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Interaction of Maleimidobenzoyl Actin with Myosin Subfragment 1 and Tropomyosin–Troponin†

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ABSTRACT: A chemical modification of G-actin with (*m*-maleimidobenzoyl)-*N*-hydroxysuccinimide ester (MBS) impairs actin polymerization [Bettache, N., Bertrand, R., & Kassab, R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6028–6032]. MBS-actin recovers the ability to polymerize when a 2-fold molar excess of phalloidin is added in 30 mM KCl/2 mM $MgCl_2$/20 mM Tris-HCl (pH 7.6). The resulting polymer (MBS–P-actin) is highly potentiated so that it activates the Mg^{2+}-ATPase of S1 more strongly than native F-actin. The affinity of MBS–P-actin for S1 in the presence of ATP (K_{ATPase}) is about four times higher than that of native F-actin, although the maximum velocity at infinite actin concentration (V_{max}) is almost the same. This high activation is not due to a cross-linking between MBS–P-actin and the S1 heavy chain, since no substantial amount of cross-linking was observed in SDS gel electrophoresis. Direct binding studies and ATPase measurements showed that the modification of actin with MBS impairs the binding of tropomyosin. Tropomyosin binding can be improved considerably by the addition of troponin. However, the regulation mechanism of the acto–S1 ATPase activity by troponin–tropomyosin is damaged. The addition of troponin–tropomyosin reduces the S1 ATPase activation by MBS–P-actin to the same level as that of native F-actin in 30 mM KCl/2.5 mM ATP/2 mM $MgCl_2$, but there is no difference in the ATPase activation in the presence and absence of Ca^{2+}. When the concentration of Mg^{2+} is increased to 5 mM, the regulation is partially restored. There is a significant difference in the ATPase activation in the presence and absence of Ca^{2+}. The properties of MBS–actin, except for its extremely high activation of S1 ATPase activity, are very similar to those of FITC-labeled actin in which Lys-61 is selectively labeled as previously reported [Miki, M. (1989) *J. Biochem. 106*, 651–655].

The interaction of actin and myosin is an essential process for muscle contraction. The presence of Mg^{2+} is required for the mechanochemical transduction of the ATP hydrolysis (Watanabe & Yasui, 1965; Weber & Murray, 1973). The ATPase activity of myosin in the presence of Mg^{2+} (Mg^{2+}-ATPase activity) is strongly depressed in comparison with the metal-free ATPase activity [(EDTA)K^+-ATPase activity]. This inhibition is released through the interaction of myosin with actin. Then, the complex of actin and myosin converts the chemical energy of the ATP hydrolysis to mechanical work performed in cell movements. In skeletal and cardiac muscle, this process is regulated by tropomyosin and troponin on the actin filament in response to a change from approximately 10^{-7} to 10^{-5} M in Ca^{2+} concentration (Ebashi et al., 1969). The regulation mechanism is closely related to the mechanism of force generation. Therefore, the localizations of the interaction sites of actin with myosin, tropomyosin, and troponin are important in elucidating the molecular mechanism of muscle contraction and regulation [for a review of localization of the interaction sites of actin, see Hambly et al. (1986)]. Chemical modifications of actin amino acids will provide useful information about its functional sites.

The modification of His-40 (Hegyi et al., 1974), Tyr-53 (Bender et al., 1976), Lys-61 (Burtnick, 1984), and Tyr-69 (Lehrer & Elzinga, 1972) impairs actin polymerization. The important functions of actin, such as myosin binding or tropomyosin binding, are closely related to its ability to polymerize. Therefore, it could not be determined whether the loss of the functions following the chemical modifications is simply because of the loss of polymerization or because of the loss of functional sites. Recently it has been shown that in the presence of the mushroom toxin phalloidin these chemically modified actins (except the Tyr-53-modified actin) recover the ability to polymerize to the same extent as native actin (Miki, 1987, 1988; Miki et al., 1987). The resulting polymers activate the S1[1] Mg^{2+}-ATPase activity as efficiently as native F-actin and we have concluded that residues His-40, Lys-61, and Tyr-69 are not directly involved in myosin binding (Miki, 1987, 1988, 1989; Miki et al., 1987). On the other hand, the modification of Lys-61 of actin impairs the regulatory mechanism of acto–S1 ATPase activity by troponin–tropomyosin, although the actin polymers bind tropomyosin or/and troponin (Miki, 1989). Recently, Bettache et al. (1989, 1990) reported that polymerization of actin induced by salt or S1 is suppressed by reaction of G-actin with (*m*-maleimidobenzoyl)-*N*-hydroxysuccinimide ester (MBS). The G-actin derivative, containing a few intramolecular cross-links (lysine–cysteine and lysine–lysine) and a free maleimide group, can be covalently cross-linked to the S1 heavy chain. However,

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[1] Abbreviations: S1, myosin subfragment 1; Tm, tropomyosin; Tn, troponin; MBS, (*m*-maleimidobenzoyl)-*N*-hydroxysuccinimide ester; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; 1,5-IAEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; FITC, fluorescein isothiocyanate; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; MBS–P-actin, MBS-modified actin that was polymerized on addition of a 2-fold molar excess of phalloidin.

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no activation of S1 Mg^{2+}-ATPase activity has been observed (Bettache et al., 1989). It is not clear whether the lack of the activation is due to the loss of polymerization or the loss of a functional site.

In the present studies, MBS–actin was polymerized in the presence of phalloidin and then the effects of MBS modification on the interaction of actin with S1, tropomyosin, and troponin were studied. The MBS–actin polymer shows different properties from normal F-actin. It stimulates the S1 ATPase activity more strongly than normal F-actin, and the regulation mechanism by troponin–tropomyosin is impaired. The results suggest that cross-linked residues of actin play an important role for the activation of the S1 ATPase activity and also for the regulation of the acto–S1 interaction by troponin–tropomyosin.

Materials and Methods

Reagents. (*m*-Maleimidobenzoyl)-*N*-hydroxysuccinimide ester (MBS) and BCA protein assay reagents were purchased from Pierce Chemicals. Phalloidin from *Amanita phalloides* and ATP were from Boehringer Mannheim Biochemica. $MgCl_2$ (4.9 M solution) and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) were purchased from Sigma Chemical. 1,5-IAEDANS was from Molecular Probes. All other reagents were analytical grade.

Protein Preparations. Actin, S1, tropomyosin, and troponin were prepared from rabbit skeletal muscle as previously reported (Miki, 1987). Protein concentrations were determined by use of absorption coefficients of $A^{1\%}_{290nm} = 6.3$ cm^{-1} for G-actin (Lehrer & Kerwar, 1972) and $A^{1\%}_{280nm} = 7.5$ cm^{-1} for S1 (Weeds & Pope, 1977), 3.3 cm^{-1} for tropomyosin (Cummins & Perry, 1973), and 4.5 cm^{-1} for troponin (Ishiwata & Fujime, 1972). The concentrations of labeled actin and tropomyosin were measured at 462 nm with BCA protein assay reagents with use of nonlabeled actin or tropomyosin as the standard. Relative molecular weights of 42 300 for actin, 115 000 for S1, 66 000 for tropomyosin, and 69 000 for troponin were used.

Labeling of Proteins. MBS–actin was prepared according to Bettache et al. (1989). G-actin (2 mg/mL) was mixed with a 20-fold molar excess of MBS (freshly prepared 50 mM solution in dimethylformamide) in 0.2 mM ATP/2 mM HEPES/0.1 mM $CaCl_2$ for 2 h at 20 °C, maintaining pH 8.0 with 0.1 M NaOH. The reaction was stopped by the addition of a 5-fold molar excess of DTT and glycine over MBS. Polymerized actin was removed through a cycle of polymerization in 0.1 M KCl/2 mM $MgCl_2$ for 1 h at room temperature followed by centrifugation at 100000*g* for 2 h. Most of the actin derivative (more than 80%) remained in the supernatant. The supernatant was dialyzed exhaustively against 0.15 mM ATP/2 mM HEPES (pH 8.0)/0.1 mM $CaCl_2$ by changing the outer solution three times. Then, the solution was clarified by ultracentrifugation at 100000*g* for 1 h. MBS-actin was lyophilized in 0.1 M sucrose and stored at −20 °C. Before experiments, lyophilized MBS–actin was dialyzed against 0.1 mM ATP/0.1 mM $CaCl_2$/1 mM Tris-HCl (pH 8.0) to remove sucrose and clarified by ultracentrifugation at 100000*g* for 1 h. IAEDANS-labeled tropomyosin was prepared as previously reported (Miki, 1990a; Lamkin et al., 1983). Tropomyosin was incubated in 10 mM DTT/50 mM Tris-HCl (pH 8.0) at 37 °C for 2 h and isolated by isoelectric precipitation at pH 4.5. The reduced tropomyosin (2 mg/mL) was reacted with a 20-fold molar excess of 1,5-IAEDANS in the dark for 24 h at 37 °C in 20 mM Tris-HCl (pH 8.0)/1 M KCl/1 mM EDTA. The reaction was quenched by the addition of 5 mM 2-mercaptoethanol, and labeled tropomyosin was precipitated in 60% saturated ammonium sulfate at 10000*g* for 20 min in order to remove free dye. The pellet was dissolved in 1 mM Tris-HCl (pH 8.0) and dialyzed exhaustively against the same buffer solution. The labeling ratio of IAEDANS to tropomyosin was determined to be 1.4 by use of the absorption coefficient of 6100 M^{-1} cm^{-1} for 1,5-IAEDANS (Hudson & Weber, 1973).

Spectroscopic Measurements. Absorption was measured with a Philips PU 8800 spectrophotometer. Fluorescence of IAEDANS-labeled tropomyosin was excited at 360 nm and measured at 480 nm with an SLM 8000 spectrofluorometer.

Binding Assays. The association of MBS–actin polymers with skeletal tropomyosin or IAEDANS-labeled tropomyosin was studied by cosedimentation at 180000*g* for 30 min in a Beckman airfuge. The amounts of bound tropomyosin and troponin were determined in a densitometric scan of an electrophoretogram by SDS–PAGE of pellets as previously reported (Miki, 1989) or by measurement of the fluorescence intensity of IAEDANS-labeled tropomyosin in the supernatant.

Other Methods. Viscosity was measured at 20 °C with an Ostwald viscometer having an outflow time of 58.9 s for water. ATPase activity was measured using a pH-stat consisting of a Radiometer TTT2 titrator with PHA943 titrigraph module, ABU12 autoburette, and SBR3 titrigraph assembly (Miki, 1989). Two millimolar potassium hydroxide was used as the titrant at 20 °C. SDS–PAGE (3% stacking gel and 7–18% gradient separation gel) was carried out according to Laemmli (1970). The densities of the protein bands were measured with a CAMAG electrophoresis scanner.

Results

Polymerization of MBS–Actin. The modification of actin with MBS strongly impaired the ability to polymerize (Bettache et al., 1989). The viscosity of MBS–actin (0.8 mg/mL) did not show any significant increase even after 24 h of incubation in 30 mM KCl/2 mM $MgCl_2$/1 mM azide/0.1 mM ATP/20 mM Tris-HCl (pH 7.6) (buffer F) at 20 °C, although the viscosity of native G-actin increased to reach a plateau within 5 min under the same conditions. However, the addition of phalloidin induced the polymerization of MBS–actin. The effect of phalloidin saturated at a 2-fold molar excess over actin. Therefore, unless especially mentioned, MBS–actin monomers were polymerized to the MBS–actin polymers (MBS–P-actin) in the presence of a 2-fold molar excess of phalloidin. Figure 1 shows the time course of the viscosity change of MBS–actin in buffer F after addition of phalloidin. In 5 mM $MgCl_2$/20 mM Tris-HCl (pH 7.6)/1 mM azide/0.1 mM ATP where no KCl was present, MBS–actin was also polymerized by the addition of phalloidin with a slightly slower rate than that in buffer F. In the absence of Mg^{2+}, the rate of polymerization was extremely slow (Figure 1). After 1 day of incubation at room temperature in 0.1 M KCl/20 mM Tris-HCl (pH 7.6)/1 mM azide/0.1 mM ATP, the viscosity of MBS–actin (1 mg/mL) increased only to 0.51. However, after 4 days of incubation, the viscosity increased to the same level as that in buffer F. Mg^{2+} is very effective but not indispensable for the recovery of polymerization in the presence of phalloidin.

The viscosities of MBS–actin at various actin concentrations were measured in the presence and absence of phalloidin. The sample solutions were incubated in buffer F for 4 days at 4 °C prior to measurement of the viscosity. In the absence of phalloidin, MBS–actin showed little increase in viscosity. The critical concentration was about 0.1 mg/mL, and the reduced viscosity was 0.1 mL/mg. On the other hand, in the presence

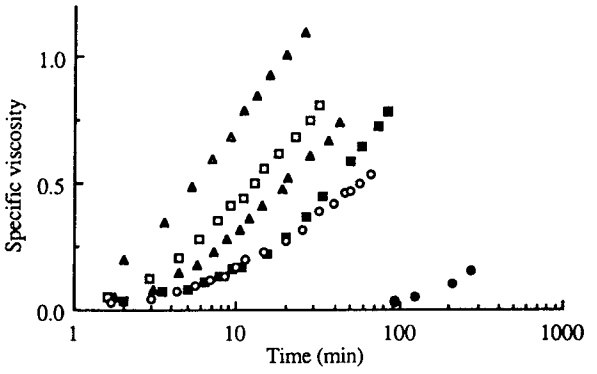

FIGURE 1: The time course of MBS–actin polymerization after addition of a 2-fold molar excess of phalloidin at various actin concentrations and various solvent conditions. A 1 mg/mL sample of MBS–actin was polymerized in 0.1 mM ATP/20 mM Tris-HCl (pH 7.6)/0.1 M KCl (●) or 5 mM $MgCl_2$ (○) or 30 mM KCl and 2 mM $MgCl_2$ (buffer F) (▲). A total of 1.5 mg/mL (△), 1.2 mg/mL (□), or 0.8 mg/mL (■) of MBS-actin was polymerized in buffer F. Temperature was 23.5 °C.

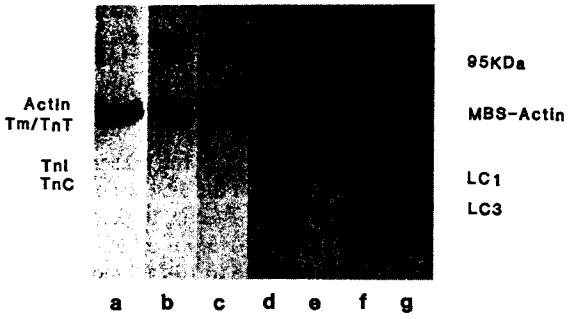

FIGURE 2: SDS–PAGE of MBS–P-actin (a), S1 (f), the complex of S1 and MBS–P-actin after 1 day of incubation in 30 mM KCl/20 mM Tris-HCl (pH 8.0)/2 mM $MgCl_2$ (g) and the sedimented pellets of MBS–P-actin and tropomyosin (b) or troponin–tropomyosin (c) and of native F-actin and tropomyosin (d) or troponin–tropomyosin (e) after centrifugation with a Beckman airfuge at 160000*g* for 30 min in 30 mM KCl/20 mM Tris-HCl (pH 7.6)/5 mM $MgCl_2$.

of phalloidin, the critical concentration was almost zero and the reduced viscosity was 1.3 mL/mg.

According to Bettache et al. (1989), MBS–G-actin suffers intramolecular cross-linking and has one free maleimide group, which can be cross-linked to the S1 heavy chain. Therefore, after polymerization of MBS–actin in the presence of phalloidin, SDS–PAGE was performed to test the possibility of intermolecular cross-linking in the MBS–actin polymers. In Figure 2 (lane a), the 42-kDa position comprised doublet bands. This feature is a characteristic of intramolecularly cross-linked monomeric actin (Lehrer, 1981; Sutoh, 1984; Bettache et al., 1989). At higher molecular size positions, only faint bands were observed. The results suggest that most of the MBS–actin protomers (87% from densitometric scan) in the MBS–actin polymers are not intermolecularly cross-linked.

S1 ATPase Activation of MBS–Actin. The effect of *MBS–actin polymers (MBS–P-actin) on the actin-activated* S1 Mg^{2+}-ATPase activity was measured in 30 mM KCl/2.5 mM ATP/1 mM Tris-HCl/2 mM $MgCl_2$ (buffer A2) or 5 mM $MgCl_2$ (buffer A5) at pH 8.0. The concentration of S1 was 0.05 mg/mL. MBS–actin in the presence of a 2-fold molar excess of phalloidin and native G-actin (2–3 mg/mL) was incubated in 30 mM KCl/2 or 5 mM $MgCl_2$/1 mM azide/1 mM Tris-HCl (pH 8.0) for more than 24 h prior to measurement of the ATPase activity. Figure 3 shows the double-reciprocal plot of the ATPase activity of S1 (in moles of ATP per moles of S1 per second) vs the concentration of

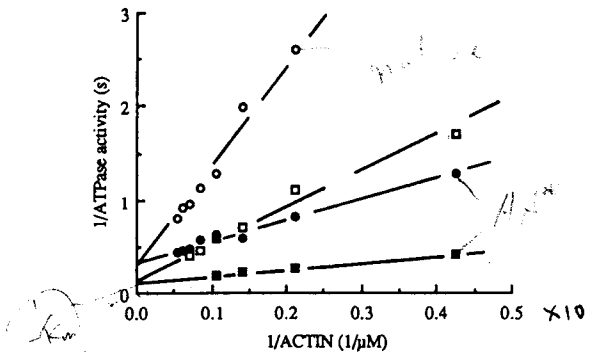

FIGURE 3: Double-reciprocal plot of the ATPase activity of S1 vs actin concentration for native F-actin (○, □) and MBS–P-actin (●, ■). The S1 concentration was 0.05 mg/mL in 30 mM KCl/1 mM Tris-HCl (pH 8.0)/2.5 mM ATP/2 mM (□, ■) or 5 mM (○, ●) $MgCl_2$ at 20 °C.

MBS–P-actin or native F-actin. K_{ATPase} and V_{max} of native F-actin in buffer A2 were 2.3×10^4 M^{-1} and 15 s^{-1}, respectively, which are almost same as the values reported previously under similar solvent conditions (Murray et al., 1980; Okamoto & Sekine, 1987; Chausspied & Morales, 1988). The actin-activated S1 Mg^{2+}-ATPase activity in 2 mM $MgCl_2$ was higher than that in 5 mM $MgCl_2$. K_{ATPase} of native F-actin in 5 mM $MgCl_2$ was almost the same as that in 2 mM $MgCl_2$, but V_{max} in 5 mM $MgCl_2$ was smaller than that in 2 mM $MgCl_2$. On the other hand, the activation of S1 ATPase by MBS–P-actin was much higher than that by native F-actin. K_{ATPase} of MBS–P-actin was 4 ± 1 times higher than that of native F-actin, while the V_{max} was not strongly affected in either buffer A2 or buffer A5.

The S1 ATPase activation by native F-actin was slightly decreased (about 10%) by the addition of phalloidin. The high activation of S1 Mg^{2+}-ATPase by MBS–P-actin may be due to cross-linking between MBS–actin and S1, since MBS–G-actin has a free maleimide group and can be cross-linked to the S1 heavy chain (Bettache et al., 1989). In order to test this possibility, MBS–P-actin (1 mg/mL) was mixed with an equimolar concentration of S1 (2.86 mg/mL) and incubated for 1 day. Then, the mixture was applied to SDS–PAGE. Figure 2 (lanes f and g) shows that no cross-linking occurred between MBS–P-actin and the S1 heavy chain. Furthermore, the S1 ATPase activation by MBS–P-actin was measured in the presence and absence of 1 mM DTT, but no difference was observed. The results indicate that the high activation of MBS–P-actin is not due to cross-linking between actin and S1.

Interaction of MBS–P-Actin with Tropomyosin and Troponin. The ability of MBS–P-actin to bind regulatory proteins was studied by centrifugation. MBS–P-actin or F-actin (1 mg/mL) was mixed with tropomyosin or troponin–tropomyosin in 30 mM KCl/20 mM Tris-HCl (pH 7.6)/5 mM $MgCl_2$. The weight ratio of actin, tropomyosin, and troponin was 4:1:1. The sample solutions were centrifuged at 160000*g* *for 30 min in a Beckman airfuge. The pellets were washed* with water and suspended in a 1% SDS/0.5% 2-mercaptoethanol solution incubated overnight at room temperature and homogenized on the following day. Equal portions of pellets were submitted to SDS–PAGE (Figure 2, lanes b–e). The densitometric scan measurements showed that the ratio of bound tropomyosin to MBS–P-actin and to native F-actin was 47 ± 20% and that the ratio of bound tropomyosin–troponin-T to MBS–P-actin and to native F-actin was 70 ± 4%. The affinity of tropomyosin and troponin for MBS–P-actin was weaker than that for native F-actin. Figure 2 (lanes b and

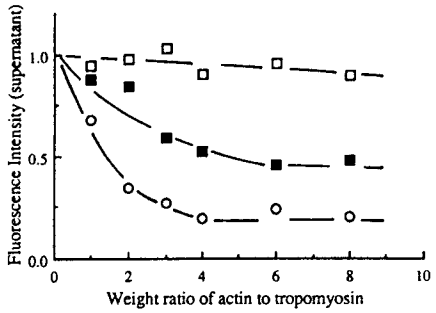

FIGURE 4: Binding of IAEDANS-labeled tropomyosin to MBS–P-actin in the absence (□) or presence (■) of troponin and to native F-actin without troponin (○). IAEDANS-labeled tropomyosin (0.25 mg/mL) was incubated at 20 °C with varying concentrations of actin in 30 mM KCl/20 mM Tris-HCl (pH 7.6)/5 mM $MgCl_2$. The samples were centrifuged with a Beckman airfuge at 160000*g* for 30 min, and the fluorescence intensities of the supernatants were measured at 480 nm. The excitation wavelength was 360 nm.

c) also shows that no substantial amount of tropomyosin or troponin was cross-linked to MBS–P-actin.

The separation of the bands of tropomyosin and MBS–actin in the densitometric scan was not good enough to get a precise value for the ratio of tropomyosin and actin. Therefore, in order to obtain more quantitative data, the binding of tropomyosin was further studied using IAEDANS-labeled tropomyosin under the same solvent conditions. IAEDANS-labeled tropomyosin (0.25 mg/mL) was mixed with various amounts of native F-actin or MBS–P-actin. The sample solutions were centrifuged at 160000*g* for 30 min with a Beckman airfuge. The fluorescence intensities of the supernatants were measured at 480 nm (Figure 4). The excitation wavelength was 360 nm. The results indicate that IAEDANS-labeled tropomyosin did not bind to MBS–P-actin, while it bound to native F-actin, and that the tropomyosin binding was improved considerably by the addition of troponin.

Regulation of the MBS–Acto–S1 ATPase by Tropomyosin and Troponin. The effects of tropomyosin or troponin–tropomyosin on the S1 ATPase activation by MBS–P-actin were measured in buffer A2 or buffer A5 in the presence (0.05 mM $CaCl_2$) and absence (1 mM EGTA) of Ca^{2+}. The concentrations of S1 and actin were 0.05 and 0.2 mg/mL, respectively. The molar ratio of troponin, tropomyosin, and actin in regulated actin was 1:1:7 (the physiological ratio). In buffer A2, where 2 mM $MgCl_2$ was present, tropomyosin alone did not inhibit the actin-activated S1 Mg^{2+}-ATPase activity considerably in either native F-actin or MBS–P-actin. In the case of regulated native F-actin (i.e., in the presence of troponin and tropomyosin), a strong inhibition of the ATPase activity was observed in the absence of Ca^{2+} (89%) but no inhibition was observed in the presence of Ca^{2+}. The addition of more troponin–tropomyosin did not affect the regulation of the ATPase activity of regulated native F-actin. On the other hand, in the case of regulated MBS–P-actin, the ATPase activity was always inhibited whether Ca^{2+} was present or not (53%). The addition of more troponin–tropomyosin (up to 3-fold more than the physiological ratio) decreased the ATPase activity by 75%, which was almost the same level as that by native F-actin, but no difference between the presence and absence of Ca^{2+} was observed (Figure 5A).

In buffer A5, where 5 mM $MgCl_2$ was present, tropomyosin alone strongly inhibited the actin-activated S1 Mg^{2+}-ATPase activity by 53% in the case of native F-actin and this inhibition was partially released by the addition of troponin in the presence of Ca^{2+} or further strengthened in the absence of Ca^{2+}. The addition of more troponin–tropomyosin did not affect significantly the regulation of the ATPase activity (Figure 5B). In the case of native F-actin and phalloidin, tropomyosin alone also strongly inhibited the actin-activated S1 Mg^{2+}-ATPase activity by 52% and this inhibition was released by the addition of troponin in the presence of Ca^{2+} or further strengthened (by 71%) in the absence of Ca^{2+}. On the other hand, in the case of MBS–P-actin, tropomyosin alone did not inhibit the S1 ATPase activation by MBS–P-actin. Further addition of tropomyosin up to 10-fold more than the physiological ratio did not affect the ATPase activation. Troponin–tropomyosin reduced the MBS–acto–S1 ATPase activity by about 30% in the presence of Ca^{2+} and by about 40% in the absence of Ca^{2+}. The addition of more troponin–tropomyosin (up to 3-fold more than the physiological ratio) decreased the MBS–acto–S1 ATPase activity by 50% in the presence of Ca^{2+} and by 83% in the absence of Ca^{2+} (Figure 5B). The results indicate that in the presence of 5 mM $MgCl_2$ the regulation mechanism was partially restored.

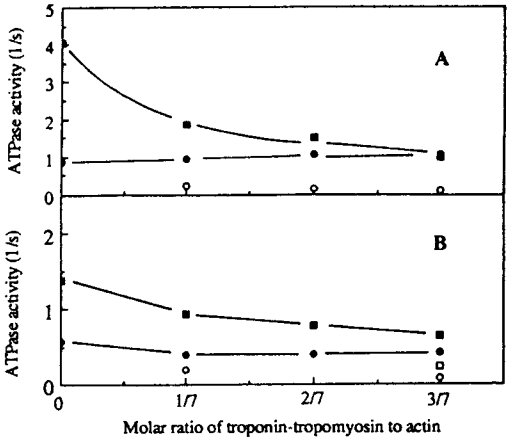

FIGURE 5: The effects of troponin–tropomyosin on the S1 ATPase activation by MBS–P-actin (■, □) and native F-actin (●, ○) in the presence (■, ●) and absence (□, ○) of Ca^{2+} in 30 mM KCl/1 mM Tris-HCl (pH 8.0)/2.5 mM ATP/2 mM (A) or 5 mM (B) $MgCl_2$. The concentrations of S1 and actin were 0.05 and 0.2 mg/mL, respectively.

DISCUSSION

Modification of Actin with MBS. The polymerization of G-actin is strongly impaired by reaction with MBS. Neither salt nor S1 induces the polymerization. Six to seven lysines and three to four cysteines in 1 mol of actin are modified with MBS (Bettache et al., 1989). However, the modified amino acids have not been identified on the primary structure. One mole of rabbit skeletal muscle actin contains five cysteines and 19 lysines (Vandekerckhove & Weber, 1979). Three cysteines are accessible to solvent (Lusty & Fasold, 1969; Kabsch et al., 1990) and can be modified with 2,2′-dicarboxy-4-(iodoacetamido)azobenzene, while the modified actin can still polymerize (Lusty & Fasold, 1969). Among the lysines in actin, Lys-61, Lys-113, and Lys-336 are the most reactive (Lu & Szilagyi, 1981). FITC reacts specifically with Lys-61 in G-actin and causes the loss of actin polymerization induced by salt or S1 (Burtnick, 1984; Miki, 1987, 1988). The atomic model of the actin filament has shown that Lys-61 and Lys-113 are located near contact regions between monomers in the filament (Holmes et al., 1990). Therefore, the loss of polymerization of actin by reaction with MBS is probably due to the modification of Lys-61 and Lys-113. However, both FITC-labeled actin (Miki, 1987) and MBS–actin recover the ability to polymerize by the addition of phalloidin, indicating that Lys-61 and Lys-113 may not directly be involved in the

contact regions. It should be noted here that the His-40-, Lys-61-, and Tyr-69-modified actins recover the ability to polymerize upon addition of phalloidin (Miki, 1987, 1988; Miki et al., 1987) but that the Tyr-53-modified actin does not recover the ability even upon the addition of phalloidin (Miki et al., 1987), whereas Tyr-53 is not itself directly part of the actin–actin interface in the atomic model of the actin filament (Holmes et al., 1990).

Polymerization of MBS–Actin. The kinetics of actin polymerization has been extensively studied by Oosawa and his colleagues (Oosawa & Asakura, 1975). According to the theory of Oosawa (Oosawa & Asakura, 1975), the half-polymerization time is proportional to $C_0^{-i_0/2}$, where C_0 is the initial concentration of G-actin, and nuclei are formed spontaneously from simple i_0-mers. They concluded that the nucleus is a helical trimer and the nucleus formation is the rate-limiting step for actin polymerization (Oosawa & Asakura, 1975). On the other hand, with use of the data in Figure 1, i_0 was calculated to be 2.9 ± 0.5. The result indicates that the nucleus of MBS–actin is also a trimer. In the absence of phalloidin, MBS–actin showed little increase (less than 10% of that in the presence of phalloidin) in the viscosity and the critical concentration was about 0.1 mg/mL in the present study. Recently, Bettache et al. (1990) reported that the critical concentration of MBS–actin is 1.8 mg/mL in 0.1 M KCl/5 mM $MgCl_2$/2 mM Hepes/0.1 mM ATP/0.1 mM $CaCl_2$/0.1 mM NaN_3 at pH 8.0. They measured the viscosity after 4 h of incubation in polymerization buffer solution, and we measured after 4 days of incubation. The rate of polymerization depends on the actin concentration, and the polymerization rate of MBS–actin is very slow even in the presence of phalloidin (Figure 1). The discrepancy between their result and ours in the critical concentration of MBS–actin may be due to a difference in the incubation time after addition of salts. The critical concentration is proportional to the ratio of the depolymerization rate to the polymerization rate (Oosawa & Asakura, 1975). In the present results, the critical concentration of MBS–actin is not so much different from that of native actin. Therefore, the nucleation rate may be strongly affected by reaction with MBS, and consequently, the ability to polymerize is impaired. Phalloidin does not bind to G-actin (Wieland et al., 1975; Estes et al., 1981; Miki et al., 1987). In the presence of salt, MBS–actin forms a trimer but it is very unstable as a nucleus. Phalloidin binds to the trimer and stabilizes it. Phalloidin also markedly reduces the rate of depolymerization, as was demonstrated by the lack of depolymerization of phalloidin-treated actin polymers (Wieland et al., 1975). Thus, MBS–actin recovers the ability to polymerize in the presence of phalloidin. This conclusion is in good agreement with the report by Estes et al. (1981).

S1 ATPase Activation. S1 does not induce polymerization of FITC–actin (Miki, 1989) or MBS–actin (Bettache et al., 1989). Moreover, the MBS–actin monomer did not activate the S1 ATPase activity even when it was cross-linked to the S1 heavy chain through the free maleimido group of MBS attached to actin (Bettache et al., 1989) or by treatment with EDC (data not shown). On the other hand, phalloidin-induced MBS–actin polymers strongly activate the S1 ATPase activity. The results indicate that the S1 ATPase activity is activated only by polymerized actin. During polymerization, a conformational change occurs in actin [see review, Oosawa (1983)]. However, it is still not clear whether one F-monomer in the polymers is enough for the S1 ATPase activation or if cooperation of neighboring F-monomers is required. Bettache et al. (1990) polymerized MBS–actin at high actin concentrations and isolated MBS–F-actin by centrifuge. They showed that MBS–F-actin activates the S1 ATPase activity to the same extent as native F-actin. However, MBS–F-actin formed in the absence of phalloidin is very unstable, and during the ATPase measurements, a large part of MBS–F-actin may depolymerize again to reach an equilibrium under their solvent conditions. Therefore, it is difficult to measure the S1 ATPase activation by MBS–actin as a function of the extent of polymerized actin. In the present experiments, MBS–actin was polymerized in the presence of phalloidin while the critical concentration was almost zero. This enabled us to measure the S1 ATPase activation by the MBS–actin polymer in comparison with the native actin polymer.

Recently, Bertrand et al. (1989) modified the N-terminal acidic region of residues 1–7 of actin with *N*-(5-sulfo-1-naphthyl)ethylenediamine (EDANS) in the presence of the carboxyl group activator, EDC. The isolated EDANS-actin polymerizes with a significantly greater rate than native actin upon addition of salt, but the resulting polymers do not activate the S1 ATPase activity significantly (the affinity of EDANS–actin for S1 in the presence of ATP is about one-twentieth of that of native F-actin). On the contrary, the modification of basic residues, lysines, of actin with MBS impairs the ability of polymerization but after the recovery of polymerization upon addition of phalloidin the resulting polymers activate the S1 ATPase activity more strongly than native F-actin. Mornet et al. (1981) first demonstrated that EDC-cross-linked acto–S1 shows an extremely high ATPase activity at a rate comparable to the maximal actin-activated ATPase rate of S1 (V_{max}). However, the high activation of the S1 ATPase activity by MBS–P-actin is not due to a cross-linking between actin and the S1 heavy chain. The results suggest that the acidic and basic residues of actin play an important role in activating the S1 ATPase activity.

Regulation of the MBS–Acto–S1 ATPase by Tropomyosin and Troponin. In a previous paper (Miki, 1989), we reported that Lys-61 is located in a region that is closely related to the regulation of the actin–myosin interaction by troponin–tropomyosin. The present paper shows that the modification of actin with MBS also impairs the interaction with tropomyosin. There was a small discrepancy in the extent of bound tropomyosin between from the densitometric scan of SDS–PAGE and fluorescence intensity measurements of supernatant. The affinity of IAEDANS-labeled tropomyosin for actin may be slightly weaker than that of native tropomyosin. However, both methods clearly showed that the modification of actin with MBS did result in impaired tropomyosin binding to actin. In fact, the S1 ATPase activation by MBS–P-actin was not inhibited even by the addition of a large excess amount of tropomyosin (up to 10 times excess over the physiological ratio) under conditions in which the physiological amount of tropomyosin inhibits the ATPase activity by 53%. The binding ability of tropomyosin to MBS–P-actin was restored by the addition of troponin. The binding of troponin–tropomyosin reduced the extra-high S1 ATPase activation by MBS–P-actin to the same extent as that by native F-actin whether Ca^{2+} is present or not in the presence of 2 mM $MgCl_2$ (Figure 5). The Ca^{2+} sensitivity in regulated MBS–P-actin was restored by increasing the Mg^{2+} concentration up to 5 mM (Figure 5). These results are very similar to the case of the interaction of actin labeled with FITC at Lys-61 (FITC–P-actin) with tropomyosin and troponin–tropomyosin except that the S1 ATPase activation by MBS–P-actin is much higher than that by FITC–P-actin. At the present, the effect of Mg^{2+} on the regulation of MBS–P-actin and FITC–P-actin cannot be ex-

plained easily. However, the present results showed that Mg^{2+} is important not only for the force-generating step but also for the regulatory step. Both steps are closely related to each other.

Potentiated State of Actin. Bremel et al. (1972) first demonstrated that at high molar ratios of S1 to actin, the ATPase activity of regulated acto–S1 is higher than that of acto–S1 in the absence of troponin–tropomyosin. The regulated actin can also be fully potentiated by adding *N*-ethylmaleimide-treated S1 (2 to 5 mol ratio of S1 to actin) (Greene et al., 1987). The K_{ATPase} of the fully potentiated regulated actin is 2.5–4.2 times higher than that of unregulated actin, while V_{max} is almost the same under the same solvent conditions (Williams et al., 1988). The states of regulated thin filament have been extensively studied (Bremel et al., 1972; Murray et al., 1982; Lehrer & Morris, 1982; Chalovich et al., 1983; Greene et al., 1987; Williams et al., 1988). However, the structural nature of the difference between these postulated states of the thin filament has not yet been established. The chemical modification of actin at Lys-238 (El-Saleh et al., 1984) or the replacement of bound ADP with adenylyl imidodiphosphate (AMPPNP) in actin (Miki, 1990b) also potentiates regulated actin. Troponin and tropomyosin are required for potentiating these actin filaments. On the other hand, even in the absence of troponin–tropomyosin, the modification by MBS in combination with phalloidin highly potentiates the actin filaments. The affinity of MBS–P-actin for S1 in the presence of ATP is about 4 times higher than that of native F-actin, while V_{max} does not change by the MBS modification. The third most reactive Lys-336 may be responsible for this potentiation, since both the extra phosphate of AMPPNP and Lys-336 are located near the cleft region between two domains of actin (Kabsch et al., 1990). More studies about the effects of chemical modifications of actin amino acids on its function will provide useful information for elucidating the molecular mechanism of muscle contraction and regulation.

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Registry No. ATPase, 9000-83-3; Ca, 7440-70-2; Mg, 7439-95-4; phalloidin, 17466-45-4.

REFERENCES

Bender, N., Fasold, H., Kenmoku, A., Middelhoff, G., & Volk, K. E. (1976) *Eur. J. Biochem. 64*, 215–218.

Bertrand, R., Chaussepied, P., Ausemard, E., & Kassab, R. (1989) *Eur. J. Biochem. 181*, 747–754.

Bettache, N., Bertrand, R., & Kassab, R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6028–6032.

Bettache, N., Bertrand, R., & Kassab, R. (1990) *Biochemistry 29*, 9085–9091.

Bremel, R. D., Murray, J. M., & Weber, A. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 267–275.

Burtnick, L. D. (1984) *Biochim. Biophys. Acta 791*, 57–62.

Chalovich, J. M., Greene, L. E., & Eisenberg, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4909–4913.

Chaussepied, P., & Morales, M. F. (1988) *Proc. Natl. Sci. U.S.A. 85*, 7471–7475.

Cummins, P., & Perry, S. V. (1973) *Biochem. J. 133*, 765–777.

Ebashi, S., Endo, M., & Ohtsuki, I. (1969) *Q. Rev. Biophys. 2*, 351–384.

El-Saleh, S. C., Thieret, R., Johnson, P., & Potter, J. D. (1984) *J. Biol. Chem. 259*, 11014–11021.

Estes, J. E., Selden, L., & Gershman, L. (1981) *Biochemistry 20*, 708–712.

Greene, L. E., Williams, D. L., & Eisenberg, E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3102–3106.

Hambly, B. D., Barden, J. A., Miki, M., & dos Remedios, C. G. (1986) *BioEssays 4*, 124–128.

Hegyi, G., Premecz, G., Sain, B., & Muhlrad, A. (1974) *Eur. J. Biochem. 44*, 7–12.

Holmes, K. C., Popp, D., Gebhard, W., & Kabsch, W. (1990) *Nature (London) 347*, 44–49.

Hudson, E. N., & Weber, G. (1973) *Biochemistry 12*, 4154–4161.

Ishiwata, S., & Fujime, S. (1972) *J. Mol. Biol. 68*, 511–522.

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) *Nature (London) 347*, 37–44.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lamkin, M., Tao, T., & Lehrer, S. S. (1983) *Biochemistry 22*, 3053–3058.

Lehrer, S. S. (1981) *J. Cell Biol. 90*, 459–466.

Lehrer, S. S., & Elzinga, M. (1972) *Fed. Proc. 31*, 502.

Lehrer, S. S., & Kerwar, G. (1972) *Biochemistry 11*, 1211–1217.

Lehrer, S. S., & Morris, E. P. (1982) *J. Biol. Chem. 257*, 8073–8080.

Lu, R. C., & Szilagyi, L. (1981) *Biochemistry 20*, 5914–5919.

Lusty, C. J., & Fasold, H. (1969) *Biochemistry 8*, 2933–2939.

Miki, M. (1987) *Eur. J. Biochem. 164*, 229–235.

Miki, M. (1988) *J. Biochem. (Tokyo) 104*, 312–315.

Miki, M. (1989) *J. Biochem. (Tokyo) 106*, 651–655.

Miki, M. (1990a) *Eur. J. Biochem. 187*, 155–162.

Miki, M. (1990b) *J. Biochem. (Tokyo) 108*, 457–461.

Miki, M., Barden, J. A., dos Remedios, C. G., Philips, L., & Hambly, B. D. (1987) *Eur. J. Biochem. 165*, 125–130.

Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306.

Murray, J. M., Knox, M. K., Trueblood, C. E., & Weber, A. (1980) *FEBS Lett. 114*, 169–173.

Murray, J. M., Knox, M. K., Trueblood, C. E., & Weber, A. (1982) *Biochemistry 21*, 906–915.

Okamoto, Y., & Sekine, T. (1987) *J. Biol. Chem. 262*, 7951–7954.

Oosawa, F. (1983) in *Muscle and Nonmuscle Motility*, (Stracher, A., Ed.) Vol. 1, pp 151–216, Academic Press, New York.

Oosawa, F., & Asakura, S. (1975) in *Thermodynamics of the Polymerization of Protein* (*Molecular Biology, An International Series of Monographs and Textbooks*) (Horecker, B., Kaplan, N. C., Marumur, J., & Scheraga, H. A., Eds.) Academic Press, New York.

Sutoh, K. (1984) *Biochemistry 23*, 1942–1946.

Vandekerckhove, J., & Weber, K. (1979) *Differentiation 14*, 123–133.

Watanabe, S., & Yasui, T. (1965) *J. Biol. Chem. 240*, 105–111.

Weber, A., & Murray, J. M. (1973) *Physiol. Rev. 58*, 612–673.

Weeds, A. G., & Pope, B. (1977) *J. Mol. Biol. 111*, 129–157.

Wieland, T., de Vries, J. X., Schäfer, A. J., & Faulstich, H. (1975) *FEBS Lett. 54*, 73–75.

Williams, D., Green, L. E., & Eisenberg, E. (1988) *Biochemistry 27*, 6987–6993.