Extensively Methylated Myosin Subfragment-1: Examination of Local Structure, Interactions with Nucleotides and Actin, and Ligand-Induced Conformational Changes†

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ABSTRACT: The atomic structure of myosin subfragment-1 (S1) has been recently solved for crystals of extensively methylated S1 [Rayment *et al.* (1993) *Science 261*, 50–58]. In this study, the effect of such a modification on S1 structure and function was examined. According to the far- and near-ultraviolet CD spectra, the methylation does not affect the secondary structure of S1 but causes limited changes in its tertiary structure. The methylation significantly decreases the affinity of S1 for actin in rigor and, to a lesser degree, that of S1 to actin in the presence of MgATPγS. This modification, like the trinitrophenylation of Lys-83, accelerates the dissociation of a nucleotide trapped on S1 either by phosphate analogs or by cross-linking of the SH_1 and SH_2 thiols. Methylation strongly impairs the coupling between the actin- and nucleotide-binding sites as revealed by the reduced effect of actin on the release of εADP from the active site. It also causes a complete loss of *in vitro* motility of actin filaments over methylated HMM. In addition to this, methylation also impairs the communication between other sites on S1 including that between the nucleotide-binding site and SH_1, and the actin-binding site and the 27/50 kDa junction and a site at 74 kDa from the N-terminus of S1. These changes are revealed in SH_1 modification, thermolysin digestion, and vanadate-dependent photocleavage experiments, respectively. The increased rate of thermal denaturation of S1 and the loss of S1 protection by ADP and actin from this process also indicate flawed communications in methylated S1. It is concluded that these relatively mild but numerous and important changes impair the function of methylated S1.

Myosin belongs to the restricted family of motor proteins which transform the chemical energy of ATP into mechanical work. The motor function of myosin is located in its two globular heads, the subfragment-1 (S1),[1] which are equipped with separate sites for actin binding and the binding and hydrolysis of ATP (Mueller & Perry, 1962). Together with ATP, S1 is sufficient for the generation of the *in vitro* movement of actin filaments (Toyoshima *et al.*, 1987). This movement depends on the coupling between the actin- and nucleotide-binding sites on S1 and, as suggested by numerous biochemical studies and a recent structural model of S1 function (Rayment *et al.*, 1993b), most likely involves structural changes induced by the attachment of actin and ATP to their respective binding sites. In order to understand the molecular mechanism of the myosin- and actin-based motility, it is essential to describe the structure and the ligand-induced structural changes in these two proteins.

In recent years, the most important progress in the field of motility and muscle biochemistry was achieved with the solution of the atomic structures of actin monomer (Kabsch *et al.*, 1990) and S1 (Rayment *et al.*, 1993a). These breakthroughs pave the way toward solving the structures of S1·nucleotide and perhaps even actin·S1 complexes, and should lead to a better understanding of structural changes which are essential for muscle contraction. In the case of S1, the achievements of Rayment's group (Rayment *et al.*, 1993a) in crystallizing and solving the structure of this protein originate in a novel and unprecedented protein treatment, extensive reductive methylation of S1. In a control study, such a methylation caused little if any change in the atomic structure of lysozyme (Rypniewski *et al.*, 1993). The methylated S1 was also examined and found to be catalytically competent and to interact with actin (White & Rayment, 1993).

However, in spite of important qualitative similarities, there are significant quantitative differences between the ATPase activities of methylated (M-S1) and unmodified S1. The V_{max} of the actin-activated ATPase activity of M-S1 decreased to less than one-tenth of that of native S1, and the maximal rate of the bond-splitting step (ATP hydrolysis) decreased about 500-fold upon methylation (White & Rayment, 1993). These changes in the kinetic parameters of S1 ATPase qualitatively resemble those induced by modifications of the single reactive lysine residue (RLR), Lys-83 (Muhlrad, 1983), and the SH_1 thiol, Cys-707 (Sleep *et al.*, 1981). In both cases, the modification-induced changes in the ATPase cycle do not result from alterations in the ATP-binding site itself, since neither

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[1] Abbreviations: S1, myosin subfragment-1; M-S1, methylated subfragment-1; HMM, heavy meromyosin; M-HMM, methylated heavy meromyosin; TNP-S1, trinitrophenylated subfragment-1; AlF_4^-, aluminum fluoride; BeF_x, beryllium fluoride; V_i, vanadate; εADP, 1,N^6-ethenoadenosine diphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]]maleimide; DTT, dithiothreitol; pPDM, *N*,*N*′-*p*-phenylenedimaleimide; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; NEM, *N*-ethylmaleimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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Cys-707 nor Lys-83 is part of the active site (Rayment *et al.*, 1993a). Instead, the kinetic changes result from propagated conformational perturbations which affect the active site and impair to some degree the ligand-induced structural changes essential for the transducer function of S1. In order to assess whether and by how much methylation affects the function and structure of S1, it was important to examine the effect of this modification on the binding of nucleotide and actin to S1, the coupling between their respective binding sites on S1, and the conformational changes induced by these ligands. Moreover, it is instructive to compare the effects of the extensive reductive methylation of lysyl residues on S1 with other modifications, especially the trinitrophenylation of Lys-83, the only other known modification of S1 which specifically affects lysyl ϵ-amino groups.

In order to pursue the above goals, we studied the effect of methylation on (1) the overall conformation of S1, (2) the stability of "trapped" S1–nucleotide complexes, (3) the binding affinity of S1 to actin in the absence and presence of nucleotides, (4) the communication between the actin- and nucleotide-binding sites, (5) ligand-induced conformational changes at sites susceptible to proteolysis and at the vicinity of SH_1, and (6) the thermal stability of the molecule. The results of these experiments show that while the overall conformation of S1 has changed very little upon methylation, there are significant localized structural changes which impair the spread of ligand-induced conformational changes and the coupling between the nucleotide- and actin-binding sites on S1.

MATERIALS AND METHODS

Reagents. ATP, ADP, IAEDANS, TNBS, pPDM, DTT, NEM, $BeCl_2$, sodium vanadate, aluminum chloride, and NaF were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-[4-[7-(Diethylamino)-4-methylcoumarin-3-yl]maleimide (CPM) was purchased from Molecular Probes (Junction City, OR). 1,N^6-Ethenoadenosine diphosphate (ϵADP) and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) were purchased from Boehringer Mannheim (Indianapolis, IN). Crystalline acrylamide was obtained from Bio-Rad (Richmond, CA). Millipore-filtered distilled water and analytical-grade reagents were used in all experiments.

Proteins. Myosin from rabbit psoas muscle was prepared according to Godfrey and Harrington (1970). Subfragment-1 (S1) and heavy meromyosin (HMM) were prepared by chymotryptic digestion of myosin as described by Weeds and Pope (1977) and Kron *et al.* (1991), respectively. S1 was used as a mixture of S1(A1) and S1(A2). The reductive methylation of S1 was carried out according to Rayment *et al.* (1993a) and White and Rayment (1993). The extent of S1 methylation was routinely checked by TNBS titration of lysine residues. This was done by adding 4 mM TNBS to 10 μM S1 or M-S1 in 50 μM borate–NaOH buffer, pH 10.2. The reaction mixture was incubated in the dark, at room temperature, for 100 min, and its absorption was measured at 345 nm (Okuyama & Satake, 1960). In all preparations, over 95% of the lysines, including Lys-83, the reactive lysine residue, were modified. The methylated S1 preparations were also checked by measurements of the actin, Mg^{2+}, Ca^{2+}, and K^+(EDTA) ATPase activities of S1. All control S1 samples were subjected to mock-methylations which included all the methylation steps except for the addition of formaldehyde. Rabbit skeletal muscle actin was prepared in G-actin buffer (0.5 mM β-mercaptoethanol, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 5.0 mM Tris-HCl, pH 7.6) by the procedure of Spudich and Watt (1971). G-Actin was polymerized by the addition of 2.0 mM $MgCl_2$. Protein concentrations were determined spectrophotometrically by using the following extinction coefficients at 280 nm: S1, $E^{1\%} = 7.5$ cm^{-1}; HMM, $E^{1\%} = 6.5$ cm^{-1}; actin, $E^{1\%} = 11.5$ cm^{-1}.

Circular Dichroism. Near-UV CD measurements were made at room temperature in a JASCO J-600 spectropolarimeter and employing a 5.0 mm pathway cell. S1 or methylated S1 (35 μM) was dissolved in 20 mM KCl and 5 mM HEPES, pH 7.0, in the presence or absence of $MgCl_2$ (2.0 mM) and ATPγS (50 μM). Far-UV (190–250 nm) spectra for S1 solutions (1 μM) were obtained in a 1.0 mm pathway cell. For each spectrum, four scans were averaged. Differential absorption data were converted to mean residue ellipticity, θ, assuming a value of 113 for the mean residue molecular weight of amino acids. The CD data for each wavelength were expressed as mean residue weight ellipticity:

$$[\theta] = \frac{\theta_\lambda M}{10lc} \text{ deg·cm}^2\text{·dmol}^{-1}$$

θ_λ is the measured ellipticity in degrees, l is the optical path length in centimeters, c is the concentration of S1 and methylated S1 in grams per cubic centimeter, and M is taken as the mean residue weight, 113.

Airfuge Binding Experiments. The binding of actin to mock-methylated and methylated S1 was determined from sedimentation experiments. S1 and methylated S1 (0–12 μM) were added to actin (4 μM) in the presence or absence of MgATPγS (3.0 mM). In the absence of nucleotide, the standard solvent contained 100 mM NaCl/10 mM Tris-HCl, pH 8.0; in the presence of nucleotide, it contained 10 mM NaCl/10 mM Tris-HCl, pH 8.0. The reaction mixture was centrifuged at 140000*g* for 15 min at room temperature in a Beckman air-driven ultracentrifuge. The pelleted proteins and the supernatant were denatured and run on SDS–polyacrylamide (10%) gels (Laemli, 1970). Molar ratios of S1 bound to actin were obtained from densitometric analysis of protein bands on SDS gels and the appropriate SDS calibration gels (Phan *et al.*, 1993). Scatchard plots for the binding of S1 to actin were constructed by expressing the amount of S1 bound to actin as a function of free S1. The apparent binding constants (K_{app}) were calculated from the slopes of the Scatchard plots.

Chase Experiments. (a) Chase of ϵADP from the S1·MgϵADP·AlF$_4^-$ Complex. The stability of the S1·MgϵADP·AlF$_4^-$ and methylated S1·MgϵADP·AlF$_4^-$ complexes was examined by monitoring the dissociation of the fluorescent nucleotide analog ϵADP from S1 in the presence of acrylamide (100 mM). This reagent preferentially quenches free ϵADP (Ando *et al.*, 1982). The fluorescence measurements were conducted at 25 °C in a Spex Fluorolog spectrophotometer (Spex Industries, Inc., Edison, NJ) at excitation and emission wavelengths of 315 and 415 nm, respectively. S1 (5.0 μM) was preincubated with $MgCl_2$ (2.0 mM), ϵADP (8.0 μM), $AlCl_3$ (500 μM), and NaF (10 mM) for 30 min at 25 °C. The standard solvent contained 20 mM KCl/5.0 mM HEPES, pH 7.0. The chase of ϵADP bound to S1 was carried out with ADP (2.0 mM) or actin (5.0 μM).

(b) EDTA Chase. The stability of S1·MgADP·V_i and S1·MgADP·BeF_x complexes containing native S1, M-S1, and TNP-S1 was studied by monitoring the dissociation of Mg^{2+} from the complexes. The released Mg^{2+} was chelated by EDTA which reacts only with free Mg^{2+} but not with Mg^{2+} trapped in the complex. The decomposition of the complexes was followed by measuring the recovery of the Ca^{2+}-activated

ATPase activity (in 2 mM ATP, 6 mM $CaCl_2$, 400 mM KCl, and 50 mM Tris-HCl, pH 8.8 at 25 °C). The complexes were formed by the preincubation of S1 (20 μM) with 1 mM $MgCl_2$, 0.2 mM ADP, 20 mM Tris-HCl, pH 8.0, and either 0.2 mM V_i or 5 mM NaF and 0.2 mM $BeCl_2$, for 30 min at 25 °C. After formation of the complexes, 4 mM EDTA was added, and aliquots were taken at various time intervals to measure their Ca^{2+}-activated ATPase activity.

All rates of nucleotide release were obtained by fitting experimental data to a single-exponential expression using the curve fitting program of the Sigma Plot Software, version 4.1.

In Vitro Motility Assays. In contrast to all other experiments, *in vitro* motility assays were carried out with HMM and not S1. The choice of HMM was dictated by the better motile function of this myosin fragment. All methylation and mock-methylation procedures were the same as for S1 except that the preparation was shortened by washing the remaining ammonium sulfate (after precipitation of the protein) on the coverslip, after HMM adsorption, rather than removing it by dialysis. Solution experiments verified that the MgATPase and actin-activated ATPase activities of M-HMM are altered in the same way as those of M-S1. The motility assays were performed as previously described (Muhlrad *et al.*, 1994). To remove ATP-insensitive heads, HMM samples were centrifuged in the presence of 0.15 mg/mL F-actin and 3 mM MgATP in a Beckman airfuge. HMM from the supernatant was applied to nitrocellulose-treated coverslips after dilution in assay buffer (25 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$, and 10 mM imidazole, pH 7.4). Movement was initiated with assay buffer containing 1 mM ATP and an oxygen-scavenging system at 25 °C. Quantification of sliding velocities was done with an Expertvision system (Motion Analysis, Santa Rosa, CA) with a minimum of 50 filaments analyzed for each sample.

SH_1 Modification of S1. (a) Modification of SH_1 by CPM. The specificity of CPM labeling at SH_1 was determined by monitoring the K^+(EDTA) and Ca^{2+}-ATPase activities of S1 at 37 °C (Reisler, 1980). Briefly, S1 (10 μM) in 20 mM KCl and 5.0 mM HEPES, pH 7.0, was reacted with CPM (25 μM) in the presence or absence of MgADP (1.0 mM) for various time intervals at 4 °C. The ATPase activities were measured after the reaction was terminated by the addition of DTT (2.0 mM). The time course of the reaction was followed by monitoring the fluorescence change of CPM in the Spex Fluorolog spectrophotometer at excitation and emission wavelengths of 390 and 475 nm, respectively.

(b) SH_1 Modification by IAEDANS. The SH_1 thiols of S1 and M-S1 were reacted with IAEDANS essentially by the method of Takashi (1979). IAEDANS (4 μM) was added to 4 μM S1 or M-S1 in 30 mM NaCl, 2 mM $MgCl_2$, and 20 mM Tris-HCl, pH 8.0, and the reaction was followed at 25 °C via changes in the fluorescence of IAEDANS (λ_{ex} = 335 nm and λ_{em} = 480 nm). Alternatively, 70 μM S1 in standard solution was reacted with 140 μM IAEDANS at 0 °C, in the dark, overnight, and the reaction was quenched by the addition of 2 mM DTT. Part of the IAEDANS-S1 was methylated (Rayment *et al.*, 1993a), and the Ca^{2+}-activated ATPase activities were measured.

pPDM Modification of S1. Inactivation of S1 (50 μM) by pPDM (75 μM) was carried out in the presence of MgϵADP (500 μM) for 2 h in 40 mM KCl/10 mM Tris-HCl, pH 8.0 at 0 °C. The extent of cross-linking was monitored by measuring the K^+(EDTA) and Ca^{2+}-ATPase activities of S1 at 37 °C. To determine the off rate of the S1-bound ϵADP, excess ϵADP was removed by passing the pPDM-modified S1 through Penefsky's column (Penefsky, 1977), and the modified S1 was subjected to ADP chase as described above.

Trinitrophenylation of S1. S1 was reacted with TNBS essentially according to Muhlrad *et al.* (1975). Briefly, TNBS was added at a 3:1 TNBS to S1 molar ratio to 30–50 μM S1 in 30 mM NaCl and 100 mM Tris-HCl, pH 8.0, and incubated at 25 °C for 10 min. The reaction was quenched by the addition of 2 mM DTT and dialyzed overnight against 30 mM NaCl and 20 mM Tris-HCl, pH 8.0, at 4 °C. The number of trinitrophenylated lysine residues was calculated from the 345 nm absorbance, assuming a $\Delta\epsilon_{345}$ of 14 500 (Okuyama & Satake, 1960).

Proteolytic Digestions. (a) Tryptic Digestion. S1, M-S1, or TNP-S1 (3 μM) in 30 mM NaCl and 20 mM Tris-HCl, pH 8.0, was digested at 25 °C with either 20:1 or 50:1 S1 to trypsin w/w ratios for 10 or 5 min, respectively. The reaction was terminated by the addition of soybean trypsin inhibitor with a 2:1 w/w ratio to trypsin. The digested samples were analyzed by SDS–PAGE.

(b) Thermolysin Digestion. S1, M-S1, or TNP-S1 (5 μM) in 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM sodium phosphate, pH 7.0, with or without 5 mM ATP or 10 μM F-actin was digested by thermolysin (S1:thermolysin ratio 10:1, w/w) at 25 °C for 20 min. The digestion was terminated by the addition of 10 mM EDTA which chelates the Ca^{2+} necessary for the digestion. The digested samples were analyzed by densitometry of SDS–PAGE.

(c) Subtilisin Digestion. S1, M-S1, or TNP-S1 (8 μM) was digested with subtilisin (S1:subtilisin ratio 10:1, w/w) in 1 mM $MgCl_2$, 30 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 2 mM ADP or 20 μM F-actin for 5–20 min at 25 °C. The reaction was terminated by the addtion of 3 mM PMSF. All samples were analyzed by SDS–PAGE.

Vanadate-Dependent Photocleavage. The cleavage was carried out essentially by the method of Ringel *et al.* (1990) on 8 μM S1, M-S1 or TNP-S1 in 2 mM $MgCl_2$, 0.4 mM V_i, and 30 mM HEPES, pH 7.0, with or without 5 mM ATP or 20 μM F-actin. A stock solution of sodium vanadate was prepared according to Goodno (1979). The samples were irradiated by a UV transilluminator (U.V.P. Inc.) on ice, using near-ultraviolet light (peak 365 nm) for 3–10 min. Following irradiation, samples were analyzed by densitometry after SDS–PAGE.

SDS–PAGE. Electrophoretic analysis of the samples was performed on 7–18% polyacrylamide gradient slab gels. The peptide bands were visualized by staining with Coomassie Brilliant Blue R-250. Molecular masses of the protein bands were estimated by comparing their mobilities with those of molecular weight markers.

Heat Treatment. This treatment was carried out essentially by the method of Setton and Muhlrad (1984). S1, M-S1, or TNP-S1 (8 μM) in 2 mM $MgCl_2$, 30 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 2 mM ADP or 20 μM F-actin, when indicated, was incubated at 37 °C. At various time intervals, samples were withdrawn to measure their Ca^{2+}- activated ATPase activity.

ATPase Activities. Ca^{2+}- and K^+(EDTA)-activated ATPase activities of S1 were determined according to Kielley and Bradley (1956). Actin-activated ATPase was measured on samples containing 0.2 μM S1, 0–40 μM actin, 2 mM ATP, 2 mM $MgCl_2$, 14 mM KCl, and 20 mM imidazole hydrochloride, pH 7.0 at 25 °C. Colorimetric Mg^{2+}-ATPase assays were carried out in 20 mM KCl/10 mM HEPES, pH

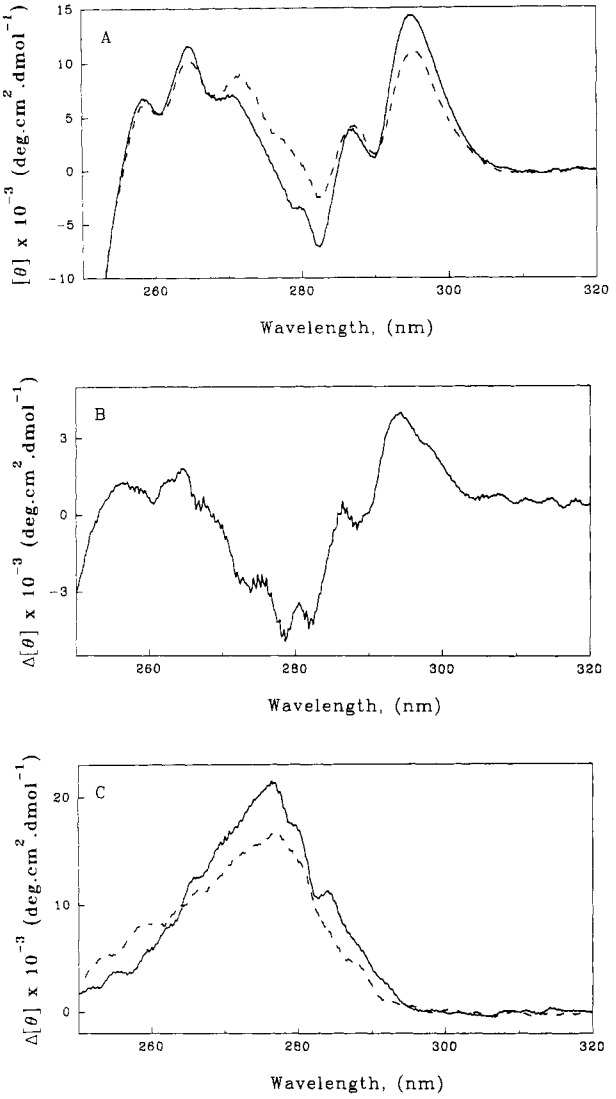

FIGURE 1: CD spectra of S1 and methylated S1. CD measurements were carried out at 25 °C in 20 mM KCl and 5 mM HEPES, pH 7.0. The protein concentration was 35 μM. The path length was 0.5 cm. (A) Near-ultraviolet spectra of S1 (—) and methylated S1 (– –). (B) Difference CD spectrum for S1 and methylated S1. The difference spectrum was obtained by subtracting the CD scan of methylated S1 from that of S1. (C) Difference spectra for S1 (—) and methylated S1 (– –) in the presence and absence of nucleotides. The difference spectra were obtained for each protein by subtracting the corresponding scans obtained in the presence and absence of ATPγS.

7.0, at 35 °C by the addition of 2.0 mM MgATP to 3.0 μM S1. The reaction was stopped with 10% trichloroacetic acid.

RESULTS

Effects of Methylation on the Circular Dichroism of S1. To determine whether the methylation of S1 perturbs its secondary structure, the CD of S1 and methylated S1 was measured. A comparison of far-UV spectra of five separate preparations of methylated and mock-methylated S1 did not reveal any significant changes in the secondary structure of the methylated S1. All pairs of methylated and mock-methylated S1 showed CD variations of ±3% (data not shown). However, methylation induced local changes in S1, as suggested by the shift in the near-UV CD signal attributed to the chromophores tryptophan, tyrosine, and phenylalanine of S1 (Figure 1A). The difference between the CD spectra of the native and methylated S1 was most pronounced in the tryptophan and tyrosine regions (Figure 1B).

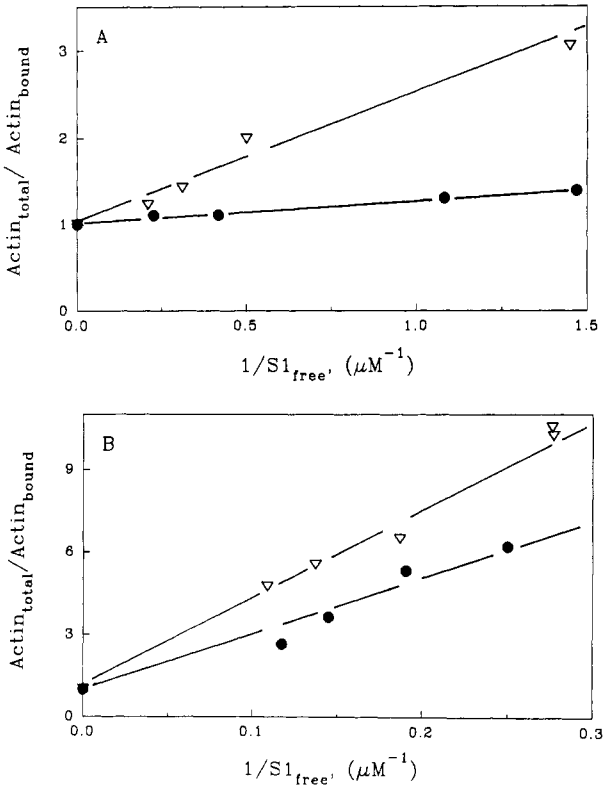

FIGURE 2: Scatchard plots for the binding of actin to S1 and M-S1 in the presence and absence of ATPγS. The binding of actin to S1 was determined by ultracentrifugation of acto–S1 solutions, and the binding constants were derived from the slope of the Scatchard plots. (A) Binding of actin to S1 (●) and M-S1 (▽) in the presence of 100 mM NaCl and 10 mM Tris-HCl, pH 8.0; the binding constants were 3.9 (±0.3) × 10^6 M^{-1} and 7.0 (±0.2) × 10^5 M^{-1}, respectively. (B) Binding of actin to S1 (●) and M-S1 (▽) in the presence of 3 mM ATPγS in 10 mM NaCl and 10 mM Tris-HCl, pH 8.0; the binding constants were 4.6 (±0.7) × 10^4 M^{-1} and 2.9 (±0.1) × 10^4 M^{-1}, respectively.

The binding of nucleotides to S1 is known to produce characteristic changes in the near-UV CD spectrum of S1 (Murphy, 1974). However, despite local differences between methylated and native S1 in the near-UV region, nucleotide binding induced almost the same spectral change in the methylated S1 as in the native protein. This is shown in Figure 1C which compares the difference spectra of control and methylated S1 obtained from CD scans of these proteins in the presence and absence of MgATPγS.

Binding of Methylated S1 to Actin. One of the sites on S1 implicated in actin binding is the 50/20 kDa junction (Mornet *et al.*, 1979; Chaussepied & Morales, 1988; Bertrand *et al.*, 1988; Miller *et al.*, 1987; DasGupta & Reisler, 1989, Yamamoto, 1989a). This junction is protected from trypsin proteolysis in the actomyosin complex (Mornet *et al.*, 1979; Chen *et al.*, 1987). The sequence contains a positively charged cluster of residues, consisting mainly of lysine and glycine amino acids. To determine to what extent methylation of lysine residues affects the interaction of S1 with actin, the binding of methylated S1 to actin was quantified by sedimentation assays. Figure 2 shows the binding plots of methylated S1 to actin in the presence and absence of nucleotide. Under rigor conditions (Figure 2A), for this set of binding experiments, methylation reduced the binding of S1 to actin by almost 6-fold [3.9 (±0.3) × 10^6 M^{-1} to 7.0 (±0.2) × 10^5 M^{-1}]. Surprisingly, in the presence of 3 mM ATPγS (weak binding) (Figure 2B), the binding was reduced only by about 2-fold [4.6 (±0.7) × 10^4 M^{-1} to 2.9 (±0.1) × 10^4 M^{-1}]. It should be noted that although a difference in

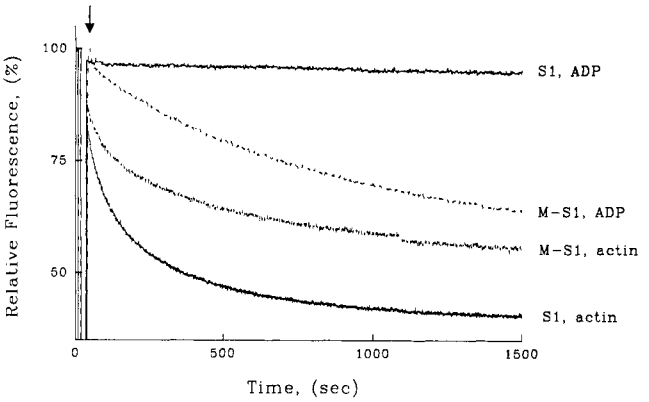

FIGURE 3: Release of ϵADP from the S1 and M-S1·MgϵADP·AlF_4^- complexes following ADP or actin addition. S1 or M-S1 (5.0 μM) and ϵADP (8.0 μM) were preincubated with $AlCl_3$ (500 μM) and NaF (10 mM) for 30 min. At the time indicated by the arrow, ADP (1 mM) or actin (5.0 μM) was added. The fluorescence intensities were measured at 415 nm in the presence of acrylamide (100 mM). The solid curves (—) correspond to the release of ϵADP from the S1·MgϵADP·AlF_4^- complex by either ADP or actin. The dashed curves (– –) correspond to the release of ϵADP from the M-S1·MgϵADP·AlF_4^- complex. For the control (—), the dissociation rate of ϵADP was not measurable in the presence of ADP (upper curve) and was estimated to be 4.93 (±0.004) × 10^{-3} s^{-1} in the presence of actin (lower curve). For the methylated S1 (– –), the dissociation rates of ϵADP were 1.24 (±0.007) × 10^{-3} s^{-1} in the presence of ADP (upper curve) and 2.80 (±0.004) × 10^{-3} s^{-1} in the presence of actin (lower curve).

actin binding was consistently observed between methylated S1 and the mock-methylated protein, the magnitude of the difference varied between methylation batches. Indeed, in the absence of nucleotides, the binding constant of methylated S1 to actin ranged from 4- to 6-fold lower than that of native S1. In the presence of MgATPγS, the difference varied from 2- to 3-fold. The variation in the binding constants of methylated S1 to actin may reflect the variation in the extent of S1 methylation, even though TNBS analysis yielded the same value for each methylation preparation.

Nucleotide Site. To determine whether methylation of S1 perturbs the environment around the active site, the dissociation of a fluorescent ADP analog, etheno-ADP, from the active site into an acrylamide-containing medium was examined. The rationale for these experiments relied on the preferential quenching of free ϵADP by acrylamide (Ando *et al.*, 1982). The release of ϵADP from the nucleotide pocket in the presence of AlF_4^- was monitored by chasing the bound ϵADP with ADP and monitoring the resulting time-dependent changes in fluorescence. The choice of AlF_4^- as a nucleotide trapping agent, which yields an analog of the M**·MgADP·P_i rate-limiting state of ATP hydrolysis (Werber *et al.*, 1992), was based on the convenient time scale of ADP release from S1 by actin in this system (Phan *et al.*, 1993). Figure 3 shows the release of ϵADP from the methylated S1·MgϵADP·AlF_4^- complex in the presence of either ADP or actin. The dissociation of ϵADP from the stable S1·MgϵADP·AlF_4^- complex was very slow, as observed earlier (Werber *et al.*, 1992). On the other hand, the dissociation of ϵADP from the methylated S1·MgϵADP·AlF_4^- complex was much faster [k_{off} under these conditions was 1.24 (±0.007) × 10^{-3} s^{-1}], indicating that the active site of the methylated protein is in a more "open" conformation. The same conclusion may be reached from quenching titrations of MgϵADP on S1 with acrylamide (not shown) which reveal slightly greater accessibility of the probe to the quenchers in methylated than in control S1 [see also Bivin *et al.* (1994)].

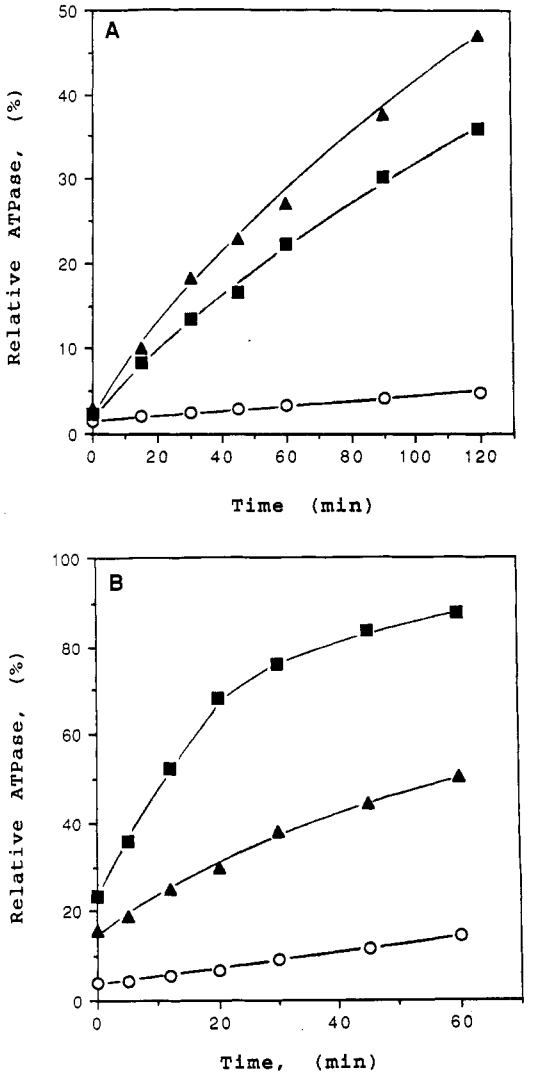

FIGURE 4: Dissociation of the S1, M-S1, and TNP-S1 complexes with MgADP·BeF_x and MgADP·V_i following EDTA addition. From the reaction mixture, which was kept at 25 °C and contained 20 μM S1, aliquots were withdrawn at given time intervals, after addition of 4 mM EDTA, for measurements of the Ca^{2+}-activated ATPase activities. For details of the complex formation and ATPase assay, see Materials and Methods. (A) and (B) are dissociation of the BeF_x- and V_i-containing complexes, respectively. ATPase activities are given relative to the activity of an analog-free S1. The dissociation rates of the S1(○), M-S1(▲), and TNP-S1 (■) complexes with MgADP·BeF_x were 8.6 × 10^{-6}, 1.0 × 10^{-4}, and 0.87 × 10^{-4} s^{-1}, respectively (A). The dissociation rates for the MgADP·V_i complexes were 3.1 × 10^{-5}, 1.6 × 10^{-4}, and 6.1 × 10^{-4} s^{-1}, respectively (B).

We examined also the effect of methylation on the stability of S1·MgADP·V_i and S1·MgADP·BeF_x complexes, which are alternative analogs of the M**·MgADP·P_i transient state of ATP hydrolysis (Goodno, 1979; Werber *et al.*, 1992; Phan & Reisler, 1992). We tested the stability of these complexes by monitoring the time-dependent restoration of the ATPase activity of S1 following the addition of EDTA to these complexes (Figure 4). The rationale of this method relies on the absolute requirement of divalent cations for the formation of the stable S1.nucleotide complexes (Peyser *et al.*, 1994). Methylation dramatically increases the rate of dissociation, and decreases the stability of both complexes, which is consistent with the observed dissociation of ϵADP from the AlF_4^--containing complex. In the EDTA chase experiments, we compared also the effects of trinitrophenylation of a single reactive lysyl residue, Lys-83, and the reductive methylation of almost all S1 lysines on the stability of the above complexes.

Figure 4 shows that the trinitrophenylation of RLR has about the same or, in the case of the V_i trap, even a stronger effect on the release of the nucleotide than the general methylation of almost all S1 lysines.

The effect of methylation on the nucleotide site is also reflected in the ATPase activities of S1. The Mg^{2+}-ATPase activity of methylated S1 was 5 times higher than that of native protein. The difference in Mg^{2+}-ATPase between the modified and unmodified proteins observed in this work was larger than that reported by White and Rayment (1993), but the discrepancy may be due to different experimental conditions or the extent of S1 methylation. The Ca^{2+}-ATPase activity of S1 was enhanced almost 4-fold by methylation, and the K^+(EDTA)-ATPase activity was abolished (8% of the control). Also, as reported before, the V_{max} value of the actin-activated ATPase activity of methylated S1 was about 10-fold lower (2.3 s^{-1}) than that of unmodified S1 (23 s^{-1}). These changes in ATPase activities are similar to those obtained upon SH_1 modification of S1.

Communication between Actin and the Nucleotide Site. In the absence of actin, the rate-limiting step in the ATPase hydrolysis cycle is the release of the products P_i and ADP from S1. Actin speeds up the release of products by at least 10^3-fold (White & Taylor, 1976; Stein *et al.*, 1979). The actin-activated ATPase of methylated S1 has been reported to be reduced about 10-fold (White & Rayment, 1993). In an attempt to understand this low activation, the dissociation of ϵADP from M-S1·MgϵADP·AlF_4^- by actin was measured. As shown in Figure 3, the dissociation of ϵADP by actin was faster from the native S1 than from the methylated S1 complex, even though in the absence of actin the native S1·MgϵADP·AlF_4^- complex was much more stable than the M-S1·MgϵADP·AlF_4^- complex. It should be noted that the difference in dissociation rates is not due solely to the lower actin-binding constant of M-S1, since under equal binding conditions (same amount of actin bound to methylated S1 as to the native) a slower product release by actin was still observed with methylated S1 (data not shown). These results suggest that the communication between actin and the active site in methylated S1 is impaired.

The flawed communication between the actin and nucleotide sites on M-S1 could result in the loss of the motor function of S1. This was directly tested by measuring the *in vitro* motility of actin filaments over methylated HMM. While the mock-methylated HMM moved actin with standard velocity (4.2 ± 0.5 μm/s), none of the actin filaments moved with the methylated HMM. The loss of movement persisted over a wide range of M-HMM concentrations (10–200 μg/mL) used for coating the surface. These observations suggest that methylation impairs the motile function of myosin either by preventing tight coupling between the actin and nucleotide sites or by other nucleotide- and actin-induced changes in S1 structure.

SH_1 and SH_2 Sites and Communication with the Active Site. The low K^+(EDTA)-ATPase and enhanced Ca^{2+}-ATPase activities of the methylated S1 are characteristic of an SH_1-modified S1 (Reisler *et al.*, 1974; Wells *et al.*, 1980a). To examine how methylation may affect the environment of the SH_1 site, the reactivity of Cys-707 toward the thiol reagent, CPM, was monitored. This reagent has a low quantum yield when free in solution, and its fluorescence intensity increases manyfold upon attachment to a thiol group (Fuchs *et al.*, 1989). Although CPM is not SH_1-specific and can also bind to the SH_2 group, it has the advantage of high reactivity and is convenient for monitoring the rates of SH_1 modification.

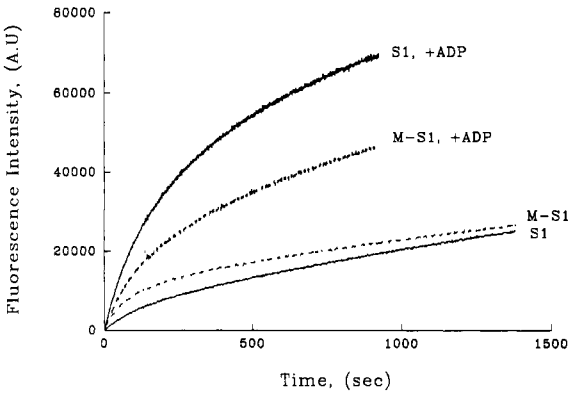

FIGURE 5: CPM modification of S1 (—) and M-S1 (– –) in the presence or absence of ADP·S1 or M-S1 (10 μM) in 20 mM KCl and 5 mM HEPES, pH 7.0, reacted with CPM (25 μM) in the presence and absence of MgADP (1.0 mM) at 4 °C. The change in fluorescence was monitored at 475 nm, and the excitation wavelength was set at 390 nm.

To determine the specificity of SH_1 labeling, the modification of the mock-methylated S1 was first monitored by K^+(EDTA)-ATPase and Ca^{2+}-ATPase activity measurements. The labeling conditions were then chosen such that the modification was well within the time in which the increase in the Ca^{2+}-ATPase activities of the modified S1 was linear with the loss of K^+(EDTA)-ATPase, indicating that only the SH_1 site was modified (Ajtai *et al.*, 1992). The incorporation of CPM into S1 was measured spectrophotometrically; it did not exceed the ratio of 1.0 CPM per S1. Figure 5 shows the time course of CPM modification of S1 in the presence and absence of ADP. In the absence of nucleotides, the CPM modification was somewhat faster for the methylated S1. This suggests that methylation renders the SH_1 group more reactive to the thiol reagent. In agreement with previous observations (Reisler *et al.*, 1974; Watterson & Schaub, 1973), nucleotide binding to S1 accelerates the SH_1 modification, as evidenced by the fast jump in fluorescence for the native S1 (Figure 5). However, for methylated S1, ADP addition had a much smaller effect on the initial rate of increase of fluorescence intensity. Thus, the slower fluorescence change suggests that nucleotide binding to M-S1 does not induce an equally large conformational change at the SH_1 site of the methylated S1 as in the native S1.

Modification of S1 with IAEDANS, which has been shown to alkylate specifically the SH_1 group (Takashi *et al.*, 1976), was used to confirm the environmental changes in the vicinity of SH_1 upon methylation. It was possible to follow the time course of the reaction also in this case by monitoring the fluorescence increase because IAEDANS, like CPM, has a lower quantum yield in its free form than in its bound form (experimental details are described under Materials and Methods). In qualitative analogy to the CPM modification, the rate of the IAEDANS reaction increased by 80% upon methylation (not shown), while the final fluorescence values were the same in S1 and methylated S1. Thus, as reported by two different cysteines probes, the vicinity of SH_1 becomes more "open" and accessible to alkylation upon methylation of S1.

One of the sequences believed to be important for the coupling between actin- and nucleotide-binding sites on S1 is the SH_1–SH_2 helix. In the presence of nucleotides, the two cysteines of S1 can be cross-linked by a variety of bifunctional reagents (Burke & Reisler, 1977, Wells *et al.*, 1980b). The SH_1–SH_2 cross-linking induces closure of the active site and consequently "traps" the nucleotide in the pocket (Wells &

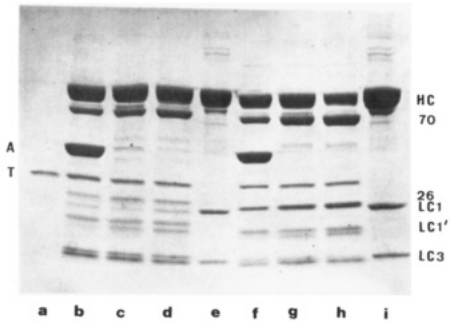

FIGURE 6: SDS–PAGE analysis of the thermolysin digestion of S1 and M-S1. S1 or M-S1 (5.0 μM) was digested by thermolysin in the presence and absence of 5.0 mM ATP or 10 μM F-actin as described under Materials and Methods. Lanes: (a) thermolysin; (b) M-S1 digested in the presence of actin; (c) M-S1 digested without additions; (d) M-S1 digested in the presence of ATP; (e) M-S1, undigested; (f) S1 digested in the presence of actin; (g) S1 digested without additions; (h) S1 digested in the presence of ATP; (i) undigested S1. Vertical symbols: numbers are apparent molecular masses in kilodaltons; HC, heavy chain; A, actin; T, thermolysin; LC1 and LC3, light chain 1 and 3; LC1′, thermolysin digested LC1.

Yount, 1979; Wells *et al.*, 1980b). To examine how methylation affects the communication between the active site and the SH_1–SH_2 sequence, S1 and methylated S1 were cross-linked with pPDM in the presence of MgϵADP. An equal amount of ϵADP was trapped in the control and methylated S1, as judged by the fluorescence intensity in the presence of 100 mM acrylamide. The release of ϵADP from the active site into an acrylamide-containing medium was then monitored. We found that the dissociation rate of ϵADP from pPDM-modified methylated S1 was 3–4-fold faster than from the pPDM-modified native S1 [2.8 ($\pm$1.2) $\times$ 10^{-3} s^{-1} versus 8.5 ($\pm$1.0) $\times$ 10^{-4} s^{-1}]. These results suggest that SH_1–SH_2 cross-linking of the methylated S1 does not induce the same conformational change as in the native protein and does not trap the nucleotide as well in the active site.

Protease-Susceptible Sites and Communication with the Actin- and Nucleotide-Binding Sites. The proteolytic sites on S1 are most frequently probed in tryptic digestion experiments (Mornet *et al.*, 1979). This option was not available after the extensive methylation of S1 which blocked the tryptic cleavage of lysine residues in the trypsin-susceptible S1 junction. Thus, subtilisin and thermolysin were employed in subsequent experiments.

Subtilisin cleaves S1 heavy chain in the absence of nucleotide at 26 and 75 kDa from the N-terminus (Applegate & Reisler, 1983). In the presence of ATP or ADP, there is an additional cut at 4 kDa from the N-terminus (Applegate & Reisler, 1984), which points to the communication between this site and the nucleotide-binding site. Methylation does not affect the subtilisin cuts either in the presence or in the absence of ADP (results not shown); i.e., the communication between the 4 kDa subtilisin-sensitive site and the active site is not affected by this modification. Thermolysin cleaves S1 heavy chain at 26 kDa from the N-terminus (Applegate & Reisler, 1983) after Glu-208 or Ala-209 (Yamamoto, 1989b). This cleavage is enhanced by nucleotides and inhibited by actin (Applegate & Reisler, 1983, 1984; Muhlrad & Chaussepied, 1990), indicating that communication exists between this site and both the nucleotide- and actin-binding sites. We found that methylation does not affect the thermolysin cut in the absence of nucleotides and actin, except for a further degradation of the 26 kDa product. However, both ATP activation and actin inhibition of the cleavage are abolished by this modification (Figure 6). In contrast to this, the trinitrophenylation of Lys-83 did not affect the ATP- or actin-induced changes in the rate of thermolysin cleavage (results not shown). These results indicate that the communication between the thermolysin-susceptible site and both the nucleotide- and actin-binding sites is impaired upon methylation. This perturbation is not due to the methylation of Lys-83, since the modification of this residue by the bulkier trinitrophenyl group does not impair the communication between Glu-208 and the ATP- and actin binding sites.

Vanadate-Dependent Photocleavage. Irradiation by near-ultraviolet light in the presence of V_i cleaves the S1 heavy chain at three sites: 23, 31, and 74 kDa from the N-terminus (Ringel *et al.*, 1990). The cleavage at the 23 and 31 kDa sites, which are a part of the "consensus" ATP-binding site, occurs at Ser-180 (Cremo *et al.*, 1989) and probably at Ser-243 (Grammer & Yount, 1991), respectively. The cleavage at these sites is inhibited by ATP (Muhlrad *et al.*, 1992). The 74 kDa site is near the lysine-rich 50/20 kDa junction which is implicated in actin binding (Mornet *et al.*, 1979; Chaussepied & Morales, 1988). Cleavage of the S1 heavy chain (95 kDa) at this site results in the formation of 21 and 74 kDa fragments. The latter product is further cleaved to a 51 kDa fragment due to the cut at 23 kDa from the N-terminus. Actin fully protects S1 against V_i-dependent photocleavage both at the 31 kDa and at the 74 kDa sites and, therefore, prevents the formation of 31, 21, and 51 kDa fragments (Muhlrad *et al.*, 1991). Methylation does not affect the V_i-dependent photocleavage of S1 in the absence of ligands (Figure 7A, lanes e and f) nor does it influence the effect of ATP on the photocleavage (Figure 7A, lanes a and b). However, methylation of S1 significantly decreases the inhibitory effect of actin on the formation of the 31, 21, and 51 kDa products (Figure 7A, lanes c and d; Figure 7B,C). This decrease in actin inhibition of V_i cleavage of S1 is not due to decreased binding of M-S1 to actin: by using $K_a = 7 \times 10^5$ M^{-1} (Figure 2A), about 90% of M-S1 is estimated to be bound to actin. (This actually underestimates the bound fraction, because V_i cleavage of S1 was carried out at a much lower ionic strength than the binding measurements shown in Figure 2, and the affinity of S1 for actin decreases with increasing ionic strength.) Thus, the methylation-induced decrease in the actin inhibition of photocleavage at 31 and 74 kDa from the N-terminus is probably due to impaired communication between the actin- and ATP-binding sites and the actin and 74 kDa sites, respectively. Trinitrophenylation of S1 did not affect the V_i-dependent photocleavage under all conditions used in Figure 7 (results not shown).

Protective Effect of ATP and Actin on Heat Denaturation of S1. Mild heat treatment of S1 between 35 and 37 °C inactivates its ATPase activity and dramatically increases the tryptic susceptibility of the 50 kDa domain. It is also known that both actin and ATP or ADP protect S1 against the heat treatment (Setton & Muhlrad, 1984). Methylation of S1 accelerates the rate of ATPase inactivation by heat treatment by over 60% and drastically decreases the protective effect of actin by almost 10-fold and of ADP by over 100-fold (Figure 8). Trinitrophenylation of Lys-83 does not affect the thermal inactivation of S1 (results not shown). The accelerated loss of the ATPase activity in methylated S1 and its greatly decreased protection by ADP and actin point to methylation-induced conformational changes and impaired communications in the S1 structure.

DISCUSSION

The effect of extensive reductive methylation of lysyl residues on various structural and functional characteristics

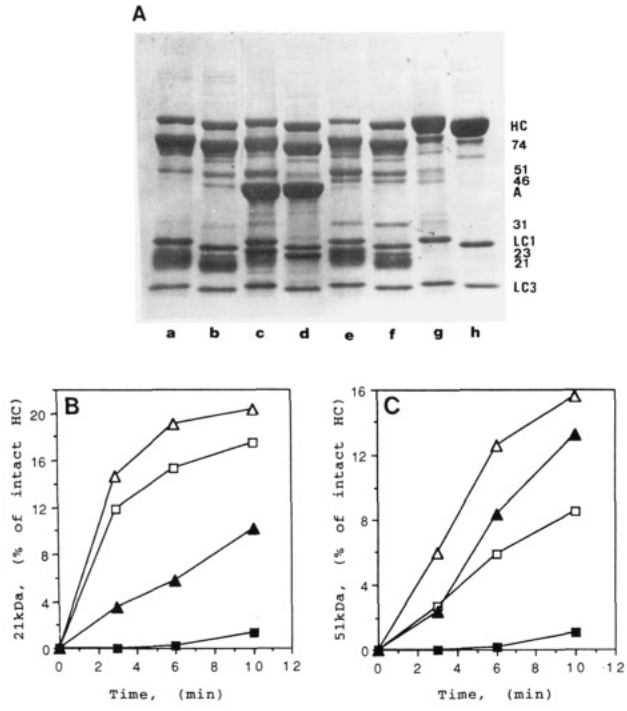

FIGURE 7: SDS–PAGE analysis of the V_i-dependent photocleavage of S1 and M-S1. S1 or M-S1 (8.0 μM) was irradiated by near-ultraviolet light in the presence of 0.4 mM sodium vanadate with and without 5.0 mM ATP or 20 μM F-actin. Samples were withdrawn at 3, 6, and 10 min and analyzed by SDS-PAGE as described under Materials and Methods. (A) Gel electrophoretogram obtained after 10 min irradiation. Lanes: (a) M-S1 irradiated in the presence of ATP; (b) S1 irradiated in the presence of ATP; (c) M-S1 irradiated in the presence of actin; (d) S1 irradiated in the presence of actin; (e) M-S1 irradiated without addition; (f) S1 irradiated without addition; (g) nonirradiated M-S1; (h) nonirradiated S1. (B and C) Formation of the 21and 51 kDa fragments, respectively, as estimated from the densitometry of peptide bands. Irradiation symbols: (■) S1, in the presence of actin; (▲) M-S1, in the presence of actin; (□) S1, without additions; (△) M-S1, without additions. Abscissa, irradiation time; ordinate, amounts of fragment formed relative to the initial amount of the intact S1 heavy chain (HC).

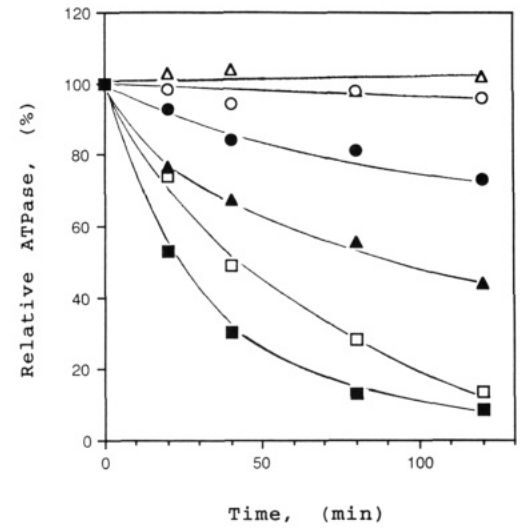

FIGURE 8: Effect of mild heat treatment on the ATPase activity of S1 and M-S1. S1 or M-S1 (8.0 μM) was incubated at 37 °C in the presence or absence of 2.0 mM ADP or 20 μM F-actin (as described under Materials and Methods). At given time intervals, aliquots were withdrawn to measure the Ca^{2+}-activated ATPase activities. ATPase activities are given relative to the activity of S1 or M-S1 before heat treatment. The heat denaturation curves follow first-order kinetics, and the rate constants were calculated accordingly. Symbols and rates of ATPase inactivation: (□), S1, 2.75×10^{-4} s^{-1}; (■), M-S1, 4.45×10^{-4} s^{-1}; (△), S1 in the presence of ADP, 1×10^{-6} s^{-1}; (▲), M-S1 in the presence of ADP, 1.07×10^{-4} s^{-1}; (○), S1 in the presence of actin, 4.33×10^{-6} s^{-1}; (●), M-S1 in the presence of actin, 4.17×10^{-5} s^{-1}.

of S1 has been studied in this work. We found that the overall secondary structure of S1 did not change upon its extensive modification. This is consistent with a preliminary report on identical low-angle X-ray scattering from S1 and M-S1 (Stone *et al.*, 1994) and the kinetic description of M-S1 (White & Rayment, 1993). On the other hand, the near-UV CD spectra revealed changes in the tertiary structure of methylated S1 which could be attributed to an altered environment of tryptophan, tyrosine, and phenylalanine residues (Strickland, 1974). The increased sensitivity of M-S1 to mild heat treatment may also reflect methylation-caused changes in the tertiary structure of the molecule.

The list of local structural changes on S1 induced by its methylation includes nucleotide and actin sites and the reactive SH_1 site. The kinetic changes in S1 ATPase together with the increased accessibility of ϵADP to collisional quenchers [see also Bivin *et al.* (1994)] and reduced trapping of nucleotide and phosphate analogs provide evidence for changes in the active site. The decrease in the affinity of S1 for actin, following S1 methylation, suggests a perturbation of the actin-binding site. Alterations in the SH_1 site are suggested by the increased rate of Cys-707 alkylation in M-S1. The fact that all of the above changes are rather small rules out the possibility of any large structural perturbations in these sites.

Measurements of S1 binding to actin have revealed that methylation causes a larger decrease in the strong (rigor) than the weak binding (+MgATP) of these proteins. This result can be rationalized by recalling that the weak acto–S1 interactions are mainly of electrostatic nature (Chaussepied *et al.*, 1988) and are probably centered around the lysine-rich cluster at the 50/20 kDa junction in S1 (Chaussepied & Morales, 1988). The methylation of these lysine residues, as evidenced by the resistance of the modified protein to tryptic digestion, does not affect their positive charge at physiological pH, and thus does not cause any major affinity change in the presence of MgATPγS. On the other hand, the strong binding between actin and S1 is based mostly on stereospecific hydrophobic interactions, in addition to electrostatic interactions which also take place in weak binding, and involves probably a larger surface area on both proteins (Rayment *et al.*, 1993b). Consequently, such interactions may be expected to be more sensitive to methylation-induced conformational changes than the weak binding.

Methylation reduced about 10-fold the V_{max} of the actin-activated ATPase activity (White & Rayment, 1993; present work), and even more dramatically the rate of bond splitting in ATP hydrolysis by S1. Actin activation of the myosin ATPase is achieved via acceleration of the rate-limiting step of product release (White & Taylor, 1976; Stein *et al.*, 1979). However, the rate of bond splitting has been so much decreased upon methylation of S1 that it limits, at least in part, the rate of the actin-activated S1 ATPase. The resulting change in the population of ATP hydrolysis intermediates is assumed to be the reason for the reduced activity (White & Rayment, 1993). This poses an important question: Is the methylated M**·MgADP·P_i complex functionally competent; i.e., can actin accelerate its dissociation by a large factor? Our results on the release of trapped nucleotide from M-S1 and S1 show that this aspect of coupling between actin and nucleotide sites is impaired in M-S1. Despite the fact that the active site appears to be more "open" in M-S1 (Bivin *et al.*, 1994) and releases trapped nucleotides at a faster rate than S1, the

opposite holds in the presence of actin (Figure 3). The lack of actin movement over methylated HMM in the *in vitro* motility assay is consistent with the hypothesis that the tight coupling between the actin and nucleotide sites on S1 and the structural changes underlying this coupling are essential for the motile function of myosin.

The impaired communication between the actin and nucleotide sites on M-S1 is also manifested in the lack of S1 protection by actin from the V_i-dependent photocleavage at Ser-243, 31 kDa from the N-terminus, which is believed to be a part of the nucleotide-binding site of myosin (Grammer & Yount, 1991; Muhlrad *et al.*, 1991, 1992). The list of other methylation-related changes in the actin-triggered effects on S1 includes (i) substantial if not complete loss of S1 protection from heat treatment; (ii) loss of actin effects on thermolysin cleavage at the 25/50 kDa junction of S1; and (iii) reduced protection of S1 from photocleavage by V_i at 74 kDa from the N-terminus of actin, i.e., about 1 kDa upstream from the lysine-rich 50/20 kDa junction.

In addition to the above-mentioned impaired communication between the actin-binding site and other sites on S1, methylation perturbs also the spread of nucleotide-induced conformational changes throughout the S1 structure. The notable changes include (i) the loss of S1 protection by nucleotides from heat inactivation; (ii) the loss of ATP activation of thermolysin cleavage at 26 kDa from the N-terminus; (iii) a reduced ADP effect on the rate of SH_1 modification by CPM; (iv) faster release of ϵADP from pPDM-cross-linked M-S1 than from cross-linked S1. The two latter effects of methylation suggest impaired communication between the SH_1–SH_2 site (the two thiols are cross-linked by pPDM) and the active site in M-S1.

The conclusion about methylation-induced damage to intersite communication in S1 merits an additional comment. It is important to note that even closely related sites on S1 do not sense the same perturbation in ligand-induced changes. Thus, for example, while methylation does not change the effect of ATP and ADP on subtilisin cleavage of S1 at the protease-sensitive regions, it impacts strongly the ability of these nucleotides to affect the cleavage of the same junctions by thermolysin. Also, while methylation abolishes the characteristic enhancement of tryptophan fluorescence by ATP (Werber *et al.*, 1972; White & Rayment, 1993), CD spectra report a similar average perturbation of the tryptophan environment by ATP on S1 and M-S1. Thus, it is possible to speculate that minor and localized alterations in the probed sites are responsible to a large degree for these variations.

In order to put the observed perturbations of S1 by its methylation in a proper perspective, it is instructive to compare this extensive modification of S1 with specific chemical modifications of the reactive SH_1 thiol (Cys-707) and Lys-83. The SH_1 modification of S1 by phenylmaleimide abolishes its motor function (Root & Reisler, 1992). Both SH_1 and Lys-83 modifications result in similar changes in the ATPase properties of myosin as the extensive reductive methylation of almost all S1 lysines (White & Rayment, 1993). These changes involve increases in the Ca^{2+}- and Mg^{2+}-activated ATPases and decreases in the K^+(EDTA) and actin-activated ATPase activities. The activity changes are mainly due to the decrease in the rate of the bond splitting step and the stability of the M**·MgADP·P_i predominant intermediate of ATP hydrolysis (Sleep *et al.*, 1981; Muhlrad, 1983). In this work, we compared some of the effects of extensive methylation of lysine residues with those of Lys-83 trinitrophenylation. We found that both extensive methylation and trinitrophenylation strongly accelerate the dissociation of the stable V_i- and BeF_x-containing S1 complexes. [In this respect, SH_1-modified S1 behaves differently, since the stability of the NEM-S1·ADP·BeF_x complex is higher than that of the native S1·ADP·BeF_x (Phan and Reisler, results not shown).] The similarity in the effect of methylation and trinitrophenylation on S1 is limited mostly to changes in the ATPase properties and at the active site, and does not include perturbations in the intersite communications on S1. Trinitrophenylation, unlike methylation, does not impair the thermostability and the communication between the actin-binding site and the 31 and 74 kDa V_i-dependent photocleavage sites, and between the thermolysin cleavage site at the 25/50 kDa junction and the nucleotide- and actin-binding sites. NEM modifications of the SH_1 group on S1 do not alter the near-UV CD spectra of this protein (Reisler *et al.*, 1977) nor do they change its thermostability and the protective effect of nucleotide against heat treatment of S1 (Shnyrov *et al.*, 1989). These considerations may lead to the speculation that the methylation-caused changes in the active site are due to the methylation of Lys-83. On the other hand, methylation causes rather extensive changes in the spread of ligand-induced conformational changes, which are related to the modification of lysine residues different from Lys-83.

In summary, we conclude that methylated S1 is a good structural model of native S1. It has more limited value as a functional model, since the methylation affects both actin and nucleotide binding, alters the coupling between actin and ATP sites and intersite communications on S1, and blocks the motor function of myosin heads. It is conceivable that the reported effects of methylation on S1 originate from numerous but minor conformational alterations at different sites on S1. Such alterations may be inconsequential in other, less dynamic proteins than the myosin head. However, this conclusion will need to be tested on a case by case basis.

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