Cross-Linking of F-Actin to Skeletal Muscle Myosin Subfragment 1 with Bis(imido esters): Further Evidence for the Interaction of Myosin-Head Heavy Chain with an Actin Dimer†

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ABSTRACT: Three bifunctional reagents with 11-Å arm length, dimethyl suberimidate, dimethyl 3,3′-dithiobis(propionimidate), and disuccinimidyl 3,3′-dithiobis(propionate), were successfully applied to the cross-linking of F-actin to myosin subfragment 1 (S1) and the tryptically fragmented S1 derivatives. A detailed analysis of native S1–F-actin cross-linking with the bis(imido esters) indicated the formation of a single cross-linked actin–S1 species of M_r 200K homologous to the 185K entity produced by carbodiimide zero-length cross-linking and that corresponds to the covalent association between a 95K heavy chain and a pair of vicinal actin subunits [Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306]. Experiments performed on rigor complexes including either fluorescent actin or fluorescent fragmented S1 led to the conclusion that the two actin monomers were cross-linked to the C-terminal 70K heavy chain segment that comprises the two actin binding fragments of M_r 50K and 20K revealed earlier by the carbodiimide reagent. However, in contrast to the latter cross-linker, the diimidates were sensitive to the structural state of the 50K–20K joint, the cleavage of which abolished cross-linking between actin and the 20K domain but not between actin and the 50K domain. Covalent binding of F-actin to S1 catalyzed by all three reagents employed induced a vast increase of the Mg^{2+}-ATPase activity. Cross-linking of F-actin to (27K–50K–20K)-S1, a derivative nonactivable by actin under reversible conditions, resulted in the restoration of its stimulated Mg^{2+}-ATPase. This finding implies that the cross-linking to actin brings the 50K and 20K fragments into a conformational state similar to that presented by the intact 70 K segment and that promotes ATPase activation. Cross-linking with the cleavable reagents tested offers a potent direct approach for the localization of the interaction sites on the surfaces of actin and S1.

Recently, we have described the use under proper experimental conditions of the bifunctional reagents dimethyl suberimidate and disuccinimidyl 3,3′-dithiobis(propionate) as valuable cross-linkers for the cross-linking of the heavy chain to the alkali light chains in myosin S1[1] from skeletal muscle (Labbé et al., 1981). The reaction has aided to establish neighbor relationships between the light subunit and the NH_2-terminal 27K segment of the heavy chain.

In a parallel study, we have employed a cardiimide zero-length cross-linker to probe the topography of the rigor complex between F-actin and S1. The findings demonstrated for the first time that the 95K heavy chain of the myosin head has intimate contacts with two adjacent actin monomers; one is bound to the 50K fragment and the other to the 20K segment of the heavy chain (Mornet et al., 1981c). Also, the "freezing" of actin and S1 in the associated state by the chemical cross-linking resulted in a considerable increase of the Mg^{2+}-ATPase activity of the complex. The identification of the major sites of cross-linking represents a further step of our work. It can give information about the location of the interaction sites on the surfaces of actin and the two heavy-chain fragments and can aid to better understand the molecular relationship of the covalent acto-S1 complex to actin activation of the myosin ATPase activity.

In the present investigation, we have exploited our cross-linking procedure involving the bis(imido ester) reagents to analyze actin–S1 association with the use of reversible cross-linkers, which offer the potential advantage over the carbodiimide reagent to permit the subsequent structural determination of the cross-linking sites on actin and S1. We have been encouraged to carry out this study by the report of Yamamoto & Sekine (1979), suggesting the cross-linking of F-actin to tryptically fragmented S1 with dimethyl suberimidate. Our results confirm the proximity of S1 heavy chain with the pair of actin subunits and further indicate that the same discrete C-terminal segments of M_r 50K and 20K that participate in the cross-linking promoted by the carbodiimide reagent also participate in the cross-linking catalyzed by the longer diimidate agents.

Material and Methods

Reagents. Disuccinimidyl 3,3′-dithiobis(propionate) and the hydrochlorides of dimethyl suberimidate and dimethyl 3,3′-dithiobis(propionimidate) were purchased from Pierce Chemical Co. Trypsin [treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone] was from Worthington Biochemical Corp. All other chemicals were of analytical grade.

Preparation of Native and Modified Proteins. Myosin was isolated from rabbit back and hind leg muscles according to Offer et al. (1973). S1 was prepared by digestion of myosin filaments with chymotrypsin (Weeds & Taylor, 1975), purified as a mixture of S1 isoenzymes by gel filtration over Sephacryl S-200 eluting with 50 mM Tris-HCl, pH 7.5, and resolved into S1 A1 and A2 isoenzymes by ion-exchange chromatography (Weeds & Taylor, 1975). F-actin was purified according to Spudich & Watt (1971). The protein pellet was resuspended in 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 2 mM

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[1] Abbreviations: ATPase, adenosinetriphosphatase (EC 3.6.1.3); S1, subfragment 1; acto-S1, actomyosin S1; LC1, alkali light chain 1; LC3, alkali light chain 3; $NaDodSO_4$, sodium dodecyl sulfate; DMS, dimethyl suberimidate; DTP, dimethyl 3,3′-dithiobis(propionimidate); DSP, disuccinimidyl 3,3′-dithiobis(propionate); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

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Tris-HCl, pH 8, containing 1 mM NaN_3 (Taylor & Weeds, 1976).

The trypsin-modified S1 derivatives, (27K–50K–20K)-S1, (27K–70K)-S1, and (75K–22K)-S1, were prepared essentially as described by Mornet et al. (1981a). F-actin and native and fragmented S1 were labeled with the fluorescent dye *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) as reported by Mornet et al. (1981b,c). Protein concentrations were determined as described previously (Mornet et al., 1981b).

Cross-Linking of F-Actin–S1 Complexes with Dimethyl Suberimidate. F-actin (2 mg/mL) and native S1 or fragmented S1 derivatives (2 mg/mL) were first combined in 1 mL of 100 mM triethanolamine–HCl buffer, pH 8.3, after centrifugation at 160000*g* for 45 min at 4 °C. The amidination reaction was initiated by addition of DMS (150-fold molar excess to S1; 0.400 mg of DMS for 1 mg of protein), dissolved immediately before use in the buffer (10 mg/mL). After incubation at 20 °C, the reaction was quenched by adjusting the solution to 100 mM glycine (pH 8). Samples containing 0.030–0.050 mg of S1 were subjected to electrophoretic analysis.

Cross-Linking of F-Actin–S1 Complexes with Dimethyl 3,3'-Dithiobis(propionimidate) and Disuccinimidyl 3,3'-Dithiobis(propionate). Cross-linking experiments with DTP were performed under similar conditions except that the reagent concentration was 75-fold molar excess to S1. Acylation with DSP (12-fold molar excess to S1) was performed under similar conditions with 100 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.5. Stock solutions of DSP (10 mg/mL) were made in dry acetone immediately prior to use.

Aliquots (100 μL) were withdrawn from the reaction medium containing DTP or DSP, mixed with an equal volume of 10% $NaDodSO_4$ (without β-mercaptoethanol), and denatured by heating at 37 °C for 10 min (Wang & Richards, 1974). Dissociation of unreacted S1 species with magnesium pyrophosphate and isolation of the covalent actin–S1 material from cross-linking reaction mixtures were carried out as reported by Mornet et al. (1981c).

Sodium Dodecyl Sulfate Gel Electrophoresis. Cross-linked species were separated by electrophoresis in 0.1% $NaDodSO_4$–polyacrylamide slab gels (Studier, 1973) containing 5–18% (w/v) gradient acrylamide (Laemmli, 1970). A 50 mM Tris–100 mM boric acid buffer (pH 8.0) was employed (Mornet et al., 1981b). The chain weights of cross-linked protein entities were determined with the following molecular weight markers: chymotryptic S1 heavy chain (95K), actin (42K), carbodiimide-cross-linked actin–S1 heavy chain (180K) (Mornet et al., 1981c), and the subunits of rabbit spinal neurofilaments of M_r 200K, 145K, and 68K, respectively (Liem et al., 1978). Gels were stained with Coomassie brilliant blue R-250 and destained according to Weber & Osborn (1969). Fluorescent protein bands were located in the gels by illumination with long-wave ultraviolet light before staining with Coomassie blue.

S1 ATPase Activity. The Mg^{2+}-dependent ATPase activity of S1 was measured as specified elsewhere (Mornet et al., 1979a). The Mg^{2+}-ATPase of the covalent actin–S1 complex was assayed in a medium (1 mL) containing 10 mM KCl, 50 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 2.5 mM ATP, and 0.050 mg of S1.

The reduction of the disulfide bonds in actin–S1 samples cross-linked with DTP was performed before Mg^{2+}-ATPase measurements as follows: At the desired time intervals 100-μL aliquots of the quenched cross-linking reaction mixture were mixed with an equal volume of 20 mM dithioerythritol in 100 mM triethanolamine, pH 8.5, and incubated at 25 °C for 3 min.

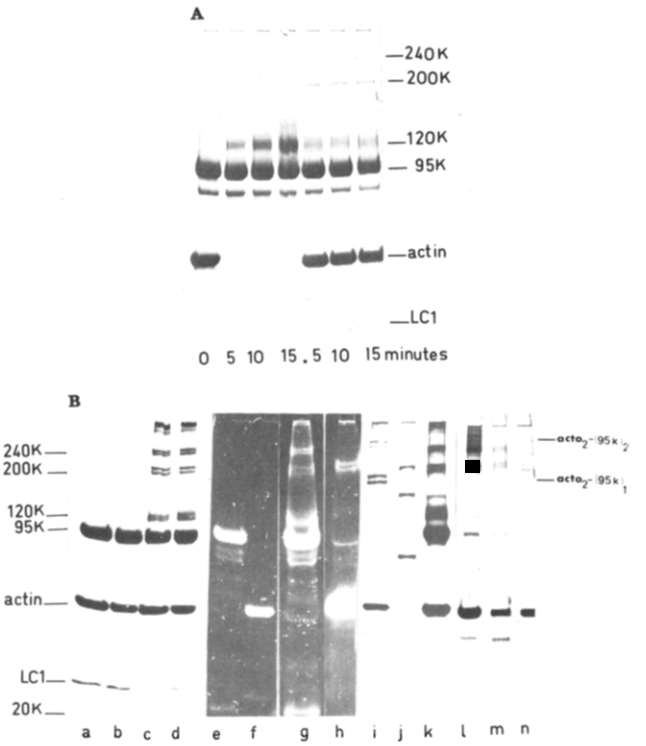

FIGURE 1: (A) Influence of F-actin on the dimethyl suberimidate induced cross-linking of the heavy and alkali light chains in S1. S1 (A1) (20 μM) was reacted with 3.6 mM DMS at pH 8.3 and 20 °C in the absence and in the prsence of F-actin (40 μM). At the time indicated, samples were subjected to electrophoresis as described under Material and Methods. (B) $NaDodSO_4$ gel electrophoretic analysis of actin–S1 cross-linking with dimethyl suberimidate. Fluorescent S1 (A1 + A2) (20 μM) complexed to F-actin (40 μM) and fluorescent F-actin complexed to S1 (A1 + A2) were reacted in parallel with 3.6 mM DMS at pH 8.3, 20 °C, for 30 min; protein samples were submitted to gel electrophoresis before (a, b, e, and f) and after addition of the reagent (c, d, g, and h). Gels were viewed under ultraviolet light (e–h) and then stained with Coomassie blue (a–d). Cross-linked species (k) were run in the presence of the following protein markers: carbodiimide-cross-linked actin dimer–S1 heavy chain [180K and 265K entities corresponding to $(actin)_2$–$(95K)_1$ and $(actin)_2$–$(95K)_2$, respectively] (i); subunits of rabbit spinal neurofilaments (200K, 145K, and 68K) (j). DMS-cross-linked actin–S1 pellet obtained after treatment of the cross-linking reaction mixture with 10 mM $MgPP_i$, pH 8.5 (l). Protein pellet (n) and supernatant (m) formed after incubation of (l) in (1 mL) 0.5% $NaDodSO_4$–100 mM triethanolamine, pH 8.5, for 5 min at 25 °C, followed by centrifugation at 160000*g* for 45 min at 4 °C.

Results

Cross-Linking of Rigor Complex of S1 and F-Actin with Dimethyl Suberimidate and Cleavable Bifunctional Reagents. When we wished to compare the diimidate-catalyzed cross-linking of the heavy and light chains in S1 (A1) in the absence and in the presence of F-actin under the optimized conditions reported earlier (Labbé et al., 1981), this study led to two striking results illustrated by the gel electrophoretic patterns presented in Figure 1A. First, the amount of 120K protein band corresponding to the heavy chain–light chain cross-linked species was markedly decreased upon addition of F-actin (molar ratio F-actin:S1 = 2). It is unlikely that the reduced production of the 120K entity was due to a competition between actin and S1 for DMS, which was reported to generate intramolecularly cross-linked F-actin (Ohara et al., 1981,

1982). The same effect was again observed when a 2-fold higher concentration of DMS was employed (0.800 mg/1 mg of S1). Second, the presence of F-actin induced also the formation of two new protein species with apparent masses of 200K and 240K, respectively.

These data encouraged us to explore the possible covalent attachment of F-actin to S1 heavy chain by means of the diimidate cross-linking reagents. The general features of the cross-linking reaction of acto-S1 with dimethyl suberimidate are detailed in Figure 1B. In our best gels each of the 200K and 240K protein bands was resolved into a sharply defined doublet. The two protein entities were formed at almost the same rate and in nearly equal proportion. For some actin–S1 preparations the 240K band was slightly more intense. As expected from the pH dependence of the reaction of imido esters with amino groups, the intensity of the two bands diminished significantly when the experiments were conducted below pH 8. The composition of the 200K and 240K species was unequivocally established by reacting DMS on rigor actin–S1 complexes in which either the actin or the S1 was labeled with the fluorophore 1,5-IAEDANS. The distribution of the fluorescence in the protein bands formed (Figure 1B, lanes e–h) demonstrated clearly that the protein doublet of M_r 200K was a cross-linked adduct of actin and S1 as label from either source was incorporated whereas the 240K product was deriving mainly from S1 as only the fluorescence of S1 heavy chain was associated with it. Because this species was observed only in the presence of F-actin, it could be a dimer of S1 possibly formed by cross-linking of adjacent S1 molecules decorating the actin filament. The additional incorporation of S1 fluorescence into the 120K band was consistent with our earlier conclusion that this material is an intramolecularly cross-linked product of the 95K heavy chain with the alkali light chain (Labbé et al., 1981).

To probe the impact of the cross-linking reaction on the association–dissociation properties of the actin–S1 complex, we supplemented the protein mixture treated with DMS for 30 min (Figure 1B, lane k) with 10 mM $MgPP_i$; after centrifugation, the pellet, analyzed by gel electrophoresis (Figure 1B, lane l), was found almost entirely free of native S1 as well as of the intramolecularly cross-linked 120K S1 species. It should be noted that in separate experiments this species was found to cosediment with F-actin. In contrast, the 240K material, which was assumed to originate from intermolecularly cross-linked S1 heavy chains noncovalently bound to F-actin, proved to be rather refractory to dissociation by either $MgPP_i$ or MgATP and remained firmly associated with F-actin. After multiple trials and errors, the 240K species could be completely removed from actin by incubation of the PP_i-treated protein pellet in 0.5% $NaDodSO_4$, pH 8.5, for 5 min at 25 °C; after centrifugation, the actin pellet formed contained only the 200K band issued from S1 covalently cross-linked to F-actin (Figure 1B, lane n) whereas the supernatant included all the 240K material together with some amount of 200K product and of G-actin (Figure 1B, lane m). The two latter components were presumably issued from an unavoidable solubilization of a portion of the covalent F-actin–S1 complex during its mild treatment with $NaDodSO_4$.

The overall impression of these results is that reaction of DMS with F-actin–S1 complexes gives rise to a single cross-linked actin–S1 unit of M_r 200K, which, according to its apparent mass, could be identical with the 180K species generated by carbodiimide-catalyzed cross-linking (Figure 1B, lane i) and which was shown to result from the association of two vicinal actin monomers with a single 95K heavy chain

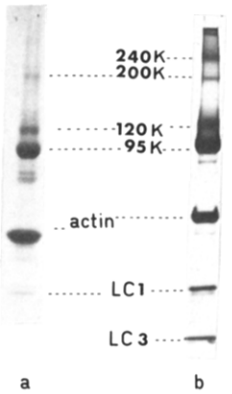

FIGURE 2: Cross-linking of actin–S1 complex with cleavable DSP and DTP. S1 (A1 + A2) (20 μM) was reacted with DSP at pH 7.5 and 20 °C for 4 min in the prsence of F-actin (40 μM) (a); S1 (A1 + A2) (20 μM) complexed to F-actin (40 μM) was treated with DTP at pH 8.3 and 20 °C for 30 min (b).

(Mornet et al., 1981c). The observed 20K difference in the apparent molecular weights of the DMS- and EDC-induced derivatives could be due either to an effect of the amidination reaction, which decreases the actual mobility of the proteins (Davies & Stark, 1970; Hitchcock, 1975), or to the involvement of the alkali light chain in the protein composition of the 200K species since this subunit undergoes also cross-linking with the heavy chain in the presence of actin and DMS. This statement holds equally well for the 240K product, the apparent mass of which was actually greater than the value expected from the molecular weight of two 95K heavy chains. To test for the eventual presence of the light chain within the 200K and 240K products, we attempted a cross-linking experiment using F-actin complexed to S1 containing LC 1 subunit labeled with 1,5-IAEDANS (Marsh & Lowey, 1980), which was obtained by the termal hybridation procedure of Sivaramakrishnan & Burke (1981). Gel analysis of the reaction mixture showed the incorporation of fluorescence into the 120K band but gave no evidence for association of the light chain with the 200K or 240K entities.

As illustrated in Figure 2, the cross-linking of actin and S1 heavy chain was also achieved upon reaction of the acto-S1 complex with the cleavable, disulfide containing cross-linkers. Acylation with DSP produced the 200K band only (Figure 2, lane a) whereas both the 200K and 240K species were formed in the presence of DTP, a disulfide analogue of DMS (Figure 2, lane b).

Cross-Linking of Actin Pairs to Intact C-Terminal 70K Segment of S1 Heavy Chain. To determine if the parts of the heavy chain that are engaged in the DMS-catalyzed cross-linking with actin are similar to those involved in cross-linking by EDC, we investigated peptide mapping of the cross-linked species formed by complexes of fluorescent actin with tryptically fragmented S1. Cross-linking experiments on fluorescent fragmented S1 combined to actin were also performed to distinguish the intramolecularly cross-linked complexes of heavy chain fragments. When (27K–70K)-S1 was used, a new protein doublet of M_r 180K was produced (Figure 3A, lanes A and B); it contained the fluorescence of either actin or 70K peptide (Figure 3A, lanes a and b). Actin dimers and trimers were also formed; their amount was generally much greater when labeled actin was employed. On the other hand, intramolecularly cross-linked 27K–70K peptide complex was also produced; a subsequent cross-linking of actin to this reconstituted heavy chain species was probably responsible for the presence of a faint 200K band. Obviously, the 180K species is homologous to the 160K doublet generated in the reaction

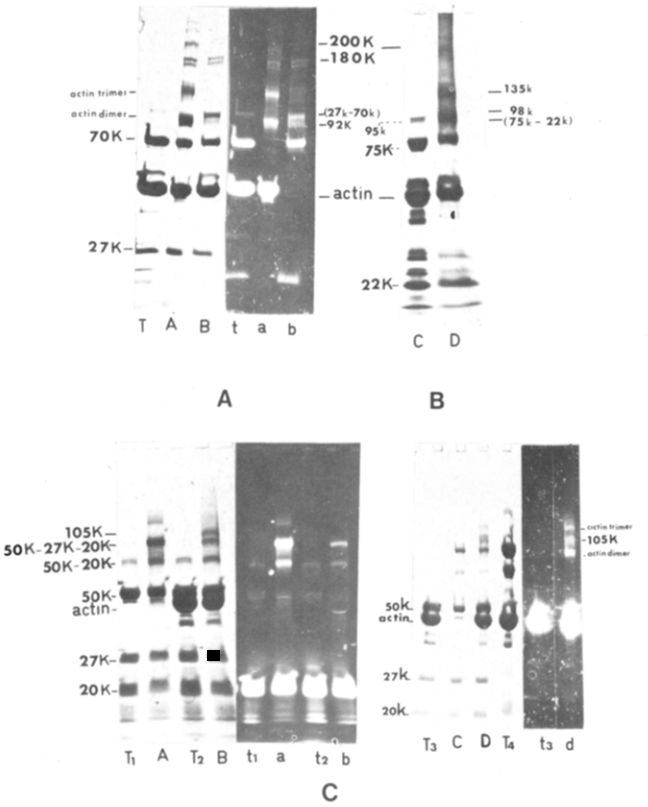

FIGURE 3: Cross-linking of actin to intact C-terminal 70K segment of S1 heavy chain. (A) Fluorescent (27K–70K)-S1 (A1 + A2) (20 μM) complexed to F-actin (40 μM) and fluorescent F-actin complexed to (27K–70K)-S1 (A1 + A2) were reacted in parallel with DMS under the conditions reported in Figure 1: protein banding pattern after staining with Coomassie blue (T, A, and B); fluorescence pattern of the gel (t, a, and b); (T and t) fluorescent actin and fluorescent (27K–70K)-S1 controls; (A and a) fluorescent actin cross-linked to (27K–70K)-S1; (B and b) actin cross-linked to fluorescent (27K–70K)-S1. (B) (75K–22K)-S1–actin complex before (C) and after (D) reaction with DMS. (C) Fluorescent (27K–50K–20K)-S1 (A1 + A2) was reacted with DMS in the absence (A and a) and in the presence (B and b) of F-actin; controls were fluorescent fragmented S1 (T_1 and t_1) and fluorescent fragmented S1–actin complex (T_2 and t_2) at zero time. (27K–50K–20K)-S1 was reacted with DMS in the absence (C) and in the presence (D and d) of fluorescent F-actin; controls were fragmented S1–fluorescent actin complex (T_3 and t_3) at zero times; 95K heavy chain, (75K–22K)-S1, and actin were run as markers (T_4).

of EDC with the same protein complex and that corresponds to two actin subunits separately bound to intact 70K segment. The present findings imply that DMS induces the cross-linking of the actin pair to the C-terminal 50K and 20K peptides of S1 heavy chain as demonstrated earlier for the EDC reaction (Mornet et al. (1981c). Cross-linking of actin to (75K–22K)-S1 yielded a major new product of M_r 135K, together with the intramolecularly cross-linked complex of 75K and 22K peptides. The 135 K species could be only the cross-linked product actin–75K peptide similar to the 120K entity that forms upon cross-linking with EDC. The obtention of this species embodies the cross-linking of actin to the 50K region of the heavy chain. However, and in contrast to the result obtained with EDC, no protein entity corresponding to actin–22K peptide could be identified in the DMS-catalyzed reaction whether or not fluorescent actin was employed. This fact suggested that tryptic cleavage of the 75K–22K junction abolishes the cross-linking of an actin monomer to the 20K segment but does not affect the cross-linking of the second actin subunit to the 50K element. A support for this proposal was provided by the results of cross-linking experiments done

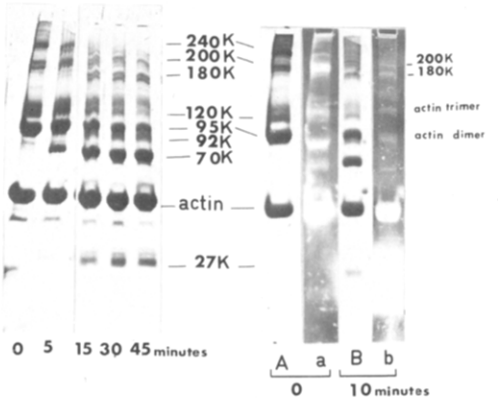

FIGURE 4: Time course of tryptic proteolysis of the 200K cross-linked $(actin)_2$–S1 complex. Fluorescent actin–S1 (A1 + A2) complex was treated with DMS under the conditions described in Figure 1; after the reaction was quenched with 0.1 M glycine, trypsin was added at a protease to S1 weight ratio of 1:10. Protein samples were analyzed by gel electrophoresis at the times indicated. The Fluorescence pattern of the gels following 0- and 10-min digestion (a and b) is shown and compared with the corresponding gel pattern after staining with Coomassie blue (A and B).

on the complex of F-actin and (27K–50K–20K)-S1. Aside from the production of intramolecularly cross-linked 50K–20K and 27K–50K–20K peptide complexes that incorporated the fluorescence of 20K peptide (Figure 3C, lanes A, B, a, and b), the reaction led to the formation of a single new species of M_r 105K, which was fluorescent when fluorescent actin was employed (Figure 3C, lanes B, D, and d); its presence was consistent with the formation of actin–50K peptide. No protein band accounting for actin–20K peptide was detected.

Finally, the association between the two actin monomers and the intact 70K heavy chain segment could be directly recognized by submitting the DMS-treated F-actin–S1 complex to limited digestion by trypsin (Figure 4). Because of the protective effect of actin on the 50K–20K junction (Mornet et al., 1979a,b), proteolysis occurred only at the 27K–70K joint and resulted in the conversion of the actin-containing 200K material into a 180K doublet similar to that obtained by cross-linking actin to (27K–70K)-S1; the fluoresence of actin initially present in the 200K band was progressively transferred to the 180K species. The tryptic reaction led also to the fragmentation of the 240K and 120K protein species. The 240K band yielded material migrating at or near the 200K position whereas the 120K entity was progressively converted into a new product of M_r 92K; the 95K polypeptide was split into the expected 27K and 70K components. Interestingly, a 92K band was also produced during the reaction of DMS with the actin–(27K–70K)-S1 complex (Figure 3A, lanes B and b); it contained the fluorescent 70K fragment, and similarly, it showed a mobility slightly higher than that of the intramolecularly cross-linked 27K–70K peptide complex. We attributed tentatively the 92K species to the possible cross-linking of the 70K fragment to a light chain.

Elevated Mg^{2+}-ATPase Activity of Acto-S1 Cross-Linked with Various Bifunctional Reagents. Figure 5 illustrates the times courses of the changes in the Mg^{2+}-ATPase activity upon reaction of acto-S1 with DMS, DTP, and DSP. All three cross-linkers induced a vast increase of the Mg^{2+}-ATPase, as observed in the EDC reaction (Mornet et al., 1981c). The extent of Mg^{2+}-ATPase stimulation was correlated with the amount of 200K product formed. With DMS and DTP the maximal extent of ATPase activation was reached after 15-min reaction whereas cross-linking with DSP activated maximally the ATPase after only 2–3-min reaction. The relative effec-

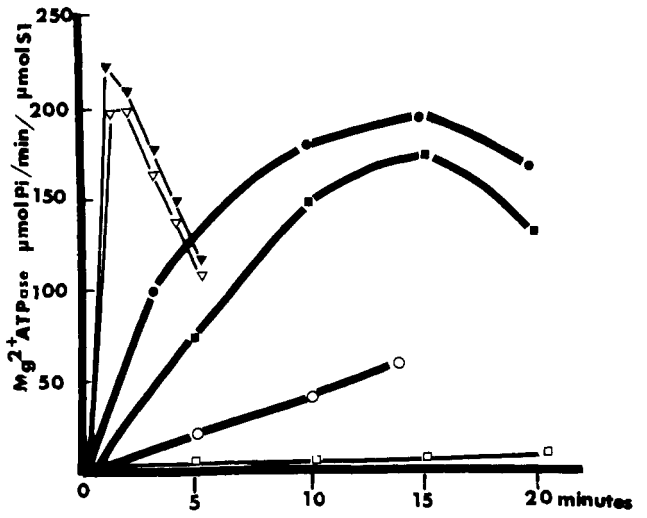

FIGURE 5: Stimulation of Mg^{2+}-ATPase of S1 covalently cross-linked to F-actin with various bifunctional reagents. S1 (A1 + A2) (20 μM) was incubated at pH 8.3 and 20 °C with 3.6 mM DMS in the presence (■) and in the absence (□) of F-actin (40 μM); S1 (A1 + A2) (20 μM) was reacted at pH 8.3 and 20 °C with 1.8 mM dimethyl 3,3′-dithiobis(propionimidate) in the presence of F-actin (40 μM). At the time indicated, 25-μL protein samples were withdrawn and submitted to Mg^{2+}-ATPase assays; for the reaction with DTP, ATPase activities were measured before (●) and after (○) 3-min incubation in 10 mM dithioerythritol, pH 8 and 25 °C. S1 (A1 + A2) (20 μM) (▼) and 27K–50K–20K)-S1 (20 μM) (▽) were treated at pH 7.5 and 20 °C with 0.3 mM disuccinimidyl 3,3′-dithiobis(propionate) in the presence of F-actin (40 μM). At the times indicated, samples were taken and assayed for Mg^{2+}-ATPase activity.

tiveness of the acylating agent was also noticed during our earlier study concerning heavy chain–light chain cross-linking (Labbé et al., 1981). The activation process generated by all the reagents employed was always followed by a progressive inactivation phase resulting, probably, from intramolecular and time-dependent secondary reactions. The use of the cleavable cross-linker DTP was convenient for providing the direct experimental evidence that the increase of the rate of S1 Mg^{2+}-ATPase was not promoted by the chemical modification of the amino groups of S1, but rather, it was caused by the nondissociable character of the covalent protein complex formed. Thus, suppression of the elevated Mg^{2+}-ATPase activity occurred upon a brief treatment of the DTP–actin–S1 complex with dithioerythritol, which splits the disulfide bonds between actin and S1 without deblocking the substituted amino groups (Figure 5). The measured residual ATPase activity was, most probably, due to an incomplete reduction of the disulfide bridges. It should be noted that reaction of DMS with S1 alone had no apparent effect on the initial Mg^{2+}-ATPase activity of the enzyme. Furthermore, as is also shown in Figure 5, cross-linking of F-actin to (27K–50K–20 K)-S1 with DSP induced a stimulation of the Mg^{2+}-ATPase of the fragmented enzyme of almost the same magnitude as that with native S1. Cross-linking with EDC produced a similar effect. This proteolytic derivative was not reversibly activable by actin because of the fission of the 50K–20K junction (Mornet et al., 1979b). Intrachain cross-linking did not have apparent influence on this property; the intramolecularly cross-linked (27K–50K–20K)-S1 peptide complex that forms in significant amount upon cross-linking in the absence of actin (see Figure 3C, lanes A and a) was not amenable to a reversible actin activation. Finally, the restoration of the ATPase activation in fragmented S1 was consequent on its covalent cross-linking to actin only and did not require the additional cross-linking of the fragments to each other; the use of EDC-activated actin led to the same result under conditions where no cross-linking between the fragments could occur (Mornet et al. 1981c).

Discussion

The amidination of S1 and its complex with F-actin by means of the bis(imido ester) reagents promotes the formation of intra- and intermolecular cross-links. Despite the diversity of the products formed, most of them could be properly characterized. Intramolecular bridges were readily established within the S1 molecule, between the three constituting 27K, 50K, and 20K segments of the heavy chains as well as between the heavy chain polypeptide and the alkali light subunit. In agreement with the results of other cross-linking investigations employing either the diimidate agent (Yamamoto & Sekine, 1979) or the carbodiimide reagent (Mornet et al., 1981b), the binding of F-actin to S1 diminished significantly the extent of the intrachain cross-linking (Figure 3C, lane A; Figure 3B, lanes a and b). Moreover, the present study suggests that it also specifically alters the covalent association of the light chain to the heavy chain; this observation is consistent with recent ^1H NMR measurements illustrating the influence of actin on the structure of both the heavy and alkali light chains (Prince et al., 1981). In this regard, the cross-linking reaction catalyzed by the bis(imido esters) can represent another sensitive probe of the actin-induced conformational changes in myosin S1. The formation of the rigor actin–S1 complex decreases the magnitude of the intramolecular cross-linking and is probably able to change the nature of the cross-linked sites between the heavy chain and the light chain. This proposal is suggested by the finding of the 92K band, which, as judged from the incorporation of fluorescence, comprises the C-terminal 70K segment of the heavy chain. Because it was formed either by direct reaction of DMS with F-actin–(27K–70K)-S1 complex or upon proteolysis of the 120K species generated through reaction of the cross-linker with acto-S1, it could include also a light chain whose cross-linking to this area of the heavy chain was directed by actin whereas, in the absence of actin, the NH_2-terminal 27K fragment is the perferred region of cross-linking (Labbé et al., 1981). Of course, additional work is needed to assess this interesting possibility.

Intermolecular cross-links were introduced between S1 molecules and between F-actin and S1. The ability of DTP and DMS to induce a covalent association between S1 particles bound to the actin filament is in agreement with the three-dimensional reconstruction of decorated thin filaments, which has revealed contacts between S1s attached to adjacent actin monomers (Vibert & Craig, 1982).

Bis(imido ester)-catalyzed cross-linking of S1 to F-actin and to the native thin filament was first attempted by Sutoch & Harrington (1977) with the use of DTP as cross-linker. Owing to the strong pH dependence we observed for the production of the 200K species by the imido esters, the reported failure of the reagent to introduce cross-links between actin and S1 was, most probably, caused by the low pH conditions employed. The reaction with the reversible DTP reagent opens the way for the chemical characterization of the amino groups between actin and S1.

Because of the 11-Å arm length of DMS, DTP, and DSP, our results established proximity but not contact of S1 heavy chain with the surface of a contiguous actin pair. However intimate contact between two vicinal actin monomers and the two C-terminal 20K and 50K was demonstrated earlier by the use of a zero-length protein cross-linker (Mornet et al., 1981b). The observation that the C-terminal 70K region of the heavy chain also participates in the cross-linking of S1 to the actin dimer with the bifunctional imidates provides another support

for the proposed topography of the rigor complex between F-actin and the myosin head from skeletal muscle. The suppression of the cross-linking between actin and the 20K segment as a result of the proteolysis of the 50K–20K joint highlights the data of Yamamoto & Sekine (1979), who have first studied the cross-linking of F-actin and tryptically fragmented S1 with dimethyl suberimidate. While they could suggest the production of an actin–50K peptide species, no derivative corresponding to actin–20K peptide could be recognized. Cross-linking of both fragments to the actin actually requires an intact 50K–20K junction. The proteolytic events taking place at this site either induce subtle changes in the structure of the 20K element with modification of the interlysine distances at the actin–20K interfaces or cause a loss of the lysine side chains invovled in the chemical cross-linking of the 20K peptide to actin.

Finally, the important enhancement of the Mg^{2+}-ATPase activity appears to be a general property displayed by S1 covalently bound to F-actin whether the two proteins are joined by a short or a long linkage. Furthermore, the cross-linking with a cleavable bifunctional agent produces a reversibly activated acto-S1 complex; it demonstrates very directly that the observed Mg^{2+}-ATPase activation is not an effect of the chemical reaction itself on the enzyme structure, but rather, it is the result of a prolonged interaction of actin with S1.

The cross-linking of actin to the 50K region was sufficient to induce the recovery of the actin-activated Mg^{2+}-ATPase of the tryptic S1 derivative. This result indicates that the covalently bound actin imposes to the 50K segment and probably also to the 20K fragment a proper configuration for acceleration of ATP hydrolysis. In conclusion, our present investigation complements our earlier cross-linking studies on the actin–S1 complex and provides a first experimental approach to the location of the actin–S1 interaction sites.

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