Binding of Heavy-Chain and Essential Light-Chain 1 of S1 to Actin Depends on the Degree of Saturation of F-Actin Filaments with S1

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ABSTRACT: The interaction of heavy-chain isoforms of myosin subfragment-1 with actin was examined by cross-linking with carbodiimide (EDC). The heavy chain of S1 could be cross-linked to a single actin molecule through sites on either 20 or 50 kDa proteolytic domains, resulting in complexes which migrated in an 8% polyacrylamide gel in the presence of Tricine buffer with an apparent molecular mass (M_{app}) of 150 or 160 kDa, respectively. Cross-linking of S1 through both sites to two actins produced a complex migrating with an M_{app} of 210 kDa. Cross-linking of the S1(A1) isoform [but not S1(A2)] to F-actin produced four additional peptides with M_{app} values of 64, 120, 185, and 235 kDa. These peptides corresponded to cross-linked complexes of A1+actin, $S1_{HC}$+actin, A1+actin+$S1_{HC}$, and A1+actin+$S1_{HC}$+actin, respectively. The production of the 64, 160, 185, 210, and 235 kDa complexes was almost inhibited at a high degree of saturation while the inhibition of the 150 kDa product was relatively small. At a low degree of saturation, the ratio of 150 to 160 kDa complexes was 1. Cross-linking between the S1 isoforms and regulated F-actin was not affected by Ca^{2+}. These data show that contact of the S1 to one actin protomer is through a site on the 20 kDa fragment and to the second actin protomer through the sites located on the 50 kDa fragment and on the essential light-chain 1. At nonphysiological conditions of full saturation of actin filaments with myosin heads, the binding of heavy chain at S1 and of A1 to the second actin could be almost abolished.

Muscle contraction occurs as a result of a cycling interaction of the myosin head (S1)[1] with the actin filament. In the absence of nucleotides, the myosin head forms a tight complex with F-actin; this rigor complex is believed to exist at the end of the power stroke of a cross-bridge in muscle. The rigor binding of myosin heads to F-actin has been extensively studied, and there have been several reports indicating that the mode of binding depends on the degree of occupancy of F-actin by myosin heads (Tawada, 1969; Harvey *et al.*, 1976; Mornet *et al.*, 1981; Yamamoto & Sekine, 1986; Yamamoto, 1990; Andreev & Borejdo, 1992; Andreeva *et al.*, 1993). Binding can be conveniently monitored by cross-linking with the water-soluble zero-length reagent EDC (1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide) which forms covalent links between S1 and F-actin. Cross-link products migrate in PAGE in the presence of glycine buffer with apparent molecular masses (M_{app}) of 175, 185, and 265 kDa (Mornet *et al.*, 1981) (in the presence of Tricine buffer, these migrate with M_{app}s of 150, 160, and 210 kDa and will be referred to here as such). Sutoh (1983) showed that under conditions of moderate excess of actin, the 150 kDa adduct was formed as a result of cross-linking of one S1 through the site located on the 20 kDa proteolytic fragment (the first site) to the N-terminus of a single actin. Similarly, the 160 kDa adduct was formed as a result of cross-linking of one S1 through the site located on the 50 kDa proteolytic fragment (the second site) to the N-terminus of a single actin. We found that during cross-linking of unsaturated acto−S1 complexes two intermediate adducts (150 and 160 kDa) were formed initially which later were transformed into a 210 kDa peptide which was a complex of one S1 with two actins (Andreev & Borejdo, 1992; Andreeva *et al.*, 1993). The 210 kDa complex was never formed when F-actin was fully saturated with S1.

The natural explanation of these results is that in fully saturated filaments S1 contacts one actin only through the site on the 20 kDa fragment (leading to formation of the 150 kDa adduct) and that in unsaturated filaments it contacts two actins, the first actin through the site on the 20 kDa fragment and the second actin through the site on the 50 kDa fragment. In unsaturated filaments, cross-linking can occur through both sites simultaneously to two adjacent actins (leading to the formation of the 210 kDa adduct). Only the N-terminus of actin is involved in cross-linking (Sutoh, 1983; Bonafe & Chaussepied, 1995). This explanation leads us to expect that the formation of 210 and 160 kDa cross-linked adducts would be dependent on the degree of saturation of actin filaments while the formation of 150 kDa complex would not. This is because in sparsely decorated filaments myosin heads suffer no interference from neighbors (and can be cross-linked through both sites) whereas in fully saturated filaments they do suffer interference from neighboring S1's and the site on 50 kDa is not close enough to actin to be cross-linked. We expected therefore that the ratio of 160/150 kDa adducts would decrease with an increase in the molar ratio (MR) of S1 to actin and that at full saturation the ratio would be equal to 1. All these expectations were fulfilled, suggesting that under nonphysiological conditions of full saturation of thin filaments with myosin heads the contact of S1 with the second actin is much weaker.

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[1] Abbreviations: S1, myosin subfragment-1; $S1_{HC}$, heavy chain of S1; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; M_{app}, apparent molecular mass; IATR, (iodoacetamido)tetramethylrhodamine; 5-IAF, 5-(iodoacetamido)fluorescein; MR, S1:actin molar ratio.

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In addition to binding to F-actin through the first and second sites, S1 can interact with F-actin through essential light-chain 1 (A1; Prince *et al.*, 1981; Henry *et al.*, 1985; Sutoh, 1982; Yamamoto & Sekine, 1983). We show that this interaction, like the formation of the 160 kDa adduct, depends on the degree of saturation of actin filaments by S1: the production of a covalent complex of actin with A1 decreases dramatically with an increase of the molar ratio of S1(A1) to actin.

In view of the above, it makes sense to expect that in muscle fibers the switching on of the regulatory system inhibits binding through the second site. However, the regulation process is more complex: Ca^{2+} does not inhibit the production of the 160 kDa complex and the 210 kDa adduct. In the absence of Ca^{2+}, the amounts of all products decreased to the same extent. The preliminary results of this work were presented earlier (Andreev, 1995).

MATERIALS AND METHODS

Materials. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), Tricine, and wide-range molecular weight markers (catalog no. M 4038) were purchased from Sigma. (Iodoacetamido)tetramethylrhodamine (IATR) isomer 5 and 5-iodoacetamidofluorescein (5-IAF) were from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade.

Proteins. Myosin was prepared from rabbit skeletal muscle by the method of Tonomura *et al.* (1966). S1 was prepared by chymotryptic digestion of myosin, and the two isoenzymes were isolated by DEAE-cellulose chromatography (Weeds & Taylor, 1975). Actin was prepared according to Spudich and Watt (1971). Troponin and tropomyosin were purchased from Sigma. The alkali light chains were prepared according to Holt and Lowey (1975). Actin was labeled with 5-IAF, and S1 was labeled with IATR as described by Andreev and Borejdo (1992). Alkali light chains were labeled by incubation with a 5 M excess of dye (IATR) for 4 h in 50 mM KCl, 2 mM EDTA, and 10 mM phosphate buffer at 5 °C. Free dye was removed by dialysis and gel filtration. Labeled light chains were exchanged with light chains of S1 as described by Wagner and Weeds (1976). Concentrations of proteins were measured by absorbance using S1 [$A^{1\%}(280) = 7.5$], G-actin [$A^{1\%}(290) = 6.3$], F − actin [$A^{1\%}(290) = 6.7$], and light chains A1 or A2 [$A^{1\%}(280) = 2.2$]. The concentration of bound IATR was determined by measuring the absorbance at 555 nm using the extinction coefficient $A(555) = 75\,000\ M^{-1}\ cm^{-1}$. The concentration of bound 5-IAF was determined by measuring the absorbance at 493 nm using the extinction coefficient $A(493) = 62\,000\ M^{-1}\ cm^{-1}$. The absorbances of labeled proteins were corrected for absorbances of bound dyes at 280 nm (at 290 nm for actin).

Cross-Linking Reaction. S1 and F-actin were mixed at different molar ratios and incubated for 1 h at room temperature; appropriate amounts of EDC were then added. Reactions were stopped by adding an equal volume of electrophoresis sample solution (4% SDS, 24% glycerol, 100 mM Tris, 4% mercaptoethanol, and 0.02% Bromphenol Blue). Unless otherwise indicated, all cross-linking experiments were done in solutions containing 0.2 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 7.5. The low concentrations of $MgCl_2$ and KCl were used to prevent actin filament bundle formation (Ando, 1984; Andreev & Borejdo, 1992). Light-scattering measurements did not detect any bundle formation in this buffer solution.

Tricine−Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis and Analysis. Gel electrophoresis was carried out according to Schagger and Jagow (1987) using 8% polyacrylamide gels. After electrophoresis and staining, the slab was dried using a Novex Gel Dryer Kit (Novex Co., San Diego, CA). The relative intensities of various bands were measured as follows: the dried slab gel was placed on a flat-bed scanner (ScanJet; Hewlett Packard, Palo Alto, CA), and the graphics image was obtained by a computer operating the Gray F/X program (Xerox Imaging, Peabody, MA). The intensity profile of the bands was measured by the Image Pro Plus program (Media Cybernetics, Silver Spring, MD) as described in detail in Andreev *et al.* (1995a,b). A calibration done using Edmund Scientific Stepped Density Filters showed that the intensities of the bands were inversely proportional to their transmittance. The 160 and 150 kDa peaks were assumed symmetrical, and the area under each was calculated as the double of the area of the half which had the smallest contribution from the neighboring peak. The background level was subtracted by eye.

RESULTS

Effect of Molar Ratio on Cross-Linking of the Heavy Chain of S1 to F-Actin. The isoforms of myosin subfragment-1, S1(A1) and S1(A2), were mixed with F-actin at different molar ratios and cross-linked with 25 mM EDC for 40 min. The molar ratio of S1 to actin was varied from 0.25 to 4.0. The concentration of either S1 or actin was fixed at 2 μM to keep the concentration of acto−S1 complexes approximately the same in each sample. Cross-linked products were analyzed by Tricine−SDS−polyacrylamide gel electrophoresis (Schagger & Jagow, 1987). It has been shown by Mornet *et al.* (1981) that cross-linking of the heavy chain of S1 with F-actin produced three major peptides. In 8% polyacrylamide in the presence of Tricine, these peptides migrated as a doublet with M_{app}s of 150 and 160 kDa and a complex of 210 kDa (Figure 1). Sutoh (1983) showed that the 150 and 160 kDa bands in the doublet corresponded to the products of cross-linking of one actin to the 20 and 50 kDa fragments of S1, respectively. We showed that the 210 kDa peptide corresponded to the complex of one S1 with two actins being cross-linked to 20 and 50 kDa fragments of S1 (Andreev & Borejdo, 1992; Andreeva *et al.*, 1993). It can be seen from Figure 1, lanes 1−7, that the intensities of the 160 and 210 kDa bands decreased dramatically with an increase in the molar ratio of S1(A1) to actin. The dependence of the relative intensities of the 160/150 kDa bands on the molar ratios is shown in Figure 3A. The fact that the ratio becomes 1 at low MR shows that S1 is equally likely to be cross-linked through the first and the second site. The fact that the ratio is not 0 at high saturation suggests that complete saturation is never achieved (see Discussion).

The intensity of the 150 kDa band, similarly to the ratio of the intensities of the 160/150 kDa bands, decreases when the molar ratio of S1/actin increases. However, this decrease is not as dramatic as that of 160 kDa. At high molar ratio, only two of the five lysines of the primary site on the S1 are involved in the cross-linking with actin (Yamamoto, 1990), so the cross-linking reaction may be less efficient (see Discussion).

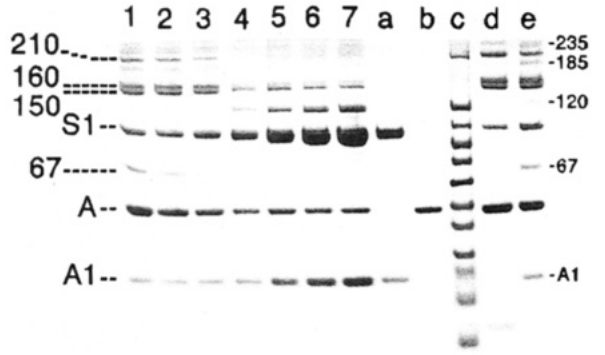

FIGURE 1: F-Actin and S1 were cross-linked with 25 mM EDC for 40 min in 50 mM KCl, 0.5 mM $MgCl_2$, 10 mM Tris-HCl buffer, pH 7.5, 23 °C. The cross-linked products were analyzed in an 8% polyacrylamide−SDS−Tricine gel. The gel was stained with Coomassie blue. Lanes 1−7 and 12 represent the cross-linked products of isoform S1(A1) with actin. The concentration of S1-(A1) was 2 μM in lanes 1−4 and 4, 6, and 8 μM in lanes 5, 6, and 7, respectively. The concentration of actin was 2 μM in lanes 4−7 and 8, 6, and 4 μM in lanes 1, 2, and 3, respectively. The molar ratio of S1(A1) to actin was varied from 0.25 to 4.0 in lanes 1−7, respectively. The other lanes: 8, 4 μM S1(A1); 9, 4 μM actin; 10, molecular mass markers (205, 116, 97, 84, 66, 55, 45, 36, 29, 24, 20, 14.2, and 6.5 kDa); 11, cross-linked products of 2 μM S1-(A2) and 8 μM F-actin; 12, same as lane 1.

Effect of Molar Ratio on Cross-Linking of A1 to F-Actin. Besides these three major products, the cross-linking of S1-(A1) to F-actin produced four additional peptides with apparent molecular masses of 64, 120, 185, and 235 kDa (Figure 1, lane e). These products were not formed upon cross-linking of S1(A2) to F-actin (Figure 1, lane d). To identify these products, we have carried out cross-linking of S1(A1*)−actin, S1*(A1)−actin, and S1(A1)−actin* where an asterisk indicates fluorescence. The label was rhodamine at Cys-177 of A1, rhodamine at Cys-707 of HC of S1, and fluorescein at Cys-374 of actin. Figure 2A shows the cross-linking pattern when A1 was fluorescently labeled and reveals that 235, 185, and 64 kDa peptides were fluorescent and therefore that all contained A1. Figure 2B shows the cross-linking pattern when S1 was fluorescently labeled and reveals that the 64 kDa band was not fluorescent and that therefore it did not contain S1. Figure 2C shows the cross-linking pattern when actin was fluorescently labeled and reveals that the 120 kDa band was not fluorescent and therefore that it did not contain actin. It follows from Figure 2, in agreement with earlier results of Yamamoto and Sekine (1983), that the 64 and 120 kDa peptides correspond to covalent complexes of A1 with actin and S1, respectively, and that the 185 and 235 kDa complexes were products of cross-linking of A1 with actin−S1 and actin−S1−actin, respectively. It is not clear whether the A1 cross-linked to actin or to the S1 in these complexes (see Discussion).

It can be seen from Figure 1, lanes 1−7, that in addition to the 160 and 210 kDa bands, the intensities of the 64, 185, and 235 kDa bands also decreased dramatically with an increase in the molar ratio of S1(A1) to actin. The dependence of the intensities of the 64 kDa band on molar ratio is shown in Figure 3B. The intensity of the bannd at MR = 0.25 was normalized to 1. These results indicated that Al, just like the second site on the heavy chain, could only bind to actin at low degrees of saturation of filament.

Effect of Ca^{2+} on Cross-Linking of S1(A1) to Regulated F-Actin. The results of a typical experiment are shown in

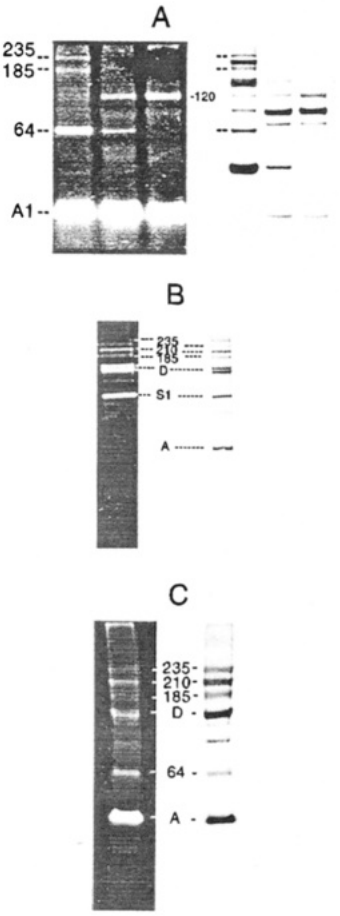

FIGURE 2: Products of EDC cross-linking of S1(A1) and F-actin. A1 was labeled with rhodamine at Cys-177, S1 was labeled with rhodamine at Cys-707 of HC, and actin was labeled with fluorescein at Cys-374. Gels on the left were photographed under UV lamp illumination and on the right after staining with Coomassie blue. (A) S1 containing fluorescent A1: 2 μM S1(A1) and 8 μM F-actin (first lane); 4 μM S1(A1) and 1 μM F-actin (second lane); and 4 μM S1(A1) and no actin (third lane). (B) 2 μM S1(A1) labeled at the heavy chain was cross-linked with 8 μM actin. (C) 8 μM fluorescently labeled actin was cross-linked with 2 μM S1(A1). Abbreviations: D, 150/160 kDa doublet; A, actin.

Figure 4. Lanes 1−3 are control experiments showing cross-linking of regulated actin without (lane 2) and with (lane 3) Ca^{2+}. Lanes 5−7 are control experiments showing cross-linking of S1(A1) without (lane 6) and with (lane 7) Ca^{2+}. Lanes 8−9 show cross-linking of S1(A2) with F-actin in the absence (lane 8) and in the presence (lane 9) of Ca^{2+}. Lanes 10−11 show cross-linking of S1(A1) with F-actin in the absence (lane 10) and in the presence (lane 11) of Ca^{2+}. The results show that while the removal of Ca^{+2} caused a small decrease in the production of each cross-linked peptide (probably because there was less bound S1 than in the presence of Ca^{+2}), troponin−tropomyosin did not specifically inhibit the interaction of heavy chain with one or two actins and of A1 with actin. Similarly, the cross-linking of heavy chain and alkali light-chain 1 of S1 to unregulated F-actin was not affected by Ca^{2+}, Mg^{2+}, or ADP (data not shown).

DISCUSSION

Sites Involved in Cross-Linking of the Heavy Chain of S1. Our results strongly support the hypothesis that 150 kDa and 160 kDa adducts are formed when S1 is cross-linked to actin through the first site and the second site, respectively.

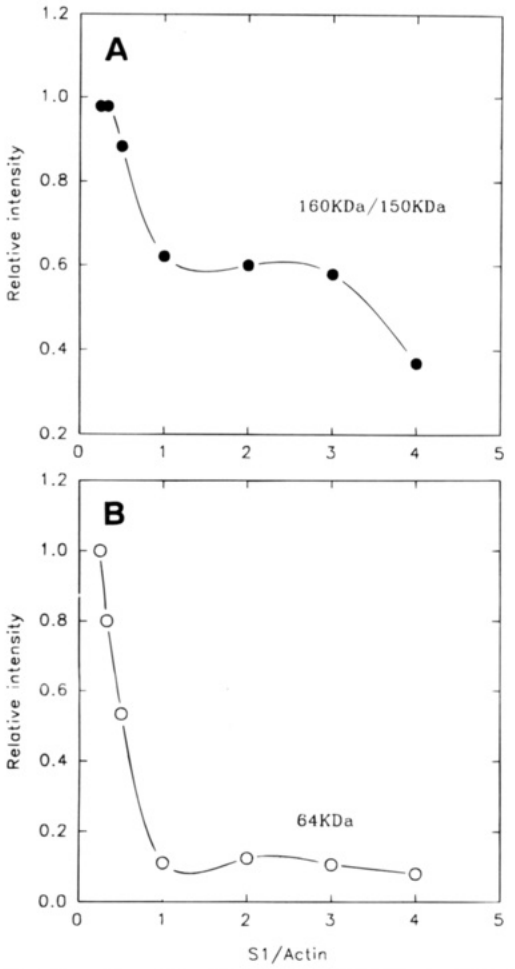

FIGURE 3: Intensities of the 64, 150, and 160 kDa bands in the gel shown in Figure 1 were measured and plotted against the molar ratio of S1 to actin. (A) Ratio of intensities of 160 to 150 kDa bands; (B) intensity of 64 kDa band normalized to the intensity of this band in lane 1 of Figure 1.

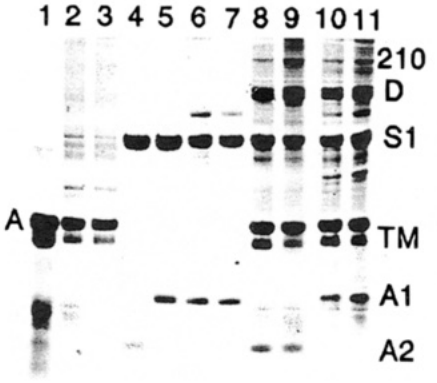

FIGURE 4: Cross-linking of S1(A1) and S1(A2) to regulated actin filament in the presence and absence of Ca^{2+}. Lane 1, 12 μM regulated F-actin; lane 2, 12 μM regulated F-actin + 30 mM EDC + 5 mM EGTA; lane 3, 12 μM regulated F-actin + 30 mM EDC + 0.2 mM Ca^{2+}; lane 4, 4 μM S1(A2); lane 5, 4 μM S1(A1); lane 6, 4 μM S1(A1) + 30 mM EDC + 5 mM EGTA; lane 7, 4 μM S1(A1) + 30 mM EDC + 0.2 mM Ca^{2+}; lane 8, 12 μM regulated F-actin + 4 μM S1(A2) + 30 mM EDC + 5 mM EGTA; lane 9, 12 μM regulated F-actin + 4 μM S1(A2) + 30 mM EDC + 0.2 mM Ca^{2+}; lane 10, 12 μM regulated F-actin + 4 μM S1(A1) + 30 mM EDC + 5 mM EGTA; lane 11, 12 μM regulated F-actin + 4 μM S1(A1) + 30 mM EDC + 0.2 mM Ca^{2+}. Abbreviation: D, 150−160 kDa doublet.

The cross-linking through both sites to two actins produced the 210 kDa complex. In fully saturated F-actin, each bound S1 interacted mostly through the first site with one actin, explaining why the formation of 160 and 210 kDa complexes was inhibited. Yamamoto (1990) showed that the first site on S1 included lysines 636, 637, 640, 641, and 642. Sutoh (1983) showed that the second site was located between Trp-510 and Trp-595 and may include the residues of flexible loop 567−578. These results were confirmed by 3D reconstruction of the myosin head which revealed that site 1 was located between residues Tyr-626 and Gln-647 (Ala-626 and Gln-647 in rabbit), and site 2 was located between Lys-567 and His-578 (Rayment *et al.*, 1993a).

On the actin side, the cross-linking site must be confined to the 1−28 N-terminal peptide (Sutoh, 1982; Bonafe & Chaussepied, 1995). Bonafe and Chaussepied (1995) proposed that in addition to the first and second site S1 has the third site located on the heavy chain near the C-terminus of the 50 kDa fragment. They proposed that 150 and 160 kDa complexes were formed as a result of cross-linking one actin to the first or third site on $S1_{HC}$. The cross-linking of the second actin to the second site of S1 transformed the doublet into the 210 kDa complex. According to their scheme, the first and third sites are separated by just a few residues, and both sites are equally available for EDC cross-linking regardless of the occupancy of F-actin. However, their proposal is in conflict with the fact that the sites on S1 involved in formation of the 150 and 160 kDa products are separated by at least a 5 kDa peptide (Sutoh, 1983; Mornet & Ue, 1985). We think that the origin of the 150, 160, and 210 kDa cross-linked peptides does not require the introduction of three different EDC cross-linking sites on $S1_{HC}$. Thus, our results do not support the 3-site model of Bonafe and Chaussepied (1995).

The ability of the S1 heavy chain to interact with the N-terminal residues of two actins in a filament suggests that the distance between the first and the second sites in S1 is about 5.0−5.5 nm. The residues of loops (626−647 and 571−574 were not observed in the S1 crystal structure (Rayment *et al.*, 1993a,b) because of a high degree of disorder of these regions, but rough estimation of the distance between these two loops gives a value of 5 nm.

While the ratio of the intensities of the 160/150 kDa bands decreases dramatically with the increase in the molar ratio, the intensity of the 150 kDa band alone also decreases. As mentioned before, this decrease is not as dramatic as that of 160 kDa and is most likely related to the fact that at high molar ratio only two of the five lysines of the primary site on the S1 are involved in cross-linking with actin (Yamamoto, 1990) so the cross-linking reaction may be less efficient. We think that at high molar ratio when the contacts through the sites on the 50 kDa and on the A1 light chain are almost abolished, the contact through the site on the 20 kDa band also becomes weaker. It is likely that the binding of S1 to the second actin strengthens the binding to the first one.

Cross-Linking of Al to F-Actin. Cross-linking of S1(A1) with F-actin gave products with M_{app} values of 64, 120, 150, 160, 185, 210, and 235 kDa. Of these, only 64, 120, 185, and 235 kDa involved A1 (Figure 2). On the basis of earlier work which has shown that A1, but not A2, could be cross-linked with EDC to the C-terminal part of actin (Sutoh, 1982; Yamamoto & Sekine, 1983) and on the basis of M_{app} and

fluorescent labeling of A1, actin, and $S1_{HC}$ (Figure 2), we think that 64 kDa corresponds to actin−A1 and 120 kDa to $S1_{HC}$−A1. Most likely only 13 N-terminal residues of A1 are involved in the interaction with actin (Hayashbara & Miaynishi, 1994). Figure 1 shows that the formation of 185 kDa adducts strongly depends on the degree of saturation of F-actin by S1. Since the formation of the cross-link between the second site and actin also strongly depends on MR, it is most likely that the 185 kDa product corresponds to cross-linking of the second site of S1 to A1 and to actin, i.e., to the A1−actin−$S1_{HC}$ complex. Cross-linking of an additional actin to the first site of S1 of this complex would transform the A1−actin−$S1_{HC}$ complex into the A1−actin−$S1_{HC}$−actin complex with an M_{app} of 235 kDa. However, we cannot exclude the possibility that A1 is cross-linked to the heavy chain in these complexes. Work is in progress to decide whether A1 can be cross-linked to one of the two actins which are in the contact with the heavy chain of S1, or to the third actin.

The fact that the 64 kDa adduct could not be formed in saturated filaments (Figure 1) suggests that A1 can only reach actin when S1 binds to two actin protomers (state 2). Since in muscle fibers most cross-bridges are in state 2 (Xiao *et al.*, 1995), it is likely that interaction of A1 with thin filaments is physiologically important (Lowey *et al.*, 1994). The present data clearly show that under conditions of full saturation there is no interaction between A1 and actin (Figure 1; note that 64 kDa is decreasing even though S1 concentration is increasing). This is in disagreement with the model of Milligan *et al.* (1990) which suggested that A1 can interact with actin in a thin filament fully saturated by S1(A1). Perhaps in saturated filaments A1 is close to the actin surface, but it cannot be cross-linked to actin by a zero-length reagent.

Conclusions. The inhibition of cross-linking of the heavy chain through the second site at high molar ratio of S1 to actin, and the fact that A1 can only cross-link in unsaturated filaments, indicates that in fully saturated filament the contact of S1 with the second actin is very weak or even absent. But the absence of contacts is nonphysiological because in fibers actin is in excess (and in the presence of ATP in a large excess!). We have provided evidence (Andreev *et al.*, 1995a,b; the manuscript with complete analysis of kinetic data is in preparation) that S1 first binds to one actin (state 1) and then two actins (state 2), and that the transition between states 1 → 2 is fast enough to be compatible with the time course of the cross-bridge power stroke in muscle. This suggests that the 1 → 2 transition might be required for force generation by acto−myosin.

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