A Single Myosin Head Can Be Cross-Linked to the N termini of Two Adjacent Actin Monomers

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ABSTRACT Myosin subfragment-1 (S1) can be cross-linked to two actin monomers by 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide only when F-actin is in excess over S1. Electron micrographs of the covalent actin$_2$-S1 complex showed that S1 was cross-linked to two adjacent monomers of the same actin filament. Cross-linking experiments with pre-proteolyzed S1 derivatives in combination with a proteolytic dissection of the intact covalent actin$_2$-S1 adduct (m = 265 kDa), revealed that two N-terminal segments of actin (residues 1–28) were covalently attached to a single S1 molecule. One was cross-linked to either the 20-kDa or the 50-kDa heavy chain fragments of S1, and the other only to the 50-kDa region. The doubly cross-linked product was formed under physiological ionic strength with S1 or with reconstituted myosin filaments, regardless of the presence of ADP or the regulatory proteins, tropomyosin and troponin. Finally, we found that this cross-linking could also take place within myofibrils in the rigor state. These results demonstrate that under nonsaturating conditions, the actin-S1 interface encompasses a much larger region than that recently proposed for the nonphysiological, fully saturated actin filaments.

INTRODUCTION

The understanding of muscle contraction and cell motility processes has taken a great step forward with the reconstitution at an atomic level of the actin-myosin head complex based on the atomic structures of the actin filament and S1 and on electron micrographs of actin filaments fully saturated with S1 (Rayment et al., 1993; Schröder et al., 1993). This reconstruction allowed the prediction of the contact sites between actin and S1. These predictions were found to fit a large number of biochemical data available on this complex. Thus, S1 would interact mainly with the bottom part of actin subdomains 1 and 3 by ionic contacts (segment 1–4 and residues 24 and 25) and at more hydrophobic sites involving actin segments 342–354, 144–148, and 332–334, as well as segment 40–42 of the top of the actin monomer below. An additional weaker interaction could also take place through electrostatic bonds with residues 91–100 of this second actin subunit. Based on the structure of the acto-S1 interface and on the anticipated flexibility of the S1 structure, a model for force production was proposed at a molecular level. In this model, the complexation of S1 to actin would consist of a multistep process that would first lead to the binding of S1 to all the contact sites except to segment 332–334. The subsequent interaction between S1 and this latter region would induce the conformational changes within the S1 structure necessary for nucleotide release from the ATPase site and for generation of the contractile force. This sequence of events turned out to be in good agreement with previously proposed models in which the powerstroke was closely linked to modifications of the contact area between actin and S1 (for reviews, see Huxley and Kress, 1985; Botts et al., 1989). A slightly different version of the same kind of model proposed that the powerstroke could be generated during the binding of S1 to the second actin subunit (Andreev et al., 1993c). This model was based on differences in the binding kinetics and orientation of S1 to F-actin depending on the degree of saturation of the filament by S1 (Andreev et al., 1993b, c). Additional evidence, such as differences in proteolytic digestion and cross-linking of the acto-S1 complex (Mornet et al., 1981a; Yamamoto, 1990; Huang et al., 1990; Andreev and Borejdo, 1992) as well as in the kinetic parameters of S1 ATPase (Tesi et al., 1990), supports different interactions in saturated and unsaturated F-acto-S1 complexes. Because these latter complexes are predominant under physiological conditions and because their reconstruction by electron microscopy is not yet possible, we have undertaken the comparison of the two types of actin-S1 interface.

We have examined the interaction of S1 with F-actin using the "zero-length" cross-linking approach. We confirmed that a single S1 can be cross-linked with 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) to two actin monomers only within the unsaturated F-actin-S1 complex, and we showed that the cross-linking sites were exclusively located on the N-terminal segments of two adjacent actin subunits. The physiological relevance of this interaction was further strengthened by the finding that it also took place in the presence of the regulatory proteins, tropomyosin (Tm) and troponin (Tn), upon using native myosin filaments instead of the soluble myosin head, as well as within myofibrils under rigor conditions.

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Abbreviations used: S1, myosin subfragment-1; S1(A1) and S1(A2), S1 isoforms with alkali light chain 1 and 2, respectively; F-actin, filamentous actin; Tm, tropomyosin; Tn, troponin; EDC, 1-ethyl-3-[3-(dimethylamino)--propyl] carbodiimide; NHS, *N*-Hydroxysuccinimide; CPI, 7-diethylamino-3-(4′-isothiocyanatophenyl)-4-methyl coumarin; 1,5 IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulpho-1-naphthyl)ethylenediamine; Arg-C, arginine-specific endoproteinase.

0006-3495/95/04/35s/09 $2.00

MATERIALS AND METHODS

Reagents

EDC, NHS, phalloidin, thrombin from human plasma (2000 units/mg), and trypsin were from Sigma Chemical Co. (St. Louis, MO). Endoproteinase Arg-C and cytochrome *c* were purchased from Boehringer Mannheim (Mannheim, Germany). CPI was obtained from Molecular Probes (Eugene, OR), and chymotrypsin was obtained from Worthington. All other chemicals were of the highest analytical grade.

Preparation of actin and S1 derivatives

Rabbit skeletal muscle actin, myosin, tropomyosin, and troponin were prepared as described (Bonafé et al., 1994). S1 was obtained by chymotryptic digestion of myosin filaments, and the purification of the isoenzymes S1-(A1) and S1(A2) was performed according to Lheureux et al. (1993).

The (75–21kDa)-S1 derivative was produced by reaction for 2 h at 25°C with endoproteinase Arg-C (10 units/mg of protein) in 20 mM HEPES, pH 8.0 as in Bonafé et al. (1993). The proteolytic reaction was terminated with 2 mM tosyl-L-lysine chloromethyl ketone.

Labeling of the central 50-kDa and C-terminal 20-kDa fragments of the S1 heavy chain was achieved using CPI (Hiratsuka, 1987) and 1,5 IAEDANS (Mornet et al., 1981b), respectively.

Protein concentrations were determined spectrophotometrically assuming extinction coefficients $A^{1\%}_{280nm}$ of 7.5, 11.0, 3.3, and 4.5 cm^{-1} for S1 (m = 115 kDa), actin (m = 42 kDa), Tm (m = 66 kDa), and Tn (m = 70 kDa), respectively.

Cross-linking reactions

F-actin and S1 (or their derivatives) were mixed in cross-linking buffer (30 mM NaCl, 5 mM $MgCl_2$, 50 mM MOPS, pH 7.0) at 25°C at an actin:S1 molar ratio of 1 or 5, in the absence or in the presence of 2 mM ADP and/or 100 mM NaCl. The cross-linking reaction was initiated by addition of EDC and NHS (freshly dissolved in the cross-linking buffer) at final concentrations of 5 and 10 mM, respectively, with an actin:S1 molar ratio of 1 or 10 mM and 50 mM, respectively, with an actin:S1 ratio of 5. For the time course analysis, the reactions were stopped by incubating for 5 min an aliquot in 3 vol of boiling Laemmli solution (50 mM HEPES, 2% (w/v) $NaDodSO_4$, 1% 2-mercaptoethanol and 50% (v/v) glycerol, pH 8.0). Alternatively, the reactions were terminated after 45 min by dissociating non-cross-linked S1 with 1 vol of 100 mM Tris, 500 mM NaCl, 1.6 M KCl, 20 mM $Na_4P_2O_7$, 10 mM $MgCl_2$ and 10 mM ATP, pH 8.0, followed by a centrifugation at 380,000 × g for 15 min at 4°C.

In the presence of the myosin filaments or Tm-Tn (with an actin:Tm-Tn molar ratio of 3), the cross-linking reaction was performed in a two-step reaction to protect the integrity of the proteins. F-actin (60 μM in cross-linking buffer containing 130 mM NaCl) was first activated by 20 mM EDC, 50 mM NHS for 15 min at 25°C. Myosin filaments or S1 were then added at an actin:myosin head molar ratio of 5. After 40 min of condensation, 10 mM EDTA and chymotrypsin (at a chymotrypsin/myosin weight ratio of 1/100) were added to the reaction mixture; after 20 min, aliquots were mixed with 3 vol of boiling Laemmli solution. In a control experiment, myosin filaments were treated under identical conditions but in the absence of actin.

Myofibrils (5–10 mg/ml) were prepared and treated by EDC as described by Herrmann et al. (1993), except that 5 mM EDC and 20 mM NHS were mixed with myofibrils in 3 mM $MgCl_2$, 3 mM EGTA, 100 mM MES, pH 7.0 for 90 min before the chymotryptic degradation.

Proteolytic analysis of the cross-linked actin-S1 complexes

Before the proteolytic reaction, the covalent actin-S1 complexes were separated from non-cross-linked S1 and depolymerized in EDTA-containing buffer as described (Bertrand et al., 1988; Bonafé et al., 1994). The samples were then clarified by centrifugation at 380,000 × g for 15 min at 4°C and treated with thrombin (at a protease to actin weight ratio of 1/15) for 45 min at 25°C. The samples were then further digested by trypsin at a protease to actin weight ratio of 1/25. Aliquots were withdrawn after 20 and 50 min, and the reaction was stopped by adding 3 vol of boiling Laemmli solution. One should note that similar gel electrophoretic patterns were obtained whether the covalent complexes were prepared in the presence or in the absence of ADP.

$NaDodSO_4$/PAGE and Western blot analysis

Gel electrophoresis was as described by Laemmli (1970) using a 5–18% gradient acrylamide gel. Fluorescent bands were located in the gel by illumination with UV light before Coomassie blue staining. Western blot analysis of the cross-linked products using rabbit polyclonal antibodies specifically directed against the N-terminal segment 1–12 of actin was performed as described (Bonafé et al., 1994). The antibodies specifically directed against the actin segment 40–113 were obtained from rat antisera after immunization with HPLC-purified thrombic peptide 40–113 (using a reverse-phase Aquapore C8 column) coupled to Keyhole limpet Hemocyanine as described (Bulinsky et al., 1983).

Electron microscopy

Samples containing at most 0.1 mg/ml F-actin were mounted on Formar/carbon-coated grids pretreated with cytochrome *c* and negatively stained with 2% uranyl acetate (Craig and Negerman, 1977). Grids were examined using a JEOL EX-2000 microscope operating at an accelerating voltage of 80 kV.

RESULTS AND DISCUSSION

Electron microscopy visualization of the covalent $acto_2$-S1 complex

Myosin subfragment-1 (S1) can be cross-linked to two actin monomers by EDC only when F-actin is in excess of S1 (Andreev and Borejdo, 1992) (our unpublished observations). Because of the S1 ability to promote actin bundles by interacting with two actin filaments at a high actin:S1 molar ratio (Ando and Scales, 1985; Ando, 1987), it was important to assess whether S1 was cross-linked to two actin monomers belonging to the same or to two different filaments. We first performed a cross-linking reaction at an actin:S1 molar ratio of 5 in the absence of nucleotide under experimental conditions that favored the formation of the $acto_2$-S1 heavy chain product (Fig. 1 *C*, lane *a*). As shown in Fig. 1 *A*, these conditions led to the formation of large, ordered bundles containing well defined strips of S1. Identical bundles were also present before the cross-linking reaction but were always absent when the actin:S1 molar ratio was equal to 1 (data not shown). In contrast, when the cross-linking was conducted under identical conditions (at a high actin:S1 molar ratio) but in the presence of 2 mM Mg-ADP, known to inhibit bundle formation (Ando, 1987), most of the filaments were separated from each other with only traces of bundles (Fig. 1 *B*) and the cross-linking pattern remained qualitatively and quantitatively unchanged (Fig. 1 *C*, lane *c*). This result clearly shows that the cross-linking reaction was not at all linked to the presence of actin bundles and that it occurred between S1 and two adjacent actin monomers within the same filament. Note that it was difficult to conclude on

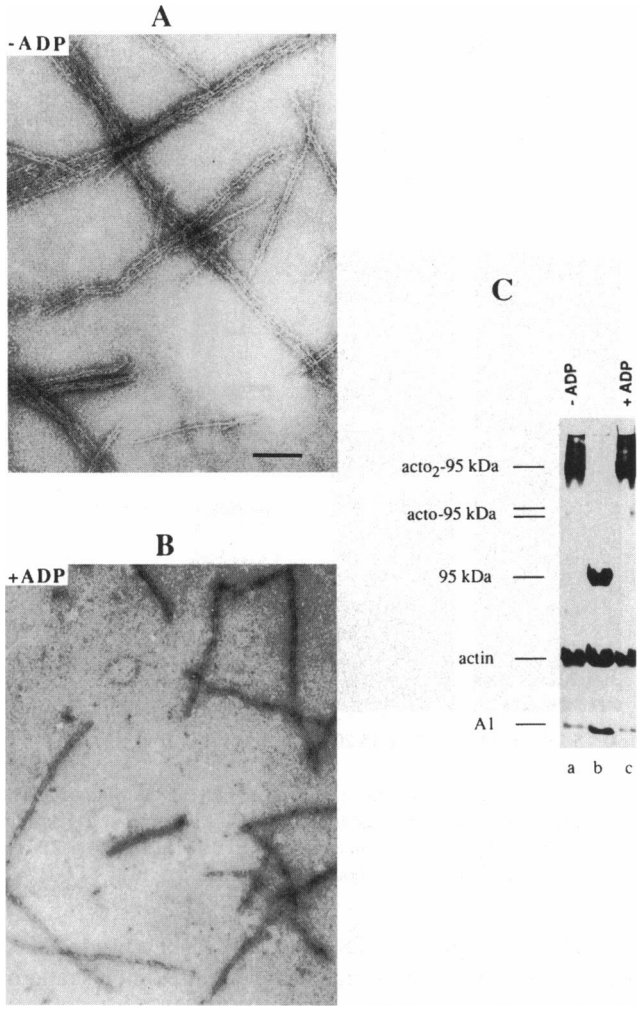

FIGURE 1 Electron micrographs of the acto-S1 complexes cross-linked at an actin: S1 molar ratio of 5 in the absence (*A*) or in the presence (*B*) of 2 mM Mg^{2+}-ADP. Scale: bar represents 0.15 μm. In *C*, the actin-S1 mixtures were subjected to $NaDodSO_4$-gel electrophoresis before (lanes *b*) and after EDC reaction with (lanes *c*) or without Mg^{2+}-ADP (lanes *a*).

the orientation of the cross-linked S1 as well as whether S1 was cross-linked to two monomers along the long pitch helix or the genetic helix because of the partial decoration of the filament by S1 (Craig et al., 1980). However, because numerous cross-linked S1s were seen at the edge of the filament (Fig. 1 *B*) in association with the geometrical constrains imposed by the structure of the actin filament (Lorenz et al., 1993), it is most likely that S1 is cross-linked to two monomers along the long pitch helix of the filament.

Identification of the S1 cross-linking sites in the $acto_2$-S1 covalent complex

When the actin-S1 complex was subjected to EDC-induced cross-linking at an actin:S1 molar ratio of 5, two kinds of products were generated as revealed by $NaDodSO_4$-gel electrophoresis (Fig. 2 *A*) (Mornet et al., 1981a; Andreev and Borejdo, 1992). At the earliest time of the reaction, the first covalent acto-S1 heavy chain adducts were formed, migrating as a doublet band (m = 165–175 kDa) and composed of 1 mole of actin linked via its N-terminal segment 1–12 to either the tryptic 20- or 50-kDa fragment of 1 mole of 95-kDa S1 heavy chain (Sutoh, 1982; Greene, 1984; Chen et al., 1985). An additional minor cross-linked product containing stoichiometric amount of actin and S1 (m = 200 kDa) (Ando and Scales, 1985; Combeau et al., 1992) was also generated. As the reaction proceeded, the initial acto-95-kDa covalent products gradually disappeared, leading to the formation of a higher M_r product (m = 265 kDa) that was found to contain 2 moles of actin bound to one mole of S1 (Andreev and Borejdo, 1992). This $acto_2$-95-kDa adduct was always absent when the reaction was performed at an actin:S1 molar ratio of 1, even after longer reaction time (Andreev and Borejdo, 1992, and data below). Because this $acto_2$-S1 species was formed at the expense of the other S1-containing bands, it was likely that it results from an additional cross-link(s) established during the EDC-catalyzed reaction.

Cross-linking experiments performed under identical conditions on a complex between actin and the Arg-C protease-split (75–21-kDa)-S1 derivative gave rise to a very similar electrophoretic pattern. Three covalent products designated (acto-21-kDa), (acto-75-kDa)^a and (acto-75kDa)^b were first generated. All three contained actin and either the C-terminal 21-kDa or the N-terminal 75-kDa fragment of S1 because these two bands reacted with an anti-actin antibody and incorporated the fluorescence of either the 21-kDa or the 75-kDa fragment (Fig. 2 *B*) (Bertrand et al., 1988). Interestingly, the (acto-75-kDa)^b entity formed in a lower yield may correspond to the faint 200-kDa (acto-95-kDa) adduct described above. A longer reaction time led to a covalent adduct of higher masses containing actin and the 75-kDa fragment of S1. By analogy with the cross-linking pattern of the actin-S1 complex, we propose this product (termed $acto_2$-75-kDa) to be composed of 2 moles of actin bound to 1 mole of the 75-kDa fragment and, therefore, that the additional covalent link generated during the reaction was located in the 75-kDa fragment of S1.

Identification of the actin cross-linking sites in the $acto_2$-S1 covalent complex

To localize the new cross-linking sites within actin, we first subjected the isolated and depolymerized acto-95-kDa and $acto_2$-95-kDa complexes to proteolysis by thrombin, which cleaves monomeric actin at residues 28, 39, and 113 but does not attack the S1 heavy chain (Fig. 4) (Muszbek et al., 1975). As shown in Fig. 3 *A* (lanes *b*), the proteolytic digestion of the acto-95-kDa complex led to the appearance of a unique product, designated 95-kDa-P, containing both S1 (as revealed by immunostaining and fluorescence labeling) and actin fragment 1–28 (as shown by immunostaining with the anti-actin segment 1–12 antibody), in agreement with previous work (Sutoh, 1982; Bertrand et al., 1988). When the thrombin degradation was conducted on the $acto_2$-95-kDa product, a new S1-containing band, named 95-kDa-P_2, with a mobility slightly higher than the 95-kDa heavy chain was formed (Fig. 3 *B*, lanes *b*). This band reacted with the an-

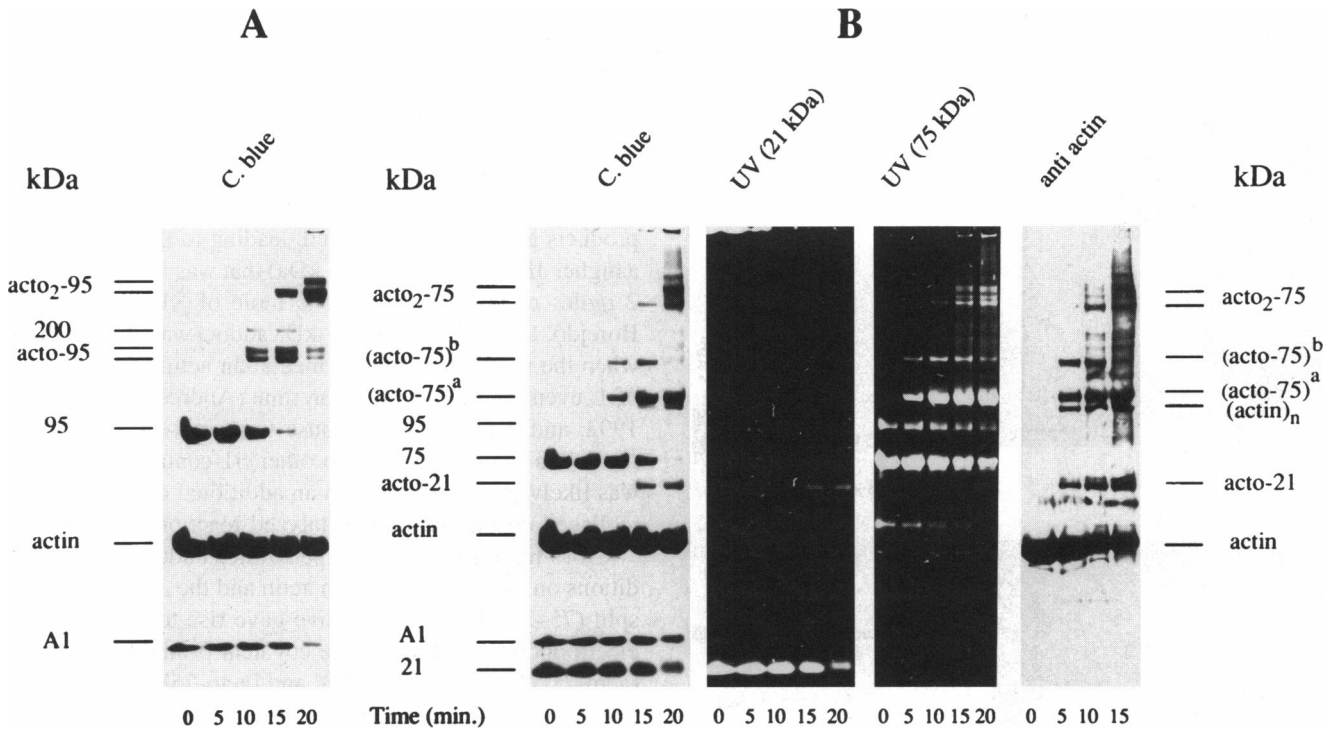

FIGURE 2 Time course of the EDC-induced cross-linking of actin to S1 (*A*) or to (75–21 kDa)-S1 derivative (*B*) at an actin:S1 molar ratio of 5. The cross-linking reactions were performed as described under Materials and Methods, and the samples were analyzed by $NaDodSO_4$-polyacrylamide gels after 5-, 10-, 15-, and 20-min reactions. Gels were stained with Coomassie blue, viewed under UV light for experiments using S1 labeled either on 21-kDa (UV(21kDa)) or 75-kDa (UV(75kDa)) fragments, and immunoblotted with the anti-actin segment 40–113 antibodies (anti-actin).

tibody to the actin fragment 1–12, but it did not react with the antibody to residues 40–113 of actin, and it did not contain the fluorescence carried by 1,5-IAEDANS-labeled Cys-374 of actin (data not shown), indicating that it included only the N-terminal actin segment 1–28. Taken together, these data demonstrate that S1 heavy chain was cross-linked to either one actin segment 1–28 within the acto-95-kDa product or to two actin segments 1–28 within the $acto_2$-95-kDa adduct.

Further proteolytic digestion of the thrombin-generated adducts of the $acto_2$-95-kDa complex was then carried out with trypsin, which cleaves the S1 heavy chain in three fragments of molecular masses 25, 50, and 20 kDa. Trypsin proteolysis produced the non-cross-linked 20- and 25-kDa fragments but not any 50-kDa material (Fig. 3 *B*, lanes *c* and *d*). Immunoblotting analysis revealed, besides residual uncleaved actin, three bands migrating at approximately 23-, 53-, and 56 kDa labeled 20-P, 50-P, and 50-P_2, respectively. The first band was assigned to a complex between the 20-kDa fragment of S1 and actin fragment 1–28 because it also included the 20-kDa fluorescence. The last two nonfluorescent bands are likely to correspond to the 50-kDa fragment of S1 cross-linked to either one or two actin fragments 1–28. This conclusion was supported by a similar, comparative experiment performed on the acto-95-kDa complex that showed the tryptic production of the 20-kDa-P and 50-kDa-P bands but none of the 50-kDa-P_2 material (Fig. 3 *A*, lanes *c* and *d*).

The overall data are consistent with the idea that the 20- or 50-kDa tryptic fragment of the S1 heavy chain is cross-linked to actin segment 1–28 in the acto-95-kDa product, i.e., when the reaction is performed at an actin:S1 molar ratio of 1 or during the earliest time of a reaction achieved at higher molar ratio (Fig. 4, cross-links A or B). When the molar ratio is higher than 1, a new covalent link is established between the 50-kDa fragment (which is part of the Arg-C-generated 75-kDa fragment) of the S1 heavy chain and a similar actin segment 1–28 leading to the $acto_2$-95-kDa product (Fig. 4, cross-links A and C or B and C). Moreover, the trypsin degradation of the covalent products also confirmed that the cross-links A (or B) and C occurred on two different actin monomers (Andreev and Borejdo, 1992). Indeed, if the three cross-links were located on the segment 1–28 of the same actin molecule, then the tryptic proteolysis of the $acto_2$-95-kDa adduct should have generated two bands of approximately 53- and 73 kDa carrying the actin segment attached to the 50-kDa fragment and to both the 50- and 20-kDa fragments, respectively. In such a case, no actin peptide-20-kDa entity could be generated, which is in contrast to the results obtained. One should notice that these results do not rule out a possible additional cross-linking between the two actin monomers, but they show that if any, it does not involve actin segments 1–28.

Our data provide evidence that when the cross-linking reaction is performed at an actin:S1 molar ratio of 5, the S1 heavy chain is first cross-linked to segment 1–28 of one actin monomer via its 20-kDa (more particularly, the ultimate N-terminal residues of this fragment; Yamamoto, 1990; Sutoh, 1983) or its 50-kDa fragment (at the C-terminal part of

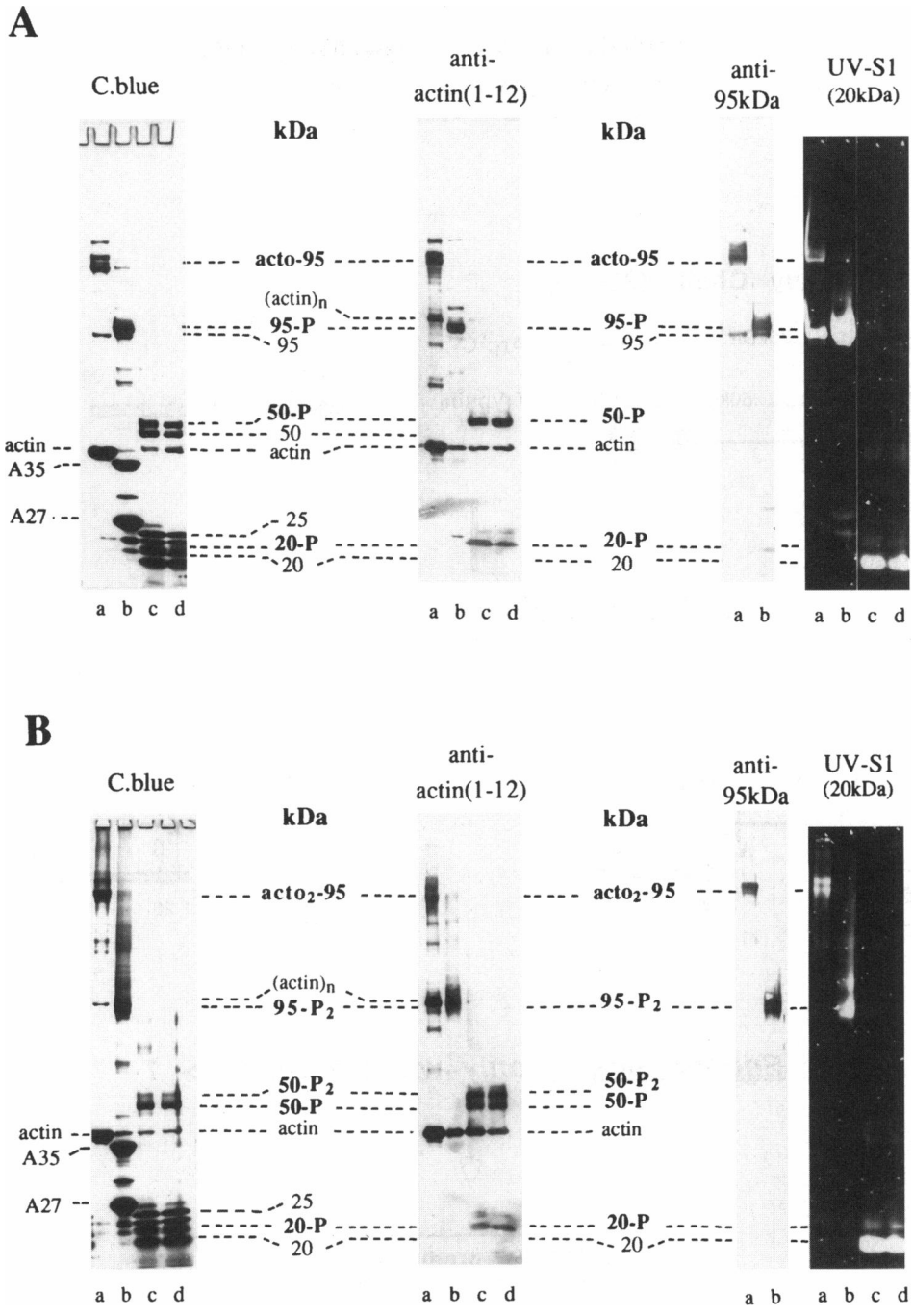

FIGURE 3 Proteolytic degradation of the covalent actin-S1 adducts. EDC-cross-linking reactions were conducted at an actin-S1 molar ratio of 1 (*A*) or 5 (*B*), and the samples were analyzed by $NaDodSO_4$ gels either stained with Coomassie blue, viewed under UV light for experiments using IAEDANS-labeled S1 (UV-S1), or immunoblotted with anti-actin (segment 1–12) or anti-S1 heavy chain (anti-95-kDa) antibodies. Cross-linked actin-S1 complexes (lanes *a*), were first digested by thrombin (lanes *b*), and subsequently cleaved by trypsin for 15 and 50 min (lanes *c* and *d*, respectively) as described under Materials and Methods. A35 and A27 stand for the thrombic fragments of non-cross-linked actin.

this fragment; Sutoh, 1983). In a second step, the same 50-kDa fragment of the S1 heavy chain is cross-linked to segment 1–28 of a second actin monomer and not with segment 91–100 as recently proposed based on the reconstruction of the fully decorated filament (Andreev et al., 1993c). This two-step reaction was proposed to be related to an isomerization of the actin-S1 interaction (Andreev et al., 1993b, c; Andreev and Borejdo, 1992). However, we obtained an identical time course pattern of the reaction whether the cross-linking was started just after mixing the two proteins or several hours later. In addition, this time course could be dramatically reduced by increasing the EDC concentration (data not shown). These results demonstrate that the two-step reaction is not linked to the isomerization of the actin-S1

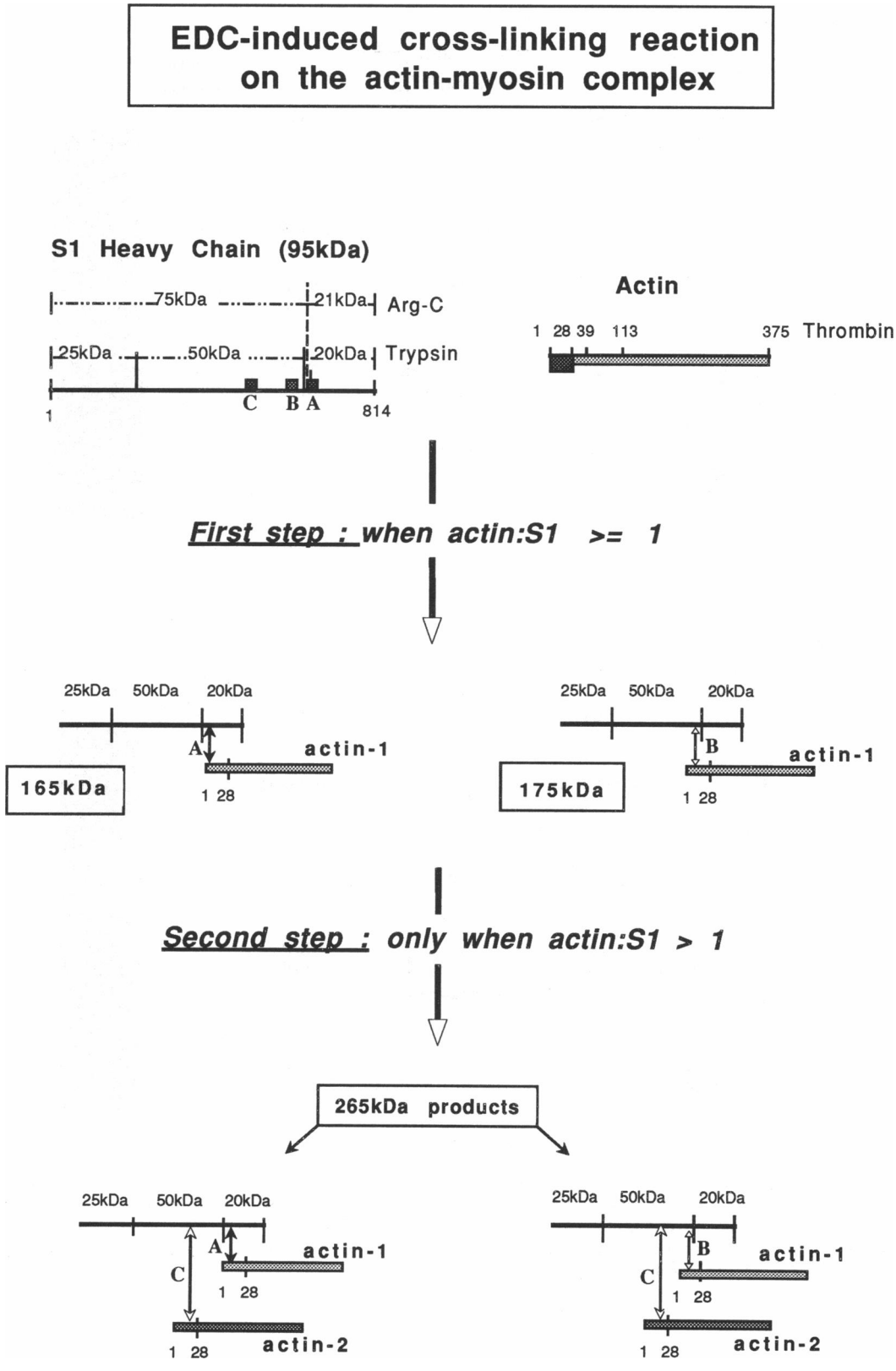

FIGURE 4 Schematic diagram illustrating the location of the EDC-induced cross-linking sites along the actin and S1 heavy chain sequences as a function of the actin-S1 molar ratio. The major peptides generated by the different proteases used in this study and the molecular masses of the cross-linked acto-S1 adducts are indicated. See text for more details.

interaction (at least in the time scale of our cross-linking reaction), but rather is related to a difference in reactivity of either the actin carboxylates reacting with EDC or the S1 amino groups involved in the isopeptidic bond. A low reactivity of these residues would indeed explain the absence of cross-linking between the S1 heavy chain and the second actin monomer earlier in the reaction time course. Because the first actin is known to be cross-linked to S1 at its first four acidic residues (Sutoh, 1982), a difference in reactivity of the actin carboxylates could be explained by cross-linking the second actin monomer via residues Asp-24 and/or Asp-25, which are the only carboxyl groups (within segment 1–28) located in an accessible loop. In agreement with this proposal, it was shown that the most severe effect of charge-reversion mutagenesis, i.e., the total loss of ATP driven sliding motion, was achieved by replacing these two acidic residues with histidine (Johara et al., 1993).

Significance of S1 binding to the N termini of two adjacent actin monomers

The Mg-ATPase activity of the cross-linked acto$_2$-95-kDa complex was found to be around 60% of the maximum velocity reached at infinite actin concentration (Andreev et al., 1993a) (our unpublished observations). If one considers that the extensive modification of actin by EDC accounts for this lower activity (Bertrand et al., 1989), it seems that the dynamics of the actin-S1 complex are not really affected by the covalent attachment of S1 to two actin molecules.

The lack of effect of Mg-ADP on the cross-linking between S1 and the N termini of adjacent actin molecules shows that this double interaction occurs not only in the rigor complex but also in the intermediate ternary actin-S1-ADP complex. The physiological significance of the cross-link of S1 to the second monomer was further established because its formation was not affected by the regulatory proteins tropomyosin (Tm) and troponin (Tn) in the "on" state. Indeed, the cross-linking experiment reported in Fig. 5 *A* shows that the yield of formation of the acto-95-kDa and the acto$_2$-95-kDa products was almost unchanged by the presence of Tm alone or of the Tm-Tn complex (Fig. 5 *A*, lanes *c* and *d*).

We then analyzed the composition of the cross-linking products obtained with the actin-myosin complex used at an actin:myosin head molar ratio of 5 and at an ionic strength of 130 mM (Fig. 5 *B*). To protect the integrity of the myosin filaments, we used a two-step reaction in which only actin was modified by EDC-NHS, leaving almost unaffected the electrophoretic pattern of myosin (Fig. 5, lanes *a* and *b*). When activated actin was added to the myosin-containing solution, high M_r cross-linked products were generated (Fig. 5 *B*, lanes *d* and *e*). Comparison of the chymotryptic digestion of myosin before (lane *c*) and after conjugation to EDC-actin (lane *f*) showed that the EDC reaction generated three products corresponding to the actin oligomers, acto-95-kDa and acto$_2$-95-kDa adducts; non-cross-linked S1 and rod subfragments were also observed. These experiments demonstrated that S1 can be cross-linked, i.e., interact, with the N termini of two actin monomers even within the double-head structure of the entire myosin molecule; of course, they do not indicate whether only one or both heads can interact with the N termini of two monomers at the same time.

Finally, the EDC reaction was performed directly on myofibrils in the rigor state, although under much milder conditions, i.e., at a lower temperature and with lower EDC and

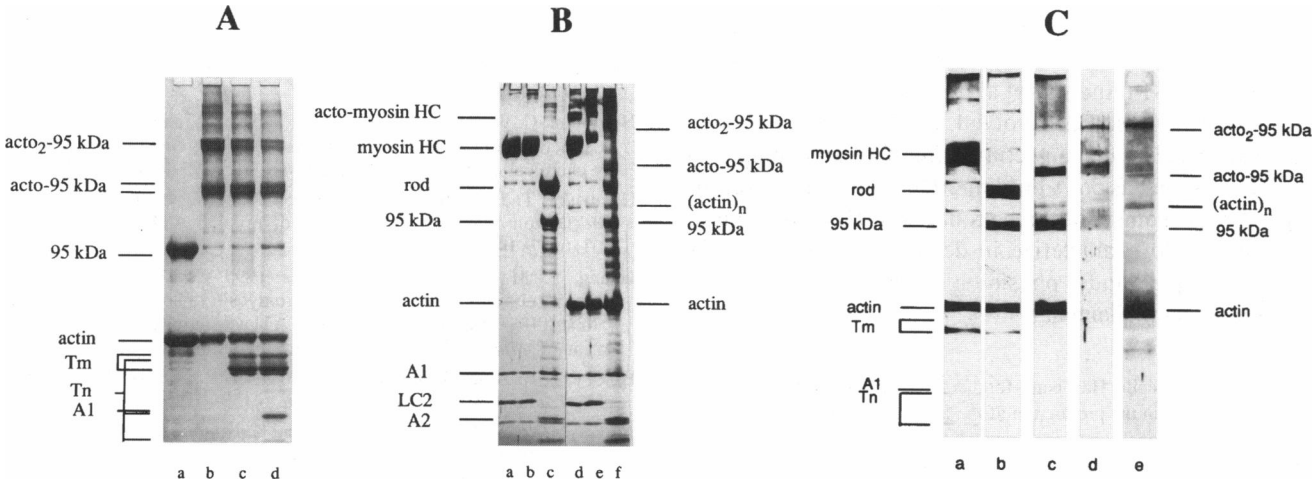

FIGURE 5 NaDodSO$_4$-gel electrophoresis analysis of EDC-cross-linking reaction between reconstituted actin filaments and S1 (*A*), between actin and myosin filaments (*B*), or within myofibrils (*C*). (*A*) EDC reactions were performed at an actin:S1 molar ratio of 5 in the absence (lane *b*) and in the presence (lane *c*) of Tm alone or associated with Tn (lane *d*). Lane *a* is a sample before reaction. (*B*) EDC reactions with myosin alone (lanes *a–c*) or mixed with actin (lanes *d–f*) were performed in a two-step reaction as described under Materials and Methods. Samples were analyzed at the beginning of the condensation step (lanes *a* and *d*), after 40-min condensation (lanes *b* and *e*), and after chymotrypsin digestion (lanes *c* and *f*). (*C*) EDC reaction on myofibrils. (lanes *a* and *b*) Non-cross-linked myofibrils before and after chymotrypsin digestion, respectively. Cross-linked myofibrils were analyzed after chymotrypsin cleavage by Coomassie blue staining (lane *c*) and by immunoblotting using anti-95 kDa (lane *d*) or anti-actin segment 1–12 antibodies (lane *e*).

NHS concentrations. As shown in Fig. 5 *C*, cross-linking of the myofibrils under these conditions led to the almost complete disappearance of the rod with little effect on the 95-kDa band (lanes *b* and *c*) and to the formation of two new cross-linked products. Because they both contained actin and S1 heavy chain (as revealed by immunostaining with the corresponding antibodies; lanes *d* and *e*) and because they had M_r of approximately 170 and 265 kDa, we concluded that these cross-linked products corresponded to the acto-95-kDa and the $acto_2$-95-kDa complexes described above with the purified proteins.

The fact that two adjacent actin monomers can be cross-linked to a myosin head in the presence of the regulatory proteins, when the myosin filaments are used under physiological ionic strength, or within myofibrils implies that the contact between S1 and the two N termini of these monomers is very likely to occur in vivo.

CONCLUSIONS

We have obtained experimental evidence demonstrating that S1 may interact with N-terminal segments 1–28 of two vicinal actin monomers. Among the various cross-linking reagent tested, EDC was the only one that was sensitive to the degree of saturation of F-actin by S1 (data not shown). Because these reagents mainly cross-link S1 to actin subdomain 1 (Bertrand et al., 1988; Bonafé et al., 1993, 1994), it seems likely that the overall binding site of S1 is not dramatically different in the unsaturated filament as compared with that recently described for fully saturated F-actin (Rayment et al., 1993; Schröder et al., 1993). Based on this latter reconstruction, it appears that a re-orientation of a few degrees of S1 around the axis of the actin filament could in principle accommodate the binding of the loop 570–576 of the 50-kDa fragment of the S1 heavy chain to actin segment 1–28 of the actin monomer below. This hypothesis in fact would agree with the difference in the myosin head orientation recently observed in myofibrils and muscle fibers depending on the ratio of bound S1 (Andreev et al., 1993b). Besides the identification of the residues involved in this interaction, however, it seems clear that an actin-S1 interface spreading over a distance as long as 55 Å has important implications for the molecular mechanism of force production. Therefore, it now seems urgent to characterize in detail the structure of the actin-S1 complex under physiological conditions, i.e., with unsaturated actin filaments.

We thank Marie-Cécile Harricane for her help in electron microscopy, Danielle Casanova in the production of the anti-actin fragment 40–113 antibodies, and Corinne Lionne for preparation of myofibrils. We are very grateful to Andrzej A. Kasprzak and Ridha Kassab for their helpful discussions and their critical reading of the manuscript.

This work was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, and the Association Française contre les Myopathies.

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DISCUSSION

Session Chairperson: Yale E. Goldman
Scribe: Jeremy Gollub

EMIL REISLER: When Julian Borejdo was first assaying the differences between the 1:1 and 2:1 conditions, not in the cross-link interactions but in simple binding experiments and fluorescence experiments, he found that the formation of this 2:1 structure depends very much on the time that is allowed for the formation of the complexes. Can you comment on that?

PATRICK CHAUSSEPIED: There is actually no relation between this time dependence of the actin binding that Julian observed and the time dependence that we observed in the cross-linking reaction, because we can keep the complex for more than an hour, 2 hours, and then 1 day, and we still have the same time course of the EDC reaction. The two-step reaction that we observe is mainly due, in our point of view, to the difference in reactivities of the carboxylate residues or lysine residues involved. I would like to make an additional point: maybe one question which is of some importance, I think, is to know how all the contacts in this complex, and more particularly, the main contact with subdomain 1, would be affected by rotating S1 around the axis of the filament.

ROGER COOKE: Instead of rotating the whole of S1, can you just have a conformational change in which that loop moves down?

CHAUSSEPIED: I don't know. I haven't done any docking experiments.

COOKE: When you cross-link myosin or myofibrils, shouldn't it act as if it's half saturated, because one of the heads would have another head below it on the filament protecting the site?

CHAUSSEPIED: We expect only one head to be capable of cross-linking.

COOKE: So you do get only one head?

CHAUSSEPIED: We cannot look that way because the conditions are quite different within myofibrils and myosin filaments. We have to use much milder conditions than with purified proteins, so we cannot do any computative analysis of data at that point.

IVAN RAYMENT: Do you plan to sequence these cross-links?

CHAUSSEPIED: We haven't sequenced yet the cross-linking site in S1.

RAYMENT: It would be enormously helpful to people like myself to know exactly which residue. Otherwise, in 10 residues you can go from one side of the enzyme to the other. So you have interesting information, but we can't use it.