Maleimidobenzoyl-G-actin: Structural Properties and Interaction with Skeletal Myosin Subfragment-1[†]

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ABSTRACT: We have investigated various structural and interaction properties of maleimidobenzoyl-G-actin (MBS-actin), a new, internally cross-linked G-actin derivative that does not exhibit, at moderate protein concentration, the salt—and myosin subfragment 1 (S-1)—induced polymerizations of G-actin and reacts reversibly and covalently in solution with S-1 at or near the F-actin binding region of the heavy chain (Bettache, N., Bertrand, R., & Kassab, R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6028–6032). The far-ultraviolet CD spectrum and α-helix content of the MBS-actin were identical with those displayed by native G-actin. $^{45}Ca^{2+}$ measurements showed the same content of tightly bound Ca^{2+} in MBS-actin as in G-actin and the EDTA treatment of the modified protein promoted the same red shift of the intrinsic fluorescence spectrum as observed with native G-actin. Incubation of concentrated MBS-actin solutions with 100 mM KCl + 5 mM $MgCl_2$ led to the polymerization of the actin derivative when the critical monomer concentration reached 1.6 mg/mL, at 25 °C, pH 8.0. The MBS-F-actin formed activated the Mg^{2+}–ATPase of S-1 to the same extent as native F-actin. The MBS-G-actin exhibited a DNase I inhibitor activity very close to that found with native G-actin and was not to be at all affected by its specific covalent conjugation to S-1. This finding led us to isolate, for the first time, by gel filtration, a ternary complex comprising DNase I tightly bound to MBS-actin cross-linked to the S-1 heavy chain, demonstrating that S-1 and DNase I bind at distinct sites on G-actin. Fluorescence measurements with pyrene-labeled MBS-G-actin were applied to monitor the solution interaction of the protein with the isolated S-1(A1) and S-1(A2) isoenzymes. The binding of either S-1 isoform did not cause polymerization of the fluorescent actin derivative and resulted in about 40% enhancement of the fluorescence emission of the pyrenyl probe. The dissociation constants (K_d) estimated at low ionic strength (2 mM) were 0.18 and 0.47 μM, for the complexes with S-1(A1) and S-1(A2), respectively. The addition of salt (100 mM) decreased the MBS-actin–S-1 affinity ($K_d \geq 60$ μM) and perturbed the structure of the actin derivative as assessed by limited proteolysis. Collectively, the data illustrate further the nativeness of the MBS-G-actin and its potential use in solution studies of the actin–myosin head interactions.

In order to assess the solution interaction of the myosin head and monomeric actin, we have recently designed a new G-actin derivative, referred to as maleimidobenzoyl-G-actin (MBS-actin),[1] containing few intramolecular cross-links between cysteine and lysine residues, which were introduced in skeletal G-actin with the bifunctional agent (*m*-maleimidobenzoyl)-*N*-hydroxysuccinimide ester (Bettache et al., 1989). This internal cross-linking led to the suppression of the polymerizability of G-actin induced by salts or skeletal myosin subfragment-1 and the G-actin derivative served for the production and preliminary characterization of soluble, reversible, and covalent binary complexes between the S-1 heavy chain and monomeric actin. Support of these results was recently provided by the identification of a soluble complex between the S-1(A2) isoenzyme and native G-actin (Chaussepied & Kasprzak, 1989a). These new G-actin–S-1 complexes may be very useful to gain further insight into the mechanism of the actomyosin interaction and potentially they may initiate the cristallography of actin-bound S-1.

Herein, we have extended our previous investigations on MBS-actin by precising some of its important structural and functional properties, including studies of the degree of nativeness of the protein derivative and quantitative studies of its interaction with DNase I and with S-1 under various experimental conditions. MBS-actin was also used to produce for the first time a ternary complex comprising both DNase I and myosin subfragment-1.

MATERIALS AND METHODS

Chemicals. Bovine pancreatic DNase I was purchased from Boehringer-Mannheim. (*m*-Maleimidobenzoyl)-*N*-hydroxysuccinimide ester (MBS) was from Pierce. L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin was obtained from Sigma. *N*-(1-Pyrenyl)iodoacetamide was obtained from Molecular Probes. Sephacryl S-300 (superfine) was from Pharmacia (Uppsala). ^{45}Ca was obtained from Amersham, France. All other chemicals were of the highest analytical grade.

Protein Preparations. Rabbit skeletal myosin was prepared as described by Offer et al. (1973). Chymotryptic S-1 was obtained according to Weeds and Taylor (1975) and was

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[1] Abbreviations: S-1, myosin subfragment-1; S-1(A1) or S-1(A2), isoenzyme of S-1 containing alkali light chain 1 or 2, respectively; Acto-S1, actomyosin subfragment-1; ATPase, adenosine-5′-triphosphatase; MBS, (*m*-maleimidobenzoyl)-*N*-hydroxysuccinimide ester; DTE, dithioerythritol; CD, circular dichroism; MBS-actin, maleimidobenzoyl-G-actin; DNase I, desoxyribonuclease I.

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further purified over Sephacryl S-200 (Chaussepied et al., 1986a). The S-1(A1) and S-1(A2) isoenzymes were separated by chromatography on SP-Trisacryl M (Trayer & Trayer, 1988). Rabbit skeletal F-actin was prepared as described by Eisenberg and Kielley (1974). G-Actin was freshly prepared by depolymerization of F-actin (2 mg/mL) in G-buffer (2 mM Hepes, 0.1 mM ADP, 0.1 mM $CaCl_2$, 0.1 mM NaN_3, pH 8.0). This solution was sonicated three times, for 1 min each time, at an amplitude of 10 μm in an MSE 150-W sonicator and then centrifuged at 180000*g* for 1 h at 4 °C.

MBS-actin was prepared as described earlier (Bettache et al., 1989), by gently mixing G-actin (1.5 mg/mL) with a 20-fold molar excess of MBS (dissolved at 8.5 mg/mL in dimethylformamide) in G-buffer for 2 h at 20 °C. The reaction was terminated by the addition of DTE and glycine (each at 5-fold molar excess relative to MBS). The glycine- and DTE-treated MBS-actin was incubated in the presence of 50 mM KCl and 5 mM $MgCl_2$ for 30 min at 20 °C. After ultracentrifugation of the protein solution at 180000*g* for 1 h, 4 °C, the supernatant was gel filtered over a Pharmacia PD10 column equilibrated in G-buffer. When desired, the protein solution could be concentrated up to 15 mg/mL at 4 °C by using a Micro-Prodicon concentrator (Bio-Molecular Dynamics). To prepare pyrene-labeled MBS-actin, native F-actin was first reacted on Cys-374 with *N*-(1-pyrenyl)-iodoacetamide according to Kouyama and Mihashi (1981). After depolymerization, pyrene–actin was mixed with native G-actin to obtain 30% extent of labeling. The protein mixture was then treated with MBS and processed essentially as described above. The protein gel filtered over the PD10 column was finally passed through a column of Sephacryl S-200 (2.5 × 150 cm) equilibrated in G-buffer.

The isolation of the covalent MBS-actin–S-1 complex was carried out as follows: KCl treated, centrifuged, and gel filtered MBS-actin, not treated with glycine and DTE, (1 mg/mL) in G-buffer, was mixed with a convenient volume of a S-1 solution (15 mg/mL) in 25 mM imidazole–HCl, pH 7.0 (final actin:S-1 molar ratio, 1:1). After 30 min at room temperature, DTE (adjusted to pH 7.5) was added to 1 mM. After ultracentrifugation at 180000*g*, 1 h, 4 °C, the supernatant was incubated for 2 h at 20 °C, with F-actin (added at a molar ratio of 2:1 relative to S-1) in the presence of 25 mM KCl and 5 mM $MgCl_2$. After ultracentrifugation at 180000*g*, 90 min, 4 °C, the pellet containing F-actin bound to un-cross-linked S-1 was discarded and the supernatant was fractionated at 4 °C on a column of Sephacryl S-300 superfine (1.9 × 150 cm) in 20 mM imidazole–HCl, 2.5 mM ATP, 25 mM KCl, and 5 mM $MgCl_2$, pH 7.5. The flow rate was 18 mL/h. The proteins of the collected fractions were identified by $NaDodSO_4$ gel electrophoresis. The fractions containing the cross-linked MBS-actin–S-1 complex were pooled, concentrated on a PM 30 Amicon membrane, and stored at 4 °C.

For the isolation of the ternary complex between DNase I, MBS-actin, and S-1, the covalent MBS-actin–S-1 complex (1.5 mg/mL) in 20 mM imidazole–HCl, 2.5 mM ATP, 25 mM KCl, and 5 mM $MgCl_2$, pH 7.5, was mixed with DNase I (2 mg/mL) dissolved in 5 mM imidazole–HCl, pH 7.0, containing 10 μg/mL leupeptin, at a molar ratio of 1:1 relative to actin. After incubation for 20 min at 4 °C, the solution was passed over Sephacryl S-300 superfine as described above. The eluted DNase I–MBS-actin–S-1 complex was identified by $NaDodSO_4$ gel electrophoresis.

Enzyme Assays. Determinations of the DNase I activity were performed by using the spectrophotometric procedure and conditions described by Blikstad et al. (1978). In the assay (1 mL) the DNA substrate (40 μg/mL) was mixed with the enzyme and its hydrolysis was followed by measuring the hyperchromicity at 260 nm. For determination of the DNase I inhibitor activity of MBS-actin or G-actin, a standardized amount of DNase I (15 μL containing 3 μg of enzyme) was mixed with 0–30 μL of protein (MBS-actin or MBS-actin–S-1 complex or native G-actin) containing 0–6 μg of actin.

The Mg^{2+}–ATPase activity of S-1 was measured in the presence of F-actin or polymerized MBS-actin (2.5 mg/mL), at 25 °C in a medium (1 mL) containing 50 mM Tris-HCl, 5 mM ATP, 10 mM KCl, and 2.5 mM $MgCl_2$, pH 8.0. S-1 was added at 0.05 mg/mL. At different times of the ATPase reaction, aliquots were taken and precipated with 15% TCA. Inorganic phosphate was measured colorimetrically as previously described (Mornet et al., 1979).

Tryptic Digestions. Glycine- and DTE-treated MBS-actin was subjected to the KCl + $MgCl_2$ treatment, ultracentrifuged, and gel filtered over a Pharmacia PD10 column. The isolated MBS-actin (1.5 mg/mL) was mixed with native S-1 in G-buffer, pH 8.0, at a molar ratio of 1:1. The reversible complex was then digested at 25 °C with trypsin (protease to substrate weight ratio = 1:100) in the absence and presence of 100 mM KCl. MBS-actin alone in G-buffer, pH 8.0, was also digested at 25 °C with trypsin (added at a weight ratio of 1:10 relative to actin) in the absence and presence of 100 mM KCl.

Spectral Procedures. Fluorescence measurements were made in a Kontron SFM 25 fluorescence spectrophotometer with the temperature controlled by a circulating water bath. The intrinsic fluorescence of MBS-actin or G-actin in G-buffer, in the absence and presence of 1 mM EDTA, was analyzed at 25 °C according to Lehrer and Kerwar (1972). The fluorescence enhancement assay for binding of myosin subfragment-1 to pyrene-labeled MBS-actin was carried out at 25 °C with excitation at 344 nm and emission at 389 nm. The binding data were fitted by using the "ENZFITTER" non-linear regression program (Biosoft-Elsevier, Cambridge, UK).

CD measurements were performed as reported previously (Chaussepied et al., 1986b) by using a computerized Jobin et Yvon Model Mark V autodichograph. The far-ultraviolet spectra were interpreted by the simplified method of Siegel et al. (1980).

Electrophoresis. $NaDodSo_4$–polyacrylamide gradient gel electrophoresis (5–18%) was carried out as described previously (Mornet et al., 1981).

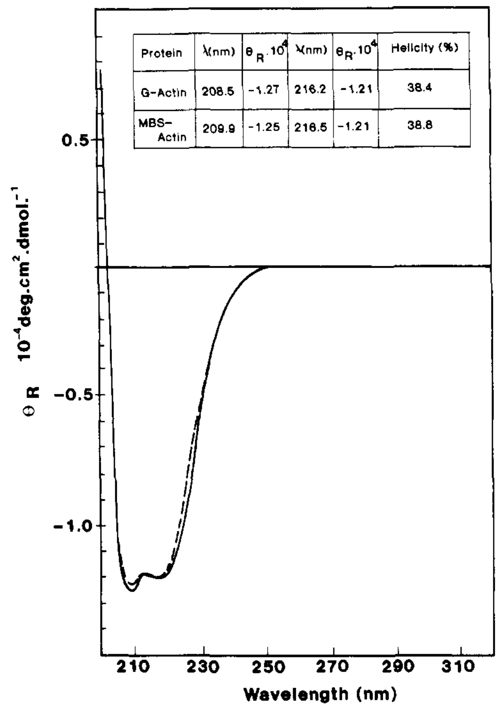
Other Measurements. The viscosity of MBS-actin (1.4–3.8 mg/mL) or G-actin (0.05–0.3 mg/mL) was measured in G-buffer at 25 °C by using an Ostwald viscometer with an outflow time of 32 s for the buffer employed.

^{45}Ca was added to a G-actin solution (17 μM), freed of excess Ca^{2+} by Chelex-100 treatment, at a final concentration of 0.1 mM (specific activity 5 μCi/mg). A 1-mL aliquot was treated with MBS as described above. Samples of both MBS-actin and G-actin labeled with ^{45}Ca were treated with Chelex-100, prepared as described by Gershman et al. (1989), for 30 min, 4 °C. The resin was removed from the actin solutions by centrifugation and aliquots of the supernatants were subjected to radioactivity measurements.

Protein concentrations were estimated by absorbance measurements assuming ${A_{280nm}}^{1\%} = 7.5$ for S-1, 11 for actin, and 12.3 for DNase I. The concentrations of the modified actins were determined by a Bradford assay (Bradford, 1976) with G-actin as the standard.

RESULTS

Conformational Properties of Maleimidobenzoyl-G-actin. To assess if the overall polypeptide conformation of G-actin



Protein	λ(nm)	$\theta_R \cdot 10^4$	λ(nm)	$\theta_R \cdot 10^4$	Helicity (%)
G-Actin	208.5	-1.27	216.2	-1.21	38.4
MBS-Actin	209.9	-1.25	216.5	-1.21	38.8

FIGURE 1: Comparative CD spectra of MBS-G-actin (···) and native G-actin (—) in the far-ultraviolet region. Conditions: 15 μM protein in 2 mM sodium phosphate, 0.1 mM ADP, and 0.1 mM $CaCl_2$, pH 7.5 at 20 °C. Inset: Molar and percentage ellipticities of MBS-actin and G-actin deduced from CD spectra.

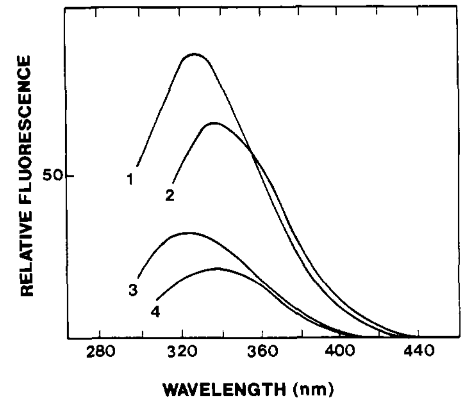


FIGURE 2: Intrinsic fluorescence spectra of MBS-actin and G-actin before and after treatment with EDTA. The spectra for G-actin (1) and MBS-actin (3) were recorded with excitation at λ = 297 nm. The proteins were at 0.8 mg/mL in G-buffer. After incubation for 15 min, 20 °C, in the presence of 1 mM EDTA, the corresponding spectra (2 and 4, respectively) were monitored under the same conditions.

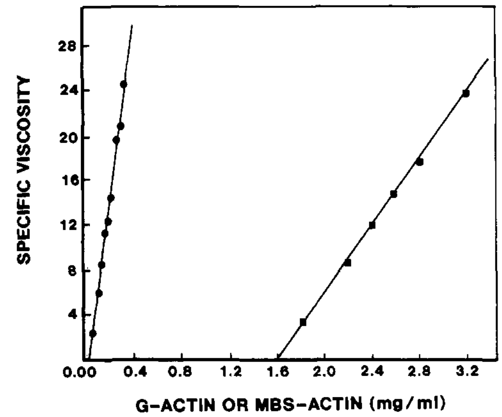


FIGURE 3: Determination of the critical monomer concentration of MBS-actin. Conditions: a concentrated protein solution in G-buffer (12 mg/mL) was diluted to 1.4–3.8 mg/mL in G-buffer containing 100 mM KCl + 5 mM $MgCl_2$; after 4 h at 25 °C, the specific viscosity was measured as indicated under Materials and Methods. (■) MBS-actin; (●) G-actin control.

was affected by its intramolecular cross-linking with MBS, we compared the far-ultraviolet CD spectrum of the isolated MBS-actin derivative with that obtained with native G-actin used as control. The results are illustrated in Figure 1. Below 250 nm the native and modified actins exhibited, under the solvent conditions employed, the same minimum and intensities of ellipticity near 210 nm. We determined an α-helix content of 38.4 and 38.8%, respectively. These values are not significantly different from those, ranging between 26 and 45%, reported earlier for native G-actin (Murphy, 1971; Nagy & Strzelecka-Golaszewska, 1972; Wu & Yang, 1976; Bertazzon et al., 1990).

These spectroscopic data were further complemented by comparing the content of G-actin and MBS-actin in tightly bound Ca^{2+}. $^{45}Ca^{2+}$ measurements indicated the presence of 0.7 and 0.8 mol of Ca^{2+}/mol of protein, respectively (mean values of at least three determinations). Moreover, Figure 2 shows that the intrinsic fluorescence spectra of the two proteins are influenced similarly upon complexation of the divalent ion with EDTA. For G-actin, the EDTA treatment is known to induce a red shift of the intrinsic fluorescence spectrum of the protein that is associated with the loss of the divalent metal and the unfolding of the actin molecule (Lehrer & Kerwar, 1972; Lehrer, 1972). As shown in Figure 2, the λ_{max} of the fluorescence emission of both G-actin and MBS-actin was found at 322 nm. It shifted to 332 nm upon 15 min of incubation of either protein with 1 mM EDTA. Thus, it appears that the environment of the Trp residues in G-actin was not changed significantly by the MBS reaction nor was the sensitivity of the protein to denaturation by EDTA affected. However, we observed that the intrinsic fluorescence emission of MBS-actin was reduced as compared to native G-actin. This was not due to a chemical modification of the tryptophanyl residues by MBS as indicated by the titration of these residues in MBS-actin. Most likely, a fluorescence quenching resulted from the proximity to tryptophanyl groups of the aromatic benzoyl moieties of MBS. In our previous study, we have also shown that the internal cross-linking of actin with MBS did not result in any loss of binding of the nucleotides ATP or ADP (Bettache et al., 1989). Collectively, these data indicate that the metal and nucleotide binding capacity as well as the overall folding of actin was not altered by the chemical modification.

To substantiate further the idea that the intramolecular cross-linking events had no deleterious effects on the actin structure, experiments were attempted to promote the polymerization of MBS-G-actin to MBS-F-actin. The viscosity measurements, depicted in Figure 3, show that the polymerization of MBS-actin in the presence of 100 mM KCl and 5 mM $MgCl_2$, pH 8.0, 25 °C, could be initiated when the protein concentration exceeded 1.6 mg/mL. Under the same experimental conditions, the conversion of the G-actin control into F-actin took place at a protein concentration near 0.020 mg/mL. Therefore, the intramolecular cross-linking of actin with MBS has not irreversibly impaired the polymerizability of the protein, but rather it has shifted the critical monomer concentration to a much higher value. The polymerized MBS-F-actin was isolated by ultracentrifugation and tested for binding to S-1 and for activation of its Mg^{2+}–ATPase in comparison with unmodified F-actin. Sedimentation studies revealed that S-1 binds tightly to the MBS-F-actin pellet; the

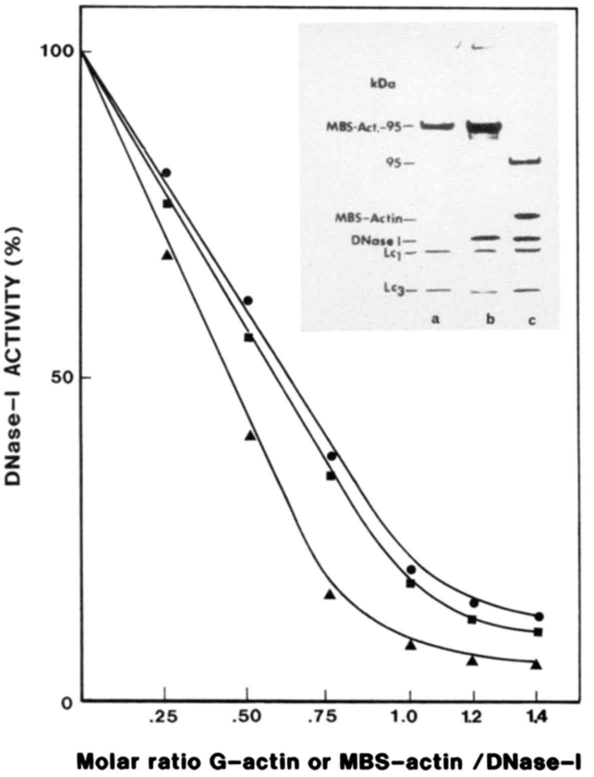


FIGURE 4: Inhibition of the enzymatic activity of DNase I by MBS-actin (■) and the purified covalent MBS-actin–S-1 complex (●). The conditions were as specified under Materials and Methods. (▼) G-actin control. Inset: electrophoretic analysis of the covalent MBS-actin–S-1 preparation isolated by filtration over Sephacryl S-300 before (lane a) and after complexation to DNase I (lane b). Lane c: protein markers (S-1, DNase I, and actin).

steady-state ATPase measurements indicated that the MBS-F-actin activated the enzymatic activity of S-1 to the same extent as native F-actin; also, decoration of MBS-actin filaments by S-1 showed typical arrowhead structures as assessed by electron microscopy (data not shown).

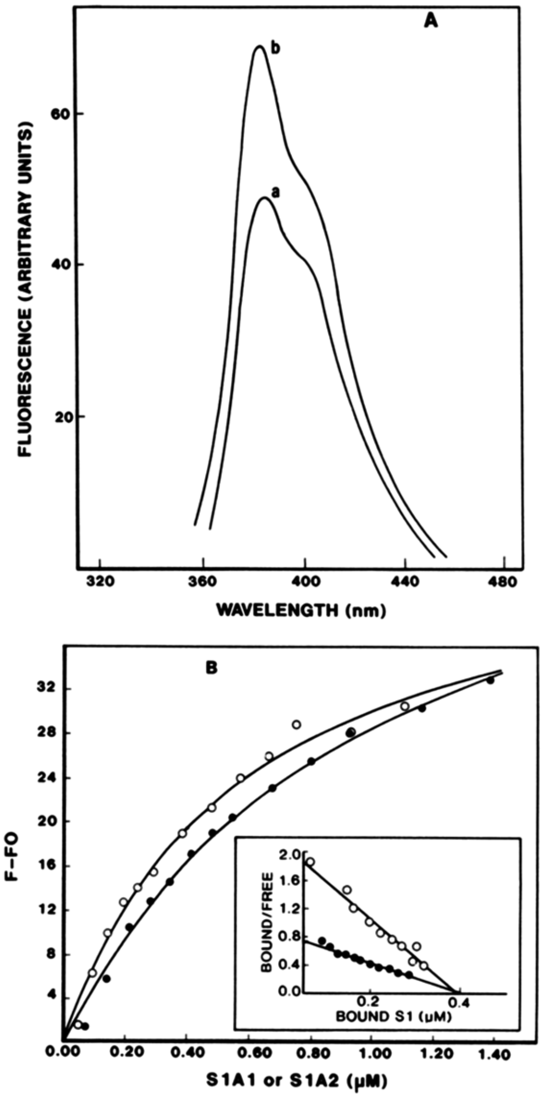


FIGURE 5: Binding of myosin subfragment-1 to pyrene-labeled MBS-G-actin. (A) Enhancement of the fluorescence emission intensity of pyrene–MBS-actin by S-1(A1 + A2). The spectrum of the fluorescent actin derivative (5 µM) in G-buffer was recorded at 25 °C in the absence (a) and presence (b) of S-1 (10 µM). (B) Dependence of the fluorescence enhancement of pyrene-labeled MBS-actin on the concentration of S-1(A1) (○) and S-1(A2) (●). Conditions: 0.5 µM 30% pyrenyl–MBS-actin, and variable concentrations of S-1 isoforms (0.05–1.5 µM) in G-buffer, 25 °C. Excitation at 344 nm; emission at 389 nm. Insets: Scatchard plots of the data in (B). The slopes give the dissociation constant.

Interactions of MBS-G-actin with DNase I and Myosin Subfragment-1. A potent property of native G-actin is its binding to DNase I and its inhibition of DNase enzymatic activity (Blikstad et al., 1978). In the experiments presented in Figure 4, we measured the DNase I activity in the presence of varying concentrations of MBS-G-actin; the same determinations were also done by using native G-actin as a control. Like G-actin, MBS-actin decreased the DNase I activity in a dose-dependent manner and 90% inhibition required about 1.5 mol of MBS-actin per mole of DNase I as compared to the 1:1 ratio observed with native G-actin. Because of the ability of MBS-actin to bind in solution to S-1 (Bettache et al., 1989), we analyzed further the influence of the S-1 interaction on its DNase I inhibitor activity. For these experiments we employed MBS-G-actin covalently coupled, via the free maleimide group, to the S-1 heavy chain (Bettache et al., 1989). The cross-linked 1:1 MBS-actin–S-1 complex was first purified by gel filtration. Protein samples essentially free of un-cross-linked MBS-actin and S-1, as analyzed by gel electrophoresis (Figure 4, inset, lane a), were tested in the DNase I activity assay. As clearly shown in Figure 4, the MBS-actin conjugated to S-1 displayed the same degree of DNase I inhibition as MBS-actin alone. A control with S-1, in the absence of MBS-actin, did not show any effect on the DNase I activity. The binding of the DNase I to the MBS-actin–S-1 conjugate was further confirmed by the isolation of the ternary complex, following gel filtration. When the purified MBS-actin–S-1 adduct was mixed with a stoichiometric amount of DNase I and gel filtered over Sephacryl S-300, a single protein peak was eluted just before the position known for the elution of the binary MBS-actin–S-1 complex. It contained all the added DNase I certainly associated to the MBS-actin covalently bound to S-1 (Figure 4, inset, lane b). Finally, we observed that the presence of DNase I during the coupling reaction between MBS-actin and S-1 did not change the rate of their cross-linking, althought it decreased somewhat the yield of the cross-linking. On the other hand, unlike S-1, the DNase I did not give rise to any cross-linked adduct with MBS-actin. Together, these data strongly suggest that S-1 and DNase I bind to different and nonoverlapping sites on MBS-actin.

Previously, we have described qualitative studies illustrating the reversible solution interaction of MBS-actin and S-1 (Bettache et al., 1989). In Figure 5, we present quantitative fluorescence measurements of this interaction using pyrene-

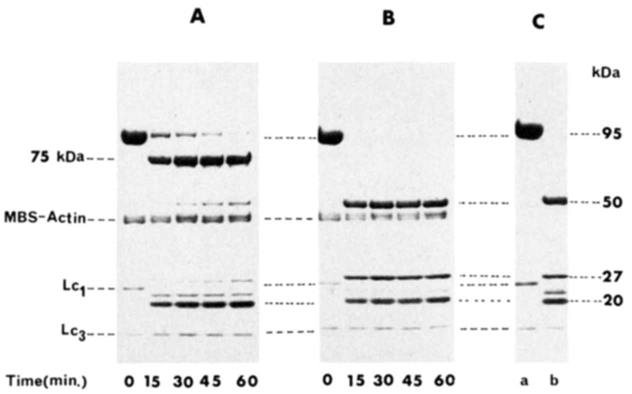


FIGURE 6: Tryptic digestion of reversible MBS-actin–S-1 complex in the absence (A) and presence of 100 mM KCl (B). (C) Electrophoretic pattern of S-1 before (lane a) and after 60-min tryptic digestion (lane b). Conditions were as indicated under Materials and Methods.

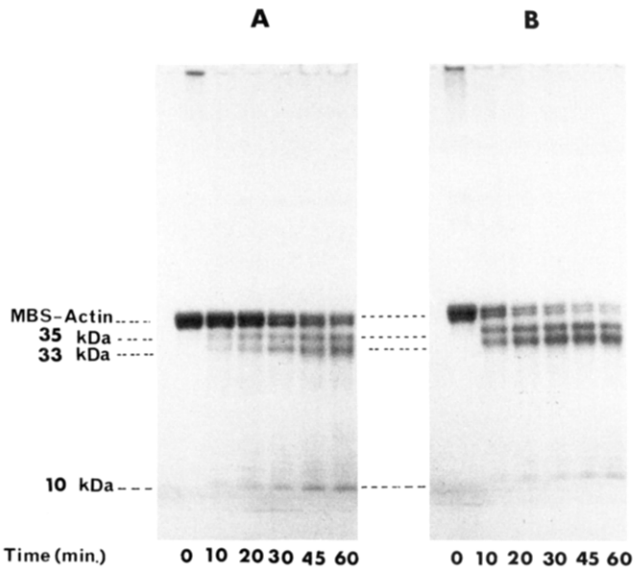


FIGURE 7: Time courses of the tryptic digestion of MBS-actin in the absence (A) and presence of 100 mM KCl (B). Conditions were as specified under Materials and Methods.

labeled MBS-actin and the isolated S-1(A1) and S-1(A2) isoenzymes. As shown in Figure 5A, the binding of S-1(A1 + A2) to the fluorescent MBS-actin in G-buffer (ionic strength 1.8 mM), at 25 °C, resulted in the enhancement of the intensity of the fluorescence emission of the pyrene moiety. Under the conditions we employed, the fluorescence increase produced by S-1 was nearly 40%. On the other hand, the fluorescence of pyrene-labeled MBS-actin was not affected by the addition of KCl to 100 mM. These features are in contrast with the S-1-induced quenching of the fluorescence observed with pyrene–F-actin and the sensitivity of pyrene–F-actin fluorescence to ionic strength (Kouyama & Mihashi, 1981; Criddle et al., 1985). They are consistent with the binding of S-1 to monomeric pyrene-labeled MBS-actin, which appears to be a species as resistant to polymerization by S-1 as the unlabeled actin derivative. Consequently, the pyrene probe can be employed to monitor the solution interaction of MBS-actin with S-1. Figure 5B shows the titration of pyrene–MBS-actin with S-1(A1) and S-1(A2). The estimated K_d values were 0.18 μM for S-1(A1) and 0.47 μM for S-1(A2). Raising the KCl concentration of the G-buffer to 100 mM increased, in both cases, the K_d value to $\geq$60 μM. This indicates that raising the ionic strength causes a major change in the affinity of MBS-actin for S-1. Support for this proposal is provided by the data presented in Figure 6, describing the limited tryptic digestion of S-1 associated with MBS-actin in the absence and presence of 100 mM KCl. Figure 6A shows that in the absence of salt and as we reported previously (Bettache et al., 1989), MBS-actin affords strong protection against the tryptic cleavage of the 27 kDa–50 kDa junction of the S-1 heavy chain, which was cut at the 50 kDa–20 kDa connector only and converted into two fragments: 75 kDa (NH_2 terminal) and 20 kDa (COOH terminal). When the proteolysis was done following the addition of 100 mM KCl, the protection effect of MBS-actin was abolished and the S-1 heavy chain was split at both junctions, giving rise to 27 kDa, 50 kDa, and 20 kDa (Figure 6B), essentially as when the digestion was performed in the absence of MBS-actin (Figure 6B). These findings are consistent with the salt-induced decrease of the binding of S-1 to pyrene-labeled MBS-actin and the formation of a less well packed complex between S-1 and MBS-actin that is more sensitive to proteolysis. Finally, we observed also that the increase of the ionic strength seems to generate a definite effect on the structure of the MBS-actin itself, which could be detected by a change in the susceptibility of the protein to trypsin. The experiments shown in Figure 7A,B compare the time courses of the limited tryptic digestion of MBS-actin in the absence and presence of 100 mM KCl, respectively. Like native G-actin, the modified protein was cut into a 35–33-kDa core and a 10-kDa fragment. The rate of the cleavage reaction was markedly accelerated by raising the ionic strength. Thus, in the time interval of 0–30 min and with added KCl, most of the protein underwent digestion, whereas very partial degradation took place in the absence of salt. Since the enzymatic activity of trypsin does not seem to be affected by the increase of the salt concentration up to 100 mM (Rich & Estes, 1976), it is likely that the observed change of the rate of MBS-actin degradation resulted from a localized conformational change promoted by KCl in the modified protein. This salt-specific alteration of the MBS-actin structure could contribute also to the observed decrease of its affinity for S-1.

DISCUSSION

The purpose of the present study was to detect additional structural or functional perturbations in actin that, besides the loss of its polymerizability, might be caused by its internal cross-linking with MBS. The nature and magnitude of the eventual changes would allow one to appreciate the extent to which the cross-linked protein could serve as a soluble analogue of G-actin in interaction studies with myosin subfragment-1.

The far-ultraviolet CD spectroscopy did not reveal a significant change in the overall conformation of the MBS-actin. The progressive unfolding of the polypeptide chain of native G-actin is known to be accompanied by a shift of the intrinsic fluorescence spectrum to longer wavelengths (Kuznetsova et al., 1988). The similarity of the intrinsic global fluorescence spectra of MBS-actin and G-actin before treatment with EDTA indicates that the chemical modification by MBS did not alter the native global structure of the actin molecule. Of course, one cannot exclude possible local conformational changes not detectable by the approaches employed. The maintenance of an intact high-affinity metal-binding site in MBS-actin was demonstrated not only by the nearly stoichiometric concentration of tightly bound Ca^{2+} measured in the protein derivative but also by the capacity of the derivative to inhibit the enzymatic activity of DNase I. Recently, indeed, it was shown that the loss of the actin-bound metal does not impair the association of the protein with DNase I but it abolishes DNase I inhibitor activity (Polzar et al., 1989).

Furthermore, lysine residues on actin, such as Lys-50, Lys-61, and Lys-68, have been implicated in the interaction of G-actin and DNase I as assessed by chemical cross-linking (Sutoh, 1984), and maleylation of a strategic lysine in actin was reported earlier to block its DNase I inhibitor activity (Ng & Burtnick, 1982). Although the reaction of MBS with actin caused the disappearance of six or seven lysine side chains (Bettache et al., 1989), none of the MBS-cross-linked lysines seems to be critical for the actin DNase I recognition.

The increase of the critical monomer concentration for actin polymerization by KCl and $MgCl_2$ was the only change so far observed in the properties of MBS-actin as compared to G-actin. Thus, the lysine–cysteine cross-linking with MBS has not suppressed the polymerization potency of G-actin. This indicates that the cross-linked residues are not essential for the establishement of the actin–actin contacts or the F-actin–S-1 interaction since the polymerized MBS-F-actin activated the S-1 ATPase as effectively as native F-actin. From a practical point of view, the use of subcritical concentrations of MBS-actin ($\leq$1.5 mg/mL) will permit any solution study to be conducted in the presence of salts without polymerization.

Recently S-1(A2) was found to bind tightly to native G-actin without inducing the polymerization of the protein, whereas the binding of S-1(A1) to G-actin was followed by its immediate conversion to F-actin (Chaussepied & Kasprzak, 1989a). This finding has revealed the involvement of the A-1 light chain in the S-1-promoted polymerization of G-actin. The present study, together with our previous investigation (Bettache et al., 1989), shows that, in contrast to G-actin, the MBS-actin derivative is resistant to polymerization by S-1-(A1), and by use of pyrene-labeled MBS-actin, the solution interaction of either S-1 isoform with monomeric actin could be quantitatively analyzed. It should be noted that cysteine-374, to which the pyrene is bound, is not essential for the production of the nonpolymerizable MBS-actin as assessed by the ability of trypsin-truncated actin (which lacks the last three C-terminal residues) to react with MBS as well as G-actin and to produce a nonpolymerizable derivative similar to that obtained with native G-actin (unpublished results). We assume therefore that the pyrene–MBS-G-actin molecule is not different from the MBS-G-actin species in its interaction with S-1. The K_d value determined by fluorescence intensity titration for the complex formation between S-1(A2) and pyrenyl–MBS-actin was essentially identical with that measured under similar ionic conditions by fluorescence anisotropy titration for the S-1(A2)–1,5-IAEDANS-labeled G-actin complex (Chaussepied & Kasprzak, 1989a). Thus, the MBS-catalyzed cross-linking has not significantly altered the strength of binding of G-actin to S-1. Moreover, the higher affinity of MBS-actin for S-1(A1) indicates that the G-actin derivative, like F-actin (Weeds & Taylor, 1975), is also sensitive to the contribution of the alkali light chain 1 in the binding process of S-1. Therefore, this sensitivity seems to be an intrinsic property of the actin monomer, most probably not dependent on the G–F conversion. In this regard, MBS-actin provides a useful tool for the study of the solution interaction between the G-actin and the alkali light chain 1. A particular feature of the interaction of MBS-actin with S-1 is its strong dependence on ionic strength. The nearly 1000-fold decrease of the affinity of MBS-actin for the S-1 isoenzymes in the presence of 100 mM KCl is much larger than the change of the affinity reported for the F-actin–S-1 complex when the salt concentration was varied in the range 60–500 mM (Greene, 1981; Marston, 1982; Criddle et al., 1985). These data suggest that electrostatic interactions may play a predominant role in the tight association of MBS-actin to S-1. This proposal is supported by the recent finding that the complexation of S-1 to native G-actin was abolished upon the binding of a negatively charged antipeptide to the positively charged stretch of residues 633–642 on the S-1 heavy chain (Chaussepied & Kasprzak, 1989b). In addition, the salt could influence directly the ability of MBS-actin to bind S-1 by promoting localized conformational transitions in the protein as suggested by the change in its susceptibility to trypsin. Earlier, KCl-induced conformational changes detectable by enzymatic proteolysis were shown to take place in native G-actin before the polymerization step (Rich & Estes, 1976). Thus, like G-actin, a structurally distinct species of MBS-actin seems to be generated in the presence of 100 mM KCl, which could display a weaker affinity of binding for S-1.

Finally, the use of the covalent MBS-actin–S-1 conjugate has revealed the ability of the actin monomer to interact simultaneously with S-1 and DNase I, suggesting that these two proteins do not share the same binding sites on the G-actin molecule. While the present study indicates, for the first time, the production of a triprotein complex with DNase I, G-actin, and S-1, the formation of another triprotein complex with DNase I–G-actin associated to the serum vitamin D binding protein was also recently reported (Mc Leod et al., 1989). The ternary complex generated by the binding of MBS-actin to both S-1 and DNase I further emphasizes the potential use of the actin derivative in developing the crystallography of actin-bound S-1.

Registry No. DNase, 9003-98-9; ATPase, 9000-83-3.

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^{51}V NMR Study of Vanadate Binding to Myosin and Its Subfragment 1[†]

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ABSTRACT: The binding of various forms of vanadate to myosin and myosin subfragment 1 (S-1) was studied by ^{51}V NMR at increasing vanadate concentrations between 0.06 and 1.0 mM. The distribution of the various forms of vanadate in the solution depended on the total concentration of vanadate. At low concentrations, the predominant vanadate form was monomeric, while at high concentration, it was tetrameric. The presence of myosin or S-1 in the solution produced a significant broadening of the signal of each form of vanadate, indicating that all of them bind to the proteins. Addition of ATP, which does not affect the ^{51}V NMR spectra in the absence of proteins, causes their significant alteration in the presence of myosin or S-1. The changes, which include the broadening of the signal of the monomeric and the narrowing of the signal of the oligomeric vanadate forms, indicate that more monomeric and less oligomeric vanadate binds to the proteins in the presence than in the absence of ATP. Irradiation by near-UV light in the presence of vanadate cleaves S-1 at three specific sites—at 23, 31, and 74 kDa from the N-terminus. The cleavages at 23 and 31 kDa are specifically inhibited by the addition of ATP. The vanadate-associated photocleavage of S-1 also depends on the total concentration of vanadate; it is observed only when the concentration of vanadate is at least 0.2 mM. This was also the lowest concentration at which oligomeric vanadate was detected in the ^{51}V NMR spectra. From the parallel concentration dependence of the photocleavage and the appearance of the tetrameric vanadate, it is concluded that photocleavage occurs only when tetrameric vanadate binds to S-1.

Myosin and actin are two major proteins of muscle whose interaction, coupled with the hydrolysis of ATP, is the molecular basis of contraction. The "head" segment, called subfragment 1 (S-1),[1] of myosin contains two distinct sites (Mueller & Perry, 1962) responsible for actin and nucleotide binding. The driving force of contraction arises as local deformations, forced by ATPase events at the nucleotide binding site, and is conducted through S-1 to the actin binding site, and there compels changes in the myosin–actin relationship (Botts et al., 1984, 1989). The binding of various intermediates of the ATPase cycle induces different structural changes and affects the affinity between actin and myosin. The occupancy of the actin binding site also causes structural changes and influences the lifetime of the intermediates of ATP hydrolysis. These results imply that there is a two-way communication between the nucleotide and the actin binding sites and that S-1 serves as a transducer in the process (Morales & Botts, 1979).

The mechanism of the myosin-catalyzed ATP hydrolysis, because of its functional significance, has been studied in great detail, and the intermediates of the hydrolysis have been described [for a review, see Taylor (1979)]. The predominant steady-state intermediate of the ATP cycle is the M·ADP·P_i complex (M denotes myosin) whose half-life is several seconds. The complex can be further stabilized by substituting phosphate (P_i) with vanadate (V_i), which increases the half-life of the complex to a couple of days (Goodno, 1979). This stable complex, which contains ADP and V_i in a "trapped" form (Okamoto et al., 1986), has proved useful in localizing the nucleotide binding site on S-1. Vanadate, which is considered to be a good analogue of phosphate (Lindquist et al., 1973), was used together with UV irradiation studies to detect the phosphate binding site(s) on S-1. Recently, it has been shown that the S-1 polypeptide chain is cleaved at 23 and 74 kDa from the N-terminus (Mocz, 1989; Cremo et al., 1988). The 23-kDa cleavage site is at Ser-180 (Cremo et al., 1989), and this serine residue participates in the "consensus" ATP binding site of myosin (Walker et al., 1982). The fragment containing

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[1] Abbreviations: S-1, subfragment 1 of myosin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LB, line broadening parameter for the exponential multiplication of free induction decay (FID).

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