Binding between Maleimidobenzoyl-G-Actin and Myosin Subfragment 1†

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Received May 21, 1992; Revised Manuscript Received July 29, 1992

ABSTRACT: It is well known that the structural interactions between S-1 moieties of myosin molecules ("cross bridges") and actin molecules in polymerized ("F") form are thought to underlie muscle contraction. It is surmised that such interactions are unitary (actin:S-1 = 1:1), but actual demonstration thereof is handicapped by intrinsic properties of the proteins. Recently, it has been reported that chemically modified [with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS)] actin maintains its monomeric ("G") form and makes a stable unitary complex with S-1 but does not activate the S-1 ATPase [Bettache, N., Bertrand, R., & Kassab, R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86,* 6028–6032]. However, we recently showed that when MBS-G-actin and S-1 are covalently cross-linked by 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC), ATPase activity is restored [Hozumi, T. (1991) *Biochem. Int. 23,* 835–843]. Here we investigated the interface between MBS-G-actin and S-1 using the techniques of tryptic digestion and EDC-cross-linking. MBS-G-actin specifically protected the N-terminal region of S-1 heavy chain against tryptic cleavage at the 25 kDa/50 kDa junction, which is different from the effect that a protomer within F-actin has on the protection of the 25 kDa/50 kDa junction. In addition, the cross-linking pattern between MBS-G-actin and S-1 was different from that between F-actin and S-1. When MBS-G actin was cross-linked to trypsin-treated S-1, no cross-linked product was observed. These results show that the MBS-G-actin and S-1 interface is different from that between actin monomer in F-form and S-1.

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

S-1 is the segment of the myosin molecule containing the active site of ATPase and also the site at which actin interacts. Because both nucleotide hydrolysis and actin binding occur on S-1, studies of changes in S-1 conformation accompanying these interactions are highly significant (Botts et al., 1984, 1989). Recently, Bettache et al. (1989, 1990) designed a new G-actin derivative, maleimidobenzoyl-G-actin (MBS-G-actin), which is prepared by treating G-actin with the heterobifunctional reagent *m*-maleimidobenzoyl-*N*-hydroxysuccin imide ester (MBS). MBS-G-actin remains monomeric even in the presence of salt or S-1 and forms a stable, soluble, and reversible unitary complex with S-1. The soluble complex of MBS-G-actin and S-1 may be a useful tool for studying the structure and function of actomyosin.

It is known that G-actin binds to S-1 (Cooke & Morales, 1971; Chantler & Gratzer, 1973; Bottomley & Trayer, 1975) and that the 633–642 region of S-1 heavy chain is involved (Chaussepied & Morales, 1988) in this contact. However, this binding does not activate S-1 ATPase (Offer et al., 1972; Chantler & Gratzer, 1976). Recently, we have observed that even MBS-G-actin can activate S-1 ATPase when it is cross-linked to S-1 with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Hozumi, 1991), similar to the actions of F-actin and S-1 (Mornet et al., 1981a). To further extend our insight into the sites of interaction between actin and S-1 heavy chain, we have examined the tryptic digestion pattern of the MBS-G-actin–S-1 complex and the EDC cross-linking of MBS-G-actin to S-1. The data presented show that the central 50-kDa fragment of S-1 heavy chain cannot be cross-linked with MBS-G-actin and that while MBS-G-actin does not protect the 50 kDa/20 kDa junction of S-1 from tryptic cleavage, it can protect the 25 kDa/50 kDa junction. Thus, the effects of MBS-G actin on the tryptic digestion of the S-1 heavy chain are different from those by F-actin. These results suggest that the structural interface between S-1 and MBS-G-actin differs from that between S-1 and monomeric actin in F-actin.

MATERIALS AND METHODS

Chemicals. L-1-Tosylamido-2-phenylethyl chloromethyl ketone treated trypsin (TPCK-trypsin), soybean trypsin inhibitor, and α-chymotrypsin were purchased from Worthington Biochemical Company. ATP and ADP were from Boehringer Mannheim Biochemica. Phalloidin was obtained from Sigma. 9-Anthroylnitrile (ANN) and *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) were from Wako. MBS and EDC were from Pierce and Nakarai, respectively. All other reagents were analytical grade.

Proteins. Myosin and actin from rabbit skeletal muscle were prepared by the methods of Tonomura et al. (1966) and Spudich and Watt (1971), respectively. S-1 was prepared by digestion of myosin filaments with α-chymotrypsin (Weeds & Taylor, 1975) and was further purified over Sephacryl S-300.

The modification of G-actin with MBS was carried out as described by Bettache et al. (1989). The chemical reaction was terminated by the addition of either dithiothreitol and

† This research was supported by a Grant-in-Aid for scientific research on priority areas from the Ministry of Education, Science and Culture of Japn.

[1] Abbrevations: MBS-G-actin, maleimidobenzoyl-G-actin; S-1, myosin subfragment 1; split S-1, trypsin-treated S-1; TPCK-trypsin, L-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin; ANN, 9-anthroylnitrile; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/92/0431-10070$03.00/0

glycine (each at 5-fold molar excess relative to MBS). After the MBS treatment, KCl and $MgCl_2$ were added to a final concentration of 60 and 2 mM, respectively, and this was followed by centrifugation at 120000*g* for 90 min. The supernatant was taken as the MBS-G-actin (Hozumi, 1991). MBS-G-actin was then dialyzed exhaustively against G buffer (2 mM Tris, 0.1 mM $CaCl_2$, 0.1 mM NaN_3, and 0.1 mM ADP, pH 8.0). The fluorescent labeling of S-1 in the N-terminal 25-kDa fragment with ANN was carried out according to Hiratsuka (1989). SH1 (Cys-707) of S-1 was fluorescently labeled with DACM as previously described (Hozumi, 1986).

Protein concentrations were determined from UV absorbance measurements, assuming $A_{280}^{1\%} = 7.5$ for S-1 (Weeds & Pope, 1977) and $A_{290}^{1\%} = 6.3$ for G-actin (Lehrer & Kerwar, 1972). The concentrations of the modified proteins were estimated from the Bradford (1976) assay using native proteins as standards.

Tryptic Digestion of S-1. S-1 (1.5 mg/mL) was digested by one hundredth its concentration (w/w) of TPCK-trypsin in G-buffer at 25 °C in the absence and presence of actin (1.5 mg/mL) for 1 h unless otherwise stated. Soybean trypsin inhibitor was added to twice the trypsin concentration (w/w) to terminate digestion. Under these conditions, actin was not digested by trypsin.

Cross-Linking Reactions. MBS-G-actin or F-actin (1 mg/mL) was mixed with S-1 or split S-1 (1 mg/mL) in G buffer at 25 °C. After 30 min, the mixture was cross-linked by EDC at a final concentration of 5 mM for 1 h at 25 °C and was quenched by the addition of excess 2-mercaptoethanol.

Electrophoresis. $NaDodSO_4$–polyacrylamide gradient gel electrophoresis (7–18%) was carried out as described previously (Hozumi, 1983). Fluorescent bands were located by illumination with a UV light before staining with Coomassie blue. 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).

ATPase Measurements. The Mg^{2+}-ATPase activities of S-1 and acto-S-1 were measured at 25 °C in G-buffer plus 0.5 mM Mg^{2+}-ATP. The concentrations of S-1 and actin (case of acto-S-1) were 0.04 mg/mL. Liberated inorganic phosphate was measured by the method of LeBel et al. (1978).

RESULTS

Effect of MBS-G-actin on the Tryptic Digestion of S-1. Limited tryptic digestion of S-1 produces three fragments of 25, 50, and 20 kDa (Balint et al., 1978). When the digestion was performed on the complex of F-actin–S-1, the heavy chain was split into only two fragments of 25 and 70 kDa (Figure 1A); F-actin binding protected the second cleavage site, the 50 kDa/20 kDa junction. However, when MBS-G-actin was used in place of F-actin during the digestion, the fragmentation of the S-1 heavy chain showed a dramatic change. The appearance of the 25-kDa band was completely abolished; instead, the parent heavy chain was slowly converted into two fragments of 75 and 20 kDa (Figure 1B), as first observed by Bettache et al. (1989).

When S-1 labeled at SH1 with the fluorescent dye, DACM, was used, a fluorescent 20-kDa band was observed after the tryptic digestion of MBS-G-actin–S-1 complex (Figure 2A,C, lanes c). Alternatively, S-1 was also labeled with ANN, because it binds specifically to the 25-kDa N-terminal segment (Hiratsuka, 1989). When ANN-labeled S-1 was used in the digestion, MBS-G-actin prevented the appearance of the

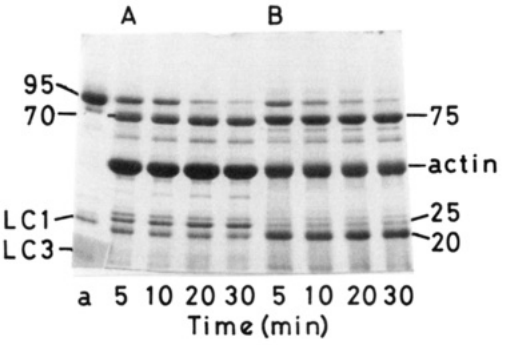

FIGURE 1: Electrophoretic patterns of the tryptic digest of S-1 heavy chain. (a) S-1. (A) Digested in the presence of F-actin. (B) Digested in the presence of MBS-G-actin. Digestion was for the times given on the bottom. LC1 and LC3 are myosin light chain 1 and 3, respectively. Numbers on the sides are molecular masses in kilodaltons.

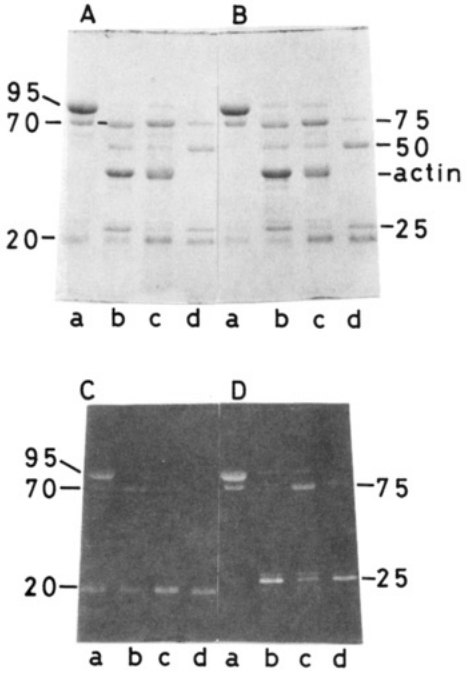

FIGURE 2: Tryptic products of the S-1 heavy chain. (A and C) S-1 labeled with DACM was used. (B and D) S-1 labeled with ANN was used. (a) S-1. (b) Digested in the presence of F-actin. (c) Digested in the presence of MBS-G-actin. (d) Digested S-1 alone. (A and B) Gels were stained with Coomassie blue. (C and D) Gels were visualized under a UV lamp. Numbers on the sides are molecular masses in kilodaltons.

fluorescent 25-kDa band; fluorescence was seen in the 75-kDa fragment (Figure 2B,D, lanes c). These results demonstrate that the 20-kDa fragment produced by tryptic digestion in the presence MBS-G-actin was the C-terminal fragment which was also obtained by tryptic digestion of S-1 alone. Furthermore, MBS-G actin protected tryptic cleavage at the N-terminal site of S-1 heavy chain.

F-actin is a potent activator for S-1 ATPase. However, MBS-G-actin cannot increase basal S-1 ATPase activity (Bettache et al., 1989). When MBS-G-actin and F-actin were added together to S-1, the ATPase activity was stimulated to the same level as F-actin alone (Table I), suggesting that MBS-G-actin did not interfere with F-actin binding to S-1. The tryptically digested MBS-G-actin–S-1 complex exhibited only basal ATPase activity (Table I). Addition of F-actin to the tryptically digested MBS-G-actin–S-1 complex could not augment the ATPase activity (Table I).

Table I: Mg^{2+}-ATPase Activities of S-1 before and after the Tryptic Digestion

protein	Mg^{2+}-ATPase (s^{-1})
S-1	0.061 ± 0.006 (1)
S-1 + F-actin[a]	2.740 ± 0.051 (44.9)
S-1 + F-actin + MBS-G-actin[a]	2.720 ± 0.073 (44.6)
tryptic (S-1–MBS-G-actin)[b]	0.061 ± 0.003 (1)
tryptic (S-1–MBS-G-actin) + F-actin[a]	0.110 ± 0.012 (1.8)

[a] The ATPase reaction was started by the addition of ATP into actin–S-1 complex which was preincubated for 10 min at 25 °C. [b] S-1–MBS-G-actin complex was digested by trypsin. Values are means ± SE for five independent measurements. Numbers in parentheses are ratios of each activity over the activity of S-1 alone.

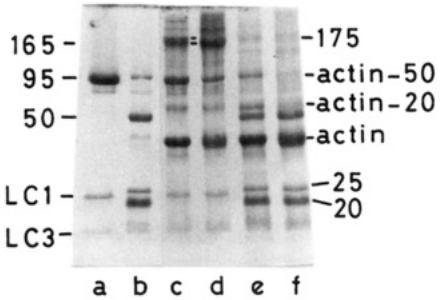

FIGURE 3: Cross-linking patterns of actin to S-1 with EDC. (a) S-1. (b) Split S-1. Cross-linking was carried out using S-1 (lanes c and d), split S-1 (lanes e and f), F-actin (lanes c and e), and MBS-G-actin (lanes d and f). Numbers on the sides are molecular masses in kilodaltons.

Cross-Linking Experiments. When F-actin and S-1 are cross-linked with EDC, cross-linked products of about 180 kDa appeared as a doublet (Figure 3, lane c). The doublet has been thought to arise from cross-linking of actin with the 20- and 50-kDa domains of S-1 heavy chain, respectively (Sutoh, 1983). However, when MBS-G-actin was cross-linked with S-1 by EDC, a cross-linked product appeared as only a singlet which corresponded to the lower molecular weight of product of the doublet (Figure 3, lane d). We also studied the cross-linking process occurring between MBS-G-actin and split S-1. Two new bands were seen when F-actin was cross-linked with split S-1 (Figure 3, lane e). One is the product with the 50-kDa fragment, and the other is the product with 20-kDa fragment (Mornet et al., 1981b). However, neither of these bands appeared when MBS-G-actin was cross-linked with S-1 (Figure 3, lane f).

Recently, we have demonstrated that MBS-G-actin is polymerized in the presence of salt and phalloidin (Miki & Hozumi, 1991). Interestingly, when the polymerized MBS-G-actin was used in cross-linking experiments, the cross-linked product between actin and S-1 appeared as a doublet (Figure 4, lane c). The molecular weights of the products were similar to those observed with the native F-actin.

DISCUSSION

The polymerization of skeletal G-actin induced by salt or S-1 is suppressed by chemically modifying G-actin with MBS (Bettache et al., 1989, 1990). Although MBS-G actin can form a stable and reversible complex with S-1 in solution, it does not activate S-1 ATPase (Bettache et al., 1989). However, we have shown that when MBS-G-actin is cross-linked to S-1 with EDC, the complex recovers ATPase activity (Hozumi, 1991). In this study, we have examined how tryptic fragments of S-1 heavy chain interact with MBS-G-actin in order to gain knowledge of the structural features of S-1 heavy chain involved in the interaction with actin.

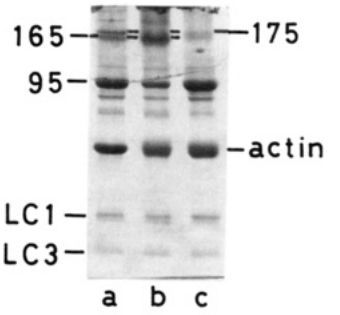

FIGURE 4: Cross-linking patterns of polymerized MBS-G-actin to S-1 with EDC. Cross-linked S-1 with F-actin (a) or with MBS-G-actin (b). MBS-G-actin was polymerized by the addition of equimolar of phalloidin, 60 mM KCl, and 2 mM $MgCl_2$ (Miki & Hozumi, 1991) and then cross-linked with S-1 (c). Numbers on the sides are molecular masses in kilodaltons.

The limited trypsinolysis splits S-1 heavy chain into three major fragments of approximately 25, 50, and 20 kDa (Balint et al., 1978). The 25-kDa fragment is generated through a 29.5-kDa precursor (Hozumi & Muhlrad, 1981), and these remain associated under nondenaturing conditions. Tryptic digestion of the F-actin–S-1 complex shows a change in the fragmentation pattern of the heavy chain. F-actin completely protects the 50 kDa/20kDa junction from cleavage; the parent heavy chain is converted into only the fragments of 25 and 70 kDa (Mornet et al., 1979). The tryptic digestion of the MBS-G-actin–S-1 complex shows a dramatic difference in the fragmentation pattern of the heavy chain. MBS-G-actin did not protect 50 kDa/20 kDa junction from tryptic cleavage but instead protected the 25 kDa/50 kDa junction, thus, producing two fragments of 75 and 20 kDa.

When F-actin and S-1 are cross-linked with EDC, two products with apparent molecular masses of 165 and 175 kDa are generated (Mornet et al., 1981a). According to Sutoh (1983), the 165- and 175-kDa products correspond to the cross-links established between actin monomer and the 20- and 50-kDa regions of S-1 heavy chain, respectively. The different molecular mass values displayed by the 165- and 175-kDa species may reflect different conformations resulting from the cross-links established. However, when MBS-G-actin is cross-linked to S-1 with EDC, only one cross-linked product is generated, which corresponds to the lower molecular mass species (165 kDa). The singlet is clearer when smaller amounts of sample are loaded to gel, and the cross-linked product which is partially purified by gel filtration shows the singlet (Hozumi, 1991). This strongly suggests that only the 20-kDa fragment is able to cross-link with MBS-G-actin. When split S-1 is used for cross-linking, two cross-linked products (actin–50-kDa fragment and actin–20-kDa fragment) (Mornet et al., 1981b) are generated with F-actin, but no cross-linking product is observed with MBS-G-actin. Bettache et al. (1989) reported that, following modification of G-actin with MBS, MBS-G-actin contains few intramolecular cross-links and a free maleimide group. These cross-links and free maleimide group may sterically disturb the contact of MBS-G-actin with 50-kDa region of the S-1 heavy chain. We have demonstrated that MBS-G-actin can be polymerized by salt and phalloidin (Miki & Hozumi, 1991). From Figure 4, it is clear that the cross-linked products of S-1 and polymerized MBS-G-actin appear as doublet, similar to native F-actin. Thus the cross-links and free maleimide group in MBS-G-actin do not disturb the contact with S-1 heavy chain. The lower yield of the overall cross-linking to S-1 by the polymerized MBS-G-actin as compared to that of the MBS-G-actin distinguishes the two actin derivatives. This

also suggests that the interface between MBS-G-actin and S-1 is different from that between actin monomer in F-actin and S-1.

It is thought that the cross-linking between actin and S-1 occurs between the carboxylate-rich N-terminal region of actin (at least in F-actin) and one or another (Mornet & Ue, 1985) of two amino-rich regions of S-1 heavy chain. The regions of S-1 were identified by Sutoh (1983) as being at the 50 kDa/20 kDa junction and the C-terminal region of the 50-kDa fragment. It is likely that residues 633–642 of the S-1 heavy chain constitute the 50 kDa/20 kDa junction (Chaussepied & Morales, 1988; Yamamoto, 1989). Botts et al. (1989) suggest that the binding site on the 50-kDa fragment may be residues 566–573. Recently, Chaussepied and Kasprzak (1989) found that an antipeptide to residues 633–642 totally blocks the binding of G-actin to S-1. These findings together with our present results suggest that the interaction between the N-terminus of actin and residues 633–642 of the S-1 heavy chain is mainly responsible for binding between G-actin and S-1. When the N-terminus of actin and this region of the S-1 heavy chain are covalently cross-linked with EDC, the adduct exhibits enhanced ATPase activity (Hozumi, 1991).

It is likely that the conformation around the 25 kDa/50 kDa junction is modulated by the binding of actin to the 50 kDa/20 kDa junctional region of the S-1 heavy chain. In native S-1, this region is exposed and is easily cut by trypsin, but the region becomes more buried when MBS-G-actin binding occurs, and the 25 kDa/50 kDa junction is protected from tryptic cleavage. Previously, we reported that the conformation around the 25 kDa/50 kDa junctional region of S-1 is dependent on ATP binding to S-1 (Muhlrad & Hozumi, 1982). It is interesting that the conformation around this region is regulated by both important events in muscle contraction, i.e., ATP binding and actin binding to S-1.

The importance of the 50 kDa/20 kDa junction of S-1 heavy chain in actin activation was demonstrated by several lines of evidence. The MBS-G-actin could not protect tryptic cleavage at this junction and the cleavage products (75–20 kDa) did not exhibit any F-actin stimulated ATPase activity. These ATPase data are consistent with findings of Bertrand et al. (1989) who used endoproteinase Arg-C on the cleavage of S-1 heavy chain (75–21 kDa). The 20-kDa fragment of split S-1 could not be cross-linked with MBS-G-actin by EDC. This may be due to a lack of significant binding between MBS-G-actin and S-1 owing to the tryptic degradation of the 50 kDa/20 kDa junction. Indeed, it has been recently shown that the interaction of native G-actin with S-1 requires the structural integrity of the 50 kDa/20 kDa junction of the heavy chain (Chen & Reisler, 1991). MBS-G-actin binding at the 50 kDa/20 kDa region is probably very weak, as it cannot protect this junction from tryptic cleavage. This is consistent with the finding that MBS-G-actin can not activate S-1 ATPase (Bettache et al., 1989). EDC cross-linking may restore ATPase activity by strengthening the binding of MBS-G-actin with 50 kDa/20 kDa region (Hozumi, 1991).

ACKNOWLEDGMENT

I am indebted to Professor Manuel F. Morales for his kind comments on the manuscript and also to Dr. Robert E. Petroski for his critical reading of the manuscript.

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Registry No. ATPase, 9000-83-3.