Tropomyosin Inhibits the Glutaraldehyde-Induced Cross-Link between the Central 48-kDa Fragment of Myosin Head and Segment 48–67 in Actin Subdomain 2[†]

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ABSTRACT: The glutaraldehyde-induced cross-linking of the F-actin–myosin head (S1) complex, previously described [Bertrand et al. (1988) *Biochemistry 27*, 5728–5736], was investigated in the presence of tropomyosin (Tm) alone or associated with troponin (Tn), at a Tm–Tn/actin/S1 molar ratio of 1:7:3. Among the two acto-S1 cross-linked products with apparent masses of 165 and 200 kDa generated in the absence of the regulatory proteins, only the 165-kDa adduct was formed in the presence of Tm. An identical result was obtained with and without Tn regardless of the presence of Ca^{2+} and/or Mg^{2+}-ADP. The abolition of the 200-kDa cross-linked acto-S1 species was independent of the S1/actin ratio since even a 3-fold excess of S1 over actin, sufficient for fully turning on the thin filament, could not restore the 200-kDa covalent complex. In addition, the acto-S1 contacts cross-linked in either the 165- or 200-kDa product were not involved in the Ca^{2+}-linked regulation of the acto-S1 ATPase activity, as the enzymatic activities of both types of complexes were regulated to the same extent by Ca^{2+}/EGTA, in the presence of the regulatory proteins. Cross-linking experiments performed with [^{14}C]glutaraldehyde showed that both covalent complexes were composed of 1 mol of actin bound to 1 mol of S1 heavy chain. The use of proteolytic actin or S1 derivatives together with the direct proteolysis of the acto-S1 covalent adducts revealed that Tm abolished the cross-link between the central 48-kDa fragment of the S1 heavy chain and Lys^{50} of actin subdomain 2 that is responsible for the formation of the 200-kDa entity, while it did not affect the cross-link between the S1 heavy chain segment of residues 636–642 and Arg^{28} of actin that generates the 165-kDa derivative. These results provide experimental clues for the interaction of S1 with actin subdomain 2 and show that this contact is implicated in the weak acto-S1 binding state. Furthermore they demonstrate the ability of Tm to affect the structure of actin subdomain 2 even in the presence of S1 bound in the rigor state.

Tropomyosin (Tm)[1] and troponin (Tn) are the actin-bound proteins responsible for calcium regulation of vertebrate striated muscle [Ebashi & Komada, 1966; for review see El-Sahel et al. (1986)]. The Ca^{2+} binding to Tn induces changes in the Tm–actin interaction enabling the head of myosin, myosin subfragment 1 (S1), to undergo the cyclic interaction with actin necessary for the tension development process. Thus, striated muscle is characterized by two extreme states: the "off" or relaxed state and the "on" or activated state, which occur in the absence and in the presence of Ca^{2+}, respectively (Huxley, 1972; Wakabayashi et al., 1975; Kress et al., 1986). The transition between these two states implies a dramatic change in the energetic properties of the actin–S1 interaction which evolves from a "weak" to a "strong" or "rigor-like" interacting state (Hill et al., 1980; Geeves & Halshall, 1987). Moreover, kinetic studies revealed that during the transition between these two states, S1 and Tm reciprocally affect their respective interactions with actin (Bremel et al., 1972; Lehrer & Morris, 1982; Williams & Greene, 1983). It is now believed that force production would take place during this transition or isomerization in the actin–S1 interaction, which depend both on the phosphorylated state of the nucleotide bound to the S1 active site and on the position of Tm on the thin filament (Geeves, 1991; McKillop & Geeves, 1993). In order to relate the Ca^{2+}-linked regulation of the kinetic properties to a structural change within the actin–S1 interaction, one needs to know the contact sites between S1 and the reconstituted thin filament (composed of actin and Tm–Tn) in the two extreme interacting states.

The S1 binding sites on actin have been established on the basis of biochemical approaches such as chemical cross-linking (Mornet et al., 1981a; Sutoh, 1982; Labbé et al., 1982; Bertrand et al., 1988; Bettache et al., 1992; Bonafé et al., 1993), immunochemical analyses (Méjean et al., 1986; Miller et al., 1987; Labbé et al., 1990; DasGupta & Reisler, 1989, 1991; Adams & Reisler, 1993), mutagenesis experiments (Aspenstrom & Karlsson, 1991; Sutoh et al., 1991; Aspenstrom et al., 1992, 1993; Johara et al., 1993; Cook et al., 1993), chemical modifications (Szilagyi & Chen Lu, 1982; Chaussepied & Morales, 1988; Miki, 1989; Bertrand et al., 1989b), and NMR studies (Moir et al., 1987; Barden & Phillips, 1990). The data obtained were recently confirmed and completed by reconstruction of cryoelectron microscopy images of the acto-S1 complex together with the 3-D structures of actin and S1 (Kabsch et al., 1990; Rayment et al., 1993a,b).

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[1] Abbreviations: S1, myosin subfragment 1; S1(A1) or S1(A2), isoenzyme of S1 containing alkali light chain 1 or 2; G-, F-actin, monomeric and filamentous actin; Acto-S1, actomyosin subfragment 1; DTE, dithioerythritol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EGTA, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; FITC, fluorescein isothiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic; Tm, tropomyosin; Tn, troponin complex; Tn C, calcium-binding component of troponin; Tn I, inhibitory component of troponin; Tn T, tropomyosin-binding component of troponin; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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The amino acid residues D^{1}–E^{4}, D^{24}, D^{25}, R^{28}, H^{40}, G^{41}, Y^{91}–E^{100} (E^{93}, R^{95}, E^{99}, E^{100}), A^{144}–T^{148}, P^{332}–E^{334}, and I^{341}–Q^{354} were identified as possible S1 binding sites. In addition to these S1 heavy chain binding sites on actin, residues 360–364 of actin were found to interact with the 41 N-terminal residues of the alkali light chain 1 (A1 light chain; Sutoh, 1982; Trayer et al., 1987; Milligan et al., 1990). Among these different binding sites, only the actin segments 1–4 and 18–29 were implicated in both the weak and the strong states of the actin–S1 interaction (Chen et al., 1985; Chaussepied, 1989; DasGupta et al., 1989; Yamamoto, 1989; Adams & Reisler, 1993).

On the other hand, the localization of the Tm binding sites on actin is not known at the atomic level. However, recent advances in the analysis of cryoelectron microscopic images and X-ray diffraction patterns of S1-decorated thin filament together with the atomic model of the actin filament have localized Tm along the interface between subdomains 3 and 4 of actin (Milligan & Whitaker, 1990; Holmes & Kabsch, 1991; Kabsch & Vandekerkhove, 1992). The amino acid residues of actin in contact with or close to Tm in the on state apparently include K^{215}, K^{238}, the α-helix D^{222}–S^{233}, P^{307}, M^{325}, K^{326}, and K^{328}. In addition, the chemical reactivities of K^{61} and K^{336} located in subdomain 2 and at the bottom of subdomain 1 of actin, respectively, were found to be sensitive to the presence of either Tm or S1 (Szilagyi & Chen Lu, 1982; Miki & DosRemedios, 1988; Barden & Phillips, 1990). Besides residues K^{61} and K^{336} of actin being indirectly implicated in both S1 and Tm binding, no extensive studies have been undertaken so far to analyze the effect of Tm–Tn on the actin–S1 interaction.

In this study, we have investigated the effect of the regulatory proteins (Tm–Tn) on the glutaraldehyde-induced cross-link between actin and S1. The dramatic effect of tropomyosin alone on one of the two major cross-linked products led us to identify the exact location of the cross-linking sites. The results obtained provide experimental evidence for the close proximity between subdomain 2 of actin and S1 (to date only based on computer image reconstruction) and establish the effect of tropomyosin on the cross-link between the central 48-kDa fragment of the S1 heavy chain and residue K^{50} of actin independent of the state of activation of the thin filament.

MATERIALS AND METHODS

Reagents. α-Chymotrypsin was from Worthington Biochemicals. DTE was obtained from Serva. Glutaraldehyde and FITC were from Merck and Fluka, respectively. Anti-rabbit IgG and 1,5-IAEDANS were supplied by Sigma. ADP, EGTA, subtilisin, and endoprotease Arg-C were from Boehringer Mannheim. All other chemicals were of analytical grade.

Protein Preparation. Rabbit skeletal myosin was prepared as described by Offer et al. (1973). S1 was obtained by chymotryptic digestion of myosin filaments (Weeds & Taylor, 1975) and purified over Sephacryl S300 according to Chaussepied et al. (1986). S1(A1) and S1(A2) were separated by ion-exchange chromatography on SP-Trisacryl (Lheureux et al., 1993). The limited proteolysis of the S1 heavy chain (4.5 mg/mL) was performed in 10 mM HEPES, pH 8.0, at 25 °C. The (75–21 kDa)-S1 derivative was produced by reaction for 120 min with the endoproteinase Arg-C (10 units of protease/mg of protein); the proteolysis was stopped by addition of 2.5 mM tosyl-L-lysine chloromethyl ketone. The (28–48–22 kDa)-S1 derivative was generated by digestion with *Staphylococcus aureus* V8 protease for 60 min (enzyme/substrate weight ratio = 1:25). Rabbit skeletal F-actin was prepared as described by Eisenberg and Kielley (1974). G-actin was obtained by depolymerization of F-actin (1.5–2 mg/mL) in buffer G (2 mM HEPES, 0.1 mM ATP, 0.1 mM $CaCl_2$, 0.2 mM DTE, and 0.1 mM NaN_3, pH 8.0). The solution was sonicated three times, for 1 min each time, at a frequency of 20 000 Hz in a Microson cell disruptor (Model XL 2005) and then dialyzed for 48 h against buffer G. The protein isolated by centrifugation at 150000*g* for 1 h at 4 °C was purified over Sephacryl S-200 equilibrated in buffer G. The (9–36 kDa)-actin derivative was generated by proteolysis of G-actin with subtilisin for 40–50 min at an enzyme to substrate weight ratio of 1:500 (Schwyter et al., 1989); the reaction was stopped with 1 mM phenylmethanesulfonyl fluoride. Polymerization of cleaved or control G-actin was achieved by the addition of 0.1 M KCl, 2 mM $MgCl_2$, and a 2-fold molar excess of phalloidin over actin for 1–2 h at 25 °C. Rabbit skeletal tropomyosin and troponin were prepared from acetone-dried muscle powder according to Smillie (1982) and Potter (1982), respectively.

Labeling of F-actin on Cys^{374} and of S1 on Cys^{707} by 1,5-IAEDANS was achieved according to Takashi et al. (1976) and Mornet et al. (1981b), respectively. Labeling of residue Lys^{61} of actin by FITC was performed on G-actin as described (Miki, 1989). The polymerization of FITC–G-actin was achieved as described above for the subtilisin-split actin derivative.

Protein concentrations were determined spectrophotometrically with extinction coefficients of $A^{1\%}_{280nm} = 7.5$ cm^{-1} for S1 or S1 derivatives, 11.0 cm^{-1} for actin or actin derivatives, 3.3 cm^{-1} for Tm, and 4.5 cm^{-1} for Tn. The molecular masses used were 115, 42, 66, and 70 kDa for S1, actin, Tm, and Tn, respectively.

[^{14}C]Glutaraldehyde Synthesis. The synthesis and the purification of radiolabeled glutaraldehyde were performed as described by McIntosh (1992). The free monomeric form of the purified product was controlled by thin-layer chromatography (silica gel; solvent: chloroform/methanol, 19/1; visualized by iodine vapor; $R_f = 0.45$). It is worth noting that the monomeric form of glutaraldehyde in dilute solutions (the concentration of labeled glutaraldehyde stock solution was at most 3 mM) was recently confirmed by light-scattering studies (Kawahara et al., 1992). The specific activity was 4800 cpm/nmol, assuming that all the glutaraldehyde was present as the free monomeric form. The prepared [^{14}C]glutaraldehyde was able to cross-link actin and S1 at a similar rate as the commercial unlabeled product.

Cross-Linking Reactions. F-actin or F-actin derivatives (2 mg/mL) and S1 or S1 derivatives (2.7 mg/mL) were first incubated in the absence or in the presence of tropomyosin (1 mg/mL) with or without troponin (1.1 mg/mL) and centrifuged at 150000*g* for 1 h in order to eliminate the excess of regulatory proteins. The pellet (containing the reconstituted thin filaments) was resuspended in 25 mM HEPES, 5 mM $MgCl_2$, and 10 mM KCl, pH 8.0. The cross-linking reaction was initiated by either 0.5 mM cold or 0.1–0.2 mM ^{14}C-labeled glutaraldehyde for 30 min or 20 h, respectively, at 20 °C. Unless otherwise indicated, all the reactions were terminated by the addition of Laemmli's sample buffer (Laemmli, 1970).

^{14}C-Labeled cross-linked and non-cross-linked protein materials were purified from preparative electrophoretic gels. After Coomassie blue staining of a narrow lane on each side of the gel, the protein-containing bands were excised and cut into small pieces with a razor blade, and the proteins were electroeluted for 10 h (100 mV) at 20 °C using BIOTRAP BT100 electroelution equipment (Schleicher & Schuell) with

50 mM Tris, 76 mM boric acid, and 0.1% SDS, pH 8.0, as electroeluting buffer. The eluted material was passed twice through a PD10 column (Pharmacia) equilibrated against the same buffer without SDS. Protein solutions were then concentrated under vacuum, and their concentrations were determined either spectrophotometrically or by the Bradford assay using actin as standard (Bradford, 1976). The amount of [^{14}C]glutaraldehyde was measured in 10 mL of scintillator using a Kontron Betamatic V counter.

The cross-linking reactions were performed in the absence or in the presence of 1 mM Ca^{2+} or 1 mM EGTA as noted in the figure captions. Alternatively, 2 mM Mg^{2+}-ADP was added to the reaction mixtures prior to cross-linking. Comparative centrifugation experiments showed that the amount of actin-bound S1 was identical in the absence and in the presence of nucleotide.

Proteolytic Cleavage of the Acto-S1 Cross-Linked Complexes. In order to digest the acto-S1 covalent complexes by chymotrypsin, the cross-linking reactions were stopped by the addition of 1 volume of buffer S (100 mM Tris, 500 mM NaCl, 1.6 M KCl, 20 mM sodium pyrophosphate, 10 mM $MgCl_2$, and 10 mM ATP, pH 8.0) and centrifuged at 150000*g* for 1 h. The protein pellet containing only the covalent acto-S1 complexes, actin, and traces of non-cross-linked S1 was then submitted to a depolymerization process employing 0.6 M KI (Bertrand et al., 1988). After incubation for 30–60 min at 4 °C in 0.6 M KI, the solutions were clarified by centrifugation at 150000*g* for 1 h, exhaustively dialyzed against buffer G supplemented with 2 mM EDTA, and recentrifuged at 150000*g* for 1 h. Digestion with chymotrypsin was then carried out at 25 °C at a protease to substrate weight ratio of 1:20 and 1:10 for 15–80 and 180 min, respectively. Aliquots were removed from the proteolytic reaction mixtures and analyzed by gel electrophoresis.

ATPase Measurements. The actin-activated Mg^{2+}-ATPase of S1 was measured in 10 mM Tris, 5 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ (or 1 mM EGTA), and 0.5 mM DTT, pH 8.0 (buffer A). When necessary, the regulatory proteins were added at an actin/Tm–Tn molar ratio of 7:3. The amount of P_i was estimated colorimetrically as described (Mornet et al., 1981b).

The Mg^{2+}-ATPase activity was measured on the glutaraldehyde-promoted actin–S1 covalent complexes obtained as described in the Cross-Linking Reactions section with the following modifications. In order to produce low-yield cross-linking, the glutaraldehyde reaction was stopped after 5 min by the addition of 1 volume of buffer S and the mixture was centrifuged at 150000*g* for 1 h. The pellet containing actin–S1 cross-linked products and traces of non-cross-linked S1 was resuspended in buffer A, supplemented (when necessary) with Tm–Tn (actin/Tm–Tn molar ratio of 7:3), and centrifuged at 150000*g* for 1 h. A first aliquot of each preparation was withdrawn and analyzed by SDS–PAGE. The amounts of cross-linked acto-S1 products (165- + 200-kDa bands) and residual non-cross-linked S1 heavy chain were quantified by densitometric scanning of the gel as recently described (Herrmann et al., 1993). The cross-linked products usually represented 7-8% and 3–4% of total S1 when the reaction was conducted in the absence and in the presence of Tm, respectively. A second aliquot was used for ATPase measurements. ATPase rates of covalent actin–S1 complexes were then calculated by subtracting the activity of residual non-cross-linked S1 which was less than 2% of the specific activities of the protein mixtures. Control experiments were performed using either untreated actin or actin modified with glutaraldehyde in the absence and in the presence of Tm under conditions identical to those described above and using an actin/S1 molar ratio of 7:1. It is important to note that our mild glutaraldehyde treatment did not impair the regulation of actin by the Tm–Tn complex (Figure 3), in contrast to the dramatic effect of a more extensive treatment which was previously observed to "freeze" the thin filament in the on or in the off state (Mikawa, 1979).

Polyacrylamide Gel Electrophoresis and Western Blot Analysis. SDS–PAGE was carried out in 3–15% gradient polyacrylamide gels (Laemli, 1970). Samples were denatured prior to electrophoresis with Laemmli's boiling buffer (50% glycerol, 5% 2-mercaptoethanol, and 1.5% SDS in 50mM Tris, pH 8.0; Laemmli, 1970). Fluorescent bands were located in the gel by illumination with ultraviolet light before staining with Coomassie Blue.

Western blot studies of cross-linked products were performed after electrophoretic transfer from acrylamide gels to nitrocellulose sheets (0.45 μm) as previously described (Towbin et al., 1979). Polyclonal antibodies specifically directed toward the N-terminal residues 1–12 of actin (Bonafé et al., 1993) revealed the presence of actin-containing bands. Protein bands first stained with the antibodies were then visualized by a goat anti-rabbit IgG conjugated to horseradish peroxidase, using α-naphthol as substrate.

RESULTS

Effect of Regulatory Proteins on Glutaraldehyde-Induced Cross-Linking between Actin and S1. Bertrand et al. (1988) found that glutaraldehyde used under mild conditions could generate cross-links between S1 and actin giving rise to only two types of covalent acto-S1 heavy chain adducts with masses of 165 and 200 kDa (Figure 1, lanes c). When Tm–Tn complex was present at saturating concentration (actin/Tm–Tn molar ratio of 7:3), only the 165-kDa product was formed regardless of the presence of Ca^{2+} (Figure 1, lanes d_1 and d_2). The abolition of the 200-kDa band occurred not only in the presence of Tm–Tn but also when Tm alone was bound to the actin filament (Figure 1, lanes e). In parallel to the formation of the acto-S1 adducts, there was no evidence of cross-linking between actin and the regulatory proteins when S1 was present, while trace amounts of an actin–Tm covalent product and of actin oligomers (seen only with fluorescently labeled actin) were found in the absence of S1 (Figure 1, lanes a and b). Although the effect of S1 on the actin–Tm cross-link would be of major interest, it is difficult to interpret its significance because of the low yield of the reaction. We did not attempt to increase the yield with, for example, the use of higher glutaraldehyde concentrations, since this would result in the modification of actin, which profoundly affects the ATPase and force-generation activities of the actin–S1 complex (Mikawa, 1979; Lehrer, 1981; Prochniewicz & Yanagida, 1990).

Finally, the presence of 2 mM Mg^{2+}-ADP in the medium during the cross-linking experiments described above did not change the cross-linking patterns obtained either qualitatively or quantitatively (data not shown).

Relationship between Abolition of the 200-kDa Acto-S1 Adduct and Inhibition of the Acto-S1 ATPase Activity Induced by Tm. It was previously shown that Tm alone (without Tn) was able to inhibit the actin-activated ATPase activity of S1 at a low S1/actin ratio (Lehrer & Morris, 1982; Williams & Greene, 1983). Indeed, when the S1/actin ratio was decreased from 3 to 0.1, the Mg^{2+}-ATPase activity of S1 was gradually reduced from 180% to 20% in the presence of Tm as compared to the activity obtained in the absence of Tm (Figure 2B). However, when the glutaraldehyde-induced

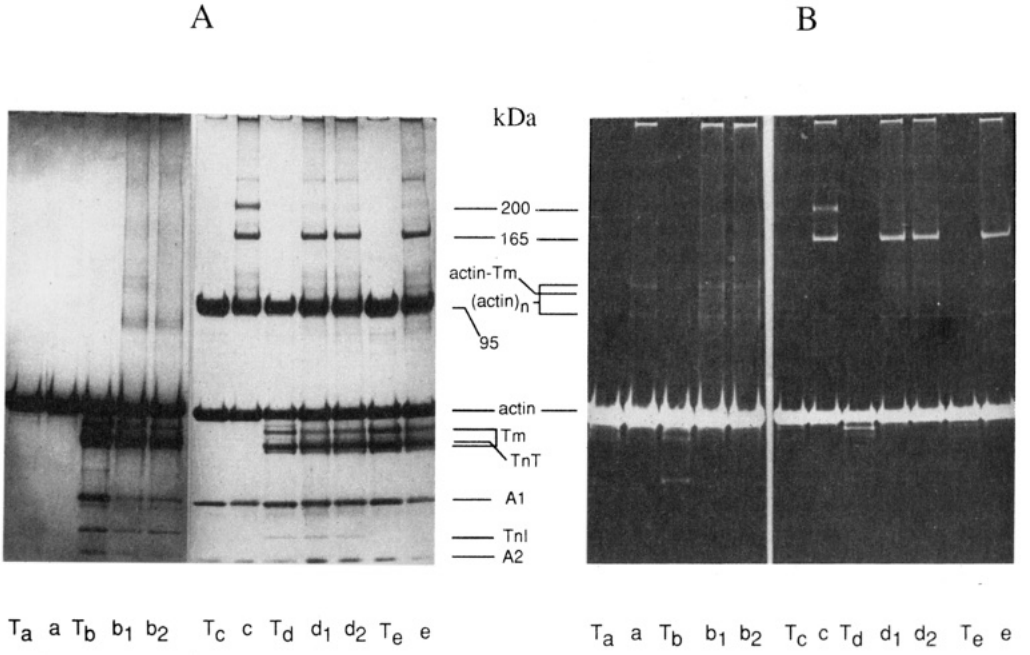

FIGURE 1: SDS–PAGE analysis of the glutaraldehyde cross-linking reaction of the actin–S1 and Tm–Tn–actin–S1 complexes. Cross-linking reactions were performed as specified under Materials and Methods. Protein bands containing fluorescent actin were viewed under UV light (B) prior to Coomassie blue staining (A) of the gels. Lanes T_a and a, actin alone; lanes T_b, b_1, and b_2, actin mixed with Tm–Tn; lanes T_c and c, actin mixed with S1; lanes T_d, d_1, and d_2, actin mixed with S1 and Tm–Tn; lanes T_e and e, actin mixed with S1 and Tm. Lanes T_a–T_e and a–e are the samples before and after cross-linking, respectively. The reactions were conducted in the presence of either 1 mM $CaCl_2$ (lanes b_1 and d_1) or 1 mM EGTA (lanes b_2 and d_2).

reaction was performed under identical conditions (with the omission of ATP), there was no detectable effect of the S1/actin ratio on the appearance of the acto-S1 cross-linked products (Figure 2A). In the presence of Tm, only the 165-kDa band was formed, while both the 165- and 200-kDa bands were produced in the absence of Tm independently of the S1/actin ratio. It is important to note that identical results were obtained whether the actin or S1 concentrations were kept constant during the experiment (data not shown). Therefore, it appears that, in contrast to the Tm-induced inhibition of the acto-S1 ATPase activity, the abolition of the 200-kDa acto-S1 product by Tm was independent of the S1/actin ratio.

To determine whether the acto-S1 contact (or proximity) which took place in the 200-kDa product was implicated in the Ca^{2+}-linked regulation of the acto-S1 ATPase activity, we compared the Ca^{2+} sensitivities of reconstituted filaments containing the acto-S1 covalent products. Since the separation of the 165- and 200-kDa products in their native forms was not possible, we compared the regulation of the filaments containing both the 165- and 200-kDa products with that of the filaments including only the 165-kDa adduct. The specific activities of the purified preparations (free from non-cross-linked S1 and modified Tm), measured at low ionic strength, were not significantly different, varying from 22 to 25 s^{-1} (Figure 3). Identical results were obtained regardless of the yield of the cross-linking reaction, in accordance with previously published data (Bertrand et al., 1988). When the 165- and 200-kDa products were produced under the usual cross-linking conditions, i.e., with a 20–30% yield, the addition of Ca^{2+} or EGTA in the presence of the regulatory proteins did not significantly change the specific activity of the two types of preparation (data not shown). Highly EDC cross-linked acto-S1 complexes were also previously found to be unregulated (King & Greene, 1985). In this latter case, it was proposed that the presence of a large number of covalent acto-S1 complexes in the rigor conformation could turn the thin filament to the on state. In contrast, when the glutaraldehyde cross-linking yield was lowered to 4–8%, by using a shorter cross-linking time, both types of cross-linked preparations were sensitive to the addition of Ca^{2+} (or EGTA). In the absence of Ca^{2+}, 42% and 47% inhibitions were reached for the 165- + 200-kDa and the 165-kDa adducts, respectively (Figure 3A). The relatively moderate extent of inhibition observed was not due to an irreversible modification of actin during glutaraldehyde treatment since control experiments showed that the extent of regulation of actin treated in the absence and in the presence of Tm was not significantly different from that of native actin (Figure 3B). Alternatively, it is possible that full regulation could have been reached only with a cross-linking yield lower than 1%, as noted by King and Greene (1985) in the case of the EDC cross-linked complexes. It is noteworthy that the extent of regulation was slightly different between the two complex mixtures (42% and 47%). However, such a small difference cannot be related to the presence of the 200-kDa product since the latter represented more than 40% of the total acto-S1 complexes in all the preparations tested. These results showed that both types of covalent complexes could be regulated to the same extent by Tm–Tn. This implies that none of the cross-linking sites involved in the 165- and 200-kDa acto-S1 complexes were implicated in the regulation of the acto-S1 ATPase activity.

Identification of the Actin–S1 Cross-Linking Site Abolished by Tropomyosin. It was previously proposed that glutaraldehyde promoted cross-links between the COOH-terminal 22-kDa or the central 48-kDa fragment of the S1 heavy chain and the actin segments including residues 1–28 and 40–113 (Bertrand et al., 1988). However, these authors did not assign these two cross-linking sites to the 165- and 200-kDa cross-linked products, nor did they establish the actin–S1 stoichiometry within the two covalent complexes. In a first set of experiments, we determined the actin–S1 stoichiometry in the two covalent complexes by estimating the amount of [^{14}C]glutaraldehyde incorporated within the modified proteins. This approach was based on two assumptions, which were apparently validated, as described under Materials and

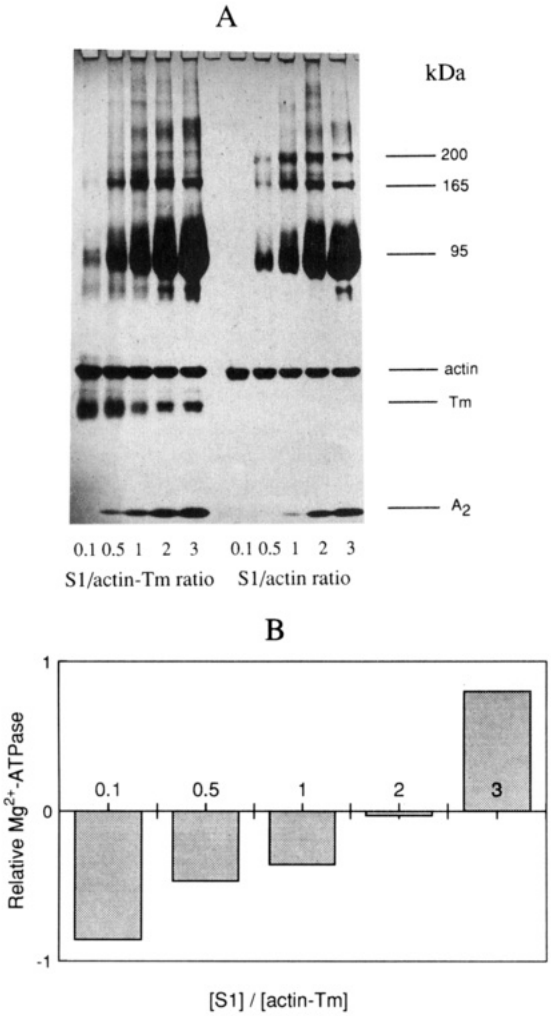

FIGURE 2: Influence of the S1/actin–Tm molar ratio on the glutaraldehyde cross-linking reaction and on the acto-S1 ATPase activity. (A) SDS–PAGE analysis of the glutaraldehyde-induced reaction performed in the absence (right panel) or in the presence (left panel) of Tm. The cross-linking reactions were conducted on protein mixtures containing different S1/actin molar ratios of 0.1, 0.5, 1, 2, and 3 ([actin] = 16 μM; actin/Tm molar ratio = 7:3), as described under Materials and Methods. (B) Measurements of the S1 Mg^{2+}-ATPase activities in the presence of various amount of actin or actin–Tm complex. The protein content was as described in part A, and the ATPase activities were determined as indicated under Materials and Methods. Relative Mg^{2+}-ATPase activities were calculated from the equation $A_{rel} = (A_{tm}/A) - 1$ where A_{tm} and A are the ATPase activities in the presence and in the absence of Tm, respectively.

Methods: first, [^{14}C]glutaraldehyde should present the same reactivity as the cold reagent, and second, [^{14}C]glutaraldehyde should react as a monomeric species. The results of the quantitation of the different products resulting from [^{14}C]-glutaraldehyde reaction with the acto-S1 mixture are summarized in Table 1. The data show that under the mild cross-linking conditions used in this study only 1.3–1.5 mol of [^{14}C]glutaraldehyde was incorporated per mole of acto-S1 heavy chain covalent products, while 0.1 and 0.5 mol reacted with the free actin and S1, respectively. Considering that at least 1 mol of glutaraldehyde was necessary to produce the covalent link between the two proteins, these results clearly demonstrate that both the 165- and the 200-kDa products were composed of 1 mol of actin bound to 1 mol of S1 heavy chain. The formation of a 1:1 acto-S1 covalent complex with an analogous mass value of 200 kDa—the theoretical mass is 135 kDa—was also previously reported for the product resulting from the cross-linking of S1 to Cys^{374} of monomeric actin (Combeau et al., 1992). Finally, it is worth noting that

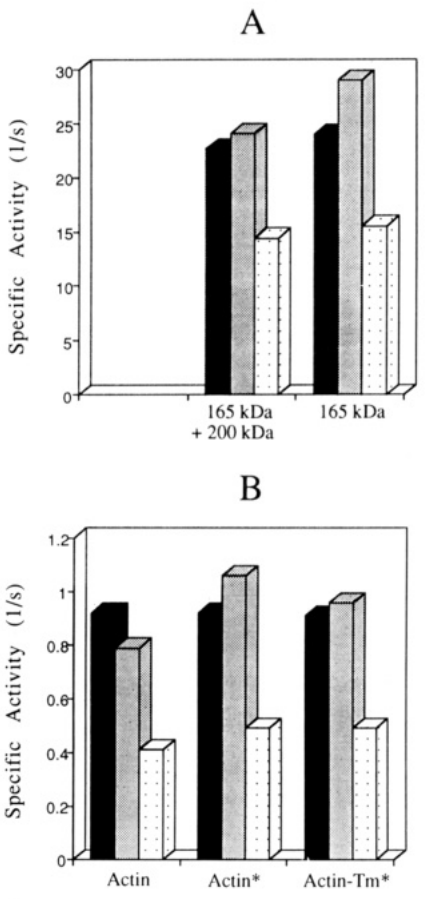

FIGURE 3: Ca^{2+}-linked regulation of the Mg^{2+}-ATPase activities of the covalent acto-S1 complexes. (A) The ATPase activities were measured for the cross-linked acto-S1 complexes obtained in the absence (165 + 200 kDa) or in the presence of Tm (165 kDa) as described under Materials and Methods. (B) Control activities were determined for S1 mixed with native actin (actin) and actin treated by glutaraldehyde in the absence (actin*) or in the presence (actin–Tm*) of Tm. The enzymatic activities were measured without (black) and with Tm–Tn in the presence of Ca^{2+} (gray) or EGTA (white).

Table 1: [^{14}C]Glutaraldehyde/Protein Molar Ratio in Actin–S1 Cross-Linked Complexes[a]

	[^{14}C]glutaraldehyde/protein (mol/mol)		
protein species	expt 1	expt 2	expt 3
A1 light chain	0.1		0.1
42 kDa	0.1	0.1	0.1
95 kDa	0.5	0.3	0.4
165 kDa	1.3	1.2	1.4
200 kDa	1.4	1.4	1.5

[a] The number of moles of [^{14}C]glutaraldehyde bound per mole of protein was determined after purification of the various protein species from preparative gel electrophoresis as described under Materials and Methods; 42 kDa, actin; 95 kDa, S1 heavy chain; 165 and 200 kDa, acto-S1 heavy chain covalent products.

under our mild cross-linking conditions only 0.1–0.5 mol of [^{14}C]glutaraldehyde was attached to other sites of S1 or actin, probably being involved in internal cross-links as proposed by Bertrand et al. (1988).

In a second set of experiments, we further identified the S1 and actin segments covalently linked by glutaraldehyde. The identification of the S1 segments was accomplished by cross-linking fluorescent actin to various proteolytic S1 species (Figure 4). The reaction between actin and the V8 protease-split (28–48–22 kDa)-S1 derivative generated predominantly actin–22-kDa and actin–48-kDa species in the absence of Tm (Figure 4A, lanes b and F_b). An additional cross-linked species designated acto-75 was issued from the covalent linkage

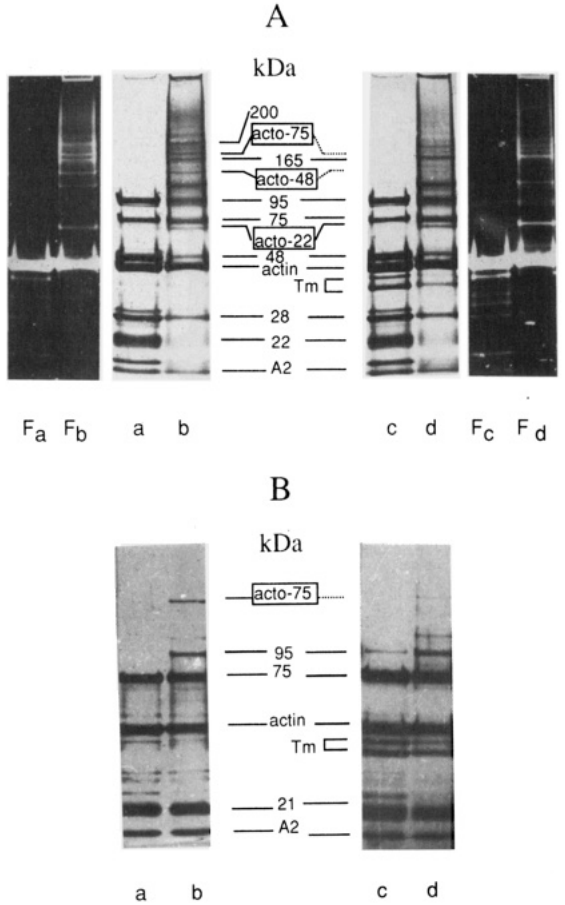

FIGURE 4: Identification of the S1 heavy chain segments cross-linked in the 200-kDa actin–S1 adduct. The glutaraldehyde cross-linking reaction was performed in the absence (a,b) or in the presence of tropomyosin (c,d) using complexes of F-actin and (28–48–22 kDa)-S1 (A) or F-actin and (75–21 kDa)-S1 derivative (B) as described under Materials and Methods. Samples were analyzed before (a,c) and after (b,d) the cross-linking reaction. Fluorescent IAEDANS-actin-containing bands were viewed under UV light (F_a–F_d), and the gels were stained by Coomassie blue (a–d).

between actin and the 75-kDa proteolytic intermediate (composed of the NH_2-terminal 28-kDa and central 48-kDa segments of the S1 heavy chain). Finally, the residual uncleaved S1 heavy chain produced the 165- and 200-kDa products described above. When the cross-linking reaction was conducted in the presence of Tm, only the 22-kDa fragments were cross-linked to actin, leading to the formation of the actin–22-kDa and actin–95-kDa (165-kDa band) adducts (Figure 4A, lanes d and F_d). The blocking effect of Tm on the cross-link between actin and the central part of the S1 heavy chain was further confirmed using the Arg-C-split (75–21 kDa)-S1 derivative. The cross-linking reaction between the (75–21 kDa)-S1 derivative and actin in the absence of Tm produced only one actin–75-kDa species as described previously (Bertrand et al., 1988; Figure 4B, lane b). A new 95-kDa band was also generated which likely results from an internal cross-linking between the 75- and the 21-kDa fragments as shown by Hiratsuka (1986). In contrast to the COOH-terminal 22-kDa fragment issued from V8 protease proteolysis, the 21-kDa fragment was unable to cross-link to actin. These two fragments differ only in their NH_2-terminal sequences; the former begins at Gly^{632}, and the latter, at Lys^{642} (Chaussepied et al., 1983; Bertrand et al., 1989a). This suggests that cross-linking takes place on the 10-residue difference segment located at the NH_2 terminus of the 22-kDa fragment. When Tm was added to the reaction mixture, the actin–75-kDa product disappeared while the putative

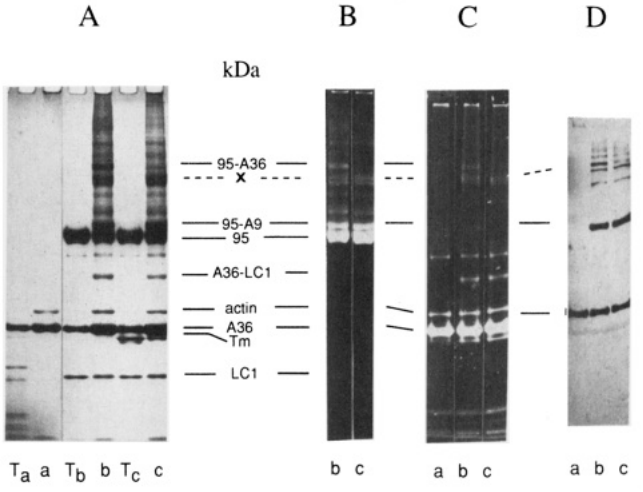

FIGURE 5: Effect of Tm on the glutaraldehyde cross-linking of subtilisin-split actin to S1. The cross-linking reactions were performed as described under Materials and Methods, and the samples were analyzed by SDS–PAGE; gels were stained with Coomassie blue (A), viewed under UV light for experiments using IAEDANS-labeled S1 (B) or IAEDANS-labeled actin (C), and immunoblotted with the anti-actin antibodies (D). Subtilisin-split actin alone [composed of the 36-kDa (A36) and 9-kDa (A9) fragments; lanes T_a and a] and mixed with S1 in the absence (lanes T_b and b) or in the presence of Tm (lanes T_c and c) was analyzed before (lanes T_a–T_c) and after (lanes a–c) the cross-linking reaction. For clarity, LC1 stands for the A1 light chain of S1.

internally cross-linked 95-kDa product remained unchanged (Figure 4B, lane d). Taken together, these results reveal that the 200-kDa actin–S1 adduct was composed of actin covalently linked to the central 48-kDa fragment of the S1 heavy chain. On the other hand, the 165-kDa product was deriving from the Tm-independent cross-linking of actin to the COOH-terminal 22-kDa fragment, most probably at the lysine-rich region located within segment 636–642 of the S1 heavy chain (as summarized in Figure 7).

In order to determine which of the actin segments 1–28 or 40–113 is sensitive to Tm binding, we analyzed the effect of Tm on the cross-linking reaction between the S1 heavy chain and the subtilisin-split (9–36 kDa)-actin derivative. Since actin was cut at Met^{47} (Schwyter et al., 1989), its cross-linked products would include either the NH_2-terminal 9-kDa fragment (A9), which contains residues 1–28, or the COOH-terminal 36-kDa fragment (A36), which includes most of the segment 40–113—actually segment 48–113. Although this actin derivative could not move during the in vitro motility assay (Schwyter et al., 1989), we found that it cosedimented with Tm and S1 to the same extent as native actin and that the S1 ATPase activated by subtilisin-split actin was fully regulated by the Tm–Tn complex (data not shown). These observations strongly suggest that the proteolytic actin derivative was capable of interacting normally with S1 and Tm and thus it represented a material suitable for testing the effect of Tm on the S1 cross-linking sites. As depicted in Figure 5, both actin fragments were cross-linked to the S1 heavy chain, generating the 95-kDa–A9 and the 95-kDa–A36 adducts. These two cross-linked entities could be unambiguously identified since both contained the IAEDANS-labeled 95-kDa species (Figure 5B, lane b) and only the 95-kDa–A9 product reacted with the anti-actin fragment A9 antibody (Figure 5D, lane b), while the 95-kDa–A36 incorporated the fluorescence of the IAEDANS-labeled 36-kDa species (Figure 5, lane b). When Tm was present in the reaction mixture, only the 95-kDa–A9 adduct was clearly formed (Figure 5A,D, lanes c), while only traces of 95-kDa–A36 could be seen (Figure 5B,C, lanes c). An additional

product, designated "X", migrating with an apparent mass of 170-kDa was also obtained during this reaction. This cross-linked product, which contained 95-kDa and both A9 and A36 fragments, was not sensitive to the presence of Tm and was apparently composed of reconstituted actin bound to the S1 heavy chain. These experiments clearly showed that Tm abolished the cross-linking reaction between S1 and the actin 48–113 segment while it did not affect the reaction between S1 and the actin 1–28 segment.

The precise localization of the S1 cross-linking sites on actin was completed by a more direct approach combining the selective proteolysis of the original covalent complexes with immunochemical and fluorescence techniques. The covalent acto-S1 complexes obtained in the absence (165 + 200 kDa) or in the presence (165-kDa) of Tm were isolated by centrifugation, depolymerized, and digested with chymotrypsin, which cuts only actin and not S1. Chymotrypsin cuts monomeric actin independently of the presence of S1 at positions Leu^{67} and Met^{44} (Jacobson & Rosenbusch, 1976; Konno, 1987; Chen et al., 1993). In order to increase the yield of depolymerization of the covalent complexes, we treated them with EDTA, which is known to denature actin partly and to induce additional cleavage sites within the protein (Strzelecka-Golaszewska et al., 1993). The analysis of the time course of the chymotryptic digestion of the two types of covalent complexes is shown in Figure 6, left and right panels, respectively. A major intermediate of apparent mass 105 kDa was first produced, in both cases, concomitantly with the degradation of actin (Figure 6A). This band was found to contain both actin segment 1–7 as revealed by immunobloting (Figure 6B) and Lys^{61} when FITC-labeled actin was used (Figure 6C). For both complexes, the prolonged proteolysis converted this band into smaller and stable S1 derivatives with a nearly identical mass of about 98 kDa (Figure 6A) which did not contain the extreme NH_2-terminal part of actin (Figure 6B) or residue Lys^{61} (Figure 6C). The straightforward interpretation of these experiments is that for both the 165- and 200-kDa acto-S1 complexes, chymotrypsin cleavage first generated a complex composed of S1 linked to the NH_2-terminal 1–67 segment of actin. The extensive digestion of the complexes produced additional cleavages within this stretch which would account for the loss of the immunoreactive NH_2-terminal residues and of the fluorescent FITC-labeled Lys^{61} and released the 98-kDa species composed of the 95-kDa heavy chain bound to a short actin peptide.

These data, in association with the results of Bertrand et al. (1988), clearly show that the S1 cross-linking sites occur within the two actin segments of residues 1–28 and 48–67 (Figure 7). Moreover, according to our cross-linking experiments employing the subtilisin-split actin derivative, only the cross-link of the S1 to segment 48–67 is abolished by Tm. On the basis of the primary structure of these two actin segments (Elzinga & Lu, 1976) and on the chemical reactivity of glutaraldehyde, which at low concentration predominantly modifies residues Lys and Arg (Peters & Richards, 1977; McIntosh, 1992), only Lys^{18} and Arg^{28} of the first segment and Lys^{50} and Arg^{62} of the second segment are candidates for glutaraldehyde reaction (Figure 7). Lys^{61} is to be excluded since its blocking by FITC did not impair the cross-link between actin and S1, neither qualitatively nor quantitatively (Figure 6). In addition, the atomic model of F-actin (Holmes et al., 1990) seems to exclude Lys^{18} and Arg^{62} from modification since Lys^{18} is shielded in the five-stranded β-sheet structure within subdomain 1 and Arg^{62} makes an ionic contact with Asp^{288} of an adjacent monomer. Since the effect of S1 on the actin structure is not known at the atomic level, these

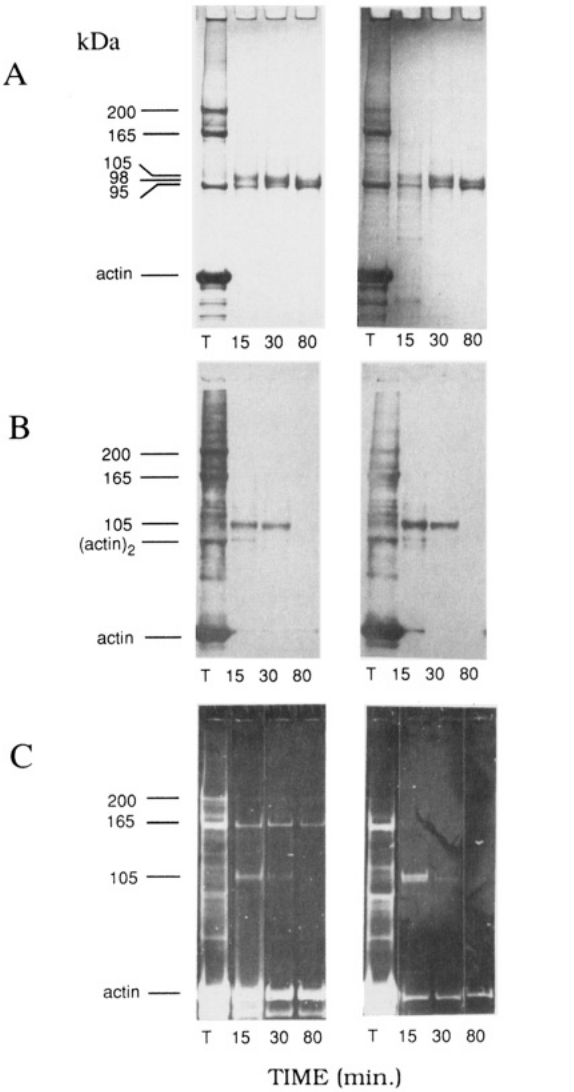

FIGURE 6: Time-course of the chymotrypsin digestion of the covalent acto-S1 complexes obtained in the absence (left panels) or in the presence (right panels) of Tm. Actin–S1 complexes were first treated by glutaraldehyde, purified, and then digested by chymotrypsin as described under Materials and Methods. Samples were analyzed on SDS–PAGE before (T) and after 15, 30, and 80 min of chymotrypsin digestion. The gels were stained with Coomassie blue (A), immunoblotted with anti-actin antibodies (B), or viewed under UV light when FITC-labeled actin was used (C).

arguments together with the results obtained during the extensive chymotrypsin digestion of the covalent complexes led us to propose that Arg^{28} and Lys^{50} are the most likely actin residues cross-linked with S1. Thus, tropomyosin would change the conformation of the actin region at or near Lys^{50}, which cross-links to the 48-kDa heavy chain fragment, while it would not affect the structure of the actin stretch at or near Arg^{28}, which cross-links to the 636–642 segment of the S1 heavy chain (Figure 7).

DISCUSSION

This study describes, for the first time, the effect of Tm on the interaction between skeletal myosin head and filamentous actin bound in the rigor state. More particularly, it shows that Tm modifies the interaction between the central 48-kDa fragment of S1 and segment 48–67 in actin subdomain 2.

Several lines of evidence tend to point toward a close proximity (if not a direct interaction) between S1 and actin subdomain 2. For example, the accessibility of probes attached to residue Lys^{61} or the proteolytic susceptibility of actin

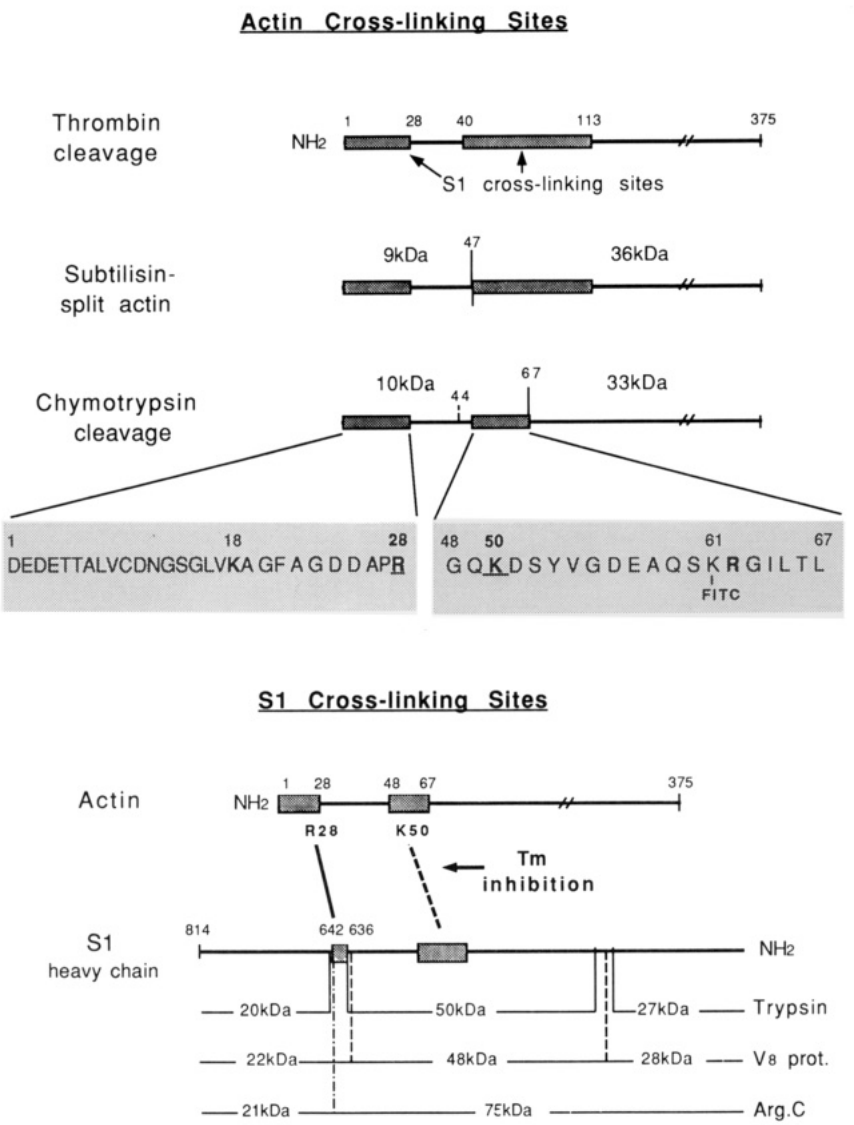

FIGURE 7: Schematic diagram illustrating the localization of the glutaraldehyde-induced cross-linking sites along the actin and S1 heavy chain sequences. The actin cross-linking sites were deduced by combining the data from cleavage of the covalent acto-S1 complexes by thrombin (Bertrand et al., 1988) with those of the present work using the chymotryptic digestion of the same complexes and the direct cross-linking reaction of S1 to the subtilisin-split actin derivative. The S1 heavy chain cross-linking sites were derived from cross-linking experiments performed between actin and proteolytic S1 derivatives. The major peptides generated by the different proteases are indicated (V8 prot., *S. aureus* protease; Arg.C, arginine-specific endoproteinase). The Tm-induced abolition of the cross-link between the Gly^{48}-Leu^{67} segment of actin and the central 48-kDa segment of the S1 heavy chain is also indicated. See text for more details.

segment 61–69 is affected by S1 interaction (Miki & dos Remedios, 1988; Chen et al., 1992; Fievez & Carlier, 1993). More recently, the reconstruction of EM pictures of the acto-S1 complex together with the 3-D structures of actin and S1 revealed a close proximity between actin loop structure 38–52 (also called DNase I binding loop) and segment 540–560 of the central 48-kDa fragment of the S1 heavy chain (Rayment et al., 1993b). However, this work presents the first direct experimental evidence for a close proximity (at most 9 Å) between actin segment 48–67 (presumably Lys^{50}) and the 48-kDa fragment of the S1 heavy chain. It is noticeable that an independent study, using maleimidobenzoic *N*-hydroxy-succinimide ester (9-Å span) as cross-linking reagent, confirmed the cross-link between Lys^{50} of actin and the 48-kDa fragment of S1 (R. Bertrand, J. Derancourt, and R. Kassab, manuscript in preparation). Three reasons could explain the effect of Tm on this actin–S1 binding subsite: (i) a steric blocking of S1 interaction, (ii) a reduction of Lys^{50} (or Arg^{62}) reactivity, or (iii) a change in the orientation of subdomain 2 within the actin filament. On the basis of the EM reconstruction of the reconstituted filaments (Milligan et al., 1990), there is no evidence that Tm could directly interact with the actin 48–67 segment located in the outer part of actin subdomain 2 since Tm seems to be located on actin subdomain 3 (at the inner edge of subdomain 2) closer to the thin filament axis. In addition Tm binding was found to stabilize the actin filament and to increase its stiffness (Hitchcock DeGregori et al., 1988; Yanagida et al., 1984), thus explaining the slight change in the actin's torsional flexibility observed by EM studies (Stokes & Derosier, 1987; Milligan & Flicker, 1987). Such effects on the actin structure could actually occur through minute changes in subdomain 2 since this domain is an important component of the actin–actin contact and since it represents the more mobile part of the molecule (Orlara & Egelman, 1993; Tirion & Ben Avraham, 1993). It is therefore reasonable to postulate that Tm changes the orientation of actin segment 48–67 and more probably in the neighborhood of residue Lys^{50}. One should note that the change in the orientation of the Lys^{50}-containing segment may be restricted to a movement of low amplitude (as short as few angstroms) and may not dramatically alter the overall relationship between actin subdomain 2 and S1.

The second acto-S1 cross-linking site, identified during this work, involves actin segment 1–18 and more probably residue

Arg^{28} located in another loop structure of actin, i.e., segment 21–29, which belongs to the outer surface of actin subdomain 1 and the S1 heavy chain segment 636–642, also known as the "50kDa–20kDa junction". It is noteworthy that a similar cross-link was recently achieved by photo-cross-linking performed with the (*p*-azidophenyl)glyoxal (APG), a photoactivable cross-linking reagent of 9-Å span (Bonafé et al., 1993). A proximity of 9 Å between these two loci is in total agreement with the recent model of Rayment et al. (1993b) which places this actin loop structure in the vicinity of loop 627–646 of the S1 heavy chain (missing in the X-ray diffraction pattern in the S1 crystal). Moreover, Arg^{28} of actin was found distant from the putative Tm binding sites (Milligan et al., 1990). Accordingly, cross-linking of Arg^{28} to S1 was not altered by Tm; neither were Tm binding capabilities reduced upon acto-S1 cross-linking induced by glutaraldehyde (this work) or by APG (data not shown). Moreover, cross-linking experiments performed with various cross-linkers directed to the segments of residues 1–7 or 93–100 of actin, located in the same outer part of actin subdomain 1, showed no effect of Tm on the proximity of S1 to this region of actin (N. Bonafé et al., personal observations).

In contrast to the Tm-linked inhibition of the acto-S1 ATPase activity, the Tm-induced inhibition of S1 cross-linking to actin subdomain 2 was independent of the S1/actin ratio. Since these two experiments were conducted under different conditions (i.e., in the absence or the presence of ATP), it is too early to conclude that the effect of Tm on the cross-linking of S1 with actin subdomain 2 is totally independent of the inhibitory state of the thin filament. However, these results reveal a real effect of Tm on a subsite of the acto–S1 interface within the rigor complex. This conclusion is further strengthened by the absence of Ca^{2+} effect on the cross-linking pattern obtained with the reconstituted filament in accordance with the small effect of this ion on the kinetics of the acto-S1 interaction in rigor conditions (McKillop & Geeves, 1993). In addition, it is interesting to note that the results were identical whether or not Mg^{2+}-ADP was present during the cross-linking reactions performed with or without the regulatory proteins. This clearly demonstrates that the weakening of the acto-S1 interaction by Mg^{2+}-ADP, also described with the reconstituted filaments (McKillop & Geeves, 1993), is not related to a change in proximity between S1 and Arg^{28} or Lys^{50} of actin. This conclusion would again agree with Rayment's model (Rayment et al., 1993b), in which the binding of nucleotide is not proposed to perturb the interaction between S1 and the outer part of actin subdomains 1 and 2 but only the binding of the "upper" domain of the central 48-kDa fragment of the S1 heavy chain to residues 332–334 located in the segment connecting the actin subdomains 1 and 3.

The cyclic transition between the weak and strong acto-S1 interacting states is necessary for the full actin-induced activation of the S1 ATPase activity [for review, see Geeves (1991)]. This transition implies that some of the acto-S1 binding subsites undergo a cyclic dissociation–association process while others, forming the weak binding state, remain constantly accessible during the entire cycle. The ATPase activities of the two acto-S1 covalent complexes, described in this work, approximate the maximum velocity obtained at infinite actin concentration, as in the case of the EDC-induced cross-linked complexes. This result suggests that the covalent linkages do not hinder the transition between the weak and the strong interacting states. One interpretation is that the interaction (or proximity) between S1 and actin residues Arg^{28} and Lys^{50} belongs to the so-called weak acto-S1 interaction. This was further confirmed by the fact that both types of acto-S1 cross-linked products are regulated to the same extent by Ca^{2+}/EGTA in the presence of the regulatory proteins. Indeed, the regulation process is thought to affect the same transition between weak and strong acto-S1 interacting states (McKillop & Geeves, 1991, 1993).

In conclusion, we have brought experimental evidence showing the interaction between S1 and actin subdomains 1 and 2. According to the 3-D model recently proposed (Rayment et al., 1993b), S1 would interact with two different actin monomers, so that it could be cross-linked to subdomain 1 of one monomer and to subdomain 2 of the adjacent monomer. However, since we could not isolate a doubly cross-linked S1, i.e., S1 simultaneously linked to two monomers, further work is needed to confirm the model definitively. We also found that the presence of Tm associated or not with Tn modifies the relationship between actin subdomain 2 and the central 48-kDa fragment of S1. This result may not indicate a dramatic change in the structure of actin subdomain 2, but it urges further analysis on the effect of the regulatory proteins on the acto-myosin interaction in the rigor state before any work is started on the structural changes associated with the isomerization of the actin–S1 interaction necessary to generate force.

NOTE ADDED IN PROOF

A recent refinement of the F-actin model at the atomic level led to a new position of the 48–67 actin segment, which is now located closer to actin subdomain 3, i.e., near one of the proposed Tm binding sites [Lorenz, M., Popp, D, & Holmes, K. C. (1993) *J. Mol. Biol. 234*, 826–836]. These new data are in total agreement with our conclusion on the effect of Tm on this actin loop.

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