Biochemistry **1992**, *31*, 389–395

Taylor, E. W. (1991) *J. Biol. Chem. 266*, 294–302.
Thomas, D. D., & Cooke, R. (1980) *Biophys. J. 32*, 891–906.
Tokunaga, M., Sutoh, K., Toyoshima, C., & Wakabayashi, T. (1987) *Nature (London) 329*, 635–638.
Tonomura, Y., Tokura, S., & Sekiya, K. (1962) *J. Biol. Chem. 237*, 1074–1081.
Wagner, P. D., & Giniger, E. (1981) *J. Biol. Chem. 256*, 12647–12650.
Warwicker, J., & Watson, H. (1982) *J. Mol. Biol. 157*, 671–679.
Yamamoto, K. (1989) *Biochemistry 28*, 5573–5577.
Yamamoto, K. (1991) *J. Mol. Biol. 217*, 229–233.

Specific Cross-Linking of the SH1 Thiol of Skeletal Myosin Subfragment 1 to F-Actin and G-Actin[†]

Nadir Bettache, Raoul Bertrand, and Ridha Kassab*

Centre de Recherches de Biochimie Macromoléculaire du CNRS, INSERM U 249, Université de Montpellier I, Route de Mende, BP 5051, 34033 Montpellier Cedex, France

Received August 2, 1991; Revised Manuscript Received October 7, 1991

ABSTRACT: Recently, we reported that (maleimidobenzoyl)-G-actin (MBS-G-actin), which was resistant to the salt and myosin subfragment 1 (S-1) induced polymerizations, reacts reversibly and covalently in solution with the S-1 heavy chain at or near the strong F-actin binding region [Bettache, N., Bertrand, R., & Kassab, R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6028–6032]. Here, we have readily converted the MBS-G-actin into MBS-F-actin in the presence of phalloidin and salts. The binding of S-1 to the two actin derivatives carrying on their surface free reactive maleimidobenzoyl groups was investigated comparatively in cross-linking experiments performed under various conditions to probe further the molecular structure of the actin–heavy chain complex before and after the polymerization process. Like MBS-G-actin, the isolated MBS-F-actin, which did not undergo any intersubunit cross-linking, bound stoichiometrically to S-1, generating two kinds of actin–heavy chain covalent complexes migrating on electrophoretic gels at 180 and 140 kDa. The relative extent of their production was essentially dependent on pH for both G-and F-actins. At pH 8.0, the 180-kDa species was predominant, and at pH 7.0, the amount of the 140-kDa adduct increased at the expense of the 180-kDa entity. The cross-linking of MBS-F-actin to S-1 led to the superactivation of the MgATPase substantiating the ability of this derivative to stimulate the S-1 ATPase as the native protein. The 140-kDa complex was suppressed by blocking Cys-707 (SH1) in S-1 but not at all by the specific modification of Cys-697 (SH2). The addition of Mg (1 mM) increased selectively the yield of the 140-kDa product with both native S-1 and SH2-blocked S-1. The cross-linking between the MBS-actins and SH1-modified S-1 in the presence of MgADP did not yield the 140-kDa species. The cross-linking of the MBS-actins to fluorescently labeled split S-1 showed the conjugation of actin to the 50-kDa fragment in the 180-kDa species and to the 20-kDa fragment in the 140-kDa derivative. The data suggest that in the G- and F-MBS-actin–S-1 complexes the cross-linkable lysine side chain on actin to which the maleimidobenzoyl arm was attached is within 0.9–1.0 nm from two different S-1 heavy-chain segments, one of which includes the SH1 thiol; these may be spatially related, forming together an unique actin recognition site in S-1.

Previously, we have described for the first time the production and some of the main structural and functional features of (maleimidobenzoyl)-G-actin (Bettache et al., 1989, 1990). This derivative, which results from the reaction of skeletal G-actin with (*m*-maleimidobenzoic acid) *N*-hydroxysuccinimide ester, contains few intramolecular cysteine–lysine cross-links which stabilize the monomeric form of actin against the salt and myosin S-1[1] induced polymerizations. This peculiarity has permitted the initiation of solution studies on the reversible complexes formed between G-actin and the two-skeletal S-1 isoenzymes (Bettache et al., 1990). The MBS-G-actin seemed to represent a useful tool to gain further insights into the mechanism of the actomyosin interaction. Most of our observations have been confirmed by a recent work of Arata (1991) conducted along similar lines of investigations. In addition, more recently, Miki and Hozumi (1991) described the ability of MBS-G-actin to polymerize in the presence of phalloidin and analyzed the activation of S-1 by the resulting MBS-F-actin as well as the regulation of the MBS-F-actin–S-1 complex by the tropomyosin–troponin system.

[†] This research was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, and the Association Française contre les Myopathies. N.B. is grateful to the Association Française contre les Myopathies for a fellowship.

[*] To whom correspondence should be addressed.

[1] Abbreviations: S-1, myosin subfragment 1; acto-S-1, actomyosin subfragment 1; 1,5-IAEDANS, *N*-(iodoacetyl)-*N′*-(5-sulfo-1-naphthyl)ethylenediamine; NaDodSO$_4$, sodium dodecyl sulfate; ATPase, adenosine-5′-triphosphatase; DTE, dithioerythritol; MBS, (*m*-maleimidobenzoic acid) *N*-hydroxysuccinimide ester; MBS-actin, (maleimidobenzoyl)actin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid MOPS, 3-(*N*-morpholino)propanesulfonic acid; AN-S-1, anthroyl-S-1; ANN, 9-anthroylnitrile; EDANS, *N*-(5-sulfo-1-naphthyl)ethylenediamine; MTMR, tetramethylrhodamine-5-(and -6)-maleimide; pPDM, *N,N′*-(1,4-phenylene)dimaleimide.

0006-2960/92/0431-389$03.00/0

In the course of our previous studies, we have also shown that MBS-G-actin contains on its surface an average of one free maleimide group generated by the monofunctional N-acylation of some lysine residue(s) (Bettache et al., 1989). We have exploited this structural property to couple covalently the MBS-actin monomer to the 50-kDa segment of the S-1 heavy chain, at or very close to the strong F-actin binding region since it has abolished the interaction of F-actin with the permanently attached S-1. To extend further our knowledge on the interaction sites between actin and the myosin head heavy chain, we have reinvestigated in the present work the cross-linking of S-1 to both MBS-G-actin and MBS-F-actin under various experimental conditions. The data presented show that in the two kinds of acto-S-1 complexes the maleimidobenzoyl group anchored in MBS-actin and spanning a cross-linking distance of 0.9–1.0 nm mediates a cross-linking reaction not only with the central 50-kDa fragment of the heavy chain but also with its COOH-terminal 20-kDa region. The covalent union between the latter domain and the MBS-actins involves specifically Cys-707 (SH1 thiol) and does not at all involve Cys-697 (SH2 thiol). Our cross-linking results provide direct structural evidence for the close proximity of the SH1 thiol region to the actin surface and strengthen the idea from other studies employing indirect approaches suggesting the interaction of actin with the heavy-chain portions flanking the SH1 thiol (Suzuki et al., 1987, 1990; Barden et al., 1989; Eto et al., 1990; Keane et al., 1990).

MATERIALS AND METHODS

Chemicals. Endoproteinase Arg-C was purchased from Boehringer-Mannheim. The sodium salt of *N*-(5-sulfo-1-naphthyl)ethylenediamine (EDANS) and phalloidin from *Amanita phalloides* were obtained from Sigma. 2-Iminothiolane hydrochloride and *N,N'*-(1,4-phenylene)dimaleimide (pPDM) were purchased from Fluka. (*m*-Maleimidobenzoic acid) *N*-hydroxysuccinimide ester (MBS) was from Pierce. 9-Anthroylnitrile (ANN) and tetramethylrhodamine-5-(and -6-)maleimide (MTMR) were purchased from Molecular Probes (Junction City, OR). All other reagents were of the highest analytical grade.

Proteins. 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 further purified over Sephacryl S-200 (Chaussepied et al., 1986a). The S-1 (A$_1$) and S-1 (A$_2$) 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 20000 cycles/s in a Microson cell disruptor Model XL 2005, and then centrifuged at 180000*g* for 1 h at 4 °C. MBS-G-actin was prepared as described by Bettache et al. (1989, 1990). The chemical reaction was terminated by gel filtration over Sephadex G-25. The fluorescent labeling of S-1 in the NH$_2$-terminal 27-kDa fragment with ANN was performed according to Hiratsuka (1989). Cys-697 (SH2) of S-1 was fluorescently labeled with MTMR following the procedure of Kasprzak et al. (1989). About 0.8 mol of MTMR was incorporated/mol of protein. S-1 was modified at Cys-707 (SH1) with 1,5-IAEDANS according to Duke et al. (1976). The S-1 internally cross-linked with pPDM between Cys-540 and SH2 was prepared as described by Chaussepied et al. (1986b). The derivative (75–21 kDa)–S-1 was produced by limited digestion of S-1 with Arg-C protease as reported by Bertrand et al. (1989).

Protein concentrations were estimated by use of absorption coefficients of $A^{1\%}_{290\mathrm{nm}}$ = 6.3 cm^{-1} for G-actin (Lehrer & Kerwar, 1972) and $A^{1\%}_{280\mathrm{nm}}$ = 7.5 cm^{-1} for S-1 (Weeds & Pope, 1977). The concentrations of the modified proteins were determined by the Bradford assay (Bradford, 1976) with the respective native proteins as standards.

Polymerization of MBS-G-Actin. The gel-filtered MBS-G-actin, prepared without treatment with glycine and DTE, was polymerized in G buffer (1.2 mg/mL) containing 10 mM KCl, 5 mM MgCl$_2$, and a 2-fold molar excess of phalloidin (Miki & Hozumi, 1991). After 30 min of incubation at room temperature, the mixture was centrifuged at 180000*g* for 30 min at 4 °C. The pellet, consisting of MBS-F-actin, was resuspended in G buffer supplemented with 2 mM MgCl$_2$.

Cross-Linking Reactions. MBS-G-actin or MBS-F-actin (1.2 mg/mL) in G buffer, pH 7.0 or pH 8.0, was mixed at 20 °C with native S-1 or modified S-1 (dissolved in 10 mM MOPS, pH 7.0, or in 10 mM Hepes, pH 8.0) at a molar ratio of 1:1. The coupling reactions were conducted in the interval time of 45 min, in the absence and presence of 1 mM Mg^{2+}-ADP or 1 mM MgCl$_2$, and were quenched by the addition of a 5-fold molar excess of DTE over MBS-actin. The cross-linking process was monitored by NaDodSO$_4$–acrylamide gel electrophoresis.

For the identification of actin in the cross-linked acto-75 kDa and acto-21 kDa peptides, a specific fluorescent labeling of the actin moiety was achieved immediately after the conjugation of MBS-G-actin and (75–21 kDa)–S-1, using the following procedure: first, a 10 mM EDANS solution in 100 mM MOPS, pH 7.5 (stock solution 100 mM in dimethylformamide) was reacted with an equimolar concentration of 2-iminothiolane (dissolved at 500 mM in 500 mM MOPS, pH 7.0) for 15 min at 40 °C. The thiolated EDANS preparation was then added at 1 mM to the protein cross-linking mixture; after incubation for 30 min at 25 °C, the thiolated dye was selectively incorporated into MBS-actin by alkylation with the available maleimide groups. The proteins were separated from excess reagent by filtration over a PD 10 column (Pharmacia) in G buffer, pH 7.0.

Electrophoresis. NaDodSO$_4$–polyacrylamide gradient gel electrophoresis (5–18%) was carried out as described previously (Mornet et al., 1981). Fluorescent bands were located in the gels by illumination with an ultraviolet light before staining with Coomassie blue (Weber & Osborn, 1969). The following proteins were used as molecular mass markers: myosin heavy chain (220 kDa), aldolase (158 kDa), S-1 heavy chain (95 kDa), bovine serum albumin (68 kDa), and soybean trypsin inhibitor (21 kDa).

ATPase Measurements. At different times of the cross-linking reaction between MBS-F-actin and S-1, samples (100 μL) containing 0.075 mg of S-1 were added to a medium (900 μL) containing 50 mM Tris-HCl, 5 mM ATP, 10 mM KCl, and 2.5 mM MgCl$_2$, pH 8.0. After 2 min of incubation at 25 °C, an aliquot was taken and precipitated with 15% TCA. Inorganic P$_i$ was measured colorimetrically as previously reported (Mornet et al., 1979).

RESULTS AND DISCUSSION

pH-Dependent Cross-Linking of the S-1 Heavy Chain to MBS-G-Actin and MBS-F-Actin. As illustrated in Figure 1, the reaction of G-actin with the bifunctional reagent MBS generates the MBS-G-actin derivative containing a free maleimidobenzoyl group that has previously served for a specific covalent coupling of the actin polypeptide to the S-1 heavy chain (Bettache et al., 1989). The rapid and extensive po-

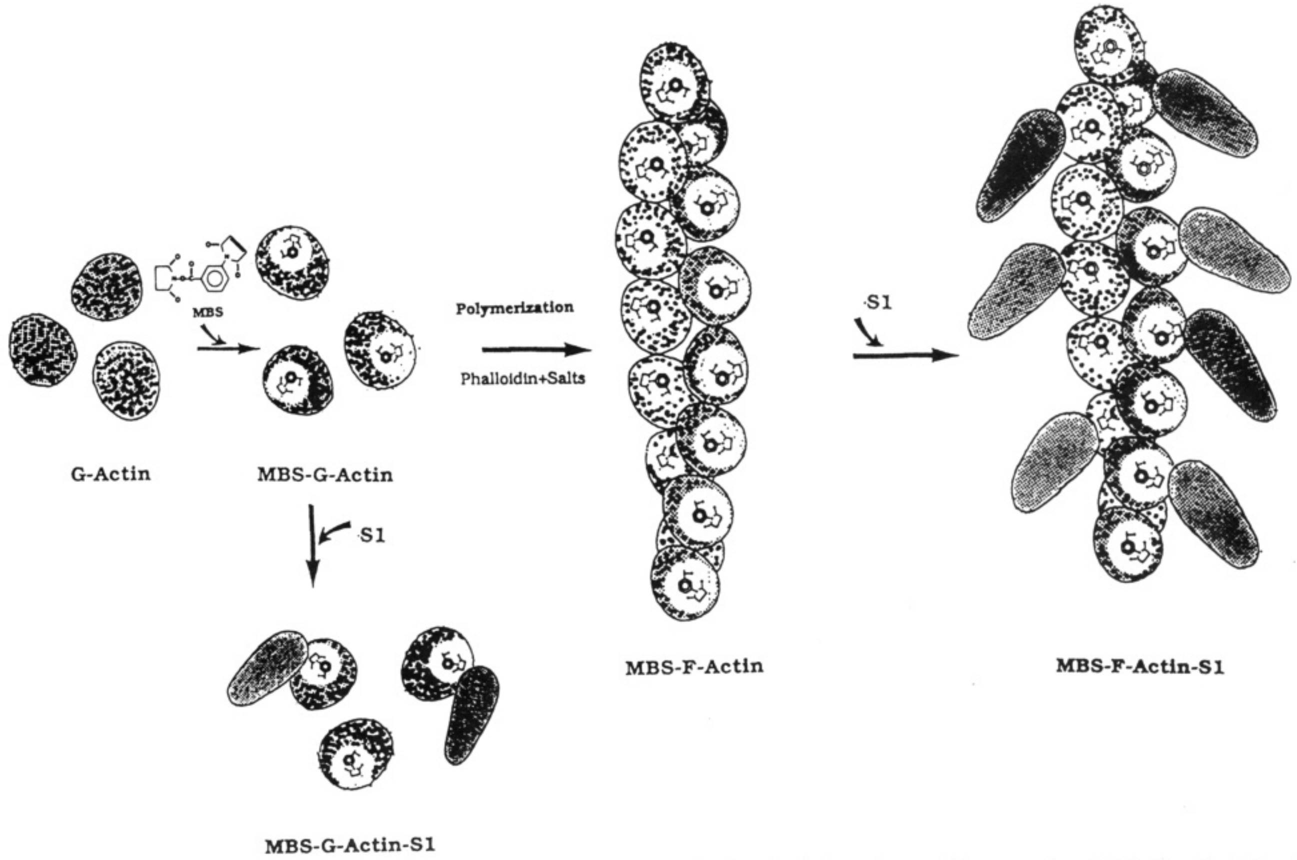

FIGURE 1: Schematic diagram illustrating the experimental approaches serving for the design of reversible or covalent MBS-G-actin–S-1 and MBS-F-actin–S-1 complexes. The reactive maleimidobenzoyl group is present within each actin monomer.

lymerization of MBS-G-actin catalyzed by phalloidin in the presence of $MgCl_2$ (Miki & Hozumi, 1991) offers the additional possibility to compare the cross-linking processes of S-1 to the monomeric and polymeric forms of MBS-actin and to assess any influence of the polymerization event on the conjugation of the two proteins. The cross-linking reactions were conducted in the pH range 7.0–8.0 in order to detect possible changes in the nature or chemical reactivity of the amino acid side chain(s) of S-1 during alkylation by the vicinal maleimide moiety of MBS-actin. Because the isolation of the MBS-F-actin takes at least 1 h after the preparation of the parent MBS-G-actin and taking into account the eventual opening of the maleimide ring which is known to occur in a time- and pH-dependent manner (Ishi & Lehrer, 1986), we have first carried out preliminary cross-linking experiments between S-1 and freshly produced MBS-G-actin to test the extent of stability of the maleimidobenzoyl group under our experimental conditions. This was done simply by incubating samples of the actin derivative at room temperature in G buffer, pH 7.0 or pH 8.0, for various time intervals before the addition of S-1. Gel electrophoresis of the reaction mixtures followed by quantitative densitometric scanning of the 180-kDa cross-link band indicated that at least 75% of the cross-linking capacity of the MBS-G-actin was conserved after its incubation at either pH for 60 min (data not shown). Assuming that the maleimide group is as stable in G-actin as in F-actin, these observations strongly suggest that the isolated MBS-F-actin material still contains a significant amount of the reactive maleimide groups initially incorporated.

In Figure 2 are presented the time courses of the coupling reactions between S-1 and either MBS-G-actin or MBS-F-actin which were performed in parallel and were analyzed by gel electrophoresis. For both MBS-actin derivatives, the pH of the cross-linking reaction had a striking effect on the gel pattern. At pH 7.0, the MBS-G-actin–S-1 condensation led to the progressive production of two new species with molecular mass values of 180 and 140 kDa (Figure 2A). The amount of the higher molecular weight product was noticeably greater than that of the other. At the alkaline pH, the 180-kDa species was rapidly and extensively accumulated whereas only a very limited amount of the 140-kDa entity was observable. The latter finding is in accordance with our previous observations describing in detail the generation of the 180-kDa band as the major actin–heavy chain adduct upon incubation of the MBS-G-actin–S-1 complex at pH 8.0 (Bettache et al., 1989). To ensure that the 140-kDa band was not just resulting from a trivial collision between S-1 and MBS-actin molecules and that it was rather deriving from a specific interaction in solution between the two proteins, the cross-linking experiments were carried out also after a 5-min treatment of MBS-G-actin with 1 mM EDTA which is known to induce the denaturation of actin but not of S-1. This unfolding of actin resulted in a total suppression of the 140-kDa band and the production of only a faint 180-kDa band (data not shown). Therefore, we conclude that the native state of the MBS-G-actin is required for the appearance of either band. The relative yield of the two products did not change by varying the actin:S-1 molar ratio between 1:1 and 3:1, and their highest yield was reached when the molar ratio was near 1:1. The S-1 isoenzymes employed separately gave essentially the same cross-linking patterns. The small amount of the higher molecular mass product (260 kDa) formed in particular with MBS-G-actin may result from the cross-linking of the S-1 heavy chain to more than one actin monomer. Indeed, this product did not form with MBS-actin alone (Figure 2A, lane T1) but formed only upon the addition of S-1.

Examination of the electrophoretic gels shown in Figure 2B clearly indicates that no oligomers were formed during either

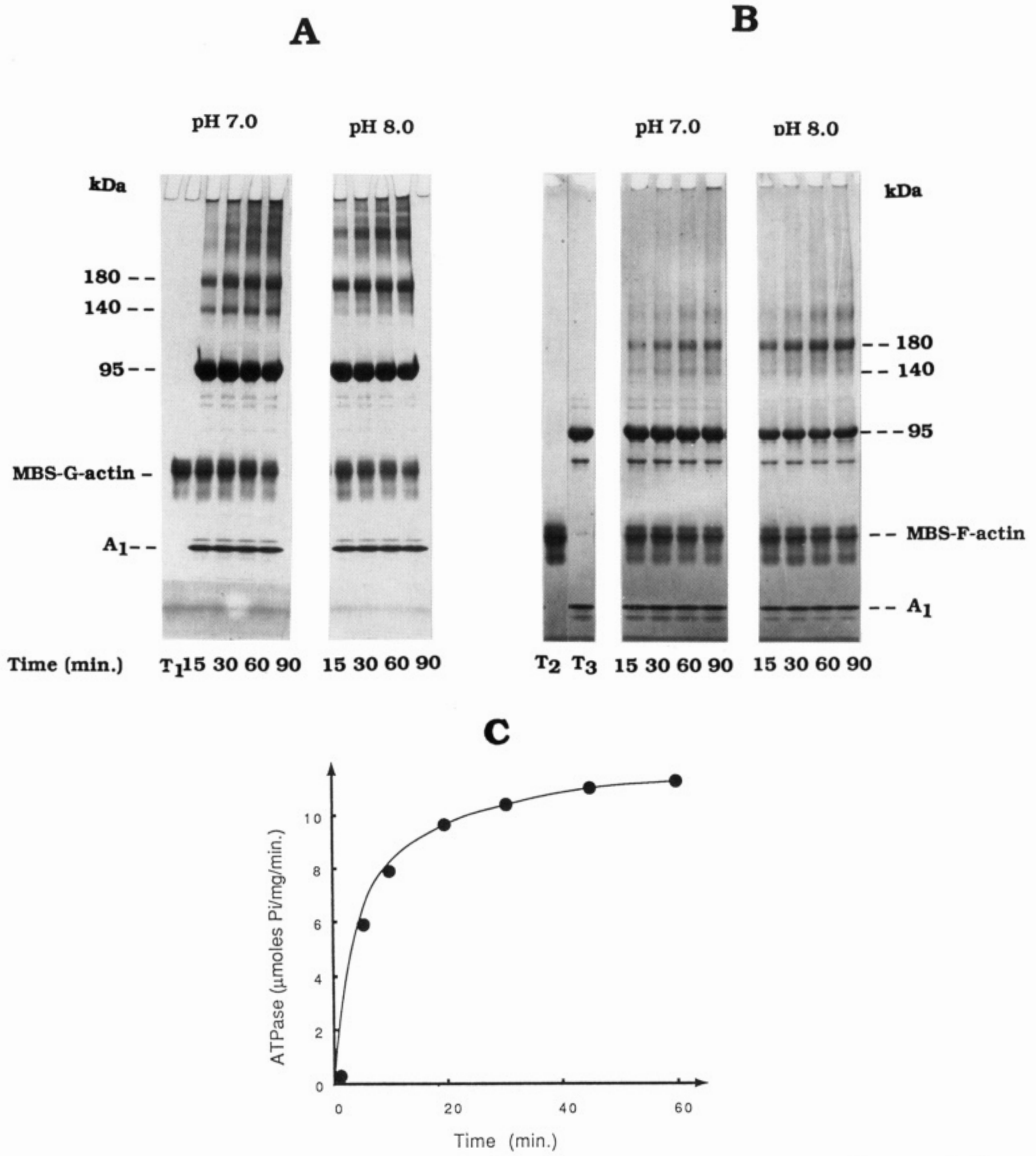

FIGURE 2: Influence of the pH on the covalent coupling of S-1 to MBS-G-actin (A) and MBS-F-actin (B). The cross-linking reactions were carried out in G buffer at pH 7.0 or pH 8.0 as specified under Materials and Methods. NaDodSO$_4$ gel electrophoresis was performed on a 5–18% gradient acrylamide. T$_1$ = MBS-G-actin, T$_2$ = MBS-F-actin, and T$_3$ = S-1 (A1) before cross-linking. (C) Time course of the superactivation of S-1 during conjugation to MBS-F-actin. The coupling reaction was conducted at pH 8.0. At various time intervals, the Mg^{2+}ATPase activity was measured on appropriate protein aliquots as indicated under Materials and Methods.

the polymerization step of the MBS-G-actin (Figure 2B, lane T2) or the incubation of the resulting MBS-F-actin with S-1 at either pH 7.0 or pH 8.0. This suggests that the maleimidobenzoyl arm present in each monomer did not undergo any cross-linking reaction with an adjacent subunit within the MBS-actin filament. In contrast, the addition of a stoichiometric concentration of S-1 generated the 180- and 140-kDa species, whose relative amounts at either pH 7.0 or pH 8.0 were apparently similar to those observed with the MBS-G-actin under the same conditions. The lower yield of the overall cross-linking process with the MBS-F-actin as compared to that with the MBS-G-actin was the first feature which distinguishes the two actin derivatives in their coupling to S-1. Moreover, Mg^{2+}ATPase determinations in the course of the conjugation of MBS-F-actin and S-1 at pH 8.0 showed that the production of the major 180-kDa adduct was accompanied by a significant elevation of the acto-S-1 ATPase activity (Figure 2C). Thus, the covalent union of S-1 with the MBS-F-actin causes also the superactivation of its ATPase as observed with the native F-actin (Mornet et al., 1981). This result is in agreement with the recent finding of Miki and Hozumi (1991) that the turnover rate of the reversible S-1 Mg^{2+}-ATPase stimulated by MBS-F-actin was similar to that promoted by the unmodified F-actin. It contrasts with the previously observed inability of the homologous 180-kDa covalent complex from MBS-G-actin and S-1 to exhibit such an enhanced Mg^{2+}ATPase (Bettache et al., 1989). Finally, another peculiarity which distinguishes the two MBS-actins was provided by the influence of the Mg^{2+} ions on the cross-linking reaction. As illustrated in Figure 3, the addition of 1 mM MgCl$_2$ to the MBS-G-actin–S-1 mixture increased selectively the yield of the 140-kDa band but it did not change the pattern of the reaction involving MBS-F-actin. The addition of Mg^{2+}ADP up to 5 mM gave essentially the same results, whereas ADP used alone was without effect on either reaction. The observed influence of Mg^{2+} on the coupling behavior of MBS-G-actin to S-1 is most likely linked with the well-documented conformational change that is specifically induced in G-actin by millimolar concentrations of this ion (Frieden, 1982; Zimmerle et al., 1987). Thus, although

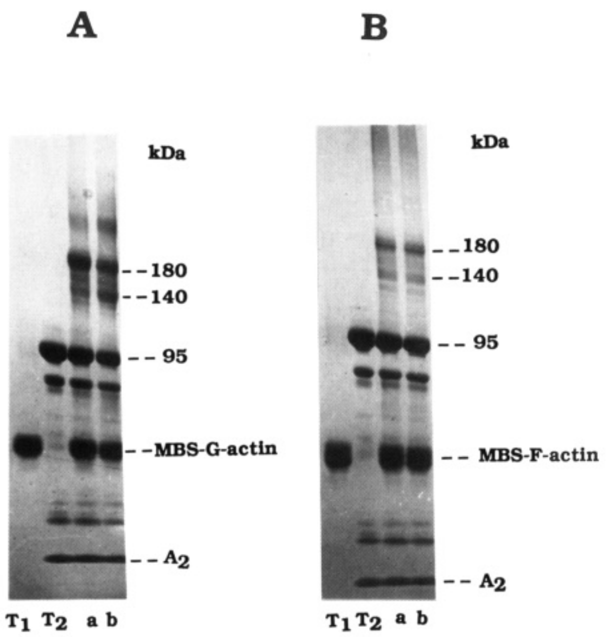

FIGURE 3: Effect of magnesium on the production of the 140-kDa MBS-G-actin–S-1 covalent complex. S-1 (A_2) was incubated at pH 7.0 for 45 min with either MBS-G-actin (A) or MBS-F-actin (B) in the absence (lanes labeled a) and presence (lanes labeled b) of 1 mM $MgCl_2$. The reaction mixtures were analyzed by gel electrophoresis as in Figure 1. T_1 = MBS-G-actin or MBS-F-actin, and T_2 = S1 (A_2) before cross-linking.

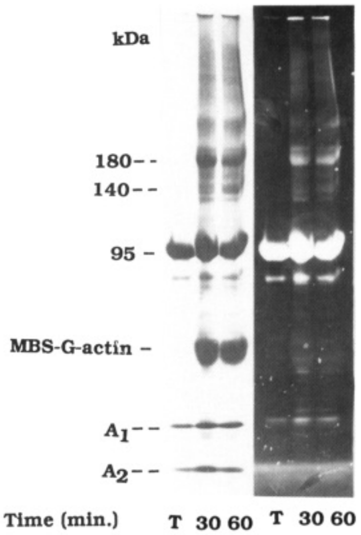

FIGURE 4: Cross-linking of MBS-G-actin to anthroyl-S-1. MBS-G-actin was reacted at pH 7.0 with the fluorescent AN-S-1 ($A_1 + A_2$). At the times indicated, aliquots were subjected to gel electrophoresis. Protein bands were viewed under UV light (right panel) and then stained with Coomassie blue (left panel). T = AN-S-1 before cross-linking.

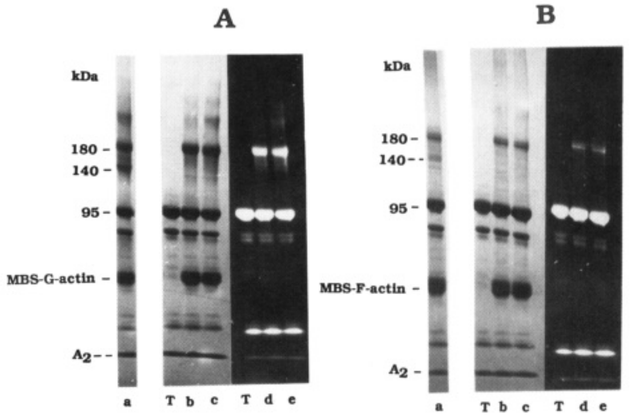

FIGURE 5: Suppression of the 140-kDa MBS-actin–S-1 heavy-chain covalent complex by blocking SH-1. AEDANS-labeled S-1 (A_2) was coupled at pH 7.0 for 45 min to either MBS-G-actin (A) or MBS-F-actin (B) in the absence (lanes b and d) and presence (lanes c and e) of 1 mM Mg^{2+}ADP. After gel electrophoresis, the protein bands were located by fluorescence (lanes d and e) or by staining with Coomassie blue (lanes b and c). As a control, native unmodified S-1 (A_2) was also incubated with MBS-G-actin or MBS-F-actin (lanes labeled a). T = AEDANS–S-1 (A_2) before cross-linking.

MBS-G-actin is resistant to the Mg^{2+}-dependent polymerization (Bettache et al., 1989), it seems to be still conformationally sensitive to the binding of the divalent metal.

Involvement of the SH-1 Thiol in the Coupling of S-1 to the MBS-Actins. While the incorporation of the 95-kDa S-1 heavy chain into the 180-kDa adduct was previously established (Bettache et al., 1989), its presence as a member of the new 140-kDa species remains to be demonstrated. This was achieved by joining, at pH 7.0, the MBS-G-actin with S-1 labeled with the fluorescent 9-anthroyl group. This fluorophore was selected as it binds specifically to the 27-kDa NH_2-terminal segment and not at all to the putative actin binding 50-kDa and 20-kDa fragments of the heavy chain (Hiratsuka, 1989). Its use avoids any possible perturbation of the cross-linking between MBS-actin and either of these two functional regions, which may result from their chemical modification. As shown in Figure 4, the cross-linking pattern displayed by the S-1 derivative was identical to that obtained with the native S-1. The fluorescence was distributed over the 180-kDa and 140-kDa bands. In some gels these migrated as protein doublets incorporating the fluorescence of S-1 and probably reflecting the electrophoretic heterogeneity of the MBS-actin itself.

Previously, the efficient cross-linking we observed at pH 8.0, between MBS-G-actin and S-1 modified at the SH-1 thiol with 1,5-IAEDANS, indicated that the major 180-kDa product formed was not deriving from a reaction of the maleimidobenzoyl group on actin with the reactive SH-1 or SH-2 thiols in S-1 (Bettache et al., 1989). In the present investigation, we have further analyzed the coupling of AEDANS–S-1 to MBS-G-actin and to MBS-F-actin at neutral pH, a condition favoring the accumulation of the 140-kDa entity. The data depicted in part A (lanes b and d) and part B (lanes b and d) of Figure 5 reveal unambiguously that the blocking of SH-1 completely abolished only the 140-kDa band from the cross-linking patterns of MBS-G-actin and MBS-F-actin, respectively. In contrast, the 180-kDa band was somewhat even more intense with the modified S-1 than with the native S-1 used as control (Figure 5A,B, lanes labeled a). The same cross-linking reactions were also carried out in the presence of 5 mM Mg^{2+}ADP, a ligand which increases the chemical reactivity of the SH2 thiol in S-1 and promotes its extensive substitution by the maleimide reagents (Reisler, 1982). However, no production of the 140-kDa derivative could be observed in the reaction mixture containing either MBS-G-actin or MBS-F-actin (Figure 5A,B, lanes c and e). In contrast, the coupling of the MBS-actins to S-1 specifically blocked at the SH2 thiol with the fluorescent tetramethylrhodamine group (Kasprzak et al., 1989; Miyanishi & Borejdo, 1989) generated a fluorescent 140-kDa band, the intensity of which increased when the MBS-G-actin–S-1 cross-linking was performed in the presence of Mg^{2+} ions (Figure 6). An identical result was obtained by reacting MBS-G-actin with S-1 intramolecularly cross-linked with pPDM between Cys-540 and SH2 (Chaussepied et al., 1986b) (data not shown). Consequently,

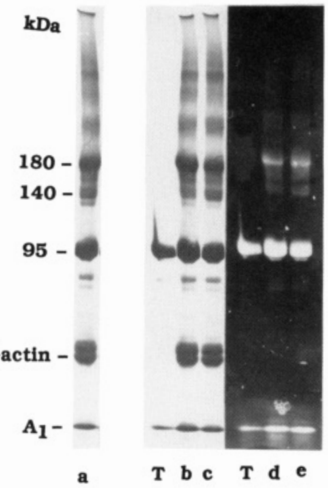

FIGURE 6: Conjugation of MBS-G-actin to SH2-modified S-1. The MBS-G-actin was incubated at pH 7.0, with fluorescent MTMR-labeled S-1 (A_1), in the absence (lanes b and d) and presence (lanes c and e) of 1 mM $MgCl_2$. A coupling reaction mixture consisting of native S-1 and MBS-G-actin (lane a) was used as control. After 45 min of conjugation, the protein solutions were analyzed by gel electrophoresis. Coomassie-stained gels (lanes b and c) were compared with corresponding fluorescent gels (lanes d and e) viewed under UV light. T = MTMR–S-1 (A_1) before cross-linking.

the sulfhydryl of either Cys-697 (SH2) or Cys-540 did not contribute to the cross-linking process and the new 140-kDa species was resulting mainly from a specific covalent coupling of the SH1 site of the S-1 heavy chain (Cys-707) to the maleimidobenzoyl group of MBS-actin.

To confirm the participation of the SH1 thiol in the acto-S-1 condensation, the MBS-G-actin was also conjugated, at pH 7.0, to (75–21 kDa)–S-1 which was split at the 50–20-kDa junction. The reaction gave rise to two new products migrating with masses of 130 and 70 kDa, which incorporated the fluorescence of actin labeled with thiolated EDANS (Figure 7, lanes b and c). They must consist of MBS-actin joined to the NH_2-terminal 75-kDa fragment and to the COOH-terminal 20-kDa fragment, respectively. The acto-75 kDa product was the only derivative detected previously when the same reaction was conducted at pH 8.0 and reflects the reported coupling of the MBS-actin to the 50-kDa domain (Bettache et al., 1989). The additional acto-21 kDa adduct, identified at pH 7.0, substantiates the conjugation between the MBS-actin and the SH1 thiol residing in the 20-kDa region of the S-1 heavy chain.

The observed labeling of the MBS-actin with thiolated EDANS after its covalent conjugation with the S-1 heavy chain indicates that only a fraction of the maleimide group in the actin derivative was attached to the cross-linkable lysine whereas the remaining part was bound to some other lysines not participating in the cross-linking process and which were substituted by the fluorescent reagent. Since the maleimidobenzoyl arm spans 0.9–1.0 nm, the actin cross-linkable lysine was located in the MBS-actin–S-1 complexes, no more than 0.9–1.0 nm from two particular heavy-chain segments. One comprises the SH1 thiol, and the other includes the cross-linkable amino acid side chain of the 50-kDa domain. The different effect of the pH on the degree of cross-linking of these two sites to the MBS-actins suggests that they have different microenvironments. The cross-linking of MBS-actin at either of these two sites gives rise to an unique species. Thus, blocking SH1 abolishes the 140-kDa species without affecting the 180-kDa product. It is unlikely that these two species are generated by the presence of heterogeneous heavy chains, and as we have shown previously (Bettache et al., 1989), the light chains are not involved in the cross-linking reaction either at pH 7.0 or pH 8.0. The different molecular mass values displayed by the 180- and 140-kDa species may result from different conformations for the cross-links established between the actin monomer and the 50-kDa peptide and between actin and the 20-kDa fragment, respectively. However, previous intramolecular cross-linking studies have indicated proximity relationships between SH1 and various peptide stretches of the 50-kDa fragment (Ue, 1987; Sutoh & Lu, 1987; Muno & Sekine, 1988). Therefore, as pictured in Figure 8, one may envision that the two sites could be located one close to the other in the three-dimensional structure of the heavy chain, forming a unique actin binding region. Because the covalent coupling of S-1 to MBS-G-actin blocks its rigor interaction with F-actin (Bettache et al., 1989), this particular area of the heavy chain must face the actin subdomain 1, which contains all the known recognition sites for S-1 (Kabsch et al., 1990) and most likely also the cross-linkable lysine of MBS-actin.

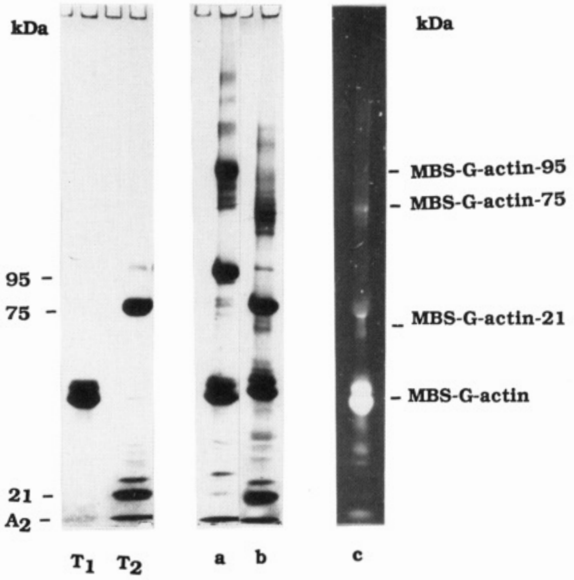

FIGURE 7: Characterization of the S-1 heavy-chain fragments cross-linked to MBS-G-actin at pH 7.0. The G-actin derivative was allowed to react at pH 7.0, for 45 min, with the Arg-C protease split (75–21 kDa)–S-1 (A_2). At the end of the condensation process, the mixture was supplemented with thiolated EDANS as described under Materials and Methods. The labeled proteins isolated over a PD 10 column were electrophoresed on a 5–18% gradient acrylamide gel. The fluorescence pattern of the gel (lane c) was compared with the corresponding Coomassie blue stained gel (lane b); a = control cross-linked MBS-G-actin–S-1 (A_2) complex; T1 and T2 = MBS-G-actin and (75–21 kDa)–S-1 (A_2), respectively, before conjugation.

In conclusion, our present cross-linking studies are in agreement with NMR results employing a spectroscopic probe attached to SH1, indicating that the actin site on S-1 involves SH1 and involves much less SH2 (Barden et al., 1989). The cross-linking distance of 0.9–1.0 nm between the SH1 thiol and MBS-actin compares well with the value of ≤1.5 nm deduced for the SH-1–actin interface by NMR analysis of the complex between F-actin and S-1 modified on SH1 with a nitroxyl spin label (Keane et al., 1990). However, while the conclusions of these NMR investigations were based on the use of S-1 substituted on SH1, a condition which may alter the interaction properties of actin with S-1 (Titus et al., 1989; Yamamoto & Sekine, 1987), our own cross-linking data were obtained mainly with S-1 having an intact SH1. They provide also a more direct support for the observations from studies using synthetic heavy-chain peptides that the immediate vi-

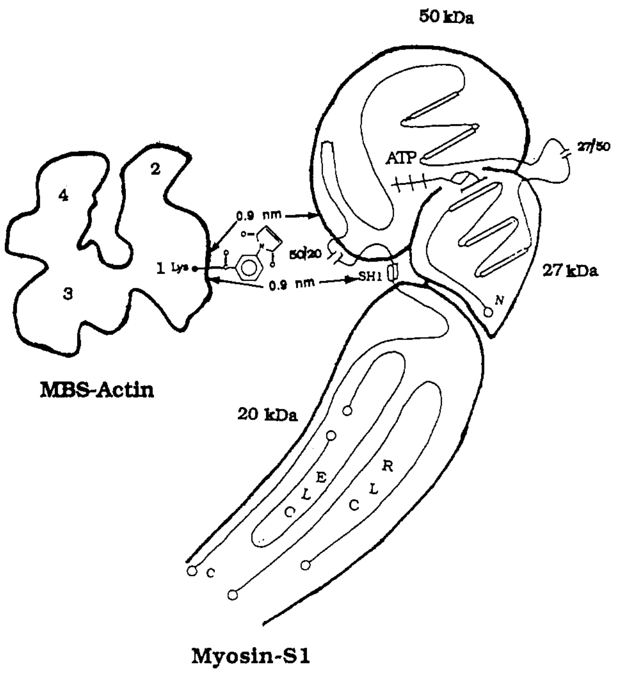

FIGURE 8: Diagram representing the putative spatial relationship of the cross-linkable (maleimidobenzoyl)lysine in the MBS-actin monomer relative to the two heavy-chain segments of the myosin head which undergo a selective coupling to the actin derivative. These include the SH1 thiol of the 20-kDa region and the reactive, unknown site of the 50-kDa domain. ELC and RLC are the essential and regulatory light chains of S-1.

cinity of SH1 may interact with actin (Suzuki et al., 1987, 1990; Keane et al., 1990; Eto et al., 1990).

Registry No. ATPase, 9000-83-3; Cys, 52-90-4, 7439-95-4.

REFERENCES

Applegate, D., & Reisler, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7109–7112.

Arata, T. (1991) *J. Biochem. (Tokyo) 109*, 335–340.

Barden, A. J., Phillips, L., Cornell, B. A., & dos Remedios, C. G. (1989) *Biochemistry 28*, 5895–5901.

Bertrand, R., Derancourt, J., & Kassab, R. (1989) *FEBS Lett. 246*, 171–176.

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.

Bradford, N. M. (1976) *Anal. Biochem. 72*, 248–254.

Chaussepied, P., Bertrand, R., Audemard, E., Pantel, P., Derancourt, J., & Kassab, R. (1983) *FEBS Lett. 161*, 84–88.

Chaussepied, P., Mornet, D., Audemard, E., Derancourt, J., & Kassab, R. (1986a) *Biochemistry 25*, 1134–1140.

Chaussepied, P., Mornet, D., & Kassab, R. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2037–2041.

Duke, J., Takashi, R., Ue, K., & Morales, M. F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 302–306.

Eisenberg, E., & Kielley, W. W. (1974) *J. Biol. Chem. 249*, 4742–4748.

Eto, M., Suzuki, R., Morita, F., Kuwayama, H., Nishi, N., & Tokura, S. (1990) *J. Biochem. (Tokyo) 108*, 499–504.

Frieden, C. (1982) *J. Biol. Chem. 257*, 2882–2886.

Hiratsuka, T. (1989) *J. Biol. Chem. 264*, 18188–18194.

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

Kasprzak, A. A., Chaussepied, P., & Morales, M. F. (1989) *Biochemistry 28*, 9230–9238.

Keane, A. M., Trayer, J. P., Levine, B. A., Zeugner, C., & Ruegg, J. C. (1990) *Nature 344*, 265–268.

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

Miki, M., & Hozumi, T. (1991) *Biochemistry 30*, 5625–5630.

Miyanishi, T., & Borejdo, J. (1989) *Biochemistry 28*, 1287–1294.

Mornet, D., Pantel, P., Audemard, E., & Kassab, R. (1979) *Eur. J. Biochem. 100*, 421–431.

Mornet, D., Pantel, P., Bertrand, R., Audemard, E., & Kassab, R. (1980) *FEBS Lett. 123*, 54–58.

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

Mornet, D., Ue, K., Chaussepied, P., & Morales, M. F. (1986) *Eur. J. Biochem. 159*, 555–561.

Muno, K., & Sekine, T. (1988) *J. Biochem. (Tokyo) 104*, 427–432.

Offer, G., Moos, C., & Starr, R. (1973) *J. Mol. Biol. 74*, 653–679.

Reisler, E. (1982) *Methods Enzymol. 85*, 84–93.

Sutoh, K., & Lu, R. C. (1987) *Biochemistry 26*, 4511–4516.

Suzuki, R., Nishi, N., Tokura, S., & Morita, F. (1987) *J. Biol. Chem. 262*, 11410–11412.

Suzuki, R., Morita, F., Nishi, N., & Tokura, S. (1990) *J. Biol. Chem. 265*, 4939–4943.

Titus, M. A., Ashiba, G., & Szent-Györgyi, A. G. (1989) *J. Muscle Res. Cell Motil. 10*, 25–33.

Trayer, H. R., & Trayer, I. P. (1988) *Biochemistry 27*, 5718–5727.

Ue, K. (1987) *Biochemistry 26*, 1889–1894.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Weeds, A. G., & Taylor, R. S. (1975) *Nature 257*, 54–56.

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

Yamamoto, K., & Sekine, T. (1987) *J. Biochem.* (Tokyo) *101*, 519–523.

Zimmerle, C. T., Patane, K., & Frieden, C. (1987) *Biochemistry 26*, 6545–6552.