50/20K junction of dissociated S-1 proceeds at a fast rate (Duong & Reisler, 1987a,b). In contrast to the un-cross-linked system, the cleavage of XLAS-1 at the 50/20K junction in the presence of MgADP, MgAMPPNP, and $MgPP_i$ (at μ = 0.5 M) is inhibited (Figures 2 and 4) and proceeds at similar rates to that of rigor XLAS-1 complexes (Figure 2). This conclusion is confirmed by comparing the respective proteolysis rates at

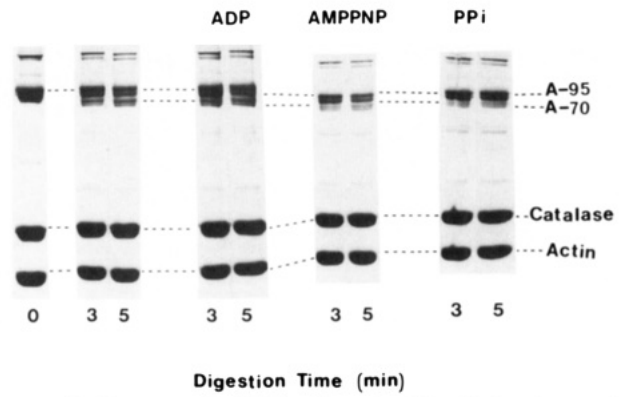

FIGURE 2: Representative SDS–polyacrylamide gels showing tryptic digestion of cross-linked acto–S-1 (1:2.5 trypsin:S-1 ratio, w/w) in the absence and presence of MgADP, MgAMPPNP, and $MgPP_i$. The digestions were carried out under ionic strength conditions of 0.16 and 0.5 M (10 mM imidazole/2 mM $MgCl_2$, pH 7.0, 4 °C; μ was adjusted with NaCl). In the absence of nucleotide, XLAS-1 was digested under μ = 0.16 M. In the presence of 3 mM MgADP, 3 mM MgAMPNP, and 4 mM $MgPP_i$, XLAS-1 was cleaved at μ = 0.5 M. Notation of protein bands is the same as in the legend to Figure 1.

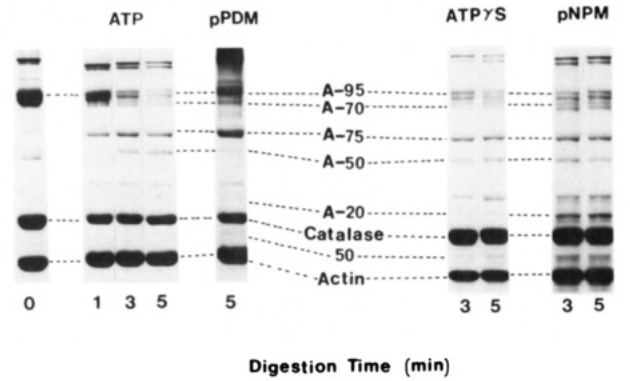

FIGURE 3: Representative polyacrylamide gels showing tryptic digestions of cross-linked acto–S-1 (XLAS-1), pPDM-modified XLAS-1, and pNPM-modified XLAS-1 (1:2.5 trypsin:S-1 ratio, w/w) in the presence of nucleotides. The digestions were carried out at 4 °C in a solution containing 2 mM $MgCl_2$/10 mM imidazole, pH 7.0, and adjusted to μ = 0.16 M with NaCl. XLAS-1 was digested in the presence of 10 mM MgATP or 2 mM MgATPγS. pPDM-bridged XLAS-1 was digested in the absence of nucleotides. For the pNPM-modified XLAS-1, S-1 was reacted at pH 8.0 with a 4-fold molar excess of pNPM and subsequently cross-linked to actin. The resulting pNPM–XLAS-1 was digested with trypsin in the presence of 2 mM MgATP. Notation of protein bands is the same as in the legend to Figure 1.

the 50/20K junction as measured by the method of Duong and Reisler (1987a,b), i.e., plotting the combined intensities of the 95K–actin and 70K–actin bands versus digestion time (Figure 4).

Due to the greatly elevated MgATPase activity of the cross-linked complexes (Mornet et al., 1981), the digestion of XLAS-1 in the presence of MgATP was conducted in the presence of high concentrations (10 mM) of this nucleotide. Gel electrophoretic patterns shown in Figure 3 indicate efficient cleavage at the 50/20K junction of XLAS-1 in the presence of MgATP to yield the 75K–actin and 50K–actin fragments. However, only minute amounts of 20K–actin and 50K fragments are generated in this digestion despite the reported cross-linking of actin to both the 20 and 50K fragments on S-1 (Mornet et al., 1981; Sutoh, 1983; Chen et al., 1985a). Thus, the simplest interpretation of the patterns shown in Figure 3 and the biphasic profiles of cleavage rates in Figure 4 could be that in the presence of MgATP trypsin preferentially cuts at the 50/20K junction in acto–S-1 cross-linked via the 50K fragment.

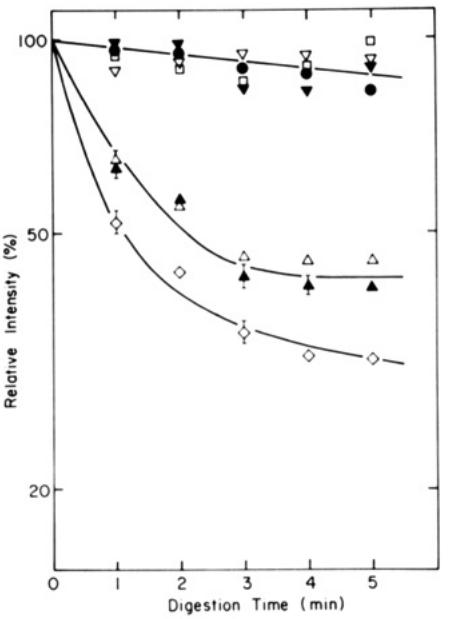

FIGURE 4: Time course of tryptic cleavage of XLAS-1 in the absence and presence of nucleotides. Combined relative intensities of the 95K–actin and 70K–actin protein bands from electrophoretic patterns similar to those shown in Figures 2 and 3 were plotted against digestion time to measure the cleavage rate at the 50/20K junction of cross-linked S-1 (band intensity = 100% at time = 0). (●) XLAS-1; (▽) XLAS-1 + 3 mM MgADP; (▼) XLAS-1 + 3 mM MgAMPPNP; (□) XLAS-1 + 4 mM $MgPP_i$; (△) XLAS-1 in the presence of 10 mM MgATP; (▲) XLAS-1 + 2 mM MgATPγS; (◇) pNPM-modified XLAS-1 in the presence of 2 mM MgATP.

As demonstrated previously (Burke et al., 1976; Chalovich et al., 1983), pPDM-modified S-1 resembles the S-1·ATP or S-1·ADP·P_i states, and pPDM–XLAS-1 appears similar to XLAS-1 in the presence of MgATP (Applegate & Flicker, 1987; King & Greene, 1987). The last analogy is verified in tryptic digestion experiments. Figure 3 shows that the cleavage patterns of cross-linked acto–pPDM–S-1 are similar to those of XLAS-1 in the presence of MgATP. In both cases, the cleavage occurs at the 50/20K junction to generate mainly the 75K–actin fragment. Due to consistent and unexplainable spreading in the electrophoretic bands of cross-linked acto–pPDM–S-1, cleavage rates at the 50/20K junction could not be determined with satisfactory precision. In general, however, these rates were similar to those observed for digestions of XLAS-1 in the presence of MgATP.

In fiber work, ATPγS has been employed as a slowly hydrolyzable ATP analogue which dissociates myosin heads from actin (Baker & Cooke, 1987). The digestion of XLAS-1 in the presence of MgATPγS appears to be similar to that in the presence of MgATP both in terms of protein band patterns and in terms of cleavage rates at the 50/20K junction (Figure 3). Both the 75K–actin and 50K–actin products are formed. Again, the appearance of only small amounts of the 20K–actin product suggests a preferential cleavage by trypsin at the 50/20K junction of S-1 cross-linked via its 50 K fragment.

In contrast to the digestion of cross-linked acto–pPDM–S-1, and XLAS-1 in the presence of MgATP and MgATPγS, the cleavage at the 50/20K junction (in the presence of MgATP) of pNPM-modified S-1 cross-linked to actin is more extensive and generates the 20K–actin products (Figures 3 and 4). The ability of trypsin to cut at the 50/20K junction of S-1 cross-linked at the 20K region suggests that pNPM modification alters acto–S-1 binding in the cross-linked complex.

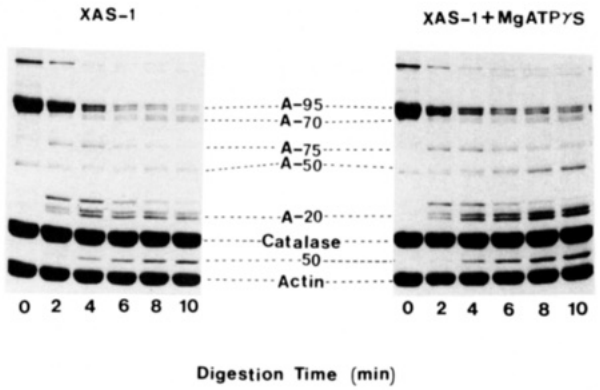

FIGURE 5: Representative SDS–polyacrylamide gels showing subtilisin digestion of cross-linked acto–S-1 (1:10 subtilisin:S-1 ratio, w/w) in the absence and presence of 2 mM MgATPγS. The digestions were carried at 22 °C in a solution containing 2 mM $MgCl_2$/10 mM imidazole, pH 7.0, and adjusted to μ = 0.16 M with NaCl. XAS-1, cross-linked acto–S-1.

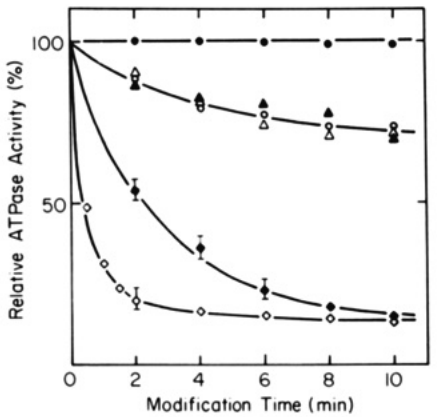

FIGURE 6: Effects of pNPM modification in the presence of nucleotides on MgATPase activity of cross-linked acto–S-1. XLAS-1 was reacted with approximately 8× molar excess of pNPM over S-1 in the absence of nucleotides (●) and in the presence of (♦) 10 mM MgATP, (◊) 3 mM MgATPγS, (○, ▲) 3 mM MgADP, or (△) 3 mM AMPPNP. The modification was carried out at 0.16 M ionic strength for XLAS-1 in the absence and presence of MgATP or MgATPγS. The ionic strength was raised to 0.5 M for the modifications carried out in the presence of MgAMPPNP. The reaction in the presence of MgADP was performed both at 0.16 M (▲) and at 0.5 M (○) ionic strength. All reactions were carried out in 25 mM Tris/2 mM $MgCl_2$, pH 8, and 4 °C (μ was adjusted with NaCl).

To investigate the slow cleavage rate at the 50/20K junction in S-1 cross-linked to actin via its 20K fragment, XLAS-1 was digested with subtilisin in the absence and presence of MgATPγS. As shown in Figure 5, subtilisin cleavage of XLAS-1 generates ample amounts of 20K–actin and 50K fragments along with the usual 75K–actin and 50K–actin products. These digestion patterns do not show any significant proteolytic "preference" for S-1 cross-linked via either its 50K or its 20K fragments. The rate of cleavage at the 50/20K junction in XLAS-1 is increased 2-fold in the presence of MgATPγS (quantification not shown). This is in good agreement with the observation that the binding of actin to S-1 slows the subtilisin cleavage rate at this junction only by a factor of 2 (Applegate & Reisler, 1983).

Effects of Nucleotides on Maleimide Modification of Cross-Linked Acto–S-1. The modification of reactive thiols on S-1 in XLAS-1 samples was monitored by measurements of MgATPase activities of acto–S-1. As shown in Figure 6, when XLAS-1 is modified with pNPM (8× molar excess) in the absence of nucleotides, its MgATPase activity remains unchanged. Under an ionic strength condition of 0.5 M, the presence of MgADP or MgAMPPNP facilitates the modification slightly, leading to about a 25% decrease in the MgATPase activity of XLAS-1. It should be noted that the rates of modifications in the presence of MgADP carried out at μ = 0.5 and 0.09 M are identical. In contrast to this, pNPM modification of XLAS-1 in the presence of MgATP or MgATPγS results in almost complete loss of the MgATPase activity (Figure 6). It is also apparent that the modification proceeds much faster in the presence of ATPγS than ATP. Since ATPγS is a slowly hydrolyzable ATP analogue (Bagshaw et al., 1972), the cross-linked S-1 is presumed to exist predominantly in the S-1·ATP state. Kinetic data have also shown that in the presence of ATP, cross-linked S-1 exists primarily in the A·S-1·ADP·P_i state (Stein et al., 1985). Thus, a slower modification rate of XLAS-1 in the presence of MgATP as compared to ATPγS suggests a relative protection of the essential thiols from modification in the cross-linked A·S-1·ADP·P_i state. Similar protection of sulfhydryls in isolated S-1 has been observed in previous studies (Reisler et al., 1977; Schaub, 1975). It should be noted also that modifications of XLAS-1 with pPDM in the presence and absence of nucleotides yield profiles of activity changes (data not shown) almost identical with those shown for pNPM reactions in Figure 6.

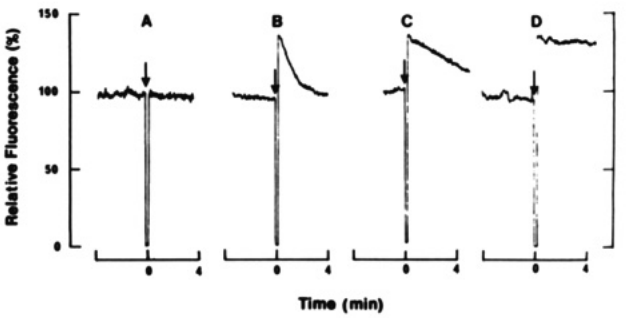

FIGURE 7: Effects of nucleotides on the fluorescence of pyrene–acto–S-1. S-1 was cross-linked to pyrene-actin as described under Materials and Methods. To remove un-cross-linked actin, the XLAS-1 preparation was recycled through five cycles of actin depolymerization and centrifugation (Greene, 1984). The recycled sample (approximately 0.1 μM) in 0.1 M KCl/25 mM Tris, pH 8.0 (22 °C), was combined with the appropriate nucleotides to measure the change in fluorescence of cross-linked pyrene-actin (λ_{ex} = 368 nm and λ_{em} = 407 nm). The addition of nucleotide is indicated by the arrow. The additions of (A) 3.0 mM MgADP, (B) 0.3 mM MgATP, (C) 1.2 mM MgATP, and (D) 0.3 mM MgATPγS are shown above.

Effects of Nucleotides on the Fluorescence of Cross-Linked Pyrene–Acto–S-1. Recent fluorescence measurements have shown the quenching of 70% of the fluorescence signal of pyrene-actin upon the binding of S-1. The fluorescence could be recovered following the dissociation of acto–S-1 complex by MgATP (Criddles et al., 1985; Kouyama & Mihashi, 1981). The present study examines the effects of nucleotides on pyrene-actin cross-linked to S-1. The fluorescent cross-linked complex was prepared as described under Materials and Methods and subsequently recycled (Greene, 1984) to deplete it from the un-cross-linked actin. As measured by the intensities of protein bands (data not shown), the concentration of free actin has been reduced to approximately 50% of total actin by this procedure. The resulting decrease in background fluorescence enables more accurate probing of the cross-linked samples. As shown in Figure 7A, the addition of MgADP to the cross-linked pyrene acto–S-1 does not alter the fluorescence intensity. In contrast to this, MgATP induces an approximately 35% transient increase in fluorescence intensity of XLAS-1 (Figure 7B). After 2 min, when the 0.3 mM MgATP is hydrolyzed by the activated XLAS-1, the fluorescence intensity returns to the original level. At a higher concentration

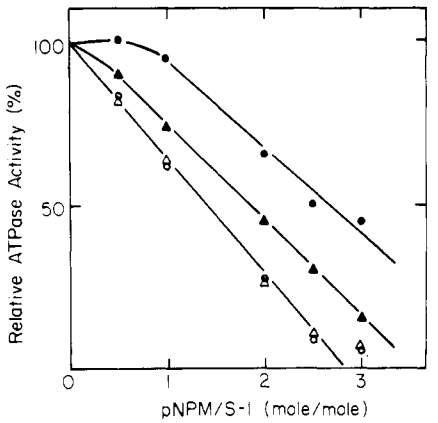

FIGURE 8: Effects of nucleotides on the modification of S-1 by pNPM at 4 °C as measured by EDTA K^+-ATPase activities of S-1. S-1 (8 μM) in 30 mM KCl/25 mM Tris (pH 8.0) was reacted for 1 min with the molar excess of pNPM indicated on the abscissa. Reactions were carried out in the absence of nucleotides (●) and in the presence of (▲) 1 mM MgATP, (○) 1 mM MgATPγS, and (△) 1 mM MgADP.

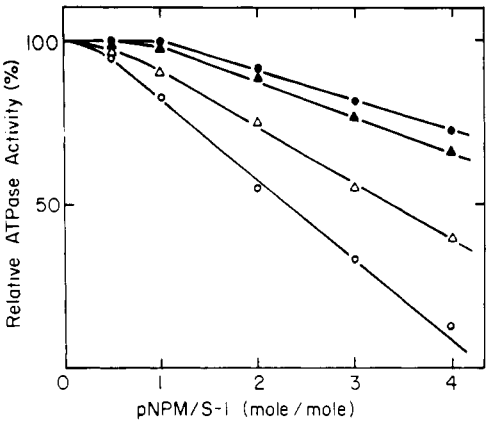

FIGURE 9: Effects of nucleotides on the modification of S-1 by pNPM at 22 °C as measured by EDTA K^+-ATPase activities of S-1. Reaction conditions and symbols are the same as those in the legend to Figure 8.

of MgATP (1.2 mM), the initial increase in fluorescent signal is similar to that in Figure 7B but decays slower to the original value (Figure 7C). The addition of ATPγS also induces a fluorescent increase of ~35% which is maintained by a virtually constant level of the nucleotide (Figure 7D). Thus, the present data indicate the release of pyrene probe on the cross-linked actin from S-1 influence by MgATP and MgATPγS.

Thiol Modification of S-1 at Different Temperatures and in the Presence of Nucleotides. The results of thiol modification experiments presented in the previous section suggest the protection of reactive SH groups on S-1 from pNPM modification in the XLAS-1·ADP·P_i state as compared to the XLAS-1·ATP state (Figure 6). To substantiate this interpretation of data, the modification of isolated S-1 with pNPM in the presence of MgATP, MgATPγS, and MgADP was investigated. The modifications were performed at two temperatures, 4 and 22 °C, and were monitored by measurements of the EDTA K^+-ATPase activity of S-1. At 4 °C and in the presence of ATPγS, when S-1 is predominantly in the S-1·ATP state, the decrease in EDTA K^+-ATPase activity of S-1 as a function of reagent concentration demonstrates the exposure of the essential thiols to maleimide modification (Figure 8). The labeling of S-1 in the presence of MgADP appears similar to that in the presence of MgATPγS. On the other hand, MgATP affords some protection to the essential thiols of S-1 against modification at 4 °C. This is evident from a smaller decrease in EDTA K^+-ATPase activity at any pNPM concentration when compared to that noted in the presence of MgATPγS. As shown earlier, the protection of reactive thiols in S-1 against modification can be linked to the presence of S-1·MgADP·P_i species in the reaction mixture (Harrington et al., 1975; Reisler et al., 1977). It was demonstrated then (Reisler et al., 1977), and reconfirmed now, that the low reactivity of essential thiols in the presence of MgATP is not due to competing reactions of other SH groups.

With the increase in the reaction temperature to 22 °C, and the consequent transition of S-1 to the predominant S-1·ADP·P_i state, the apparent protection of S-1 thiols by MgATP is rather obvious (Figure 9). The EDTA K^+-ATPase of S-1 modified in the presence of MgATP at 22 °C decreases only slightly faster than in the control reaction in the absence of nucleotides. However, the SH groups of S-1 remain reactive in the presence of MgATPγS. The binding of MgADP to S-1 at this temperature produces a conformation in which thiol reactivity is between that in S-1·ATP and S-1·ADP·P_i states. Thus, the present data are consistent with the claim that essential thiols are protected from modification in the S-1·ADP·P_i state and are more exposed to reaction in the S-1·ATP state. Such protection could explain the greater modification of cross-linked acto–S-1 in the presence of MgATPγS in comparison to that in the presence of MgATP (Figure 6).

Discussion

The obvious interest in the structural properties of weakly bound actomyosin complexes, which play a key role in the contractile cycle (Huxley & Kress, 1985), was frustrated by experimental difficulties in resolving between the features of detached and weakly attached S-1 molecules. The cross-linked acto–S-1 complexes offered the means of studying active (near V_{max}), attached S-1, free from the contribution of the detached protein (Mornet et al., 1981). Indeed, by using cross-linked acto–S-1 preparations, Craig et al. (1985) and Applegate and Flicker (1987) revealed the disordered arrangement of myosin heads on actin in the presence of ATP. Consistent with these findings, solution EPR experiments detected considerable rotational mobility of cross-linked S-1 in the presence of ATP (Swensson & Thomas, 1986).

The EPR results and electron microscopy evidence for unrestricted geometry of the weakly bound acto–S-1 suggest ATP-induced changes along a significant portion of the acto–S-1 complex. Some changes have been already detected in fluorescence energy-transfer experiments (Trayer & Trayer, 1988). Yet, this interpretation of data depends on the assumption that the orientational freedom of S-1 on actin is inherent to the weakly bound states and does not result from covalent tethering of detaching proteins. The former possibility is favored mainly by kinetic studies which document the prevalence of the AM·ADP·P_i state during ATP hydrolysis by XLAS-1 (Stein et al., 1985) and experiments which probe the effect of XLAS-1 on the troponin–tropomyosin–actin complex (King & Green, 1987). Chemical modifications carried out in this work provide new evidence for state-specific changes in cross-linked acto–S-1 and the predominance of AM·ADP·P_i species in the presence of ATP. This conclusion is based on characteristic effects of ATP on the modification of the reactive thiols on S-1 as monitored through ATPase activity measurements.

It has been shown earlier that the protective effect of MgATP on the labeling of SH_1 and SH_2 groups in S-1, when compared to their modification in the presence of MgADP, can be linked to changes in the population of M·ADP·P_i species

with variations in temperature and ionic strength conditions (Harrington et al., 1975; Reisler et al., 1977). In contrast to isolated S-1, the modification of acto–S-1 in the presence of ATP cannot be calibrated against reactions done in the presence of ADP, which does not dissociate the complex. However, control modifications of S-1 with pNPM carried out in the presence of ATPγS, ADP, and ATP (Figures 8 and 9) reveal that the thiol protection in the M·ADP·P_i state is even more pronounced when measured against M·ATP (S-1 + ATPγS) than M·ADP species. Thus, the considerably faster thiol modification of cross-linked acto–S-1 in the presence of ATPγS than with ATP is consistent with the respective prevalence of AM·ADP·P_i and AM·ATP states of the acto–S-1 complex. It may be noted that the choice of pNPM, a monofunctional analogue of pPDM, as a thiol reagent stems from the previous demonstration of SH_1 and SH_2 labeling of cross-linked acto–S-1 by pPDM (Applegate & Flicker, 1987). In addition, very similar modification profiles to those shown for pNPM were observed also for pPDM reactions (not shown).

Further support for the special ATP- and ATPγS-induced states of cross-linked acto–S-1 is provided by the inability of ADP and PP_i in the presence of 0.5 M KCl to promote thiol modification, tryptic digestion, and changes in the fluorescence of pyrene XLAS-1. Clearly, as observed earlier by Craig et al. (1985) and Applegate and Flicker (1987), even under conditions of dissociating power equivalent to that of ATP (Greene, 1982), these agents do not "open up" the actomyosin interface. Taken together, these results and the above-discussed state-specific modification of XLAS-1, as well as earlier kinetic studies (Stein et al., 1985), are consistent with the formation of weakly bound complexes and do not agree with the explanation on tethering of XLAS-1 by ATP and ATPγS.

The "opening" of cross-linked acto–S-1 by ATP and ATPγS was monitored at three separate locations, at the reactive thiols and the 50/20K junction of S-1, and at Cys-374 on actin. As observed before (Applegate & Flicker, 1987), the reactive SH_1 and SH_2 groups are practically inaccessible to thiol reagents (pNPM) in the absence of nucleotides. ATP and ATPγS (but not ADP and PP_i) change that situation dramatically and expose the thiol groups to labeling reactions. Similarly, ATP and ATPγS enable tryptic digestion of cross-linked acto–S-1 at the 50/20K junction, an effect which is not duplicated by ADP and PP_i. As might be expected from previous work (Applegate & Flicker, 1987), pPDM-bridged XLAS-1, which is considered analogous to AM·ADP·P_i or AM·ATP, is equally open to tryptic attack even in the absence of nucleotides.

From the equal digestion rates of acto–S-1 in the presence of ATP and ATPγS, it may be deduced, that in contrast to reactive thiols, the 50/20K junction on S-1 does not "sense" the structural transition between the AM·ADP·P_i and AM·ATP states. In the same way, the acto–S-1 contact responsible for quenching of pyrene-actin fluorescence (Criddle et al., 1985) is abolished equally well by ATP and ATPγS and not by ADP or PP_i. The transient "unquenching" of fluorescence can be easily explained in terms of a transition between the tight, rigorlike XLAS-1 complex and the weakly bound states in the presence of ATP and ATPγS. As shown by Geeves et al. (1986) and Geeves and Jeffries (1988), the quenching of pyrene-actin fluorescence by S-1 is not associated with the binding step but rather the isomerization between weakly and strongly bound acto–S-1 complexes. The relatively small extent of unquenching by ATP observed in this work can be accounted for by at least 2-fold excess of actin over S-1 in the cross-linked preparations.

The results of digestion experiments merit particular attention. Although ATP and ATPγS expose the 50/20K junction in XLAS-1 to tryptic attack, the proteolysis occurs mainly if not only in S-1 cross-linked to actin via the 50K fragment. This is evidenced by the formation of 50K–actin and the lack of 20K–actin and 50K products in tryptic digestion reactions. In fact, just the opposite result is expected in view of the preferential cross-linking of actin to the 20K over 50K fragment on S-1 (Chen et al., 1985a,b). These results suggest that the linking of actin to the cross-linking sites on the 20K fragment either chemically blocks the susceptible lysine residues or sterically hinders their accessibility to trypsin. The simplest interpretation of this observation would be to postulate that one or more lysines in the cluster of junctional lysine residues (Lys-636, -637, -640–642) constitute the actin cross-linking site. Alternatively, indirect steric or conformational effects could be transmitted to the 50/20K junction from the adjacent 18/20K segment (on the 20K fragment) identified by Sutoh (1983) to contain the actin cross-linking site.

It is instructive to note that very little if any constraints are placed on subtilisin cleavage of the 50/20K junction in cross-linked acto–S-1. As expected (Chen et al., 1985a), the major cleavage products both in the presence and in the absence of ATPγS are the 20K–actin, 50K, and 50K–actin materials. Earlier studies showed that actin does not inhibit greatly the rate of subtilisin attack on S-1 (Applegate & Reisler, 1983). In agreement with that finding, ATPγS accelerates only 2-fold the proteolysis of cross-linked acto–S-1. The striking differences between tryptic and subtilisin digestion reactions appear to be caused by a shift in the cleavage site, from the lysines attacked by trypsin (Lys-640/641/642) to the subtilisin cut between Gly-632/Gly-633 (M. Elzinga, personal communication). Thus, either the acto–S-1 contact does not extend to the subtilisin cleavage site or the indirect actin effects are not transmitted there.

Another point of interest in the tryptic digestion results is related to the appearance of 20K–actin and 50K products in S-1 modified with pNPM and then cross-linked to actin. Since the modification of SH_1 and SH_2 groups does not alter the tryptic cleavage of free S-1 (Chaussepied et al., 1986), the results shown in Figure 3 suggest some changes in either the cross-linking of acto–S-1 or the geometry of the complex following the labeling of the reactive thiols. This material is to be distinguished from acto–S-1 which has been cross-linked prior to pPDM modification and thus does not yield the 20K–actin and 50K products upon tryptic proteolysis. The actual relationship between the SH_1 and SH_2 groups and the acto–S-1 cross-linking sites is yet to be explored.

In conclusion, this work provides additional support for the view that cross-linked acto–S-1 in the presence of ATP and cross-linked acto–pPDM–S-1 represent weakly bound states of acto–S-1. The results of our experiments characterize also the weakly bound states as more "loose" and open than the rigor acto–S-1 complexes and are consistent with the idea of rotational mobility of S-1 weakly bounud to actin.

Acknowledgments

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