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Maleimidobenzoyl Actin: Its Biochemical Properties and *In Vitro* Motility[1]

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Muscle G-actin treated with the hetero-bifunctional cross-linking reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), denoted as MBS-G-actin, is not induced to polymerize into F-actin by salt and myosin subfragment 1 [Bettache, N., Bertrand, R., and Kassab, R. (1989) *Proc. Natl. Acad. Sci. USA* 86, 6028-6032]. However, the addition of salt and phalloidin together allowed MBS-G-actin to polymerize and the resulting polymer (P-MBS-G-actin) could activate the Mg^{2+}-ATPase of S-1 [Miki, M. and Hozumi, T. (1991) *Biochemistry* 30, 5625-5630]. When F-actin was treated with MBS (MBS-F-actin), unlike MBS-G-actin, intercross-links between monomers in F-actin occurred. The MBS-F-actin could activate the Mg^{2+}-ATPase of heavy meromyosin (HMM): its maximum turnover rate, V_{max}, was almost the same as that of native F-actin, but the affinity of HMM for MBS-F-actin in the presence of ATP, K_m, was about 3 times higher. Electron microscopy showed that both P-MBS-G-actin and MBS-F-actin had the double stranded structures of F-actin and formed the arrowhead structures when combined with HMM. By *in vitro* motility assay, the sliding velocities of P-MBS-G-actin and MBS-F-actin were found to be slightly slower than that of native F-actin. But the critical concentration of KCl, over which the sliding movement was not observed, for MBS-modified actins was considerably higher than for native F-actin. When MBS-modified actins were regulated by tropomyosin-troponin complex, they were less sensitive to the Ca^{2+} concentration for HMM ATPase activation and sliding movement. These results showed that the modification of some of the lysine residues in the actin molecule leads to change in the biochemical properties of F-actin.

Key words: actin-actin interface, actin-myosin interaction, Ca^{2+}-regulation, hetero-bifunctional reagent, *in vitro* motility assay.

The cyclic interaction of the myosin subfragment 1 (S-1) moieties protruding from the thick filament with the actin subunits forming the thin filament is essential for muscle contraction. The mechanical force is generated at the S-1-actin interface and is coupled to the Mg^{2+}-ATP hydrolysis catalyzed by the myosin-actin complex.

In order to clarify the solution interaction between S-1 and monomeric actin, Bettache *et al.* (*1, 2*) designed a new G-actin derivative, referred to as maleimidobenzoyl-G-actin (MBS-G-actin), which was prepared by treating G-actin with the hetero-bifunctional reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) which is lysine-cysteine or lysine-lysine cross-linker. The treatment with MBS led to an inhibition of polymerization of G-actin induced by salts or S-1. MBS-G-actin formed a stable, soluble, and reversible unitary complex with S-1 (*1*). Most of their results have been confirmed by Arata (*3*) who conducted similar lines of investigation. Recently, we described the ability of MBS-G-actin to polymerize in the presence of salt and phalloidin, and analyzed the activation of S-1 ATPase by the polymerized MBS-G-actin with phalloidin (P-MBS-G-actin) as well as the regulation of the P-MBS-G-actin-S-1 complex by the tropomyosin-troponin system. It has also been found that the S-1 Mg^{2+}-ATPase activity was stimulated more strongly by P-MBS-G-actin than native F-actin, and the Ca^{2+}-sensitivity of P-MBS-G-actin combined with tropomyosin-troponin was impaired (*4*).

In the present studies, MBS has been used to modify F-actin. Among the 19 lysine residues (positively charged residues) in G-actin (*5*), Lys-61 has been shown to be the main residue labeled by fluorescein isothiocyanate (FITC). FITC-modification impaired the polymerization of G-actin

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Abbreviations: HMM, heavy meromyosin; MBS-F-actin, maleimidobenzoyl-F-actin; MBS-G-actin, maleimidobenzoyl-G-actin; P-MBS-G-actin, polymerized MBS-G-actin with salt and phalloidin; S-1, myosin subfragment 1; DTT, dithiothreitol; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FITC, fluorescein isothiocyanate; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; kDa, kilodaltons; SDS-PAGE, PAGE in the presence of SDS.

(*6, 7*). Because the atomic model of the actin filament suggests that Lys-61 is located near contact regions between monomers in the filament (*8*), it would be hard to modify Lys-61 in monomer in F-actin. So, the MBS-modification of F-actin (MBS-F-actin) is important to establish the roles of other lysine residues except Lys-61 in the functions of F-actin. Although MBS cross-linked intermolecularly between actin monomers in F-actin, the biochemical behaviors of MBS-F-actin were qualitatively the same in the activation of heavy meromyosin (HMM) ATPase and in its regulation by the tropomyosin-troponin system as those of P-MBS-G-actin (*4*). We also measured, in this paper, the *in vitro* motility of MBS-F-actin and P-MBS-G-actin over HMM. Both MBS-actin derivatives could slide on HMM attached to a glass surface at a slightly slower velocity than native F-actin did. However, both actin derivatives could slide even at a higher concentration of KCl under which condition native F-actin could not slide at all. From *in vitro* motility and ATPase assays it was observed that MBS-modification of F-actin or G-actin resulted in reduced sensitivity to the Ca^{2+}-regulation by tropomyosin-troponin. These results suggest that other lysine residues in actin play an important role in the interaction between actin and myosin and also in the regulation of its interaction by tropomyosin-troponin.

MATERIALS AND METHODS

Reagents—α-Chymotrypsin was purchased from Worthington Biochemical. Glucose oxidase, catalase and phalloidin were from Sigma Chemical. Rhodamine-phalloidin was from Molecular Probes. ATP and MBS were from Boehringer Mannheim and Pierce Chemicals, respectively. All other reagents were analytical grade.

Preparation of Proteins—Myosin and actin from rabbit skeletal muscle were prepared by the methods of Tonomura *et al.* (*9*) and Spudich and Watt (*10*), respectively. HMM was prepared according to Weeds and Pope (*11*) with slight modifications (*12*). Tropomyosin and troponin were prepared as reported previously (*7*).

The modification of F-actin with MBS was carried out under the conditions used for G-actin by Bettache *et al.* (*1*). The chemical reaction was terminated by the addition of dithiothreitol (DTT) and glycine (each at 5-fold molar excess relative to MBS), followed by dialysis against 30 mM KCl and 30 mM imidazole, pH 7.6, to remove free MBS. MBS-G-actin was prepared and polymerized with phalloidin as described previously (*4*).

Protein concentrations were determined by use of UV absorption coefficients of $A^{1\%}_{280}=6.0$ for HMM (*13*), $=3.3$ for tropomyosin (*14*), $=4.5$ for troponin (*15*), and $A^{1\%}_{290}=6.3$ for G-actin (*16*). The concentrations of modified actins were estimated by means of the Bradford (*17*) assay using native actin as a standard. Relative molecular masses of 42 kDa for actin, 340 kDa for HMM, 66 kDa for tropomyosin, and 69 kDa for troponin were used.

ATPase Measurements—The ATPase activities of HMM and acto-HMM were assayed at 23°C in a solution containing 1 mM $MgCl_2$, 0.5 mM ATP, 30 mM KCl, and 30 mM imidazole buffer pH 7.6, in which the liberated inorganic phosphate was measured by the method of LeBel *et al.* (*18*). The reaction was initiated by addition of ATP.

In Vitro Motility Assay—MBS-G-actin was polymerized in the presence of rhodamine-phalloidin. Intact actin or modified actin filaments were labeled with rhodamine-phalloidin at a molar ratio of 1 to 1 (*19*). Motility was assayed at 23°C in a solution containing 1 mM $MgCl_2$, 0.5 mM ATP, 30 mM KCl, and 30 mM imidazole buffer pH 7.6, unless otherwise mentioned. Twenty millimolar DTT, 0.22 mg/ml glucose oxidase, 0.036 mg/ml catalase, and 4.5 mg/ml glucose were added to prevent rhodamine from bleaching (*20*). The sliding movement of F-actin filaments on HMM attached to a coverslip coated with collodion film (*21*) was observed with a fluorescence microscope (Olympus Kogaku, BH-2 type), and was recorded on video tape by using an SIT camera (Hamamatsu Photonics C2400) attached to the microscope (*12*). The velocity was measured by monitoring the sliding movement recorded on the video tape by use of a program (*12*).

Electron Microscopy—F-actin filaments at a concentration of about 0.1 mg/ml were mounted on a carbon-coated grid and stained negatively with uranyl acetate. The structures were observed with an electron microscope (JEOL, JEM 100C) operated at 80 kV.

Electrophoresis—SDS-polyacrylamide gradient gel electrophoresis (7-18%) with 3% stacking gel was carried out as described previously (*22*). The following proteins were used as molecular mass markers: phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa), which are Low Range Standards from Bio-Rad.

RESULTS

Modification of F-Actin with MBS—According to Bettache *et al.* (*1*), MBS-G-actin suffers intramolecular cross-linking and has one free maleimide group, but the MBS-G-actin is not intermolecularly cross-linked (Fig. 1, lane c) In contrast, the treatment of F-actin with MBS does not generate only intracross-links within the actin subunit, but also induces intercross-linked products between actin subunits (Fig. 1, lane d), as first observed by Sutoh (*23*). As can be seen in Fig. 1, the cross-linked dimer and trimer showed doublet bands, as previously reported for the

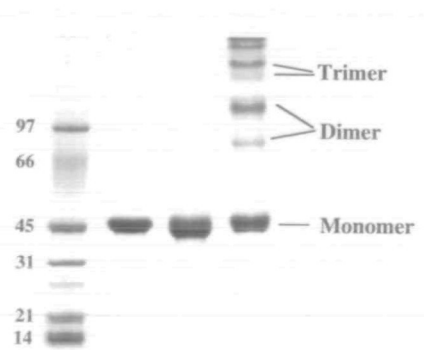

Fig. 1 **SDS-PAGE pattern of actin after modification with MBS.** Lane a, marker proteins (see "MATERIALS AND METHODS"); lane b, native F-actin; lane c, MBS-G-actin; lane d, MBS-F-actin. Molecular mass of actin monomer is 42 kDa. Numbers on the left side are molecular masses in kDa.

cross-linking of F-actin with *p*-phenylenebis(maleimide) (p-PDM) (*24*), though the intensities of the minor bands were much weaker than those of the major bands. These minor bands may represent cross-linked species that contain both intersubunit and intrasubunit cross-links (*24*), since the monomer actin showed doublet bands as well upon MBS treatment (Fig. 1, lane c). The intermolecular cross-linking reaction is rather limited since most of the actin still migrates as a 42 kDa species (about 63%) (Fig. 1, lane d).

Activation of HMM ATPase by MBS-F-Actin—Figure 2 shows a double-reciprocal plot of the ATPase activity of HMM *vs.* the concentration of native F-actin or MBS-F-actin. The maximum turnover rate (V_{max}) and the affinity to HMM (K_m) of native F-actin were 20 s^{-1} and 4.6×10^4 M^{-1}, respectively. The activation of HMM ATPase by MBS-F-actin was much higher than that by native F-actin at a given fixed concentration of actin. However, the V_{max} was almost the same as that of native F-actin (17 s^{-1}) and the K_m of MBS-F-actin to HMM was about 3 times higher than that of native F-actin (13.3×10^4 M^{-1}).

Regulation of the MBS-F-Actin-HMM ATPase by Tropomyosin and Troponin—Tropomyosin alone did not affect the actin-activated HMM ATPase in the case of native F-actin, MBS-F-actin and also P-MBS-G-actin (Fig. 3A). When troponin was added as well, the HMM ATPase was slightly activated by native F-actin and Ca^{2+}, but substantially inhibited by removal of Ca^{2+}. As shown in Fig. 3A, regulated MBS-F-actin and P-MBS-G-actin did not fully activate the HMM ATPase even in the presence of Ca^{2+} (60 and 65% activation for MBS-F-actin and P-MBS-G-actin, respectively), and the activity was not inhibited completely by removal of Ca^{2+} (70 and 65% inhibition for MBS-F-actin and P-MBS-G-actin, respectively). But when excess amounts of tropomyosin-troponin were added to MBS-F-actin or P-MBS-G-actin, the ATPase activity of acto-HMM was reduced to about 18 and 15% by removal of Ca^{2+} for MBS-F-actin and P-MBS-G-actin, respectively (82 and 85% inhibition, respectively) (Fig. 3B).

Fine Structure of MBS-F-Actin—In observation by electron microscopy, both MBS-F-actin and P-MBS-G-actin showed the double-stranded helical structure of native F-actin filaments (Fig. 4, a and c). P-MBS-G-actin existed as short filaments of 80-200 nm long, as well as long filaments (Fig. 4a). The rigor complex of HMM and MBS-F-actin or P-MBS-G-actin showed the characteristic arrowhead structure (Fig. 4, b and d). When HMM and actin were mixed at a weight ratio of 2 : 3 (present conditions), the long filaments were specifically decorated by HMM (Fig. 4d). The reason for this is unclear.

In Vitro Motility Assay—The sliding of fluorescently labeled F-actin filaments on HMM *in vitro* can be observed by fluorescence microscopy. During the sliding, the actin filaments were fragmented into shorter filaments. The sliding velocity varied slightly from preparation to preparation. Figure 5 shows the average sliding velocities of native F-actin, MBS-F-actin, and P-MBS-G-actin on

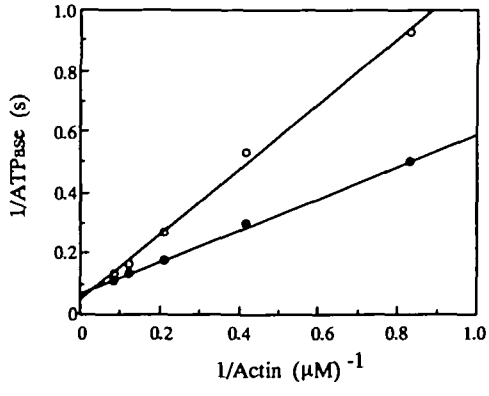

Fig. 2. **Double-reciprocal plot of the ATPase activity of HMM *vs.* actin concentration.** Open circles, native F-actin; closed circles, MBS-F-actin. The concentration of HMM was 0.05 mg/ml, and that of actin ranged from 0.3 to 1 mg/ml.

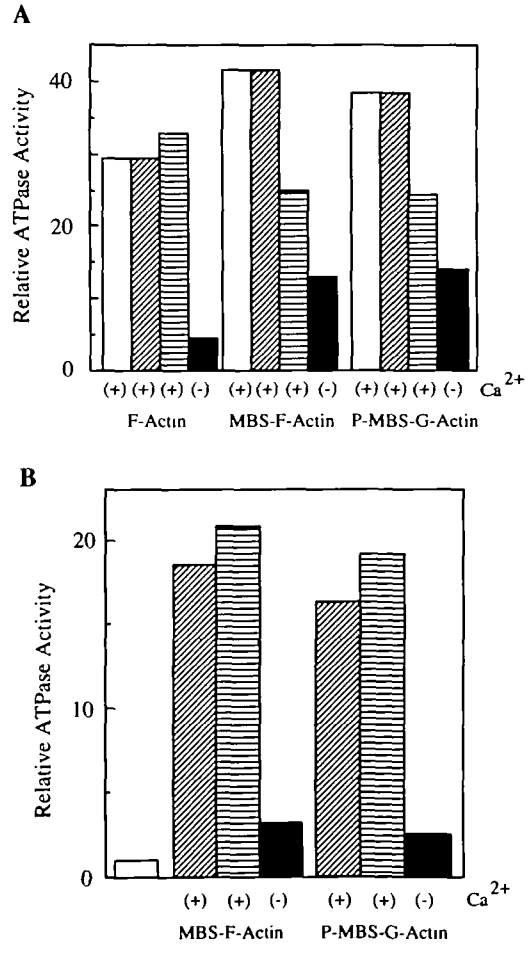

Fig. 3. **Effect of tropomyosin and troponin on the ATPase activity of acto-HMM.** A: The molar ratio of actin, tropomyosin, and troponin is 7:1:1 (the physiological ratio). HMM ATPase activity in the presence of actin (open bars), in the presence of actin and tropomyosin (hatched bars), in the presence of actin and tropomyosin-troponin (striped bars, closed bars). ($+Ca^{2+}$), in the presence of 0.2 mM $CaCl_2$; ($-Ca^{2+}$), in the presence of 0.5 mM EGTA. The concentrations of HMM, actin, tropomyosin, and troponin are 0.1, 0.4, 0.2, and 0.2 mg/ml, respectively. B: The molar ratio of actin and tropomyosin-troponin is 1:5. The ATPase activity of HMM alone (open bar); in the presence of MBS-F-actin (hatched bar); in the presence of MBS-F-actin, tropomyosin-troponin and 0.2 mM $CaCl_2$ (striped bar); in the presence of MBS-F-actin, tropomyosin-troponin and 0.5 mM EGTA (closed bar). The concentrations of HMM, actin, and tropomyosin-troponin were 0.05, 0.05, and 0.7 mg/ml, respectively. Relative ATPase activity is defined as the ratio of the ATPase activity to that of HMM alone.

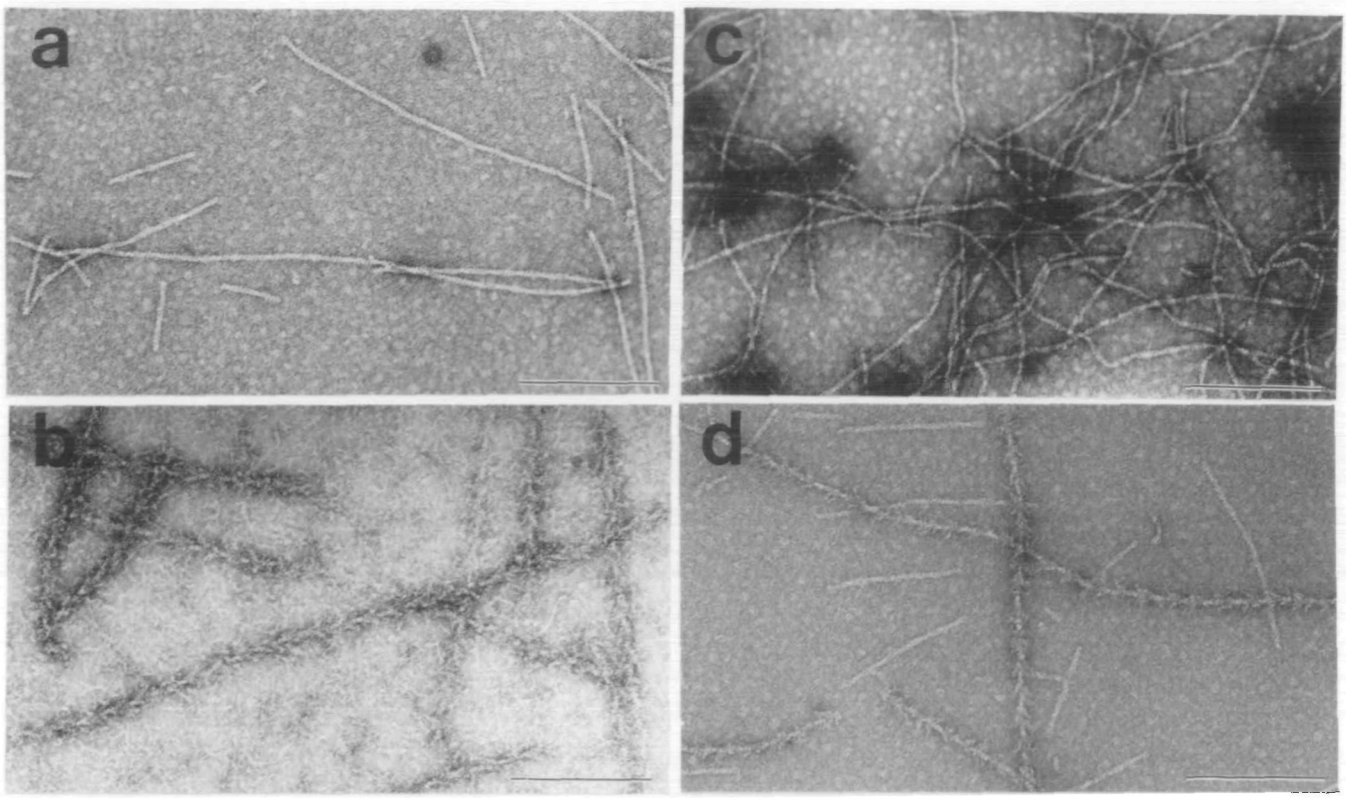

Fig. 4. **Electron micrographs of MBS-actin filaments.** (a) and (b), P-MBS-G-actin polymerized in a solution containing 30 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 30 mM imidazole buffer, pH 7.6, and phalloidin at a molar ratio to actin of 1 to 1. (c) and (d), MBS-F-actin in the same solution as in (a) and (b) but without phalloidin. (a) and (c), actin filaments. (b) and (d), actin filaments decorated with HMM in the absence of ATP. The bars indicate 0.2 μm.

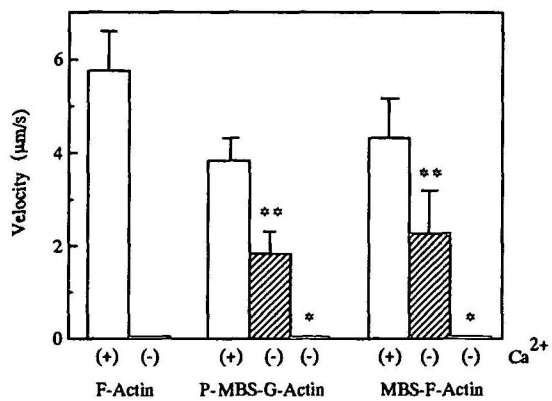

Fig. 5. **Effect of tropomyosin-troponin on sliding velocity of actin filaments on HMM.** Open bars, in the presence of Ca^{2+} (0.2 mM $CaCl_2$); hatched bars and symbol (*), in the absence of Ca^{2+} (0.2 mM EGTA). The line associated with each bar indicates standard deviation ($n=15$). For F-actin, velocity was measured at 4-fold molar excess of tropomyosin-troponin to actin. For P-MBS-G-actin and MBS-F-actin; open and hatched bars, velocity at 4-fold molar excess of tropomyosin-troponin to actin and symbol (*), velocity at 20-fold molar excess of tropomyosin-troponin to actin. Average velocity of short filaments (**).

HMM: 5.86±1.12, 4.34±1.00, and 3.68±0.69 μm/s, respectively. The maximum KCl concentration, which supported sliding on HMM (referred to as the critical concentration), was 60 mM for native F-actin (*12*). However, MBS-F-actin and P-MBS-G-actin could slide on HMM at 1.27±0.49 and 3.22±0.81 μm/s, respectively, even at 90 mM KCl (Table I).

TABLE I. **Dependence of sliding velocity on KCl concentration.** Sliding velocity is expressed in the unit of μm/s (mean±SD) ($n=15$).

Actin	KCl (mM)			
	0	30	60	90
F-actin	n.d.	4.81±1.14	n.d.	0
P-MBS-G-actin	3.20±0.07	4.06±0.99	3.53±0.89	3.22±0.81
MBS-F-actin	2.38±0.42	3.10±0.80	3.12±1.14	1.27±0.49

As observed in the HMM ATPase activation by MBS-F-actin, its Ca^{2+}-regulation by tropomyosin-troponin was impaired by MBS-modification. In accordance with this, sliding movement of MBS-modified actin filaments (MBS-F-actin and P-MBS-G-actin) was less sensitive to Ca^{2+} when tropomyosin-troponin was added. In the *in vitro* motility assay, the concentration of F-actin in the assay medium was very low (about 3 nM), when compared with the case of ATPase assay. So, an excess amount of tropomyosin-troponin is needed for the accurate measurement of Ca^{2+}-regulation using the motility assay. When a 4-fold molar excess of tropomyosin-troponin over actin was added, all native F-actin filaments stopped sliding completely in the absence of Ca^{2+}. Under the same conditions, the short filaments of MBS-modified actin less than 2 μm long remained moving, while the long actin filaments of

MBS-modified actin did not move, although both filaments could move with the same velocity in the presence of Ca^{2+}. The sliding velocities of these short filaments of MBS-actin were about half of those in the presence of Ca^{2+} (Fig. 5). When the molar ratio of tropomyosin-troponin to actin was increased to 20, both P-MBS-G-actin and MBS-F-actin filaments stopped sliding completely (* in Fig. 5), indicating that the affinity of tropomyosin-troponin for those short actin filaments was reduced.

DISCUSSION

Intramolecular cross-links between cysteine and lysine residues in G-actin by MBS led to the suppression of the polymerizability of G-actin induced by salts or S-1 (*1, 2*). However, we found that MBS-G-actin can polymerize upon addition of salt and phalloidin together, that the P-MBS-G-actin augmented the S-1 ATPase activity more strongly than native F-actin, and that the regulation by tropomyosin-troponin was impaired by the MBS-modification (*4*).

When F-actin was modified with MBS, the MBS-F-actin could enhance the ATPase activity of HMM more strongly than native F-actin, although the maximum turnover rate at infinite actin concentration was almost the same (Fig. 2). The affinity of MBS-F-actin for HMM in the presence of ATP was higher than that of native F-actin. The modification of F-actin with MBS impairs the Ca^{2+}-regulation of the ATPase activity of HMM with tropomyosin-troponin (Fig. 3), possibly due to a reduction of the binding affinity of tropomyosin-troponin to F-actin. These biochemical properties of MBS-F-actin were qualitatively similar to those of the P-MBS-G-actin (*4*), suggesting that additional inter-cross-links between actin monomers with MBS did not cause any major change of the biochemical properties. The intermolecular reaction is rather limited since most of the actin still migrates as 42 kDa species (Fig. 1, lane d).

The critical concentration of KCl for sliding movement of native F-actin on HMM *in vitro* is 60 mM (*12*), but MBS-F-actin and P-MBS-G-actin can slide on HMM even at 90 mM KCl (Table I). These findings may be consistent with higher affinity constants for HMM of MBS-F-actin and P-MBS-G-actin than that of native F-actin in the presence of ATP (Fig. 2).

Recently Cook *et al.* (*25*) reported that the addition of negatively charged residues to the N-terminus of actin increased the V_{max} value, but did not affect the K_m value or the velocity of actin sliding over myosin *in vitro*. Here we have shown that the reduction of positive charges of lysine residues in actin by MBS increased K_m without changing V_{max}, but slightly decreased the sliding velocity of actin on HMM.

After G-actin was cross-linked by glutaraldehyde or 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), it was polymerized by salt, and the resulting F-actin activated the HMM ATPase but did not move on HMM *in vitro*, suggesting that the ATPase activation is not always coupled with motility (*26*). However, in the case of cross-linking of G-actin with MBS, which could polymerize only in the presence of phalloidin and salt, motility and ATPase activation were roughly correlated, as described in this paper, indicating close coupling between them.

When tropomyosin-troponin were added to the medium, the native F-actin could not slide on HMM at all in the absence of Ca^{2+}. On the other hand, MBS-F-actin and P-MBS-G-actin could slide on HMM even in the absence of Ca^{2+} (Fig. 5). However, when excess amounts of tropomyosin-troponin were added, both P-MBS-G-actin and MBS-F-actin filaments stopped sliding completely. These results indicate that the modification of F-actin with MBS reduces the affinity of tropomyosin-troponin for actin filaments, as shown in the case of P-MBS-G-actin (*4*) and also as suggested from the changes in ATPase activity. The electron micrographs of P-MBS-G-actin and MBS-F-actin show that these filaments form the typical arrowhead structures, when decorated with HMM (Fig. 4). So, the weak binding of tropomyosin-troponin to MBS-F-actin or P-MBS-G-actin may not be due to any change in the overall structure of actin filaments after the modification.

When G-actin was treated with MBS, six to seven lysine residues and three to four cysteine residues in the G-actin molecule are modified with MBS (*1*). However, it has not been identified which amino acids are modified with MBS in the primary structure. Such knowledge is important for elucidating the molecular mechanisms of muscle contraction and regulation. Among the lysine residues in G-actin, Lys-61, Lys-113, and Lys-336 are the most reactive (*27*). It was reported that Lys-61 is closely related to the regulation of the actin-myosin interaction by tropomyosin-troponin (*28*). The modification of Lys-61 leads to the loss of actin polymerization induced by salt or S-1 (*6, 7*). However, the modified actin regains the ability to polymerize upon addition of salt and phalloidin (*7*), as in the case of MBS-G-actin (*4*). Recently it has been reported that the lysine-specific reagent pyridoxal 5′-phosphate modifies Lys-61 in G-actin and that the resulting G-actin derivative has a greatly reduced ability to polymerize into filaments (*29*). The atomic model of the actin filament suggests that Lys-61 is located near contact regions between monomers in the filaments (*8*; and see Ref. *30* for a review). Chemical modifications of lysine residues at positions 50, 61, 68, 113, 284, and 291 in G-actin inhibited the polymerization to F-actin and, therefore, these lysine residues might be in a monomer-monomer contact region (*27, 31*). Upon modification with MBS, F-actin retains a filamentous structure (Fig. 4). Therefore, other lysine residues in actin monomer are modified with MBS in the case of F-actin. According to Sutoh (*23*), peptide maps of the MBS cross-linked actin dimer revealed that the cross-linking sites of MBS between actin dimer in F-actin are Cys-374 and a lysine residue in the CB-17 segment (Lys-191, Lys-213, or Lys-215). Hori and Morita (*32*) found that the region between Arg-177 and Tyr-198 (containing Lys-191) is in the monomer-monomer interface. So, these findings, together with our present results, suggest that Lys-213 and/or Lys-215 are important for the biochemical and motile properties of F-actin, and for tropomyosin-binding to actin.

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