FEBS Letters 345 (1994) 113–119

FEBS 14012

The covalent maleimidobenzoyl-actin-myosin head complex

Cross-linking of the 50 kDa heavy chain region to actin subdomain-2

Raoul Bertrand, Jean Derancourt, Rhida 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 7 January 1994; revised version received 18 March 1994

Abstract

We have identified the region of actin involved in the covalent coupling of maleimidobenzoyl-G-actin to the central 50 kDa segment of the myosin-S-1 heavy chain by analyzing the structure of the maleimidobenzoyl-G-actin-S-1 conjugate using selective proteolytic digestions, amino acid sequence determinations and novel cross-linking reactions between S-1 and different maleimidobenzoyl-G-actin derivatives. The cross-linking is shown to occur only on the stretch of residues 48–67 in actin subdomain-2 with Lys-50, residing on the outer part of the DNase-I-binding loop, as the most likely site of cross-linking. Because the maleimidobenzoyl-F-actin-S-1 complex undergoes the same coupling process, the data provide experimental evidence in favor of the recent model of the rigor F-actin-S-1 complex suggesting a close contact between structural elements of the lower domain of the 50 kDa fragment and the top of actin subdomain-2.

Key words: F-actin; G-actin; Actomyosin interaction; Actin-myosin head cross-linking; Actin subdomain-2

1. Introduction

Previously, we described the production and characterization of maleimidobenzoyl-G-actin (MBS-G-actin) and maleimidobenzoyl-F-actin (MBS-F-actin) [1,2]. The former derivative was generated by the reaction of skeletal monomeric actin with the heterobifunctional agent, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MB-S), which resulted in the establishment of few intramolecular cross-links and the incorporation of an average of one maleimidobenzoyl group by the monofunctional acylation of a lysine residue of the protein. Although resistant to the salt-and myosin-S-1-induced polymerization, the MBS-G-actin could be readily converted into MBS-F-actin in the presence of phalloidin and $MgCl_2$ [2,3]. Both modified actins formed reversible and ATP-sensitive complexes with rabbit skeletal myosin-S-1 which were earlier investigated [1,3–5]. Most importantly, the free maleimidobenzoyl group in the G- or F-MBS-actin was in a position that permits the actin subunit to couple covalently to the S-1 heavy chain at the same region at which native F-actin binds to the heavy chain during rigor and the major covalent cross-link was shown to involve the central 50 kDa segment of the S-1 heavy chain [1,2]. Hence, the covalent MBS-actin-S-1 complex represents a useful tool for further assessing the molecular contacts between F-actin and myosin-S-1.

In the present study we have identified the lysine of actin engaged in the MBS-promoted conjugation process by analysing the structure of the major 180 kDa adduct consisting of the MBS-actin molecule selectively bridged to the 95 kDa S-1 heavy chain at the 50 kDa region [1]. The data show that the cross-linking takes place exclusively on the actin stretch of residues 48–67 and most likely involves Lys-50. This segment makes part of the actin subdomain-2. The findings directly demonstrate the spatial proximity of this subdomain to the S-1 heavy chain in both complexes of S-1 with G- or F-actin. They also provide an experimental support to the recent model of the rigor F-actin-S-1 complex suggesting a close interaction between residues of the 50 kDa fragment and amino acids in the loop region at the top of actin subdomain-2 [6].

2. Materials and methods

2.1. Chemicals and protein preparations

m-Maleimidobenzoic acid-*N*-hydroxysuccinimide ester (MBS) was from Sigma. α-Chymotrypsin and subtilisin carlsberg were purchased from Worhington and Serva, respectively.

G-actin, F-actin and chymotryptic myosin subfragment-1 (S-1) from rabbit skeletal muscle were prepared as described [1,2]. S-1 was labeled at SH-1 thiol with 7-diethyl-amino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) as in [7]. G-actin specifically modified at Lys-61 with fluorescein isothiocyanate (FITC) was obtained as reported earlier [8]. MBS-G-actin or FITC-labeled MBS-G-actin were prepared as previously described [4] by mixing G-actin or FITC-G-actin with 20-fold molar excess of MBS (dissolved at 8.5 mg/ml in dimethylformamide) in G-buffer (2 mM HEPES, 0.1 mM ADP, 0.1 mM $CaCl_2$ and 0.1 mM NaN_3, pH 8.0). After 2 h at 25°C, the protein solution was incubated with 25 mM KCl and 5 mM $MgCl_2$ for 15 min at 25°C and ultracentrifuged at 180,000 × *g* for 1 h at 4°C; the supernatant was then gel filtered over a Pharmacia PD-10 column equilibrated in G-buffer. MBS-G-actin split at Met-47 with subtilisin was produced as described for

* Corresponding author.

SSDI 0014-5793(94)00398-F

G-actin [9] by treatment of the actin derivative in G-buffer, pH 8.0 for 60 min at 25°C with subtilisin (protease to substrate weight ratio = 1:250).

2.2. Cross-linking reactions and proteolytic cleavages of the covalent complexes

The purified MBS-G-actin, FITC-labeled MBS-G-actin or subtilisin-split MBS-G-actin (1.5 mg/ml) was supplemented with a convenient volume of S-1 solution (15 mg/ml) in 20 mM MOPS, pH 7.5 (final actin/S-1 molar ratio = 1:1). After incubation for 45 min at 25°C the coupling process was terminated by adding dithioerythreitol (adjusted to pH 7.5) to 1 mM and the reaction mixture was ultracentrifuged at 180,000 × *g* for 1 h at 4°C. The supernatant containing the covalent complex of S-1 and either MBS-G-actin or FITC-labeled MBS-G-actin was treated at 25°C with α-chymotrypsin (protease to actin weight ratio = 1:50) in the presence of 2 mM EDTA. The same complexes were also digested in the absence of EDTA with subtilisin added at a protease to actin weight ratio of 1:250. The proteolytic reactions were monitored over the time interval 0–60 min by gel electrophoresis.

2.3. Electrophoresis and sequence analyses

SDS-PAGE was carried out using a 5–18% acrylamide gradient and a 50 mM Tris, 100 mM borate buffer pH 8.0 [10]. Fluorescent bands were located in the gel by ultraviolet illumination before staining with Coomassie blue. Molecular weight markers were: myosin heavy chain (200 kDa), S-1 heavy chain (95 kDa), bovine serum albumine (68 kDa) and actin (42 kDa). Protein bands containing the intact NH_2-terminal segment of actin were identified by Western blotting using polyclonal antibodies specifically directed against the actin sequence of residues 1–12 [11]. Isolation of pure protein material for sequence analyses was achieved either by electroelution from gel slices or by reverse-phase HPLC on a C-4 Aquapore column (2 × 100 mm) eluted with 0–90% acetonitrile gradient containing 0.1% trifluoacetic acid. NH_2-terminal sequencing was performed on an Applied Biosystems 470-A instrument connected to an online phenylthiohydantoin amino acid analyzer [11].

2.4. Binding assays

The association of F-actin with the covalent 105 kDa S-1-actinopeptide complex was studied by cosedimentation at 180,000 × *g* for 30 min in a Beckman Airfuge at 20°C in 20 mM MOPS, pH 7.5.

3. Results

3.1. MBS-promoted cross-linking of S-1 to the actin sequence of residues 1–67

The cross-linked MBS-G-actin-S-1 preparation includes, besides the uncross-linked MBS-actin-S-1 complex, the prominent 180 kDa species resulting from the covalent union between the maleimidobenzoylactin and the 95 kDa S-1 heavy chain (Fig. 1A, lane 0). It was first subjected to a controlled digestion with α-chymotrypsin in the presence of EDTA. This protease is known to cleave specifically native G-actin at Met-44 and Leu-67 [12] but it does not act at all on native S-1 [13]. The same sites are also split upon addition of EDTA [14] which unfolds the remaining actin core of residues 68–375 and induces its proteolytic degradation without affecting the structural integrity of S-1. Therefore, as shown in Fig. 1A, under the employed conditions, both the un-cross-linked actin and the covalently attached actin moiety of the 180 kDa adduct were selectively attacked by chymotrypsin. The former was rapidly cut into small peptides whereas the 180 kDa band was progressively converted into a new 105 kDa entity which incorporated the fluorescence of the S-1 heavy chain when the fluorescent CPM-labeled S-1 was employed (Fig. 1B). The 105 kDa product was eluted from the electrophoretic gel and after further purification by reversed-phase HPLC, it was submitted to NH_2-terminal microsequencing and to immunostaining with antibodies directed to the extreme NH_2-terminus of actin. The sequence analyses did not reveal any free NH_2-terminal amino acid and the immunoblot depicted in Fig. 1C, lanes c and d, demonstrated that the actin antibody strongly reacts with the 105 kDa derivative but not with the 95 kDa S1 heavy chain. It recognized also the MBS-actin and 180 kDa bands as well as faint bands in the 85–90 kDa position which presumably correspond to traces of intermolecularly cross-linked MBS-actin dimers. Collectively, these findings clearly indicate that the 105 kDa species is composed of the intact 95 kDa S-1 heavy chain cross-linked to the chymotryptic NH_2-terminal actin fragment spanning residues 1–67. Both components are known to bear an acetylated NH_2-terminus. The complete absence of peptide bond cleavage at Met-44 of the covalently bound actin peptide is in agreement with the recently described ability of S-1 to protect the segment of residues 44–50 of G-actin against chymotryptic hydrolysis [15]. Actually, the protection afforded by S-1 was reported to extend to the actin stretch 60–69. However, the use we made of EDTA during the digestion has certainly facilitated peptide bond scission at Leu-67 since the 105 kDa material was also released by chymotrypsin in the absence of EDTA but at a much slower rate.

3.2. Localization of the cross-linking site on the actin subdomain-2 within the segment 48–67

The cross-linked actin fragment contains three lysines at positions 18, 50 and 61. To delineate the MBS-substituted lysine mediating the cross-linking, we coupled S-1 to subtilisin-cleaved MBS-G-actin. This protease was shown to clip native G-actin at Met-47 into 9 kDa N-terminal and 35 kDa C-terminal fragments which remain noncovalently associated [9]. The proteolytic MBS-actin derivative displayed an identical electrophoretic profile except that the 35 kDa peptide migrated as a doublet band reflecting the doublet composition of the parent MBS-actin (Fig. 2A, lane O). Amino acid sequencing of the latter isolated fragment identified Gly-48 as the unique NH_2-terminal residue as well as its first 17 residues between Gly-48 and Ile-64 (Table 1). The conjugation of the subtilisin-split MBS-actin to S-1 led to a single new 170 kDa species (Fig. 2A) which must be the adduct of the 95 kDa heavy chain and the 35 kDa actin fragment. The generation of this product excludes the participation of Lys-18 in the coupling reaction and, combined with the data presented above, it is consistent with the location of the cross-linking site on the actin stretch 48–67. It is worth noting that the yield of the 170 kDa material was noticeably lower as compared to the 180

kDa species issued from the condensation of S-1 with the intact MBS-actin (Fig. 2A, lane T2), suggesting that cleavage at Met-47 affects the efficiency of the cross-linking between the adjacent actin segment and S-1. To confirm that the actin region 48–67 is involved in the cross-link between S-1 and the intact MBS-actin, we directly treated the covalent MBS-G-actin-S-1 complex with subtilisin (Fig. 2B). The digestion led to the breakdown of the 180 kDa adduct and the concomitant formation of the 170 kDa band with very little influence on the S-1 heavy chain structure whereas the uncross-linked MBS-actin was split into the 9 kDa and 35 kDa peptides. The 180–170 kDa conversion was only partial although the conditions used ensure the complete hydrolysis of the subtilisin-sensitive bond of MBS-actin in the absence of S-1. This feature is also in agreement with the reported protection of G-actin by binding to S-1 against the subtilisin cut at Met-47 [15].

Finally, we investigated the cross-linking of S-1 to FITC-G-actin stoichiometrically modified at Lys-61 [8] and then converted into (FITC)-MBS-actin. The incorporation of the fluorophore into native G-actin does not impair the interaction with S-1 [16] and the latter MBS-actin derivative could be coupled to S-1 with an efficiency as high as for unlabeled MBS-actin (Fig. 3A,B). Furthermore, the fluorescence of the resulting 180 kDa product was transfered by chymotryptic digestion to the corresponding 105 kDa entity (Fig. 3A,B, lanes a), hence, confirming the sequence 1–67 we assigned to the cross-linked, chymotryptic NH_2-terminal actin fragment. These results rule out the involvement of Lys-61 and point towards Lys-50 as the residue most likely engaged in the formation of the cross-link from MBS-actin to S-1. In the tertiary structure of actin, this side-chain resides on the outer part of subdomain-2 at the end of the DNAse I-binding loop [17].

Earlier, we showed that the coupling of S-1 to MBS-G-actin at the 50 kDa heavy chain fragment inhibits its cosedimentation with F-actin suggesting that the cross-linking took place at or very close to the strong F-actin-binding region of the heavy chain [1]. Following the chymotryptic digestion of the MBS-G-actin-S-1 complex and exhaustive dialysis to remove the small actin peptides, the resulting protein solution, which included only the covalent 105 kDa S-1-actin peptide complex and uncross-linked S-1 (Fig. 4A, lane c), was ultracentrifuged

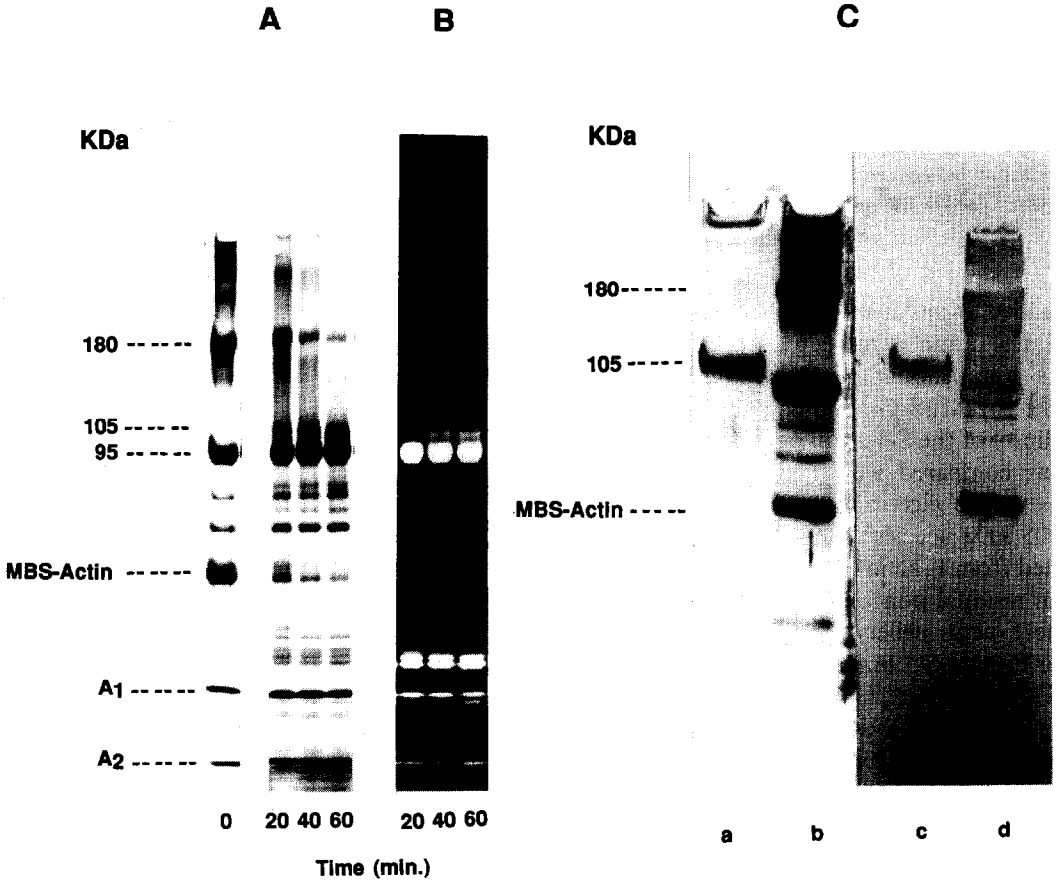

Fig. 1. Chymotryptic conversion of the 180 kDa MBS-actin-S-1 heavy chain adduct into a 105 kDa S-1 heavy chain-actinopeptide complex. (A) The MBS-G-actin-S-1 conjugate was digested with α-chymotrypsin in the presence of EDTA, as described in section 2; at the times indicated, samples were analyzed by gel electrophoresis. (B) The proteolytic reaction was also carried out on the complex of MBS-G-actin and fluorescent CPM-labeled S-1. (C) The isolated 105 kDa band (lane c) and the cross-linking reaction mixture before digestion (lane d) were submitted to immunoblotting with antibodies directed to the actin sequence 1–12 and to staining with Coomassie blue (lanes a and b, respectively).

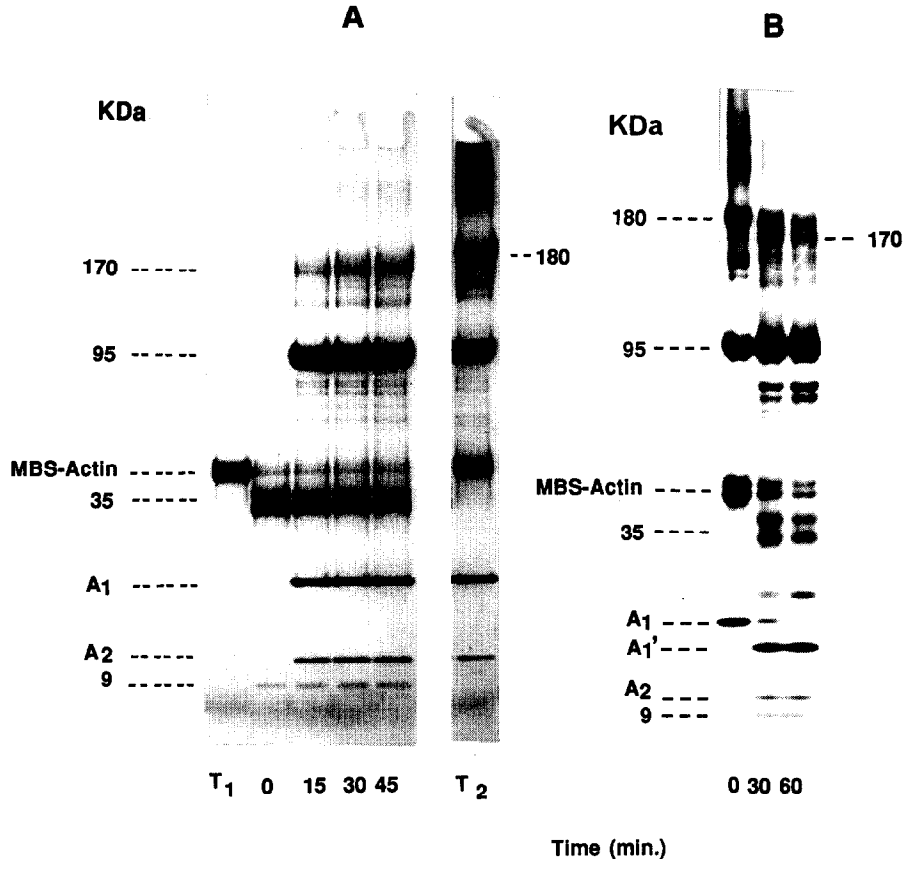

Fig. 2. Covalent coupling of the S-1 heavy chain to the 35 kDa COOH-terminal fragment of subtilisin-cleaved MBS-G-actin. (A) Isolated MBS-G-actin (lane T1) was first split by subtilisin into the (9–35 kDa) derivative (lane O) which was then conjugated to S-1, as described in section 2; lane T2 = the 45-min coupling reaction between MBS-G-actin and S-1, used as control. (B) Breakdown by subtilisin of the 180 kDa MBS-actin-S-1 heavy chain conjugate into a new 170 kDa species; the digestion of the covalent MBS-G-actin-S-1 complex (lane O) was done as reported in section 2.

with F-actin added at molar ratios to total S-1 of 1:1 or 2:1 (Fig. 4B, lanes d and g, respectively). These F-actin concentrations were similar to those previously employed with the S-1-MBS-G-actin conjugate [1] so that the F-actin affinity of the S-1-actin peptide adduct could be qualitatively compared with that observed for the former complex. The electrophoretic analyses showed most of the 105 kDa species to remain in the supernatants dissociated from F-actin (Fig. 4B, lanes e and h) in contrast to the residual nonconjugated S-1 which accumulated in the F-actin pellets (Fig. 4B, lanes f and i). Thus, the rigor F-actin-S-1 binding appears also blocked within the 105 kDa complex. However, a similar chymotryptic 105 kDa derivative produced from the EDC-cross-linked MBS-actin-S-1 complex which involves the EDC-cross-linkable sites flanking the 50–20 kDa junction of the heavy chain [1], did fully cosediment with F-actin under identical experimental conditions (data not shown). This indicates that the observed inhibition of F-actin binding is dependent not on the actin peptide by its own but rather on the location of the heavy chain sites to which it has been attached. Therefore, the proper access to and positioning on F-actin of the subregion of

Table 1
Sequence analysis of the C-terminal 35 kDa fragment isolated from subtilisin-split MBS-G-actin

Cycle no.	Residue identified	Yield (pmol)[a]
1	Gly	265
2	Gln	182
3	Lys	115[b]
4	Asp	158
5	Ser	ND[c]
6	Tyr	155
7	Val	167
8	Gly	144
9	Asp	129
10	Glu	112
11	Ala	180
12	Gln	113
13	Ser	ND
14	Lys	113
15	Arg	144
16	Gly	123
17	Ile	150

[a]Sequencing was performed with an average yield per cycle of 97%.
[b]The yield of Lys-50 is about 40% lower than the theoretical value and must result from its partial conversion into maleimidobenzoyllysine.
[c]ND means not determined by sequence.

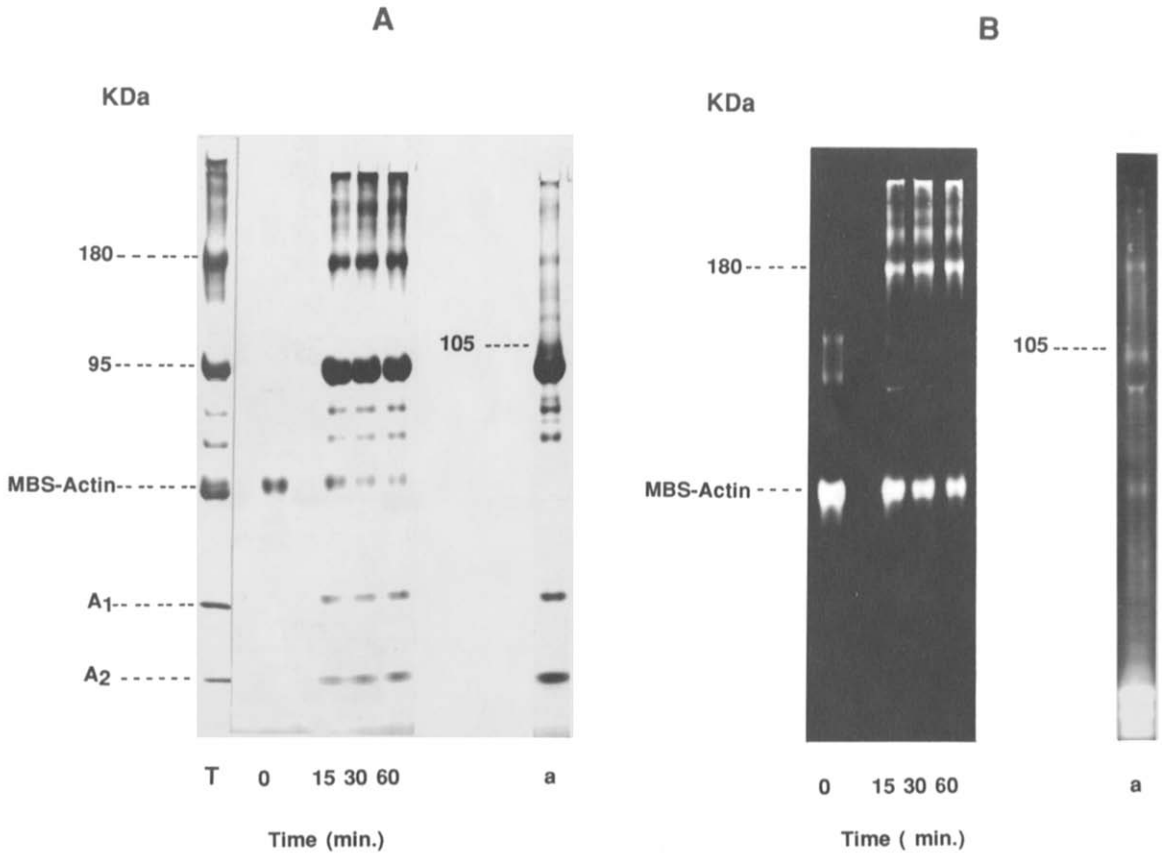

Fig. 3. Cross-linking of S-1 to MBS-G-actin fluorescently labeled at Lys-61. (A) Isolated FITC-G-actin was derivatized into (FITC)-MBS-G-actin (lane O) which was conjugated to S-1, as outlined in section 2. After 60-min cross-linking, the protein mixture was treated with α-chymotrypsin + EDTA, as in Fig. 1A, and the 60-min digest was electrophorezed (lane a); lane T = control MBS-G-actin-S-1 conjugate. (B) The gels in (A) were viewed under ultraviolet light.

the 50 kDa segment that was cross-linked by MBS to the actin stretch of Lys-50, seem to be crucial for determining the high-binding affinity of S-1.

4. Discussion

The MBS-catalyzed coupling of actin subdomain-2 to S-1 rationalizes the decreased levels of cross-linking previously observed [2] for MBS-F-actin relative to MBS-G-actin, which may reflect the conformation changes known to occur in this domain during the G-F-conversion [18–20] as well as the change in the environment of Lys-50 earlier noticed upon actin polymerization [21]. The much lower chemical reactivity of the latter residue in F-actin could explain, at least in part, the failure of the preformed F-actin-S-1 complex to be cross-linked by direct reaction with MBS [2]. It is worth noting that although the cross-linking of MBS-actin to S-1 appears to involve only Lys-50, this side-chain is not the unique lysine in actin which carries a free maleimidobenzoyl group. This is indicated by the incorporation of fluorescence we observed within the 180 kDa MBS-actin-heavy chain adduct upon reaction of the conjugate with an excess of thiolated *N*-(5-sulfo-1-naphthyl) ethylenediamine (EDANS) [2] which readily attaches to the maleimide moiety of some other(s) MBS-lysine(s) not participating in the cross-linking. Thus, the various structural analyses we performed on the 180 kDa product are required to ensure the identification of the cross-linked actin region.

Most importantly, the conjugation of the actin subdomain-2 to the 50 kDa heavy chain fragment provides an experimental support to the recently proposed structural model of the F-actin-S-1 complex [6]. In this model S-1 spans two longitudinal actin monomers and bears a set of primary actin recognition sites with binding to subdomain-1 of an actin monomer and interaction with the top of subdomain-2 on the next actin subunit. The latter close contact is thought to involve the actin residues 40–42 and the peptide stretch 552–558 located in the lower domain of the 50 kDa fragment. The length of the maleimidobenzoyl arm on the ε-amino group of Lys-50 (0.9–1.0 nm) which is located in the model very near the lower domain of the 50 kDa region, makes possible the cross-linking at this heavy chain segment. The conjugation at this cricital subregion of the 50 kDa fragment may explain the observed ability of the covalently at-

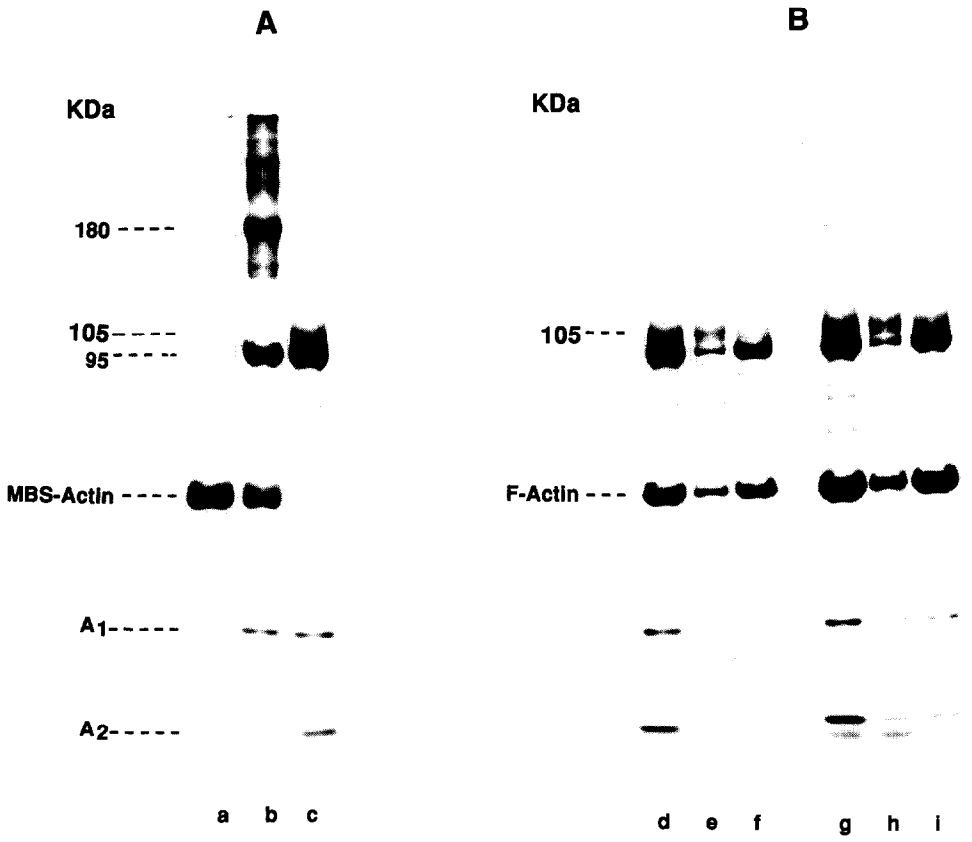

Fig. 4. Blocking of F-actin binding to S-1 cross-linked at the 50 kDa heavy chain region to the chymotryptic actin peptide of residues 1–67. (A) Purified MBS-G-actin (lane a) was conjugated to S-1 (lane b) and the resulting protein mixture was incubated for 60 min with α-chymotrypsin + EDTA, as described in section 2, to produce the 105 kDa S-1-actinopeptide complex (lane c). (B) The chymotryptic digest was supplemented with F-actin added at a molar ratio to S-1 of 1:1 (lane d) or 2:1 (lane g); the supernatants (lanes e and h, respectively) and pellets (lanes f and i, respectively) obtained by ultracentrifugation were analyzed by gel eletrophoresis.

tached intact MBS-G-actin [1] or its 67-residue chymotryptic peptide to interfere with further tight binding of S-1 to F-actin. Because the maleimide group can react with cysteines or lysines of proteins, the nature of the cross-linked heavy chain site cannot be envisioned from the reconstructed acto-S-1 complex; current structural studies on the 180 kDa species are underway for its characterization.

The MBS-G-actin monomer was reported to undergo an equilibrium with a dimer form [5], similarly to native G-actin [22] to which S-1 could bind, and interaction of S-1 at subdomain-1 of MBS-G-actin was earlier demonstrated [1]. Therefore, the solution binding of S-1 to the dimeric MBS-G-actin species might have driven its specific cross-linking to subdomain-2 as with MBS-F-actin. Parallel investigations from our laboratory [23] have also identified within the native F-actin-S-1 complex the cross-linking of the 50 kDa fragment to the actin sequence 48–67, using glutaraldehyde as cross-linker. Thus, the spatial relationships of these two regions are similar in the complexes of S-1 with G- or F-actin. The interaction of S-1 with either MBS-G-actin [4] or native G-actin [24] is highly salt-sensitive involving mainly electrostatic bonds but not the strong hydrophobic contacts that operate in the rigor F-actin-S-1 complex. These are represented by stereospecific bonds between the lower domain of the 50 kDa fragment and hydrophobic residues at the bottom surface of actin subdomain-1 facing the subdomain-2 loop of the actin protomer below [6]. The dual association of S-1 to these two adjacent subdomains within a stable F-actin dimer appears essential for the precise orientation of the actin binding cleft in the 50 kDa fragment to its hydrophobic recognition sites on the actin molecule.

Acknowledgements: 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.

References

[1] Bettache, N., Bertrand, R. and Kassab, R. (1989) Proc. Natl. Acad. Sci. USA 86, 6028–6032.
[2] Bettache, N., Bertrand, R. and Kassab, R. (1992) Biochemistry 31, 389–395.

[3] Miki, M. and Hozumi, T. (1991) Biochemistry 30, 5625–5630.
[4] Bettache, N., Bertrand, R. and Kassab, R. (1990) Biochemistry 29, 9085–9091.
[5] Arata, T. (1991) J. Biochem. (Tokyo) 109, 335–340.
[6] Rayment, I., Holden, H.M., Whittacker, M., Yohn, C.B., Lorenz, M., Holmes, K.C. and Milligan, R.A. (1993) Science 261, 58–65.
[7] Bertrand, R., Derancourt, J. and Kassab, R. (1992) Biochemistry 31, 12219–12226.
[8] Burtnick, L.D. (1984) Biochim. Biophys. Acta 791, 57–62.
[9] Schwyter, D., Phillips, M. and Reisler, E. (1989) Biochemistry 28, 5889–5895.
[10] Mornet, D., Bertrand, R., Pantel, P., Audemard, E. and Kassab, R. (1981) Biochemistry 20, 2110–2120.
[11] Bonafé, N., Chaussepied, P., Capony, J.P., Derancourt, J. and Kassab, R. (1993) Eur. J. Biochem. 213, 1243–1254.
[12] Konno, K. (1987) Biochemistry 26, 3582–3589.
[13] Wagner, P.D. and Weeds, A.G. (1977) J. Mol. Biol. 109, 455–473.
[14] Strzelecka-Golaszewska, H., Moraczewska, J., Khaithina, S.Y. and Mossakowska, M. (1993) Eur. J. Biochem. 211, 731–742.
[15] Fievez, S. and Carlier, M. (1993) FEBS Lett. 316, 186–190.
[16] Miller, L., Phillips, M. and Reisler, E. (1988) Eur. J. Biochem. 17, 23–29.
[17] Kabsch, W., Manherz, H.G., Suck, D., Pai, E.F. and Holmes, K.C. (1990) Nature 347, 37–44.
[18] Holmes, K.C. and Kabsch, W. (1991) Curr. Opin. Struct. Biol. 1, 270–280.
[19] Orlova, A. and Egelman, E.H. (1992) J. Mol. Biol. 227, 1043–1053.
[20] Lorenz, M., Popp, D. and Holmes, K.C. (1993) J. Mol. Biol. 234, 826–836.
[21] Hitchcok-De Gregori, S.E., Mandala, S. and Sachs, G.A. (1982) J. Biol. Chem. 257, 12573–12580.
[22] Valentin-Ranc, C., Campeau, C., Carlier, M.-F. and Pantaloni, D. (1991) J. Biol. Chem. 266, 17872–17879.
[23] Bonafé, N., Mathieu, M., Kassab, R. and Chaussepied, P. (1994) Biochemistry, in press.
[24] Lheureux, K., Forné, T. and Chaussepied, P. (1993) Biochemistry 32, 10005–10014.