Molecular Movements in the Actomyosin Complex: F-Actin-Promoted Internal Cross-Linking of the 25- and 20-kDa Heavy Chain Fragments of Skeletal Myosin Subfragment 1†

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ABSTRACT: We describe, for the first time, the F-actin-promoted changes in the spatial relationship of strands in the NH_2-terminal 25-kDa and COOH-terminal 20-kDa heavy chain fragments of the skeletal myosin subfragment 1 (S-1), detected by their exclusive chemical cross-linking in the rigor F-actin–S-1 complex with *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS). Quantitative electrophoretic analysis of the reaction products showed extensive conversion of the 95-kDa heavy chain of the actin-bound S-1 into a new species with an apparent mass of 135 kDa (yield = 50–60%), whereas the heavy chain mobility remained unaffected when actin was omitted. The 135-kDa entity retained the fluorescence of AEDANS–S-1 but not of AEDANS–actin, indicating that it was not a cross-linked acto–heavy chain adduct. Its extent of production depended markedly on the S-1:actin molar ratio and was maximum near a ratio of 1:4. The MBS treatment of acto-S-1 led also to some covalent actin–actin oligomers which could be suppressed by using trypsin-truncated F-actin lacking Cys-374, without altering the generation of the 135-kDa heavy chain derivative. The MBS reaction on the complex of F-actin and tryptic (25–50–20 kDa)-S-1 resulted in a new 40-kDa band, comigrating with actin, which was composed of the N-terminal 25-kDa and C-terminal 20-kDa fragments since it incorporated the fluorescence of the anthroyl group specifically attached to the former peptide and the fluorescence of AEDANS selectively bound to the SH-1 thiol in the latter segment. Blocking SH-1 and SH-2 thiols did not abolish this interfragment cross-linking. However, the addition of millimolar concentrations of MgADP led to its total suppression without dissociation of the acto-S-1 complex as assessed by cosedimentation. Peptide mapping of the 40-kDa adduct indicated that the cross-linking of the 20-kDa region was only to the COOH-terminal stretch of the 25-kDa fragment between amino acids 145 and 204, where resides the flexible γ-phosphate-binding glycine-rich loop of the S-1 ATPase site. The data suggest that the relative movements of the cross-linked heavy chain segments which are reciprocally modulated by F-actin and nucleotides may contribute to the mechanism of interactivity between the actin and ATPase sites of myosin during force generation.

The production of force and motion during muscle contraction requires the cyclic and ATP-dependent interaction between F-actin and the heavy chain of the myosin head or S-1.[1] During ATP hydrolysis by the actomyosin complex, transient changes in the conformation of the S-1 ATPase site take place and are thought to be transmitted to the remote actin-binding site, thereby determining the mechanical attitudes of S-1 relative to the actin filament (Botts et al., 1984; Huxley & Kress, 1985; Vibert & Cohen, 1988). On the other hand, the interaction of F-actin with the S-1 heavy chain is also supposed to profoundly influence the conformation of the adjacent ATPase site, facilitating the release of the tightly bound ADP·P_i intermediate of the ATPase cycle (Taylor, 1979). The overall reciprocal internal displacements of segments or domains of the heavy chain upon binding of nucleotides and actin are made possible by the well-known mobile structure of the S-1 heavy chain (Highsmith & Jardezky, 1981; Highsmith & Eden, 1986) and provide a molecular basis for the intersite communication in S-1 during energy transduction (Botts et al., 1984).

Nucleotide-mediated distortions of the S-1 heavy chain were previously characterized through nucleotide-dependent intramolecular chemical cross-linking within or between particular regions of the tryptic 25-, 50-, and 20-kDa heavy chain fragments (Wells et al., 1980; Chaussepied et al., 1986a, 1988; Lu et al., 1986; Sutoh & Lu, 1987; Rajasekharen et al., 1987, 1989). In contrast, the nature and location of the heavy chain movements specifically induced by F-actin have not yet been precisely identified. Their characterization appears to be important for the knowledge of the internal structural dynamics of the acto-S-1–nucleotide system and the molecular mechanism of the catalytic function of F-actin. Recently, a spectrofluorometric investigation of the acto-S-1–ADP complex has led to the suggestion that F-actin induces relative movements of the N-terminal 25-kDa and C-terminal 20-kDa segments of the skeletal S-1 heavy chain, which were reversed upon binding of MgADP to the acto-S-1 complex (Lin & Cheung, 1991). In the present study, we directly demonstrate the F-actin-promoted deformation of the 25- and 20-kDa heavy chain regions revealed by their selective internal

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[1] Abbreviations: S1, myosin subfragment 1; acto-S-1, actomyosin subfragment 1; MBS, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester; ATPase, adenosine-5′-triphosphatase; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTE, dithioerythritol; AN-S-1, anthroyl-S-1; ANN, 9-anthroylnitrile; AEDANS, *N*-acetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; CPM, 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin; NCS, *N*-chlorosuccinimide.

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cross-linking in the rigor acto-S-1 complex with *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS). This new interfragment cross-linking, which does not involve the SH-1 and SH-2 thiols, was abolished when MgADP bound to the acto-S-1 without dissociation of the complex. The cross-linkable strand of the 25-kDa segment whose relative position is brought by F-actin to approximately 0.9 nm from the 20-kDa domain was identified as residing between residues 145 and 204, a region including the flexible nucleotide-binding glycine-rich loop of the S-1 heavy chain (Cremo et al., 1989).

MATERIALS AND METHODS

Chemicals. *m*-Maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine (1,5-IAEDANS), and *N*-chlorosuccinimide were obtained from Sigma. Dithioerythritol (DTE) and *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) were from Serva Heidelberg. 9-Anthroylnitrile (ANN) and 7-(diethylamino)-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) were obtained from Molecular Probes (Eugene City, OR). TPCK-treated trypsin was from Worthington. Endoproteinase Arg-C was purchased from Boehringer Mannheim.

Proteins. Rabbit skeletal myosin was prepared as described by Offer et al. (1973). Chymotryptic S-1 was obtained according to the method of Weeds and Taylor (1975) and was further purified over Sephacryl S-200 according to the procedure of Chaussepied et al. (1986b). The S-1 (A1) and S-1 (A2) isoenzymes were separated as previously described (Bettache et al., 1992). Rabbit skeletal F-actin was prepared as described by Eisenberg and Kielley (1974). G-Actin was obtained 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 a frequency of 20 000 Hz in a Microson cell disruptor (Model XL 200S) and then centrifuged at 180000*g* for 1 h at 4 °C. The G-actin solution was repolymerized by adding KCl and $MgCl_2$ to final concentrations of 10 and 2 mM, respectively. Glycine- and DTE-treated MBS–G-actin was prepared as described by Bettache et al. (1990). S-1 labeled at SH-1 with 1,5-IAEDANS was produced as described by Duke et al. (1976); S-1 blocked at SH-1 with CPM or at both SH-1 and SH-2 with DACM was obtained according to the method of Sutoh (1981). About 2.1 mol of DACM was incorporated/mol of protein. The labeling of S-1 in the NH_2-terminal 25-kDa fragment with the fluorescent ANN was carried out as reported by Hiratsuka (1989). F-Actin was labeled with 1,5-IAEDANS as described by Mornet et al. (1981). The derivative (75–21 kDa)-S-1 was produced by digestion of S-1 with the endoproteinase Arg-C, at 25 °C, in 20 mM MOPS, pH 7.5 (Bertrand et al., 1989). The trypsin split (25–50–20 kDa)-S-1 and (25–70 kDa)-S-1 were obtained by digesting S-1 in the absence and presence of F-actin, respectively, according to the procedure of Mornet et al. (1980). F-Actin truncated at residues 374–375 was produced by limited proteolysis of native F-actin with trypsin following the procedure of O'Donoghue et al. (1992) and using protease:actin weight ratios varying between 1:50 and 1:10.

Protein concentrations were determined spectrophotometrically with extinction coefficients of $A^{1\%}_{280nm}$ = 7.5 cm^{-1} for S-1 and $A^{1\%}_{280nm}$ 11 for actin. The concentrations of the modified proteins were estimated according to the Bradford assay (Bradford, 1976) with the respective native proteins as standards.

Intramolecular Cross-Linking Using MBS. F-Actin (2 mg/mL) in F-buffer (2 mM HEPES, 10 mM KCl, 0.1 mM ADP, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM NaN_3, pH 8.0) was incubated with 0.25 mM MBS (dissolved at 8.5 mg/mL in dimethylformamide) for 30 min at 20 °C. It was then mixed with native S-1 or modified S-1 (dissolved at 6–10 mg/mL in 20 mM MOPS, pH 7.5) at a molar ratio of 3:1. The intramolecular cross-linking reaction was conducted at a final pH value of 7.6 for 45 min in the absence and presence of 2 mM MgADP. It was terminated by the addition of DTE and glycine (each at a 4-fold molar excess over MBS). The cross-linking process was analyzed by $NaDodSO_4$–acrylamide gel electrophoresis. Similar experiments were also carried out by directly reacting MBS with the preformed F-actin–S-1 complex.

Hydrolytic Cleavages and Sequence Analyses. The tryptic digestion of the MBS-treated complexes between F-actin and S-1 or (75–21 kDa)-S-1 (2 mg/mL) was performed in F-buffer, pH 7.6, at an enzyme:S-1 weight ratio of 1:20, at 25 °C, for 30 min (molar ratio actin:S-1 = 3:1).

The hydrolysis of the 40-kDa species at the tryptophan residues was carried out as follows: the complex of F-actin and MBS-cross-linked CPM-labeled (25–50–20 kDa)-S-1 was first collected by centrifugation. The pellet was resuspended in a volume of F-buffer, pH 7.6, equal to half the initial volume of the reaction mixture. After sonication, the protein solution was supplemented with MgATP and NaCl to 10 and 500 mM, respectively. Following centrifugation, the supernatant, containing the internally cross-linked S-1 devoid of actin, was electrophoresed and the separated fluorescent 40-kDa band was excised and in-gel-digested with *N*-chlorosuccinimide according to the method of Lischwe and Ochs (1982). After a second-dimension gel electrophoresis, the released fluorescent 28-kDa peptide band was electroblotted onto a poly(vinylidene difluoride) membrane (Matsudaira, 1987) and subjected to NH_2-terminal sequencing using an Applied Biosystems Model 470-A sequanator with an "on-line" Model 120-A phenylthiohydantoin amino acid analyzer (Hewick et al., 1981).

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). Densitometric scanning of the gels was performed with a Shimadzu Model CS-930 high-resolution gel scanner equipped with a computerized integrator. The following proteins were used as molecular weight markers: MBS-cross-linked actin upper dimer (120 000), S-1 heavy chain (95 000), actin (42 000), and the heavy chain fragments of (25–50–20 kDa)-S-1 and (75–21 kDa)-S-1.

ATPase Assays. The Ca^{2+}- and K^+-EDTA–ATPase activities were measured at 25 °C in 2.5 mM ATP, 0.5 mM KCl, and 50 mM Tris-HCl, pH 8.0, in the presence of 5 mM $CaCl_2$ and 5 mM EDTA, respectively. The actin-activated ATPase was assayed in 5 mM ATP, 2.5 mM $MgCl_2$, 10 mM KCl, and 50 mM Tris-HCl, pH 7.5, in the presence of 1 mg of F-actin. P_i liberated was determined colorimetrically as previously reported (Mornet et al., 1979).

RESULTS

F-Actin-Dependent Internal Cross-Linking of the S-1 Heavy Chain with MBS. When S-1 was exposed to submillimolar concentrations of MBS at pH 7.6, for a few minutes at room temperature, there was production of a 110-kDa species in a small amount, without apparent change in the electrophoretic mobility of the residual 95-kDa S-1 heavy chain (Figure 1A).

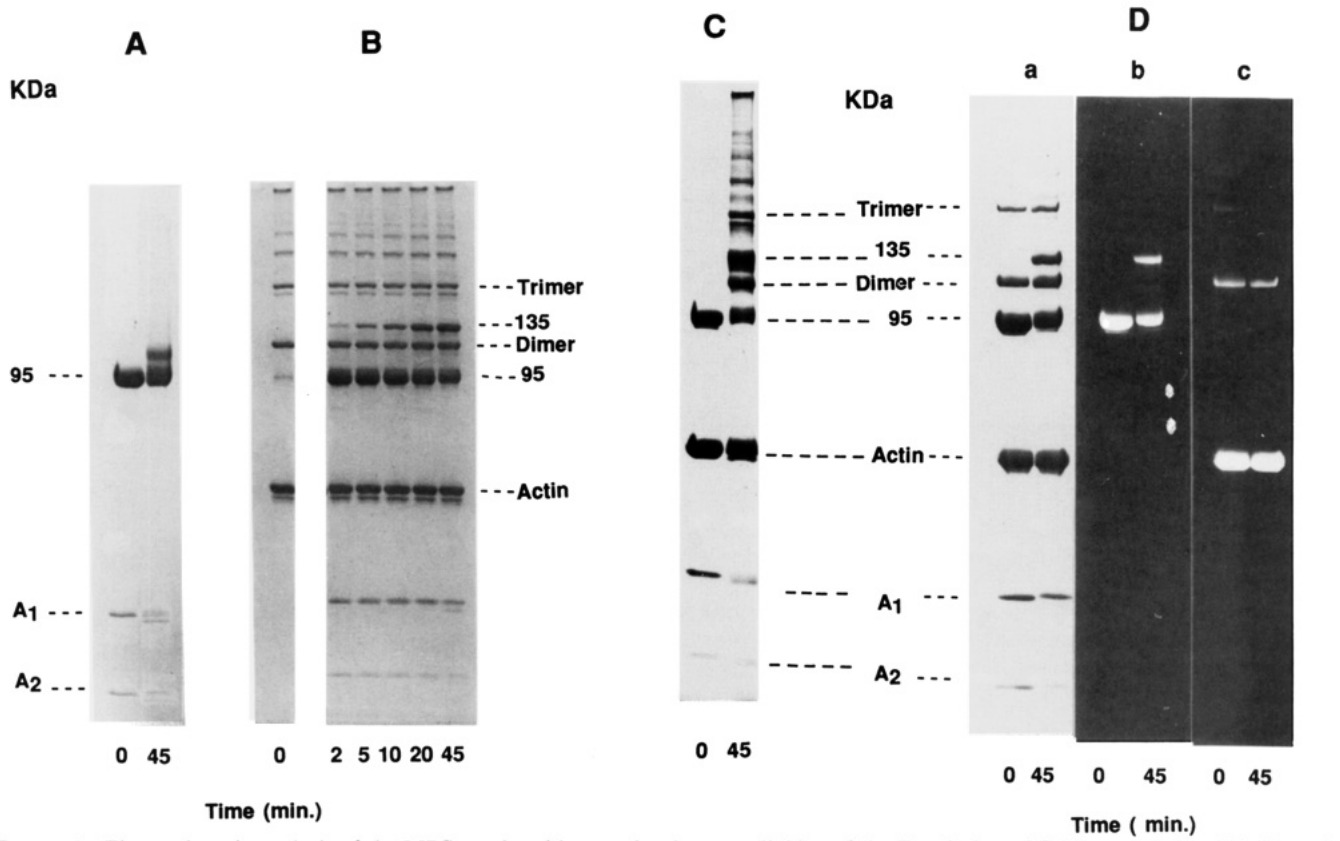

FIGURE 1: Electrophoretic analysis of the MBS-catalyzed intramolecular cross-linking of the F-actin-bound S-1 heavy chain. (A) Control S-1 (2 mg/mL) in 2 mM HEPES, 10 mM KCl, 0.1 mM ADP, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.1 mM NaN_3, pH 7.6, was subjected to $NaDodSO_4$ gel electrophoresis on a 5–18% gradient acrylamide before and after 45-min treatment with 0.25 mM MBS at 20 °C. (B) F-Actin (2 mg/mL) in HEPES buffer, pH 8.0, was first incubated with MBS for 30 min (lane 0). It was then supplemented with S-1 (actin:S-1 molar ratio = 3:1), and at the indicated time intervals, protein aliquots were analyzed by gel electrophoresis. (C) Electrophoretic pattern of the preformed acto-S-1 complex before and after 45-min incubation with 0.25 mM MBS, at pH 7.6, 20 °C. (D) Comparative fluorescence incorporation into the 135-kDa species upon reaction of MBS with acto-S-1 complexes including either fluorescent AEDANS–S-1 (panel b) or fluorescent AEDANS–actin (panel c). Protein bands were viewed under UV light (panels b and c) and then stained with Coomassie blue (panel a).

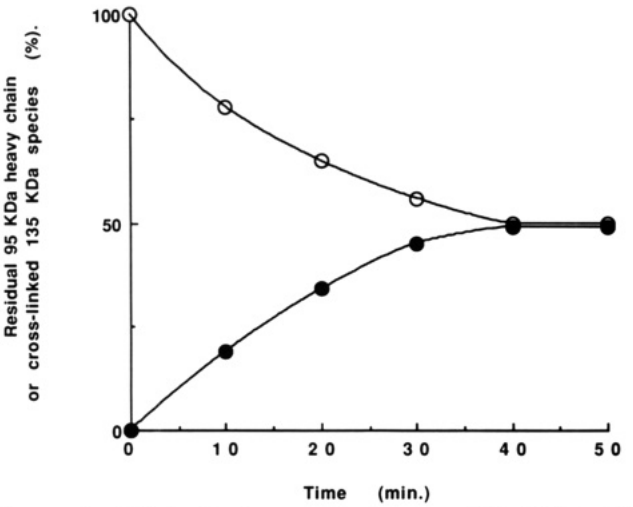

FIGURE 2: Relationship between the decrease of the 95-kDa S-1 heavy chain (○) and the increase of 135-kDa band production (●) during the reaction of MBS with the F-actin–S-1 complex. The amount of 95- and 135-kDa species was determined by densitometry of the gel as specified under Materials and Methods.

This derivative could represent a heavy chain–alkali light chain adduct as its mobility was similar to that observed for other authentic conjugates of the heavy and light subunits identified earlier (Labbé et al., 1982; Yamamoto & Sekine, 1983; Bertrand et al., 1988; Onishi et al., 1992). As illustrated in Figure 1B, lane 0, a 30-min treatment of F-actin with MBS under the same experimental conditions resulted, as expected, in the generation of cross-linked actin oligomers (upper dimer, trimer, etc.), the formation of which was previously described (Sutoh, 1984). However, Figure 1B shows also that the incubation of MBS with both F-actin and S-1 (molar ratio = 3:1) led to the suppression of the 110-kDa product and to the progressive appearance of a major new species with an apparent molecular mass value of 135 kDa. This additional band was sharper and more homogeneous when it derived following the addition of S-1 to F-actin preincubated for 30 min with the cross-linking agent (Figure 1B) than when it derived from the direct reaction of MBS with the preformed F-actin–S-1 complex (Figure 1C). Under the latter condition, the major 135-kDa band was broader and comprised one or two additional closely migrating faint bands which also form at MBS concentrations lower as well as higher than the optimum concentration of 0.25 mM. Furthermore, the incubation of S-1 with isolated, MBS-treated F-actin did not yield any 135-kDa material unless fresh MBS was added to the protein mixture. In this case also, the 135-kDa band produced was rather broad. It is possible that reversible interactions between the hydrophobic MBS and F-actin prior to the addition of S-1 favor some particular orientation of the reagent which subsequently reacts more specifically. Therefore, in routine work, the former procedure was selected for the accumulation and further study of the 135-kDa entity. This product incorporated the fluorescence of the AEDANS–S-1 heavy chain but not at all the fluorescence of AEDANS–actin (Figure 1D, panels b and c, respectively), indicating unambiguously that it does not represent a cross-linked adduct of actin with the 95-kDa heavy chain. The quantitative

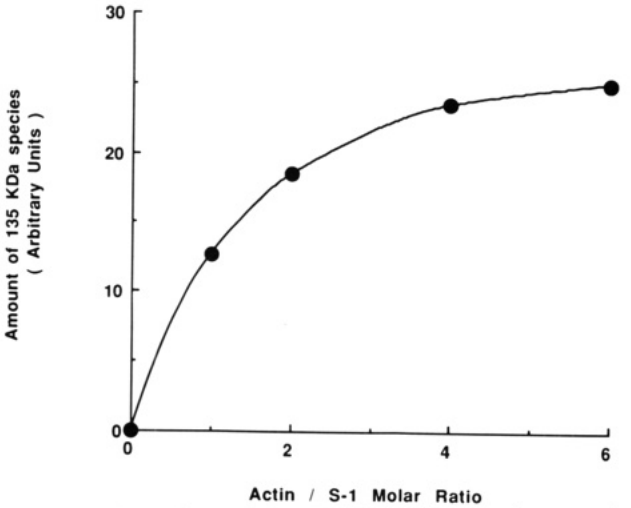

FIGURE 3: Dependency of the 135-kDa band formation upon the F-actin:S-1 molar ratio. The amount of 135-kDa band was determined as in Figure 2.

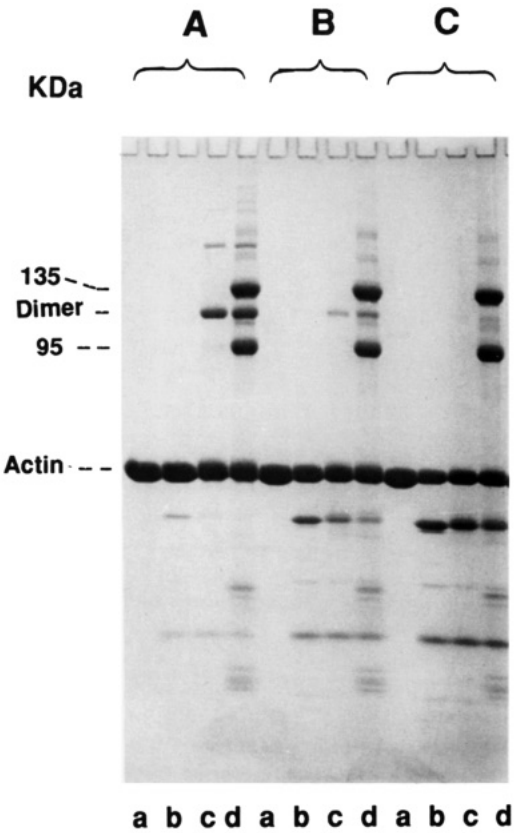

FIGURE 4: Intramolecular cross-linking of the S-1 heavy chain in the presence of truncated F-actin. Native F-actin (lanes a) was digested with trypsin at an enzyme:substrate weight ration of 1:50 (A), 1:25 (B), or 1:15 (C) as indicated under Materials and Methods. The isolated trucated F-actin (lanes b) was treated with 0.25 mM MBS for 30 min (lanes c) and then incubated in the presence of S-1 (actin: S-1 molar ratio = 3:1) for 45 min(lanes d) under the conditions reported in Figure 1.

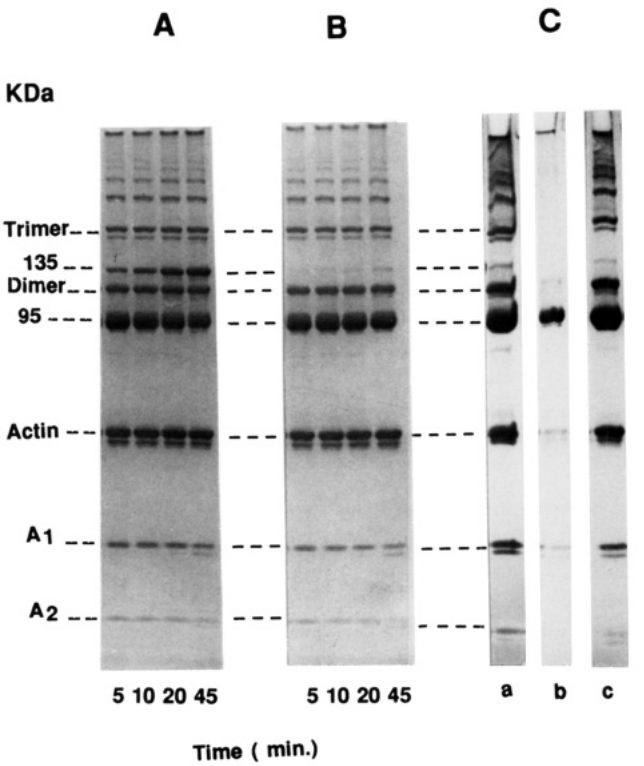

FIGURE 5: Influence of MgADP on the extent of 135-kDa band production in subfragment 1. The reaction of MBS on the F-actin–S-1 mixture (actin:S-1 molar ratio = 3:1) was carried out as in Figure 1B, in the absence (A) and presence (B) of 2 mM MgADP. (C) The F-actin–S-1 complex treated with MBS in the presence of MgADP was subjected to ultracentrifugation at 150000*g* for 1 h at 4 °C; samples corresponding to the whole protein solution (a) and to the resulting supernatant (b) and pellet fractions (c) were analyzed by gel electrophoresis.

estimation of the 135-kDa band formation by densitometric analysis of the Coomassie blue stained gel showed a close correlation between the extent of production of the 135-kDa species and the decrease of the band intensity corresponding to the 95-kDa S-1 heavy chain during the entire course of the MBS reaction (Figure 2). The production of the 135-kDa band reached its maximum value of approximately 50% at 45 min. It was formed even at neutral pH, but its amount substantially increased when the pH of the reaction was varied from 7.0 to 8.0. The isolated S-1 isoenzymes, S-1 A1 and S-1 A2, generated equal amounts of the 135-kDa band, indicating that the particular nature of the alkali light chain is not essential for its production. In addition, the yield of the 135-kDa derivative was essentially dependent on the F-actin:S-1 molar ratio; the maximum value was observed at a ratio near 4:1 (Figure 3). Replacing F-actin by the soluble monomeric MBS-G-actin which also interacts with S-1 (Bettache et al., 1989) did not generate the 135-kDa band (data not shown). On the other hand, the appearance of the 135-kDa product had no relationship with the MBS-induced cross-linking of the actin subunits within F-actin. This was demonstrated by the data depicted in Figure 4. F-Actin was first subjected to limited digestion with trypsin using increasing protease:actin weight ratios. This proteolysis leads to the selective removal of the C-terminal actin segment of residues 374–375, without affecting the affinity of actin binding to S-1 (O'Donoghue et al., 1992). The resulting F-actin preparations were isolated by centrifugation and then submitted to the MBS treatment in the presence of S-1. As shown in Figure 4, the amount of actin dimer formed decreased progressively, reflecting the degree of proteolytic loss, in each actin preparation, of Cys-374, which represents one of the sites involved in actin–actin cross-linking by MBS (Sutoh, 1984). However, the extent of production of the 135-kDa band remained unchanged for all of the tryptically cut F-actins employed. Collectively, our results suggest that the 135-kDa species derives mainly from an intramolecular cross-linking of the 95-kDa S-1 heavy chain which occurs only upon tight binding of S-1 to F-actin. As a result of this internal cross-linking, the heavy chain displayed an abnormal mobility on $NaDodSO_4$ gel electrophoresis. This proposal is also consistent with the observation that the presence of MgATP, which dissociates acto-S-1, suppressed

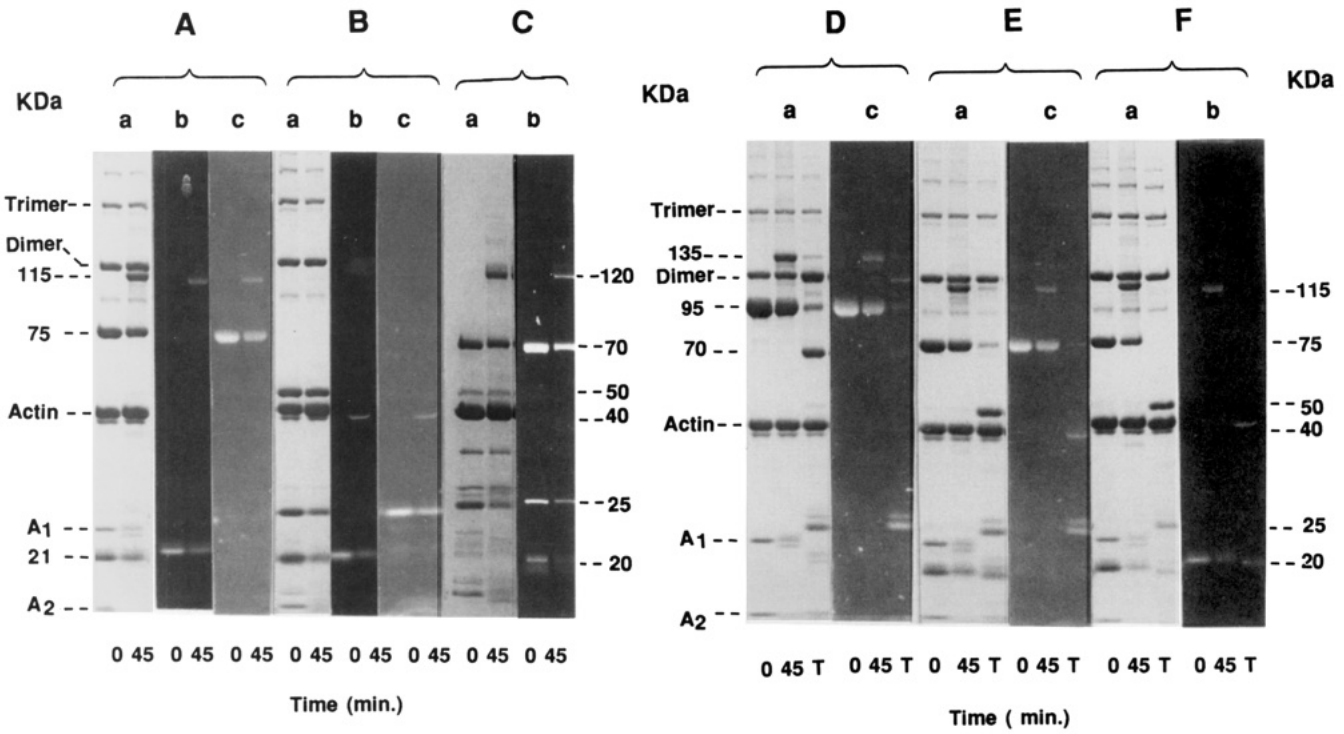

FIGURE 6: F-Actin-dependent cross-linking of the 25- and 20-kDa fragments of the S-1 heavy chain by MBS. Complexes of actin and fluorescent (75–21 kDa) S-1 (A), (25–50–20 kDa) S-1 (B), or (25–70 kDa) S-1 (C) were treated with MBS as described in Figure 1B and under Materials and Methods. The protein solutions were analyzed by gel electrophoresis before and after a 45-min reaction. The S-1 derivatives were labeled either in the 20-kDa fragment with 1,5-IAEDANS (lanes b) or in the 25-kDa fragment with ANN (lanes c). After a 45-min incubation with MBS, the complexes of F-actin and AN-S-1 (D), AN-(75–21 kDa)-S-1 (E), or AEDANS–(75–21 kDa)-S-1 (F) were digested with trypsin (lanes T) as specified under Materials and Methods. Protein bands were located by fluorescence (lanes b and c) or by staining with Coomassie blue (lanes a).

the 135-kDa band, whereas the increase of the ionic strength (≥100 mM), which weakens the acto-S-1 affinity, lowered the yield of its production (data not shown). Most interestingly, Figure 5 shows also that the addition of MgADP (2 mM) caused a dramatic decrease of the 135-kDa species without significant dissociation of S-1 from actin as assessed by parallel cosedimentation experiments illustrated in Figure 5C (the initial presence of 0.1 mM ADP in the F-buffer employed for the reaction was checked to be without apparent effect on the cross-linking). The latter binding studies did not reveal any noticeable alteration of the affinity of S-1–ADP for actin treated with MBS. These findings indicate that the internal heavy chain cross-linking is sensitive not only to the binding of F-actin to S-1 but also to the interaction of nucleotides with the S-1 ATPase site. Unfortunately, ATPase measurements during the MBS treatment did not provide useful information concerning the influence of the internal heavy chain cross-linking on the enzymatic properties of S-1. All S-1 ATPases were more or less inactivated in the absence and presence of either F-actin or MgADP, probably because monofunctional reactions are also taking place, masking any particular effect of the cross-link(s) on the S-1 ATPases. However, after the MBS reaction, the 135-kDa entity could be pelleted with actin and the resulting complex was dissociated by MgATP, although not as completely as observed with the residual un-cross-linked 95-kDa heavy chain (data not shown).

F-Actin-Induced Cross-Linking between the NH_2-Terminal 25-kDa and the COOH-Terminal 20-kDa Fragments of the S-1 Heavy Chain. To identify the peptide segments involved in the internal cross-linking of the S-1 heavy chain, the MBS reaction was carried out using F-actin complexed to various proteolytic S-1 derivatives which were fluorescently labeled either in the NH_2-terminal 25-kDa fragment or in the COOH-terminal 20-kDa domain of the heavy chain. The overall data are presented in Figure 6. The (75–21 kDa)-S-1 gave rise to a new band migrating at the 115-kDa position which incorporated the fluorescence of both the 20- and 25-kDa fragments (Figure 6A, lanes b and c, respectively). This finding strongly suggests that the 115-kDa band derives from the intramolecular covalent union of the 75- and 20-kDa segments of the S-1 heavy chain. This conclusion was unequivocally confirmed by the distribution of the fluorescence in the gel pattern corresponding to the (25–50–20 kDa)-S-1. This derivative yielded a new 40-kDa band, migrating just ahead of the actin position, which incorporated the 20- and 25-kDa fragment fluorescence (Figure 6B, lanes b and c, respectively). The fluorescent cross-linked 40-kDa peptide was also obtained upon tryptic digestion of the cross-linked 115-kDa species deriving from (75–21 kDa)-S-1 carrying the fluorescence either on the 25-kDa fragment or on the 20-kDa peptide (parts E and F, respectively, of Figure 6). The digestion of the cross-linked intact 135-kDa species (which occurs only at the 25–50-kDa junction due to the presence of F-actin) yielded a 120-kDa product comigrating with the actin dimer (Figure 6D). This material was also formed by directly reacting MBS with the actin-bound (25–70 kDa)-S-1 (Figure 6C). Thus, as schematized in Figure 7, the F-actin-induced covalent union of the 25- and 20-kDa regions takes place similarly within the native and the proteolytically split S-1 heavy chain. In the latter case also, the addition of millimolar concentrations of MgADP or the omission of F-actin abolished all of the cross-linked protein bands (data not shown). However, the apparent masses of the cross-linked heavy chain derivatives (135, 120, or 115 kDa) depended on the structural integrity of the two protease-sensitive connector segments of the heavy chain. Moreover, we observed that the cleavage of the 25–50-kDa junction lowers noticeably the yield of the

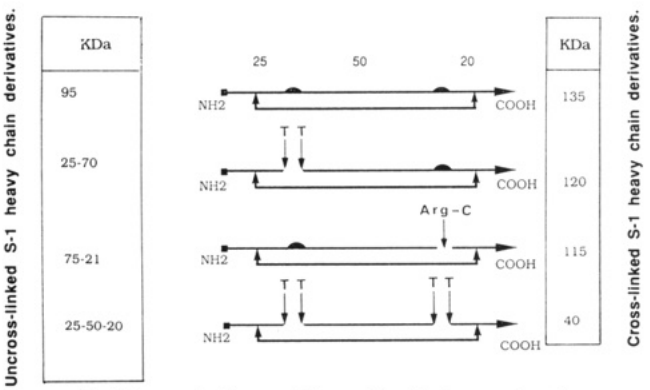

FIGURE 7: Schematic diagram illustrating the intramolecular cross-linking pattern of the 95-kDa S-1 heavy chain as deduced from the distribution of the 25- and 20-kDa fragment fluorescence within the cross-linked species deriving from the proteolytic S-1 derivatives. Note the different apparent sizes of the whole cross-linked S-1 heavy chain depending on the state of the two connector segments. Cleavage at the latter sites was achieved with trypsin (T) or the arginine-directed endoproteinase (Arg-C).

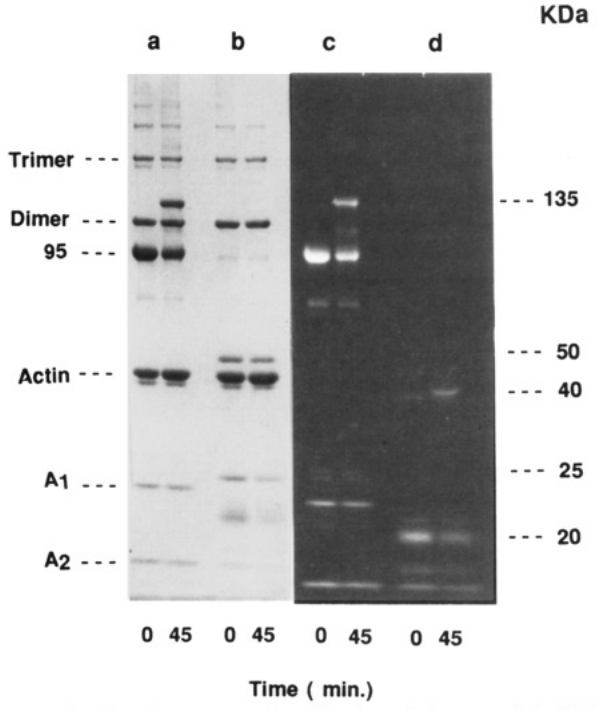

FIGURE 8: F-Actin-promoted production of the cross-linked 135- and 40-kDa bands in S-1 with blocked SH-1 and SH-2 thiols. Complexes of F-actin and DACM-labeled S-1 (lanes a and c) or DACM-labeled (25–50–20 kDa)-S-1 (lanes b and d) were treated with MBS as in Figure 1B. The protein mixtures were subjected to gel electrophoresis before and after a 45-min reaction. Protein bands were viewed under UV light (lanes c and d) and then stained with Coomassie blue (lanes a and b).

cross-linking. Finally, no adduct was detected between the central 50-kDa region and either of the two other frgments of the heavy chain.

Identification of the Cross-Linking Site(s) within the 25-kDa Fragment. The observation of the 25–20-kDa fragments cross-linking in the above experiments employing S-1 modified at the SH-1 thiol with 1,5-IAEDANS clearly indicates that the process did not involve a reaction of the maleimidobenzoyl group of MBS with the reactive SH-1 thiol. Furthermore, Figure 8 shows that blocking both SH-1 and SH-2 with DACM did not significantly affect the nature or the extent of the heavy chain cross-linking in either native S-1 (Figure 8, lanes a and c) or split (25–50–20 kDa)-S-1 (Figure 8, lanes b and d). Because the F-actin-mediated cross-linking of the 25-kDa region is canceled by nucleotides, the binding of which to S-1 is known to involve the latter NH_2-terminal heavy chain segment (Okamoto & Yount, 1985; Yount et al., 1987; Cremo et al., 1989), our efforts were directed to the structural characterization of the peptide stretch in the 25-kDa fragment participating in the cross-linking to the 20-kDa region. The tryptic 25-kDa peptide, which encompasses residues 1–204 of the rabbit skeletal S-1 heavy chain, contains two tryptophans at positions 112 and 130, whereas the 20-kDa fragment, representing residues 643–809, does not include any tryptophan side chain (Tong & Elzinga, 1990). Following the internal cross-linking of (25–50–20 kDa)-S-1 fluorescently labeled at the SH-1 thiol with CPM and its dissociation from F-actin with MgATP, the produced fluorescent 40-kDa band was excised from the gel after electrophoresis and subjected to chemical cleavage with *N*-chlorosuccinimide (NCS), which selectively splits the tryptophanyl peptide bonds (Shechter et al., 1976). This reaction was expected to release the intact fluorescent 20-kDa fragment covalently attached to either of the three peptides deriving from the hydrolysis of the 25-kDa fragment and spanning residues 1–112, 113–130, and 131–204, respectively. Un-cross-linked 20- and 25-kDa bands isolated from the same gels were also treated with NCS as controls. The data are illustrated in Figure 9. The 20-kDa material was little affected by NCS (Figure 9C, lanes b and d), whereas two major cleavage products were generated from the gel slices of the 25-kDa fragment incubated with NCS; their apparent masses were approximately 13 and 8 kDa (Figure 9A, lane b). Each product was accompanied by a closely migrating minor species. From this pattern and the knowledge of the covalent structure of the 25-kDa fragment (Tong & Elzinga, 1990) we infer that they span the segments of residues 1–130 and 131–204, respectively, whereas the two minor bands represent residues 1–112 and 113–204, respectively, indicating a more extensive NCS hydrolysis at Trp-130 than at Trp-112. The cleavage of the cross-linked 40-kDa band gave rise to a single new fluorescent 28-kDa entity as well as to a nonfluorescent 13-kDa species, but no product was observed at the 8-kDa position (Figure 9B, lanes b and d). This cleavage profile suggests that the fluorescent 20-kDa region was covalently joined mainly to the COOH-terminal 8-kDa moiety of the 25-kDa fragment between residues 131 and 204 and not at all to its NH_2-terminal 13-kDa segment of residues 1–130. The NH_2-terminal sequencing of the electroblotted 28-kDa band confirmed unambiguously this conclusion by identifiying the sequence Leu-Pro-Val adjacent to Trp-130. These data exclude the participation of the reactive lysine-83 in the cross-linking reaction. The cross-linked segment of residues 131–204 contains no cysteine but five lysines at positions 145, 146, 184, 189, and 204. Thus, the ϵ-amino group of at least one of these residues should mediate the MBS cross-linking process of the 25-kDa fragment. The spatial proximity of the 25–50-kDa junction (residues 204–213) to these lysines may explain the observed influence of its proteolysis on the extent of the cross-linking. Moreover, upon reaction of MBS on acto-S-1 in the presence of an excess of thiolated *N*-(5-sulfo-1-naphthyl)ethylenediamine (EDANS) which readily reacts on the maleimide moiety of the reagent (Bettache et al., 1992), only the 25-kDa fragment became fluorescent with abolition of the internal heavy chain cross-linking (data not shown). This observation strongly suggests that the latter event involves lysine acylation(s) in the 25-kDa peptide with the succinimide ester group and cysteine or lysine alkylation(s) in the 20-kDa fragment with the maleimide function of MBS.

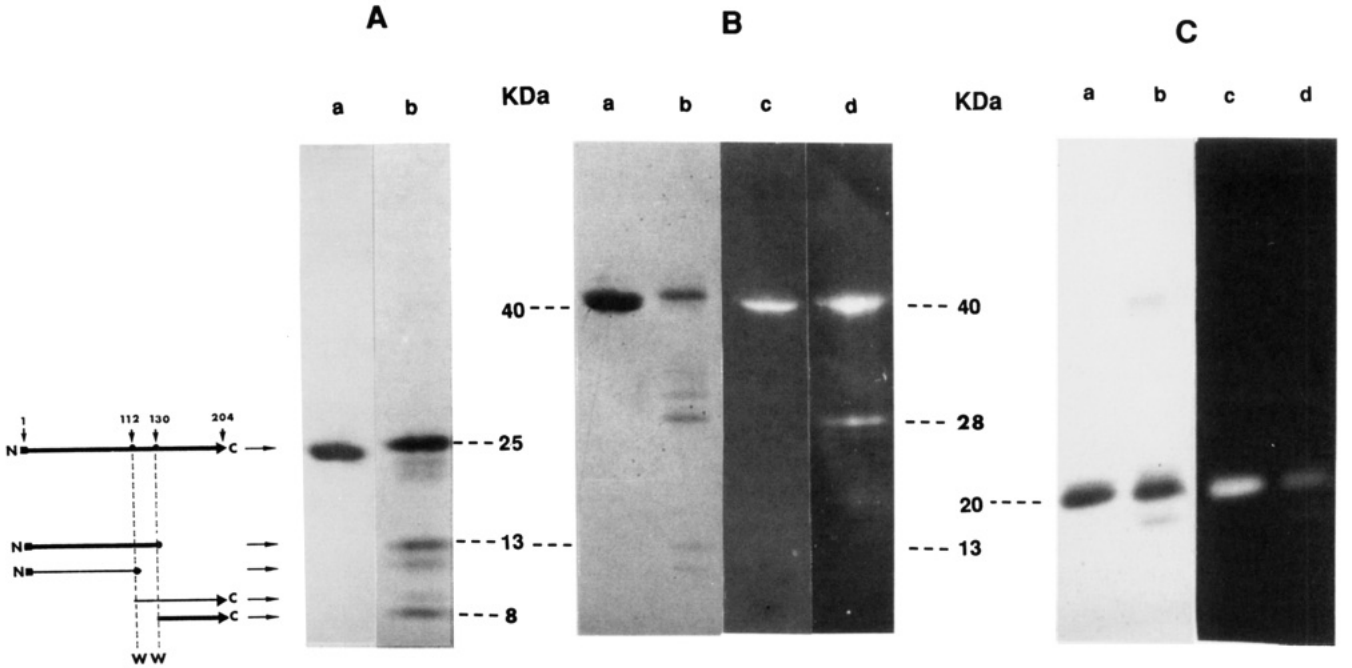

FIGURE 9: NCS peptide map of the 25- and 20-kDa fragment adduct visualized by fluorescence distribution and Coomassie blue staining. Following gel electrophoresis of the MBS cross-linked (25–50–20 kDa)-S-1 fluorescently labeled on the SH-1 thiol with CPM, the bands containing free N-terminal 25-kDa fragment (A), the fluorescent 40-kDa adduct (B), or free fluorescent C-terminal 20-kDa fragment (C) were excised and digested with NCS to cleave at tryptophans, as described under Materials and Methods. The gel slices before (lanes a and c) and after (lanes b and d) NCS treatment were run on a second-dimension gel (9–18% gradient acrylamide), and peptide bands were located by fluorescence (lanes c and d) or by staining with Coomassie blue (lanes a and b). The diagram of the 25-kDa fragment and its peptides in (A) is drawn to scale on the basis of the sequence (Tong & Elzinga, 1990). The cleavage of the standard and of the cross-linked 25-kDa fragments by NCS does not go to completion. The NCS reaction causes a decrease of the fluorescence intensity probably due to the oxidative properties of the reagent. In lane b of (B), the faint nonfluorescent band migrating with a lower mobility than the 28-kDa species is a cleavage product of the central 50-kDa heavy chain fragment which unavoidably contaminates slightly the 40-kDa adduct, as assessed by NCS cleavage of a control 50-kDa peptide band. W = tryptophan.

DISCUSSION

Recently, we have used MBS-derivatized F-actin and G-actin to achieve, by a two-step methodology, their specific coupling to the S-1 heavy chain at or near the rigor binding site of the myosin head (Bettache et al., 1992). The present study shows that no such covalent conjugation of F-actin and S-1 can be accomplished by directly reacting MBS with the preformed acto-S-1 complex. Instead, an intramolecular cross-linking of the S-1 heavy chain was the main event occurring under this condition. The reported intermolecular acto-S-1 cross-linking required the initial substitution of G-actin with free maleimidobenzoyl group(s) at specific sites located at or near the strong binding surface. This critical step makes the resulting MBS–G-actin and its polymerized from, MBS–F-actin, able to covalently join to S-1. However, with the preformed, native F-actin, no significant reaction between MBS and the potentially cross-linkable actin sites seems to take place, probably because of the conformational changes accompanying the G–F conversion. The intramolecular cross-linking process displayed the striking feature to be essentially dependent on the strong attachment of S-1 to F-actin. The 50–60% yield of the cross-linked 135-kDa heavy chain is quite similar to that observed earlier by others for S-1 internally cross-linked with another bifunctional agent, dibromobimane (Mornet et al., 1986). The fact that the reaction plateau was near 50% is unlikely to be due to heterogeneity of the S-1 preparation or to some kind of cooperativity. Rather, monofunctional reactions favored by the hydrolysis of either of the two reactive functions of MBS could have restricted the final extent of heavy chain cross-linking. Although the affinity between F-actin and S-1 is high, the extent of cross-linking did not saturate at an actin:S-1 molar ratio of 1, as would be expected, but rather at a noticeably higher molar ratio. Since the cosedimentation analyses did not show a reduction of the affinity of MBS-treated actin for S-1, it may be possible that the internal heavy chain cross-linking was further increased by the different mode of S-1 binding to actin, which was thought to occur when the actin sites are in excess (Yamamoto, 1990). By analogy with the well-known nucleotide-directed internal cross-linking of the S-1 heavy chain which reflects nucleotide-dependent conformational changes in flexible segments of the heavy chain, our cross-linking data also provide direct structural evidence for F-actin-induced movements at the interface of mobile portions of the 25- and 20-kDa heavy chain fragments of S-1. These two regions contain functionally essential structural determinants related to the binding of nucleotides in the former fragment (Yount et al., 1987; Cremo et al., 1989) and to the interaction of actin and possibly also of the nucleotides in the latter fragment (Mornet et al., 1981; Sutoh, 1982; Burke et al., 1990). The observed reciprocal influence of F-actin and ADP on their cross-linking by MBS is in line with the overall biological activities of these peptides and with the suggestion that they are intervening in the intersite communication within the S-1 molecule (Lin & Cheung, 1991).

Previous intramolecular cross-linking investigations on the native S-1 have revealed the spatial close proximity of strands in the 25- and 20-kDa segments (Hiratsuka, 1984; Lu et al., 1986; Sutoh & Lu, 1987; Sutoh & Hiratsuka, 1988; Maruta et al., 1990). The reported very short distance between Lys-184 or Lys-189, which make part of the flexible ATP-binding glycine-rich loop in the 25-kDa fragment, and Cys-697 (SH-2 thiol) in the 20-kDa domain (Sutoh & Hiratsuka, 1988), is of particular relevance to our findings locating the former amino acid side chains in the stretch of residues 145–204 engaged in the F-actin-promoted cross-linking to the 20-kDa region. In both cases, the binding of nucleotides to the S-1 ATPase site suppresses the cross-linking reaction. Collectively,

the data point to the F-actin- and nucleotide-modulated changes in the orientation and/or environment of the COOH-terminal portion of the 25-kDa fragment, which includes a major phosphate-binding subsite of the S-1 ATPase site (Cremo et al., 1989). Our present cross-linking study further underlies the dynamic aspects internal to S-1.

The F-actin effect on the COOH-terminal domain of the 25-kDa fragment may have a potent functional signifiance as it could represent at least part of the molecular mechanism allowing F-actin to enhance the rate of P_i release during the transition from the weakly attached acto-S-1-ADP·P_i to the strongly bound state and thereby to promote force generation. In this regard, it was recently reported that a monoclonal antibody directed to the COOH terminus of the 25-kDa fragment strongly inhibited the sliding of actin filaments not by inhibiting the ATPase activity but rather by uncoupling the ATP hydrolysis from movement, suggesting the flexibility of this region is important for conversion of ATP hydrolysis to mechanical force (Kinose et al., 1992). Also, F-actin was previously found to affect the conformation of the adjacent 25–50-kDa connector region as inferred from the change in its proteolytic susceptibility in the acto-S-1 complex (Applegate & Reisler, 1983; Muhlrad & Chaussepied, 1990). Because, to our knowledge, F-actin was not shown to bind to the 25-kDa fragment in the native S-1, its proposed influence on the ATPase subsite(s) in this segment could be transmitted through specific peptide stretches of the COOH-terminal 20-kDa domain, which is known to bind directly to actin and which was selectively participating in the F-actin-dependent MBS cross-linking. The identification, currently underway, of the site of cross-linking in the 20-kDa region will provide further insights into the structural and dynamic properties of the acto-S-1 complex. Also, the combination of our cross-linking data with the knowledge of the tertiary structure of the S-1 heavy chain, which will provide essential information on the actual separation in native S-1 of the residues involved in the MBS cross-linking, should be valuable for a firm appreciation of the amplitude of the F-actin and nucleotide-induced changes in S-1.

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