Structure of the 265-Kilodalton Complex Formed upon EDC Cross-Linking of Subfragment 1 to F-Actin†

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ABSTRACT: The conventional model of force generation in muscle requires the presence of at least two different contact areas between the myosin head (S1) and the actin filament. It has been found that S1 has two sites available for carbodiimide cross-linking, but it is generally believed that the myosin head can be cross-linked to only one actin through either site. We provide here, for the first time, evidence that one S1 can be cross-linked to two separate actin molecules. The covalent complex of one S1 with two actins was found to have an apparent molecular mass of 265 kDa. The formation of the 265-kDa acto–S1 complex was strongly dependent on the ratio of S1 to actin. Limited tryptic digestion converted the 265-kDa product into the 240-kDa complex by releasing a 27-kDa N-terminal S1 fragment. Limited subtilisin digestion of the 265-kDa covalent acto–S1 complex yielded 29-, 93-, and 66-kDa peptides which corresponded to the 29-kDa N-terminal domain of S1, actin–44-kDa (central domain of S1) and actin–22-kDa (C-terminal domain of S1) complexes, respectively. These peptides could be generated only if a single S1 has been cross-linked to two separate actins. The 265-kDa acto–S1 complex (S1:actin ratio = 0.5) had 60% of the ATPase activity of the 175–185-kDa acto–S1 complex (S1:actin ratio = 1). The ability of the myosin head to bind to one or to two actins suggests that during an active stroke the myosin head may first bind to one and then to two monomers in F-actin, producing a ~10-nm shift between thick and thin filaments.

Muscle contraction is caused by the ATP-driven cyclic interaction of myosin heads (subfragment 1, S1) and actin filaments. It is widely believed that force is generated when the attached myosin heads execute a "power stroke" during which they change orientation with respect to actin filaments (Huxley, 1969). It has been suggested that at the beginning and at the end of the power stroke, different contact areas are involved in the interaction of the myosin head with the actin filament (Huxley & Simmons, 1971). The zero-length cross-linker EDC has been used extensively to localize the binding sites in acto–S1 complexes. Mornet *et al.* (1981) showed that cross-linking of acto–S1 with EDC produced complexes with apparent molecular masses of 175, 185, and 265 kDa. Sutoh (1983) using cleavage of 175–185-kDa peptides by trypsin, formic acid, BNPS-skatole, or hydroxylamine found that one cross-linking site in S1 was located between 18 and 20 kDa from the C-terminus, leading to the formation of a 175-kDa peptide The second cross-linking site was located between 27- and 35-kDa fragments from the C-terminus, leading to the formation of a 185-kDa peptide. Just one site on actin (residues 1–12) was found to be cross-linked to S1 in 175–185-kDa peptides. Sutoh found that the 175–185-kDa doublet contained one actin and one S1, which was confirmed by others (Heaphy & Tregear, 1984; Greene, 1984; Chen *et al.*, 1985). It was found that the Mg-ATPase activity of 1:1 cross-linked complexes (175–185 kDa) was near a maximum value expected at infinite actin concentration (Mornet *et al.*, 1981). However, there is little information available about the structure and activity of the 265-kDa cross-linked complex. Heaphy and Tregear (1984) and Mornet *et al.* (1989) suggested that the 265-kDa product might correspond to the cross-linked complex of one S1 with two actins. On the other hand, Lu and Wong (1991) proposed that 265 kDa could have been a product of cross-linking of both sites of S1 to one actin.

Recently, using fluorescently labeled S1 and F-actin, we have shown that the 265-kDa band contained two actins and one S1. The production of 265-kDa complex was strongly dependent on the S1:actin molar ratio. When the S1:actin molar ratio was low, cross-linking with high concentrations of EDC at first produced 175–185-kDa peptides, most of which were later converted to a 265-kDa peptide. When the molar ratio was high, only a 175–185-kDa doublet was formed (Andreev & Borejdo, 1991, 1992).

In the present paper, we extend these studies to investigate the structure of the actin–S1 interface in the 265–kDa complex by mild tryptic and subtilisin digestions. The results show that the 265-kDa complex is a product of cross-linking of one S1 with two separate actins. The Mg-ATPase activity of the 265-kDa complex is about 60% of that of 175–185-kDa complexes. The results confirm our earlier suggestion that S1 can bind to two actin monomers in a filament.

MATERIALS AND METHODS

Materials. ATP, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), phenylmethanesulfonyl fluoride (PMSF), β-mercaptoethanol, phalloidin, subtilisin Carlsberg (type VIII bacterial protease), and α-chymotrypsin were from Sigma (St. Louis, MO). Trypsin and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. (Freehold, NJ). (Iodoacetamido)tetramethylrhodamine (IATR), 5-[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (1,5-IAEDANS), and 9-anthroylnitrile (9-ANN) were purchased from Molecular Probes (Eugene, OR). Sephadex G-50 gels was from Pharmacia (Pharmacia, Piscataway, NJ).

Proteins. Myosin was prepared from rabbit skeletal muscle by the method of Tonomura *et al.* (1966). S1 was obtained by chymotryptic digestion of myosin according Weeds and Taylor (1975), and actin was prepared according to Spudich

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and Watt (1971). To remove free ATP, G-actin was passed through a Sephadex G-50 column equilibrated with a buffer containing 0.2 mM $CaCl_2$, 0.1 mM NaN_3, and 10 mM Tris-HCl, pH 7.5, and was later polymerized for 2 h at room temperature by addition of 50 mM KCl, 2 mM $MgCl_2$, and 1.2 molar excess of phalloidin. Concentrations of proteins were measured by using the following extinction coefficients: for S1, $A^{1\%}(280) = 7.5$: for G-actin, $A^{1\%}(290) = 6.3$: for F-actin, $A^{1\%}(290) = 6.7$.

Labeling of S1 and F-Actin. S1 was labeled by 9-ANN according to the method of Hiratsuka (1989). Labeling of S1 and F-actin by IATR or IAEDANS was described previously (Andreev & Borejdo, 1992).

Cross-Linking of S1 to F-Actin. S1 and F-actin were mixed at different molar ratios and incubated at room temperature for 1 h in 50 mM KCl, 0.2 mM $MgCl_2$, and 10 mM Tris-HCl buffer, pH 7.5. The cross-linking reaction was initiated by adding an appropriate volume of freshly prepared water solution of EDC (concentration of EDC in stock solution was 1 M). The final concentrations of EDC and the time of the cross-linking reaction are indicated in the text. The reaction was stopped by adding β-mercaptoethanol to a final concentration of 0.2 M.

Tryptic and Subtilisin Digestion. Acto–S1 or S1 was digested by trypsin or subtilisin at a weight ratio of protease to S1 of 1:20. The trypsin or subtilisin digestion reactions were stopped by adding soybean trypsin inhibitor or PMSF, respectively. All digestion experiments were done with F-actin stabilized by phalloidin, which increased the resistance of F-actin to subtilisin or tryptic digestion. Trypsin was able, however, to cleave two C-terminal residues (374 and 375) in actin even in the presence of phalloidin. The cleavage of residues 374 and 375 did not affect the interaction of F-actin with S1.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out according to Laemmli (1970) using 15% (w/w) or 6–15% gradient polyacrylamide gels.

ATPase Measurements. The Mg-ATPase activity of cross-linked complexes was determined by following the time courses of P_i liberation at 25 °C at high salt concentration, where the Mg-ATPase of non-cross-linked S1 is not activated by actin (Huang *et al.*, 1990). The cross-linking reaction was stopped by adding 0.2 M β-mercaptoethanol, and then samples were diluted to a final concentration of 0.006 μM S1 in buffer solution containing 0.5 M KCl, 3 mM $MgCl_2$, 2 mM ATP, and 10 mM Tris-HCl buffer pH 7.5. The concentration of inorganic P_i was measured by the colorimetric method of Ohno and Kodama (1991).

RESULTS

Tryptic Digestion of Cross-Linked Acto–S1 Complexes. Cross-linking of S1 to F-actin with EDC produces complexes with apparent molecular masses of 175, 185, and 265 kDa (Mornet *et al.*, 1981). Results of limited tryptic cleavage of S1 and of cross-linked acto–S1 complexes are shown in Figure 1. S1 was specifically labeled near the N-terminus by 9-ANN or near the C-terminus by 1,5-IAEDANS. Limited tryptic digestion of S1 alone confirmed that 9-ANN was associated with the 27-kDa N-terminal domain of S1 and 1,5-IAEDANS with the 20-kDa C-terminal domain (Figure 1A, samples 1–3). Staining the gel with Coomassie Blue did not reveal any difference in digestion patterns between samples containing S1 labeled with 9-ANN or with 1,5-IAEDANS (Figure 1B). The patterns of fluorescence of cross-linked products after digestion of trypsin revealed that when S1 was labeled with

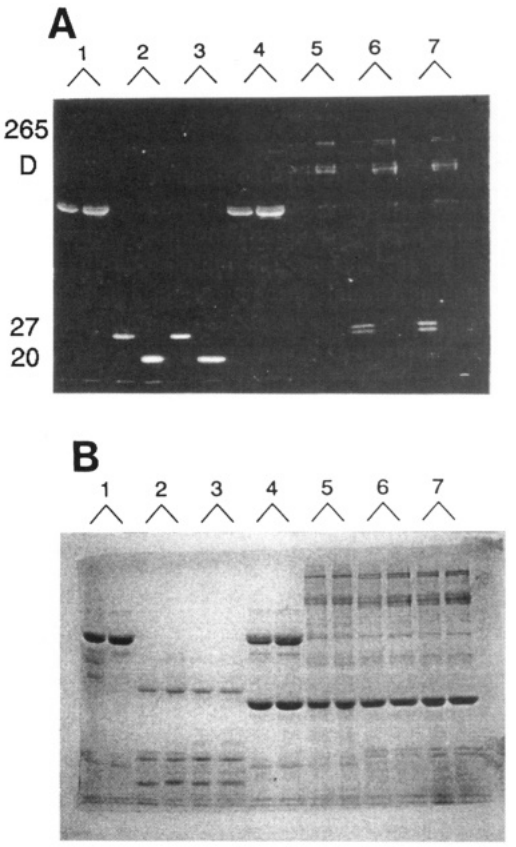

FIGURE 1: Tryptic digestion of fluorescently labeled S1 alone and cross-linked to F-actin. S1 was labeled by 9-ANN at 27 kDa (Hiratsuka, 1989), or by 1,5-IAEDANS at 20 kDa (Takashi *et al.*, 1976). S1 (4 μM) and F-actin (12 μM) were cross-linked by EDC (50 mM) for 1 h; the reaction was stopped by adding β-mercaptoethanol (200 mM). Tryptic digestion was carried out at a 1:20 weight ratio of trypsin to S1 at 20 °C. Buffer solution: 50 mM KCl, 0.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5. Samples were applied in pairs: in the first sample of each pair, S1 was labeled with 9-ANN, and in the second, it was labeled with 1,5-IAEDANS (the numbers refer to the pairs). (A) Fluorescent picture of gel (15% polyacrylamide). (B) The same gel stained with Coomassie Blue. Samples 1–3: S1 was digested by trypsin for 0, 5, and 15 min. Sample 4: S1 and actin before cross-linking. Samples 5–7: S1 and actin were cross-linked and digested by trypsin for 15 min (sample 6) and for 30 min (sample 7). No difference was found in the appearance of digestion products in the gel stained with Coomassie Blue. 15% polyacrylamide gel.

9-ANN, 27–25-kDa fragments were intensely fluorescent (Figure 1A, left lanes 6 and 7), suggesting that digestion released the 27-kDa N-terminal fragment of S1 (to which 9-ANN was bound). The 175–185- and 265-kDa fragments were fluorescent before the digestion (Figure 1A, left side of lane 5) but decreased in intensity as the 27-kDa terminal fragment of S1 was released (Figure 1A, left lanes 6 and 7).

When S1 was labeled with 1,5-IAEDANS, tryptic digestion yielded 20-, 64-, 175–185-, 160–170-, 265-, and 240-kDa fluorescent products (Figure 1A, right side of lanes 6 and 7). The minor products with masses of 20 and 64 kDa corresponded to the C-terminal domain of S1 and to the cross-linked complex of this domain with one actin, respectively (Mornet *et al.*, 1981; Sutoh, 1983). The 175–185-kDa doublet was converted into 160–170 kDa, which corresponded to a cross-linked complex of the 70-kDa, which corresponded to a cross-linked complex of the 70-kDa C-terminal domain of S1 with one actin (Mornet *et al.*, 1981; Duong & Reisler, 1989). The 240-kDa product was fluorescent only when S1 was labeled with 1,5-IAEDANS, indicating that limited tryptic digestion of the 265-kDa product converted it into 240 kDa by releasing the 27-kDa N-terminal domain of S1. Otherwise,

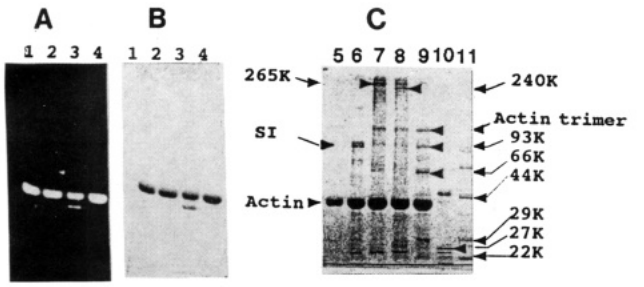

FIGURE 2: (A and B) Effect of subtilisin digestion on F-actin labeled by IATR. (C) Subtilisin and tryptic digestion of S1 and cross-linked acto–S1. (A and B) Fluorescent and Coomassie Blue stained pictures of the same gel (6–18% gradient polyacrylamide gel), respectively. Lanes 1 and 3, 5 μM IATR–F-actin (no phalloidin) before and after treatment by subtilisin, respectively; lanes 2 and 4, the same, but in the presence of 6 μM phalloidin. (C) Lane 5, F-actin (4 μM) was incubated with 50 mM EDC for 1 h; lane 6, actin (8 μM) and S1 (2 μM); lane 7, F-actin (8 μM) and S1 (2 μM) were cross-linked with 50 mM EDC for 1 h; lane 8, cross-linked acto–S1 was digested by trypsin; lane 9, cross-linked acto–S1 was digested by subtilisin; lane 10, S1 digested by trypsin; lane 11, S1 digested by subtilisin.

trypsin was ineffective in hydrolyzing the 265-kDa complex. The 27-kDa fragment degraded later to 25 kDa. Other results indicated that fluorescently labeled F-actin could not be used to map the peptides of the 265-kDa product, because trypsin cleavage released fluorescent probe together with the 374- and 375-kDa terminal residues of actin (data not shown). Tryptic cleavage of the 374- and 375-kDa residues of actin did not affect S1 binding to F-actin.

Subtilisin Digestion of Cross-Linked Acto–S1 Complexes. For further analysis, we used subtilisin to map the 265-kDa complex. Subtilisin cleaves S1 into three domains: 29, 44, and 22 kDa starting from N-terminus of S1 (Applegate & Reisler, 1983; Mornet *et al.*, 1984). The 175–185-kDa cross-link products are not protected by F-actin from subtilisin cleavage (Duong & Reisler, 1989), and we thought the same may be true for the 265-kDa product. Figure 2C shows the pattern of subtilisin cleavage of the 265-kDa product which was formed when 2 μM S1 and 8 μM F-actin were cross-linked using 50 mM EDC for 1 h at 23 °C. Under these conditions, almost all S1 was cross-linked with actin forming 265-kDa complex (Figure 2C, lane 7). When F-actin alone was incubated with 50 mM EDC for 1 h, a small amount of actin trimer with an apparent molecular mass of 130 kDa was produced (Figure 2C, lane 5). Tryptic digestion of the 265-kDa complex produced 240- and 27-kDa peptides (Figure 2C, lane 8) while subtilisin digestion produced three peptides with apparent molecular masses of 93, 66, and 29 kDa (Figure 2C, lane 9). The 29-kDa peptide corresponded to the N-terminal fragment of S1. Subtilisin digestion of S1 alone yielded 29-, 44-, 22-, and 73-kDa (=29+44) products (Figure 2C, lane 11). Subtilisin did not cleave F-actin stabilized by phalloidin (Figure 2A,B, lane 4). In the absence of phalloidin, there appeared a minor 35-kDa band (Figure 2A,B, lane 3). Most likely, the sample contained a small amount of G-actin which was less resistant to subtilisin attack than F-actin. As reported before (Schweyter *et al.*, 1989), subtilisin cleaves G-actin between Met-47 and Gly-48, splitting actin into 35- and 9-kDa (from the N-terminus) fragments.

To determine the composition of the 93- and 66-kDa peptides, we labeled either actin or S1 with IATR and studied the subtilisin digestion pattern of the cross-linked complexes. As shown in Figure 3, when actin was labeled with IATR, both 93- and 66-kDa products were fluorescent (Figure 3, lane 2a) while just 66 kDa was fluorescent when S1 was labeled with IATR (FIgure 3, lane 4a). The 66-kDa peptide could not be generated by subtilisin from the 69-kDa actin–light-

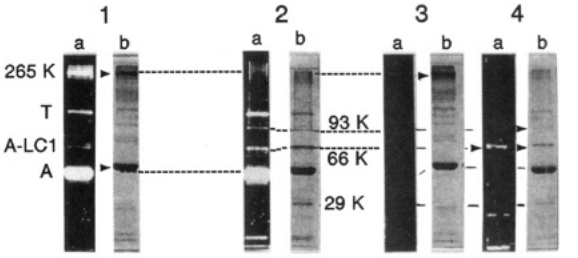

FIGURE 3: Subtilisin digestion of cross-linked acto–S1 where actin (gels 1 and 2) or S1 (gels 3 and 4) was labeled with IATR. S1 (3 μM) and F-actin (12 μM) were cross-linked with 50 mM EDC for 1 h at 23 °C (1 and 3) and then digested by subtilisin (2 and 4). The 265-kDa complex was a predominant product. Lanes a and b indicate fluorescent and Coomassie Blue stained images of the same gel (6–18% gradient polyacrylamide gel). Lanes 1a, 2a, and 4a were contrast-enhanced to show fluorescent bands better. Abbreviations: 265 K, 265-kDa cross-linked complex; T, actin trimer; A-LC1, 69-kDa cross-linked complex of actin and alkali chain 1; A, actin.

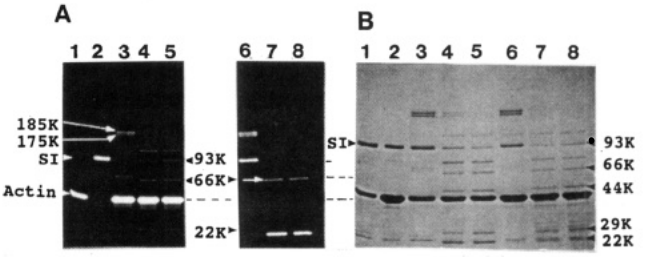

FIGURE 4: Representative electrophoretic patterns of S1 cross-linked to actin under limited conditions and patterns of subtilisin digestion of cross-linked complex. 3 μM native or IATR-labeled S1 and native or IATR-labeled F-actin was cross-linked with 5 mM EDC for 30 min. Panel A is a fluorescent picture of the 6–18% gradient polyacrylamide gel of the cross-linked complex. Panel B is the same gel stained with Coomassie Blue. Lanes: 1, F-actin–IATR and unlabeled S1 before cross-linking; 2, S1–IATR and unlabeled F-actin before cross-linking; 3, cross-linked acto–S1 where F-actitn was labeled by IATR; 6, cross-linked acto–S1 where S1 was labeled by IATR; 4 and 5, 7 and 8, subtilisin digestion of acto–S1 for 20 and 40 min, respectively.

chain 1 cross-linked complex (Figure 3, line 1a) which was only fluorescent when actin was labeled by IATR. The same products (93 and 66 kDa) were found when 175–185-kDa cross-linked complexes were digested by subtilisin (Figure 4), which corresponded to covalent complexes of one actin with a central domain of S1 (93 kDa) or with a C-terminal domain of S1 (66 kDA), in agreement with Duong and Reisler (1989). Subtilisin digestion of non-cross-linked S1 and or 175–185-kDa complexes generated 73- (N-terminal), 44-, 29-, and 22-kDa fragments (Figure 4, lanes 4, 5, 6, and 8). Since 73-, 44-, and 22-kDa fragments were not observed in the subtilisin digestion pattern presented in Figure 3, it follows that most of S1 was cross-linked to F-actin through both 44- and 22-kDa domains. The 93- and 66-kDa peptides were also observed when S1 was first cleaved by subtilisin and then cross-linked to F-actin (not shown).

We note that a small amount of actin trimer was produced at cross-linking conditions used in our experiments (e.g., Figure 3). We could not find evidence for covalent complexes of actin trimer with S1 or its fragments. Probably, actin trimer was generated from actin unoccupied by S1.

Mg-ATPase Activity of Cross-Linked Acto–S1 Complexes. F-Actin (8 μM) and S1 (2 μM) were cross-linked with 5 mM EDC for 30 min or with 50 mM EDC for 5, 30, 60, 90, and 120 min in 50 mM KCl, 0.2 mM $MgCl_2$, and 10 mM Tris-HCl buffer, pH 7.5, at 23 °C. The cross-linking reaction was stopped by adding 0.2 M β-mercaptoethanol. The Mg-ATPase activity of cross-linked complexes was determined by time courses of P_i liberation at 25 °C at high salt concentration,

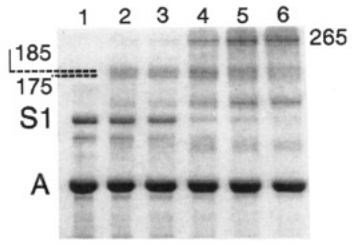

FIGURE 5: Time course of cross-linking of S1 to F-actin with high concentrations of EDC. 3 μM S1 and 12 μM F-actin (lane 1) were cross-linked with 50 mM EDC for the following times: lane 3, 5 min; lane 4, 30 min; lane 5, 60 min; lane 6, 120 min. In lane 2 is a control in which S1 and F-actin were cross-linked with 5 mM EDC for 30 min. S1, actin, and 265-kDa and 175–185-kDa cross-linked complexes are indicated.

where the non-cross-linked S1 did not contribute to Mg-ATPase activated by actin (Huang *et al.*, 1990). The amounts of non-cross-linked S1 and S1 included in the 175–185-kDa or in the 265-kDa complexes were determined by measuring the intensities of the corresponding bands in a polyacrylamide gel stained with Coomassie Blue (Figure 5). The fractional contributions of actin and S1 to the intensities of the 175–185- and 265-kDa bands were calculated assuming S1:actin ratios in these bands of 1:1 and 1:2, respectively. The relative uptake of Coomassie Blue by actin and S1 was estimated as in Sutoh (1983). The amount of S1 in the 175–185-kDa band ($[S1]_{doub}$) and in the 265-kDa band ($[S1_{265}$) was calculated as

$$[S1]_{doub} = [S1]_T I_{doub} C_{doub} / I_T$$

$$[S1]_{265} = [S1]_T I_{265} C_{265} / I_T$$

where $[S1]_T$ and I_T are the total S1 concentration and the intensity of the 95-kDa band before cross-linking, respectively, and C'_{doub} and C'_{265} are the uncorrected fractional contributions of S1 to the intensities of the 175–185- and 265-kDa bands, respectively, and are $[95/(95 + 42)] = 0.69$ and $[95/(95 + 42 + 42)] = 0.53$. After correction for the uptake of Coomassie Blue, the corrected fractional contributions C_{doub} and C_{265} were 0.71 and 0.56, respectively. I_{doub} and I_{265} were the intensities of the 175–185- and 265-kDa bands. The total Mg-ATPase activity of each sample was a sum of the ATPase activities, V_{doub} and V_{265}, of the 175–185-kDa doublet and 265-kDa complexes. The total amount of phosphate liberated increased linearly with time, t, according to the equation: $[P_i]/t[S1]_T = V_{doub}[S1]_{doub}/[S1]_T + V_{265}[S1]_{265}/[S1]_T$. The V_{doub} was determined in samples containing only doublet (5 mM EDC for 30 min, or 50 mM EDC for 5 min of cross-linking). Then V_{265} was determined in samples containing different amounts of doublet and 265-kDa complexes (50 mM EDC for 30, 60, 90, and 120 min of cross-linking). Eight gels having four different protein preparations were analyzed, and average values were $V_{doub} = 35 \pm 3\ s^{-1}$ and $V_{265} = 21 \pm 5\ s^{-1}$. The total Mg-ATPase activity decreased when the products with molecular masses higher than 265 kDa begun to appear.

DISCUSSION

Cross-linking of S1 to F-actin with EDC is known to produce three complexes with apparent molecular masses of 175, 185, and 265 kDa (Mornet *et al.*, 1981). Masses varied with gel concentration (Heaphy & Tregear, 1984). The 175–184-kDa doublet appeared in the initial period of EDC cross-linking, but as the reaction proceeded a 265-kDa band became more abundant [Figure 5 and see Mornet *et al.* (1981) and Sutoh (1983)]. Recently, we found that the formation of the 265-kDa complex depended strongly on the molar ratio of bound S1 to actin. At a low molar ratio of S1 to actin and after prolonged cross-linking with high concentrations of EDC, the 175–185-kDa doublet was converted into 265 kDa. This band indicated a product which contained 2 times more actins per S1 than 175–185-kDa product (Andreev & Borejdo, 1992). While a lot of data exist about proteolytic and chemical cleavage of the 175–185-kDa doublet [e.g., see Mornet *et al.* (1981), Sutoh (1983), Chen *et al.* (1985), Bertrand *et al.* (1988), and Yamamoto (1990)], nothing was known about the peptide map of the 265-kDa product. In this paper, we used limited tryptic and subtilisin digestion of cross-linked acto–S1 complexes to examine if the 265-kDa complex is a cross–linked product of S1 with two actins or with one actin which is cross-linked to another actin. The limited tryptic digestion of the 265-kDa cross-linked complex revealed that the 27-kDa N-terminal domain of S1 was not cross-linked to actin. The junction between the central and C-terminal domains of S1 was protected by F-actin from tryptic attack. In contrast to tryptic digestion, subtilisin cleavage was not inhibited by actin. This difference between tryptic and subtilisin digestion may be caused a shift of the cleavage site by 10 residues toward the N-terminus, from the lysines which are attacked by trypsin (Lys-640/641) to glycines which are attacked by subtilisin (Gly-632/Gly-633) (Duong & Reisler, 1989). Subtilisin digestion of cross-linked acto–S1 complexes, when the 175–185-kDa doublet or the 265-kDa product were predominant products, gave the same fragments with apparent molecular masses of 93 and 66 kDa. The covalent complex of actin with the 73-kDa N-terminal S1 fragment could be observed in the beginning of subtilisin digestion of the 265-kDa fragment product or of the 175–185-kDa doublet, but it was degraded into 29-kDa and actin–44-kDa fragments as it was verified using S1 labeled with 9-ANN (data not shown).

We considered three possible modes of creation of 265-kDa: (i) Both sites of S1 could be cross-linked to the same actin (Lu & Wong, 1991). (ii) Two actins could be cross-linked to each other and to either fragment of S1. (iii) One actin could be cross-linked to the central domain of S1 and the other to the C-terminal domain of S1. The first possibility could be ruled out because the 265-kDa product contained twice as many actins as the 175–185-kDa doublet (Andreev & Borejdo, 1992). Further, it cannot be true because subtilisin cleavage of the 265-kDa complex would have produced 29 kDa and a peptide containing actin+44+22 fragments. Actin+44+22 peptide would have been fluorescent when either S1 or actin was labeled by IATR, and its apparent molecular mass would have been different from 93 or 66 kDA (actin+44 and actin+22 peptides). Figures 3 and 4 show that there is no such peptide among the products of subtilisin digestion of 265- or 175–185-kDa cross-linked complexes. If (ii) were true (i.e., the 265-kDa complex was a result of cross-linking of two actins to each other and to S1), the subtilisin cleavage would have generated actin+actin+22, actin+actin+44, or actin+actin+44+22 peptides, which were not found. This leaves (iii) as the only plausible explanation of our results. It can be expected that subtilisin cleavage of junctions between 29-, 44-, and 22-kDa domains of S1 would generate 29-kDa, 44+actin (93-kDa), and 22+actin (66-kDa) peptides. The 93-kDa peptide should be fluorescent when actin is labeled with IATR. The 66-kDa peptide should be fluorescent when actin or S1 is labeled with IATR. Such peptides were indeed found (Figure 3); 66- and 93-kDa fragments were therefore identified as actin+44-kDa and actin+22-kDa covalent complexes. The fact that subtilisin digestion of 265-kDa and 175–185-kDa cross-linked complexes generated the same peptides (93 and 66 kDa) confirms the suggestion that the

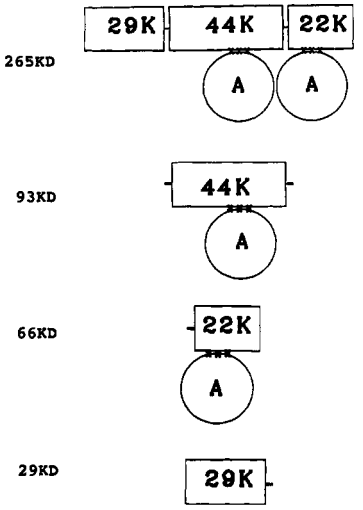

FIGURE 6: Schematic diagram of the 265-kDa covalent acto–S1 complex and its subtilisin digestion products. S1 contains three fragment shown as 29, 44, and 22 kDa.

265-kDa peptide was produced as a result of cross-linking of one actin to the central domain of S1 and other actin to the C-terminal domain of S1. This conclusion is illustrated schematically in Figure 6.

Recent model of the acto–S1 complexes by Rayment *et al.* (1993a,b) and by Schroder et al. (1993) indicated that at least three sites on S1 were involved in making contact with a single actin molecule, and one site was involved in making contact with a neighboring actin molecule. Sites involved in making contact with a single protein actin were (1) the region between Tyr^{626} and Glu^{647} on S1 ("missing loop" containing five lysines); this region is thought to make ionic contact with the negatively charged NH_2 terminal of actin; (2) the S1 region between Pro^{529} and Lys^{553}; it is thought to interact hydrophobically with Ala^{145}–Ser^{148} and with Iso^{341}–Gln^{354} regions on actin; (3) the S1 region Arg^{405}–Lys^{415} (so-called "second loop"); it is thought to interact ionically with the Pro^{332}–Glu^{334} region of actin. In addition, Rayment *et al.* (1993b) identified a region on S1 which interacts with a neighboring actin monomer, namely, (4) the S1 region Lys^{567}–His^{578}, which is thought to interact ionically with the Tyr^{91}–Glu^{100} region of actin. We think that this contact (of S1 with the neighboring actin monomer) is weak in F-actin which is fully saturated with S1. We think that only at low molar ratios of S1 to actin this contact becomes stronger. The fact that the 265-kDa complex was formed only at low molar ratios of S1 to actin indicates that the interface between S1 and actin is different in F-actin which is fully or partially saturated with S1. In fact, we have recently shown that the orientation of S1 bound to actin filaments in muscle fibers and myofibrils was different at high and low molar ratios of S1 to actin (Andreev *et al.*, 1993). The identification of binding sites in acto–S1 complex formed at low molar ratios is of particular interest because in working muscle actin is always in excess over myosin heads.

In view of the fact that in the 265-kDa complex S1 is cross-linked to actin at two sites, it is not surprising that it has a lower ATPase activity than the 1:1 complex. It is thought that for efficient release of P_i, S1 must have freedom of motion. When S1 is cross-linked through two sites, this freedom is restricted.

The present result showing that the two separate actins are cross-linked to a single S1 molecule when the S1:actin ratio is <0.5 supports our previous findings that depending on the molar ratio of bound S1 to actin two different rigor complexes can be formed: A·S1 (one actin and one S1), when S1 is in molar excess over actin, or A·S1·A (two actins and one S1), when actin is in molar excess over S1. The ability of the myosin head to bind to one or two actins suggests that during an active stroke the myosin head may first bind to one and then to two monomers in F-actin, producing a shift between thick and thin filaments of ~10 nm (average size of the actin dimer).

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