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Mapping of Actin-Binding Sites on the Heavy Chain of Myosin Subfragment 1†

Kazuo Sutoh

ABSTRACT: When the rigor complex of actin and myosin subfragment 1 (S1) was treated with a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, covalently linked complexes of actin and S1 heavy chain with apparent molecular weights of 165 000 and 175 000 were generated. Measurements of the molar ratio of actin to S1 heavy chain in the 165K and 175K products showed that they were 1:1 complexes of actin and S1 heavy chain. Chemical cleavages of the cross-linked products followed by peptide mappings revealed that two distinct segments of S1 heavy chain spanning the 18K–20K region and the 27K–35K region from its C terminus participated in cross-linking with actin. Cross-linking of actin to the former site generated the 165K peptide while the latter site was responsible for generating the 175K peptide.

A detailed knowledge of the structure of the actin–myosin complex during a cyclic ATP hydrolysis by the complex is required to understand the molecular mechanism of muscle contraction. As a model system of the actin–myosin interaction, the structure of a stable actin–myosin subfragment 1 (S1)[1] complex formed in the absence of MgATP (the "rigor" complex) has been extensively investigated. One promising approach to reveal the structure of the actin–S1 rigor complex is to reconstitute its three-dimensional image from its electron micrographs (Moore et al., 1970; Toyoshima & Wakabayashi, 1979; Wakabayashi & Toyoshima, 1981; Taylor & Amos, 1981). Another complementary approach is to identify peptide segments participating in the actin–S1 contact. The latter approach, by employing the chemical cross-linking of the actin–S1 rigor complex with a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), has provided fruitful results (Mornet et al., 1981a,b; Sutoh, 1982a,b).

It was shown that EDC treatment of the rigor complex of actin and trypsin-treated S1 generated covalent cross-links between actin and the 20K and 50K tryptic fragments of S1 heavy chain, indicating that these two tryptic fragments of S1 heavy chain contain actin-binding sites (Mornet et al., 1981a). Detailed mapping of the actin-binding site was carried out on the 20K fragment of the heavy chain by employing the peptide mapping technique (Sutoh, 1982b). The peptide mapping showed that the N-terminal cyanogen bromide peptide of the 20K fragment spanning residues 1–20 participated in binding with actin. Similar cross-linking and peptide mapping experiments revealed that the N-terminal acidic segment of actin spanning residues 1–12 participated in binding with both of the 20K and 50K fragments of S1 heavy chain and, moreover, that a cluster of acidic residues close to the C terminus of actin was a binding site of alkaline light chain 1 of S1 (Sutoh, 1982a).

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[1] Abbreviations: S1, myosin subfragment 1; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-methyl-3-bromoindolenine.

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In the present study, similar experiments were carried out on the rigor complex of actin and intact S1 to locate actin-binding sites along S1 heavy chain. Two distinct heavy chains segments were shown to participate in the actin binding: one segment spanning the 18K–20K region from the C terminus of the heavy chain and the other spanning the 27K–35K region.

Materials and Methods

Preparation of Proteins. Myosin subfragment 1 (S1) was prepared by the method of Weeds & Taylor (1975) from rabbit skeletal muscle myosin. Actin was prepared by the method of Spudich & Watt (1971) from acetone powder of rabbit skeletal muscle.

DACM Modification of Actin and S1. Cys-373 of actin was selectively labeled with a fluorescent dye, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM), as previously described (Sutoh, 1982a) with slight modifications. Actin (3 mg/mL) in 1 mM imidazole, 0.1 mM $CaCl_2$, and 0.1 mM ATP (pH 7.0) was reacted with 1.0 molar equiv of DACM for 2 min at 0 °C. The reaction was quenched by addition of 0.01 volume of 0.1 M *N*-acetylcysteine (pH 7.0). Under these conditions, about 80% of the thiols at position 373 were labeled with the fluorescent dye.

S1 (3 mg/mL) in 30 mM KCl, 20 mM Tris-HCl, and 1 mM MgADP (pH 8.0) was reacted with 1.2 molar equiv of DACM for 5 min at 0 °C (Sutoh, 1981, 1982b). The reaction was again quenched by addition of *N*-acetylcysteine. Under these conditions, one of the reactive thiols of S1, SH_1, is completely blocked by DACM with a contaminating amount of incorporation of the fluorescent dye into the other reactive thiol, SH_2 (Sutoh, 1982b).

The ability of the DACM-labeled proteins to form the rigor complex was checked by the use of an analytical ultracentrifuge (Beckman Model E).

Cross-Linking of the Actin–S1 Complex. Varying amounts of the cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) (0.2–5 mM) were added to mixtures of F-actin (0.5 mg/mL) and S1 (0.5 mg/mL) in 0.1 M NaCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0). The cross-linking reaction was allowed to proceed for 2 h at 25 °C and then quenched by addition of excess 2-mercaptoethanol. In some cases, reaction time was varied.

Fluorometric and Densitometric Measurements. Cross-linked products of the actin–S1 complex, in which one of the components was labeled with DACM, were electrophoresed in the presence of $NaDodSO_4$ (Laemmli, 1970) at different protein loadings (10–40 μg). After electrophoresis, fluorescent intensities of fluorescent bands on acrylamide gels were recorded by the use of a fluorescence gel scanner as described previously (Sutoh, 1980; Sutoh & Matsuzaki, 1980). Relative fluorescent intensities were determined by measuring areas under peaks. Since separation of the 165K and 175K peaks was rather poor, areas under these peaks were counted together.

The same cross-linked products as described above were loaded on acrylamide gels at lower loading concentrations (1–4 μg). After electrophoresis, gels were stained with Coomassie Blue and then scanned by a conventional densitometer at 600 nm. Areas under peaks were measured. Peak areas of the 165K and 175K bands were counted together.

Estimation of the Relative Uptake of Coomassie Blue by Actin and S1 Heavy Chain. A mixture of actin (0.8 mg/mL) and S1 (0.8 mg/mL) was electrophoresed in the presence of $NaDodSO_4$ (Laemmli, 1970) at different loading concentrations, and resulting acrylamide gels were stained with Coomassie Blue. The stained gels were scanned at 600 nm, and peak areas were recorded. Peak areas of actin and those of S1 heavy chain were plotted against loading concentrations, and the adherence to Beer's law was checked. Regression lines of loading concentrations vs. peak areas for the heavy chain and actin gave a ratio of Coomassie stain present in the actin band to that in the heavy chain band. Molecular weights of 120 000 for S1 (Weeds & Taylor, 1975) and 95 000 for the heavy chain (Mornet et al., 1979; Yamamoto & Sekine, 1979) were used for the calculation of the relative uptake of the dye by actin and S1 heavy chain.

Isolation and Purification of Fluorescent Peptides. The rigor complex of actin and DACM-labeled S1 was cross-linked with EDC (1 mM) for 2 h at 25 °C as described above. Cross-linked products were electrophoresed on slab gels containing 10% acrylamide and 0.15% bis(acrylamide) in the presence of $NaDodSO_4$. Fluorescent 95K, 165K, and 175K bands were cut out under illumination with a UV lamp. Care was taken to avoid cross-contamination of the 165K and 175K peptides. The peptides were eluted out of acrylamide gels electrophoretically into small dialysis tubes attached to bottoms of short acrylamide gels. The eluted peptides were again electrophoresed, and fluorescent bands were cut out. They were washed with 20% methanol and then with 50% methanol. They were dried in vacuo.

The rigor complex of DACM-labeled actin and trypsin-treated S1 was cross-linked with EDC as previously described (Sutoh, 1982a). The cross-linked complex of the DACM-labeled actin and the 50K tryptic fragment of S1 heavy chain was isolated and purified as described above.

Chemical Cleavages of Peptides and Electrophoresis of Cleavage Products. Dried acrylamide gels trapping fluorescent peptides were soaked in 70% formic acid to cleave the acid-labile bond. The cleavage reaction was allowed to proceed for 16 h at 37 °C. The acid-treated gels were thoroughly washed with 50% methanol and then dried in vacuo.

These dried gels were incubated in 1% $NaDodSO_4$, 10 mM Tris-HCl, 1% 2-mercaptoethanol, and 10% glycerol (pH 8.0) for 6 h at 37 °C and then directly layered on an acrylamide slab gel [15% acrylamide–0.45% bis(acrylamide)] with a stacking gel system (Laemmli, 1970). After electrophoresis in the presence of $NaDodSO_4$, fluorescent bands were detected under illumination with a UV lamp.

Dried gels were also soaked in freshly prepared 6 M guanidine hydrochloride–1 M hydroxylamine (pH 9.0) for 4 h at 45 °C (Bornstein & Balian, 1977; Sutoh, 1982a,b) or in 60% acetic acid containing BNPS-skatole (1 mg/mL) for 2 h at 37 °C. Resulting cleavage products were electrophoresed as above.

For double cleavages of the heavy chain with BNPS-skatole and hydroxylamine, acrylamide gels trapping the fluorescent heavy chain were treated with BNPS-skatole at first. After electrophoresis of the cleavage product on a slab gel containing 15% acrylamide–0.45% bis(acrylamide) in the presence of $NaDodSO_4$, the gel was washed and dried. The dried gel was then treated with 6 M guanidine hydrochloride–1 M hydroxylamine, washed, and dried again. The gel was finally soaked in 1% $NaDodSO_4$, 10 mM Tris-HCl, 1% 2-mercaptoethanol, and 10% glycerol (pH 8.0). Fluorescent bands detected on the gel (16K, 18K, 26K + 27K, and 35K bands) were cut out and layered on a slab gel containing 15% acrylamide and 0.45% bis(acrylamide).

Densitometric Analysis of Negative Films. For quantitation of the fluorescent intensities of fluorescent peptides generated from the 95K, 165K, and 175K peptides by various chemical

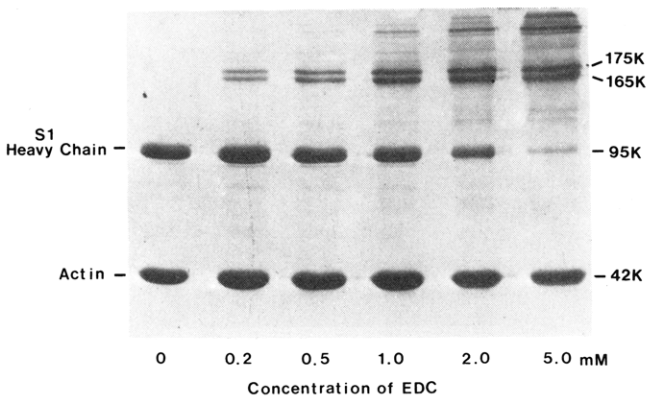

FIGURE 1: Cross-linking of the rigor complex of actin and S1 with EDC. Cross-linking conditions: protein concentrations, 0.5 mg/mL F-actin and 0.5 mg/mL S1; solvent, 0.1 M NaCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0); cross-linker concentrations, 0.2–5 mM; reaction time, 2 h; temperature, 25 °C. Electrophoresis was carried out on a $NaDodSO_4$ gel containing 10% acrylamide and 0.15% bis-(acrylamide). The gel was stained with Coomassie Blue.

treatments, negative films of $NaDodSO_4$–acrylamide gels taken under illumination with a UV lamp were scanned by a densitometer.

Protein Concentrations. Protein concentrations were determined by measuring the absorbance at 280 nm with an extinction coefficient of $E^{1\%}_{280nm} = 7.5$ cm^{-1} for S1 (Wagner & Weeds, 1977) and 11.0 cm^{-1} for actin (West et al., 1967).

Estimation of the Apparent Molecular Weights of Peptides by $NaDodSO_4$ Gel Electrophoresis. Apparent molecular weights of peptides were estimated by $NaDodSO_4$ gel electrophoresis (Weber & Osborn, 1969). Myosin heavy chain (M_r 200 000), heavy chain of S1 (M_r 95 000), bovine serum albumin (M_r 68 000), actin (M_r 42 000), troponin T (M_r 30 500), troponin I (M_r 21 000), troponin C (M_r 18 000), myoglobin (M_r 17 200), and cytochrome *c* (M_r 11 700) were protein standards.

Results

Cross-Linking of Actin and S1. A rigor complex of F-actin and S1 in 0.1 M NaCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0) was cross-linked with varying amounts of EDC (0.2–5 mM) for 2 h at 25 °C. As shown in Figure 1, the cross-linking reaction generated peptides with apparent molecular weights of 165 000 and 175 000 as major cross-linked products. These 165K and 175K peptides have been previously shown to be covalently linked complexes of actin and S1 heavy chain by the use of the labeled actin or S1 (Mornet et al., 1981b; Sutoh, 1982a). On the acrylamide gel shown in Figure 1, no cross-linked product of actin and S1 heavy chain was detected below the 165K and 175K doublet bands. Therefore, the 165K and 175K peptides seem to be cross-linked products of actin and S1 heavy chain with minimum molecular weights under the cross-linking conditions used here. Cross-linked peptides with higher molecular weights appeared when the actin–S1 complex was treated with a high concentration of the cross-linker. The same cross-linking pattern as shown in Figure 1 was obtained when cross-linking time was varied instead of varying cross-linker concentration. Similar results were previously reported by Mornet et al. (1981b).

Determination of the Actin to S1 Heavy Chain Ratio in the 165K and 175K Peptides. Cys-373 of actin was selectively labeled with the fluorescent dye DACM (Sutoh, 1982a), and then the DACM-labeled actin was cross-linked to S1 with EDC. When analyzed by $NaDodSO_4$ gel electrophoresis, the 165K and 175K doublet bands as well as the 42K actin band were fluorescent as shown in Figure 2. For determination of the weight ratio of actin present in the doublet bands to that in the 42K actin band, the fluorescent intensities of these bands were quantitated by scanning acrylamide gels with a fluorescence gel scanner (Sutoh, 1980; Sutoh & Matsuzaki, 1980).

As shown in Figure 2, fluorescent intensities of the fluorescent bands were quantitated at four different loading con-

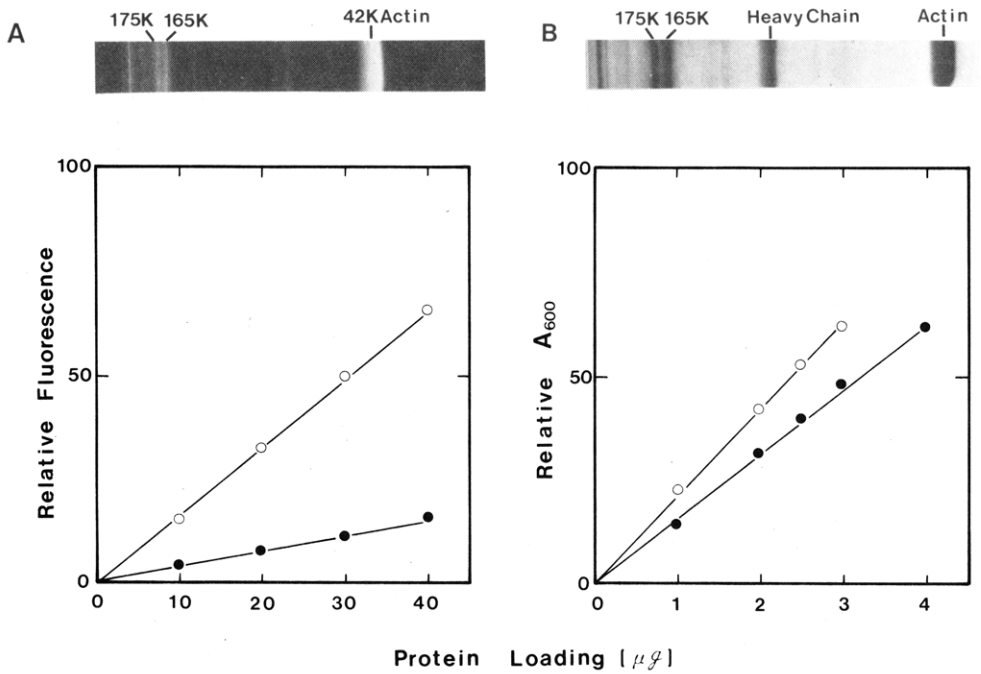

FIGURE 2: Fluorometric and densitometric measurements of intensities of the 42K actin band and those of the 165K + 175K bands. (A) Relative fluorescent intensities of the 42K actin band (○) and those of the 165K + 175K bands (●) at different loading concentrations. Fluorescent intensities were determined by the use of a fluorescence gel scanner. Fluorescent band patterns are shown in the upper part of the figure. (B) Relative intensities of Coomassie stain on the 42K actin band (○) and those on the 165K + 175K bands (●) at different loading concentrations. Acrylamide gels were stained with Coomassie Blue and then scanned at 600 nm by the use of a conventional gel scanner. Areas under peaks were measured (relative A_{600}). Band patterns after the Coomassie staining are shown in the upper part of the figure.

centrations (10–40 μg). The 165K and 175K peaks were counted together, since these peaks could not be separated well. Data points of relative fluorescent intensity vs. protein loading are linear both for the 42K actin and for the 165K and 175K doublet bands. The regression lines in Figure 2 gave a ratio of A_m/A_x, where A_m is the weight of actin peptides present in the 42K actin band and A_x is that present in the 165K and 175K bands. A_m/A_x was estimated to be 4.44.

Acrylamide gel electrophoresis was carried out in a similar way at lower loading concentrations (1–4 μg), and the resulting gels were stained with Coomassie Blue. Densitometer traces of these gels were recorded, and peak areas of the 42K actin band and those of the 165K and 175K doublet bands were measured (the 165K and 175K peaks were counted together as above). Data points of peak area (relative A_{600}) vs. loading concentration are linear as shown in Figure 2, indicating the adherence to Beer's law over the range of loading concentrations used here. The regression lines gave the ratio $A_m/(A_x + 1.14S_x)$, where S_x is the weight of S1 heavy chain present in the 165K and 175K doublet bands and 1.14 is a factor to correct for the relative uptake of Coomassie stain by acin and S1 heavy chain (Materials and Methods). The ratio thus determined was 1.33.

The above values gave a ratio of 2.05 for S_x/A_x. Taking molecular weights of 42 000 for actin (Elzinga et al., 1973) and 95 000 for S1 heavy chain (Mornet et al., 1979; Yamamoto & Sekine, 1979), we estimated the molar ratio of actin to S1 heavy chain in the 165K and 175K doublet bands to be 1.10. Another independent cross-linking experiment gave a value of 0.90.

Similar fluorometric and densitometric measurements were carried out by using the DACM-labeled S1 (Materials and Methods) in place of the DACM-labeled actin. Three independent cross-linking experiments of the rigor complex of actin and DACM-labeled S1 gave values of 0.94, 0.88, and 0.97 for the molar ratio of actin to S1 heavy chain in the 165K and 175K cross-linked products. Thus, it is concluded that one actin and one S1 heavy chain are cross-linked to form covalently linked peptides with apparent molecular weights of 165K and 175K.

Mapping of Cross-Linking Sites of Actin along S1 Heavy Chain by Formic Acid Cleavages. The DACM-labeled S1 in which the SH_1 thiol was selectively labeled with the fluorescent dye (Sutoh, 1981) was cross-linked to actin with EDC as above. The fluorescent 165K and 175K peptides as well as the fluorescent 95K heavy chain were separated and purified electrophoretically as described under Materials and Methods. Dried acrylamide gels trapping the 165K, 175K or 95K fluorescent peptide were soaked in 70% formic acid for 16 h at 37 °C.

The formic acid treatment of the fluorescent 95K heavy chain generated a fluorescent fragment with an apparent molecular weight of 26 000 as shown in Figure 3 (lane 1) as a major cleavage product. Bearing in mind that the DACM label is incorporated in the SH_1 thiol located in the C-terminal 20K segment of S1 heavy chain as shown in Figure 7 (Gallagher & Elzinga, 1980; Sutoh, 1981) and that the 20K segment does not contain the Asp–Pro bond which is susceptible to formic acid cleavage (Landon, 1977), it is possible to identify the fluorescent 26K fragment as the C-terminal segment of the 95K heavy chain. The acid cleavage site on the heavy chain is denoted as "F" in Figure 7.

Formic acid treatment of the fluorescent 175K peptide again released the fluorescent 26K fragment as a major cleavage product as shown in Figure 3 (lane 2), indicating that the

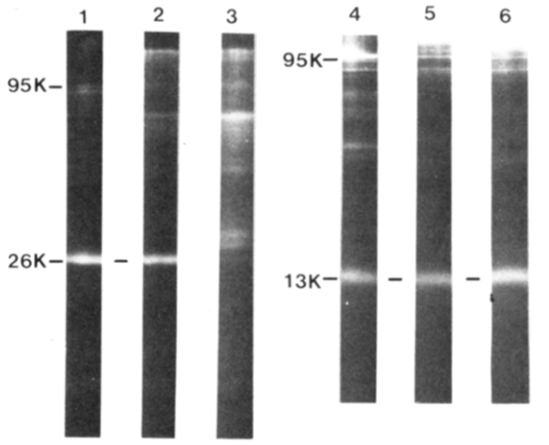

FIGURE 3: Formic acid or hydroxylamine cleavages of the 95K, 165K, and 175K peptides. These peptides were treated with formic acid (1–3) or with hydroxylamine (4–6). (1 and 4) Cleavage products of the control 95K heavy chain. (2 and 5) Cleavage products of the 175K peptide. (3 and 6) Cleavage products of the 165K peptide. Conditions for formic acid cleavages: solvent, 70% formic acid; reaction time, 16 h; temperature, 37 °C. Conditions for hydroxylamine cleavages: solvent, 6 M guanidine hydrochloride–1 M hydroxylamine (pH 9.0); reaction time, 4 h; temperature, 45° C. Electrophoresis was carried out on $NaDodSO_4$ slab gels containing 15% acrylamide–0.45% bis(acrylamide). Fluorescent peptides were detected by illuminating acrylamide gels with a UV lamp.

C-terminal 26K segment of the heavy chain did not significantly participate in the cross-linking reaction generating the 175K product. The cross-linking site of actin on the heavy chain in the 175K product is, therefore, located outside its C-terminal 26K segment as shown in Figure 7. The fluorescent 26K fragment was, however, never released on formic acid treatment of the fluorescent 165K peptide as shown in Figure 3 (lane 3). Thus, the cross-linking site of actin on the heavy chain in the 165K product is located within its C-terminal 26K segment as shown in Figure 7.

Mapping of Cross-Linking Sites of Actin along S1 Heavy Chain by Hydroxylamine Cleavages. It was previously shown that hydroxylamine treatment of the tryptic 20K fragment of the DACM-labeled heavy chain resulted in cleavage of the Asn–Gly bond (Bornstein & Balian, 1977) present in the fragment (Gallagher & Elzinga, 1980) to release the fluorescent 13K peptide spanning its C-terminal 111 residues (Sutoh, 1981). When the intact DACM-labeled heavy chain was incubated in 6 M guanidine hydrochloride–1 M hydroxylamine (pH 9.0) for 4 h at 45 °C, the fluorescent 13K fragment was released as shown in Figure 3 (lane 4), consistent with a previous report (Sutoh, 1981). The hydroxylamine cleavage site on the heavy chain (Gallagher & Elzinga, 1980; Sutoh, 1981) is denoted as "H" in Figure 7.

When the fluorescent 165K and 175K peptides were subjected to hydroxylamine cleavage, the fluorescent 13K peptide was again released as shown in Figure 3 (lanes 5 and 6). Relative fluorescent intensities of the 13K fragment in lanes 4, 5, and 6 were similar to each other, indicating that the C-terminal 13K segment of the heavy chain did not significantly participate in cross-linking with actin. Thus, cross-linking sites of actin on the heavy chain generating the 165K and 175K peptides are located outside its C-terminal 13K segment as shown in Figure 7.

Mapping of Cross-Linking Sites of Actin along S1 Heavy Chain by BNPS-skatole Cleavages. BNPS-skatole modifies tryptophan residues and cleaves tryptophanyl peptide bonds on prolonged incubation (Fontana, 1972). When the fluorescent 95K heavy chain was treated with BNPS-skatole (1 mg/mL) in 60% acetic acid for 2 h at 37 °C, several fluor-

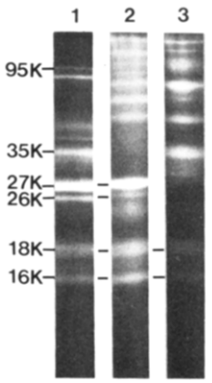

FIGURE 4: BNPS-skatole cleavages of the 95K, 165K, and 175K peptides. (1) Cleavage product of the control 95K heavy chain. (2) Cleavage product of the 175K peptide. (3) Cleavage product of the 165K peptide. Cleavage conditions: solvent, 60% acetic acid; concentration of BNPS-skatole, 1 mg/mL; reaction time, 2 h; temperature, 37 °C. Fluorescent fragments were electrophoresed and detected as in Figure 3.

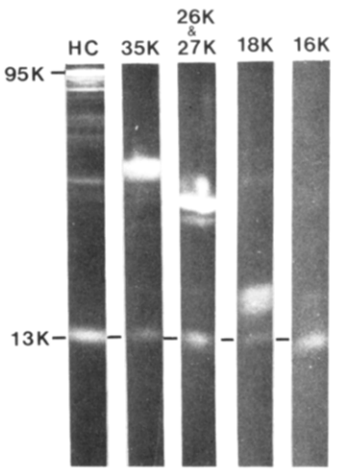

FIGURE 5: Double cleavages of the 95K heavy chain with BNPS-skatole and hydroxylamine. After the 95K heavy chain was cleaved with BNPS-skatole, its 16K, 18K, 26K + 27K, and 35K fluorescent fragments as well as the control 95K heavy chain were further digested with hydroxylamine. Conditions for the BNPS-skatole and hydroxylamine cleavages were the same as those in Figures 3 and 4. Fluorescent peptides were detected after $NaDodSO_4$ gel electrophoresis as in Figure 3.

escent fragments were generated as shown in Figure 4 (lane 1). Apparent molecular weights of major cleavage products were 16000, 18000, 26000, 27000, and 35000. By following the time course of the cleavage reaction, it was shown that all of these fluorescent fragments appeared even at an early stage of the reaction. If one considers the fact that the fluorescent label is attached to the SH_1 thiol which is located at the 103rd residue from the C terminus of the heavy chain (Gallagher & Elzinga, 1980), the result implies that these fluorescent fragments contain the intact C-terminal region of the parent heavy chain. The notion was supported by the double cleavage experiment (Sutoh, 1982b). When the fluorescent BNPS-skatole fragments (16K, 18K, 26K + 27K, and 35K fragments) were further digested by hydroxylamine (Materials and Methods), all of them released the fluorescent 13K fragment as a major cleavage product as shown in Figure 5. Since the fluorescent 13K peptide released on hydroxylamine cleavage of the 95K heavy chain is its C-terminal segment spanning 111 residues (Gallagher & Elzinga, 1980; Sutoh, 1981), the result indicates that these BNPS-skatole fragments have the same C-terminal segment as the parent heavy chain, consistent with the above notion. Thus, molecular weights of the fragments can be directly related to positions of cleavage sites on the heavy chain as shown in Figure 7, where BNPS-skatole cleavage sites are denoted as "B".

Although BNPS-skatole cleaves tryptophanyl bonds very selectively under well-controlled conditions (Fontana, 1972), cleavage reactions of the heavy chain with the reagent seem to occur at other amino acid residues as well, since two BNPS-skatole cleavage sites are located within the C-terminal 20K segment of the heavy chain even though the 20K fragment does not contain a tryptophan residue (Gallagher & Elzinga, 1980). The nature of cleavage reactions at theses sites remains to be clarified.

BNPS-skatole treatment of the fluorescent 175K peptide generated the fluorescent 16K, 18K, 26K, and 27K fragments as shown in Figure 4 (lane 2). Densitometric quantitation revealed that relative fluorescent intensities of these fragments were similar to those of the fragments generated from the control heavy chain (lane 1). The 35K fluorescent fragment was, however, not released from the 175K peptide by BNPS-skatole treatment (lane 2). The results indicate that the C-terminal 35K segment of the heavy chain participates in cross-linking with actin while the C-terminal 16K, 18K, 26K, and 27K segments do not. Thus, a cross-linking site of actin on the heavy chain is located in a segment spanning the 27K–35K region from the C terminus.

When the fluorescent 165K peptide was subjected to BNPS-skatole treatment, the fluorescent 16K and 18K fragments were again generated (lane 3). Densitometric quantitation showed that relative fluorescent intensities of the 16K and 18K fragments were similar to those of the fragments generated from the control heavy chain (lane 1) (the 16K and 18K bands in lane 3 contain 16% of the total fluorescence while the bands in lane 1 contain 19%), indicating that the C-terminal 16K and 18K segments of the heavy chain do not significantly participate in cross-linking with actin. On the other hand, the fluorescent 26K and 27K fragments were missing in the cleavage product of the 165K peptide (lane 3), suggesting that the C-terminal 26K and 27K segments of the heavy chain participate in cross-linking with actin. Therefore, it seems likely that a heavy chain segment spanning the 18K–26K region from the C terminus contains a cross-linking site of actin.

A fluorescent peptide migrating near the 35K position was detected in the cleavage product of the 165K peptide (lane 3). Since the C-terminal 26K and 27K segments of the heavy chain participate in cross-linking with actin as shown above, it is likely that the fluorescent peptide is not the free C-terminal 35K peptide of the heavy chain but a cross-linked complex of a heavy chain fragment and an actin fragment.

Cross-Linking of Actin and Trypsin-Treated S1. When S1 was digested with trypsin, its 95K heavy chain was cleaved into three fragments (25K, 50K, and 20K fragments) (Mornet et al., 1979; Yamamoto & Sekine, 1979). The 25K, 50K, and 20K fragments are aligned in this order on the parent heavy chain from its N terminus (Mornet et al., 1979) as schematically illustrated in Figure 7. Cross-linking of the trypsin-treated S1 to the DACM-labeled actin with EDC generated fluorescent cross-linked products of actin–20K fragment (M_r 65K), actin–alkaline light chain 1 (M_r 68K), and actin–50K fragment (M_r 95K) as shown in Figure 6 (lane 1). The same results have been reported previously (Sutoh, 1982a).

The cross-linked complex of the DACM-labeled actin and the 50K fragment of the heavy chain was electrophoretically purified and then subjected to formic acid treatment. As controls, the DACM-labeled actin and the DACM-labeled heavy chain were treated with formic acid at the same time.

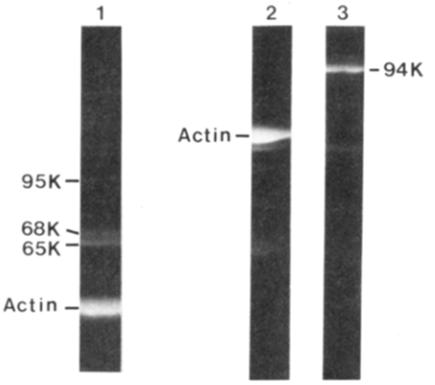

FIGURE 6: Cross-linking of DACM-labeled actin and trypsin-treated S1. (1) Fluorescent peptides generated by cross-linking the DACM-labeled actin and the trypsin-treated S1. Fluorescence of DACM covalently attached to actin was detected after the cross-linked product was electrophoresed on a $NaDodSO_4$ slab gel containing 10% acrylamide and 0.15% bis(acrylamide). Three fluorescent cross-linked peptides (M_r 65K, 68K, and 95K) were detected. (2) DACM-labeled actin treated with formic acid. Notice that the actin peptide is quite resistant to formic acid. (3) Cross-linked complex of DACM-labeled actin and the 50K fragment cleaved with formic acid. Fluorescent peptides in lanes 2 and 3 were detected after cleavage products were electrophoresed on a slab gel containing 10% acrylamide and 0.3% bis(acrylamide). Conditions for formic acid cleavages were the same as those in Figure 3.

As shown in Figure 6 (lane 3), formic acid treatment of the cross-linked complex of the 50K fragment and the DACM-labeled actin generated a fluorescent peptide (M_r 94K) migrating slightly ahead of the intact 50K fragment–actin complex. Under the same cleavage condition, actin was quite resistant to formic acid (lane 2 in Figure 6), consistent with the fact that actin does not contain an Asp–Pro bond susceptible to formic acid (Elzinga et al., 1973). On the other hand, the DACM-labeled heavy chain was readily cleaved to generate the fluorescent 26K fragment as shown above (lane 1 in Figure 3).

When the alignment of three tryptic fragments of the heavy chain is considered, the cleavage site generating the fluorescent 26K fragment from the heavy chain (the site denoted as "F" in Figure 7) is located in the tryptic 50K fragment. Thus, formic acid treatment is expected to cleave the 50K fragment into two peptides, the N-terminal 44K peptide and the C-terminal 6K peptide. The 94K fluorescent peptide generated by formic acid treatment of the 50K fragment–actin complex would be a cross-linked complex of the 44K fragment and actin, considering its apparent molecular weight.

The above cross-linking experiment of trypsin-treated S1 and actin followed by formic acid treatment indicates that two distinct regions of the heavy chain (the tryptic 20K fragment and the 44K segment of the tryptic 50K fragment) participate in cross-linking with actin, consistent with previous studies (Mornet et al., 1981a; Sutoh, 1982a,b).

Discussion

Chemical cross-linking of the rigor complex of actin and S1 with EDC generated two types of cross-linked peptides as major products. One type has an apparent molecular weight of 165K and the other 175K. The same result has been previously reported by Mornet et al. (1981b), who claimed that these cross-linked products were covalently linked complexes of two actins and one S1 heavy chain. However, from the following lines of evidence, it is concluded that the 165K and 175K peptides actually are cross-linked complexes of one actin and one S1 heavy chain. (1) Under the various cross-linking conditions examined here, no cross-linked complex of actin and S1 heavy chain smaller than the 165K and 175K peptides could be detected. (2) Direct measurements of the molar ratio of actin to the heavy chain in the 165K and 175K peptides gave a ratio of 1:1. (3) Mapping of cross-linking sites of actin

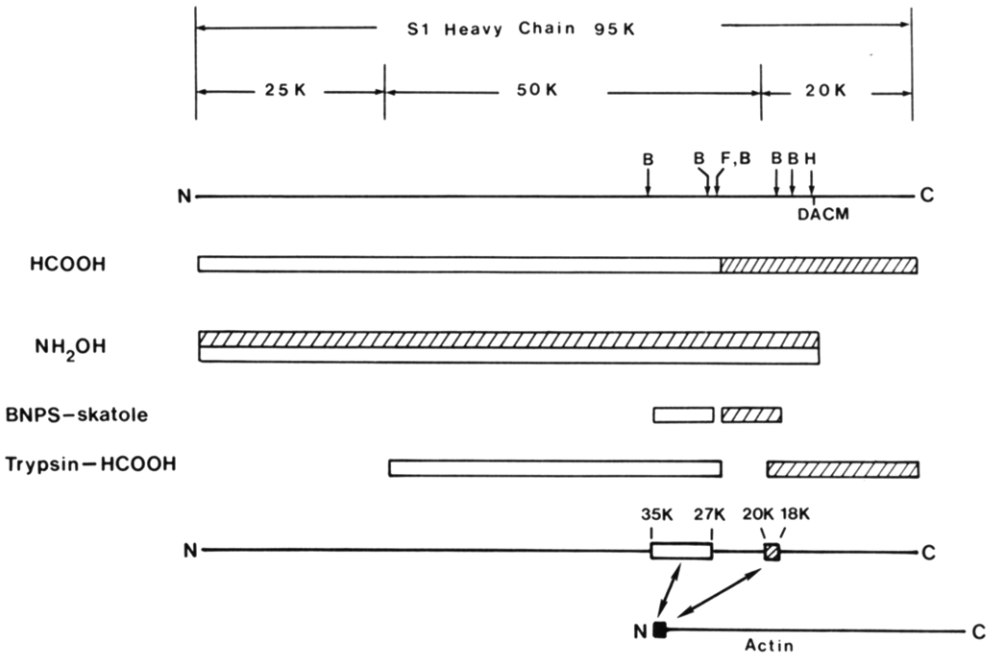

FIGURE 7: Mapping of cross-linking sites of actin on the heavy chain. In the upper part of the figure, the locations of heavy chain sites cleaved by various chemicals as well as those of three tryptic fragments of the heavy chain (20 K, 50K, and 25K fragments) are depicted. Cleavage sites are indicated by the following symbols: F, formic acid cleavage; H, hydroxylamine cleavage; B, BNPS-skatole cleavage. The position of the DACM label on the heavy chain is also indicated. Horizontal bars indicate peptide segments identified as those participating in cross-linking with actin. Cross-hatched areas are heavy chain segments participating in cross-linking to generate the 165K peptide while open areas are those for generating the 175K peptide. The shortest heavy chain segments participating in cross-linking with actin are deduced from these peptide mappings and shown in the lower part of the figure. These segments contain the actin-binding sites. Interactions between the two heavy chain segments (open and cross-hatched boxes) and the N-terminal acidic segment of actin (closed box) are indicated by arrows.

on the heavy chain revealed that two distinct heavy chain segments participated in cross-linking with actin. It is most likely, as discussed below, that one segment is located in the tryptic 20K fragment of the heavy chain and that the other is in its 50K fragment. Cross-linking of actin to the former site seems to generate the 165K peptide while the latter site is responsible for generating the 175K peptide.

Heavy chain segments participating in cross-linking with actin were identified by the peptide mapping technique (Sutoh, 1982a,b). When the cleavage reaction with formic acid was employed, a cross-linking site of actin generating the 165K peptide was mapped within the C-terminal 26K segment of the heavy chain. On the other hand, a cross-linking site generating the 175K peptide was mapped within the N-terminal segment of the heavy chain spanning the 26K–95K region from its C terminus. Hydroxylamine cleavages of the 165K and 175K peptides revealed that the C-terminal 13K segment of the heavy chain did not significantly participate in cross-linking with actin. Furthermore, when the cleavage reaction with BNPS-skatole were employed, a cross-linking site of actin generating the 165K peptide was mapped within a segment spanning the 18K–26K region from the C terminus of the 95K heavy chain, while the other cross-linking site of actin generating the 175K peptide was located within a heavy chain segment spanning the 27K–35K region from the C terminus. These results (summarized in Figure 7) indicate that two distinct heavy chain segments spanning the 18K–26K and 27K–35K regions contain cross-linking sites of actin. The former site is responsible for generating the 165K cross-linked product while the latter for generating the 175K product.

By the use of trypsin-treated S1 in place of intact S1, it was shown that the tryptic 20K fragment as well as the 44K segment of the tryptic 50K fragment contained cross-linking sites of actin. The cross-linking site of actin in the 20K fragment seems to be identical with that in the 18K–26K heavy chain segment, considering overlap of peptide segments. Therefore, it seems likely that cross-linking of actin to a heavy chain segment spanning the 18K–20K region from the C terminus generates the 165K peptide or the 20K fragment–actin complex (Figure 7). On the other hand, the cross-linking site of actin in the tryptic 50K fragment seems to be identical with that in the 27K–35K heavy chain segment. Cross-linking of actin to a heavy chain segment spanning the 27K–35K region from the C terminus would, therefore, generate the 175K peptide or the 50K fragment–actin complex (Figure 7).

By analyzing the cross-linked complex of actin and the 20K fragment, it has been previously shown (Sutoh, 1982b) that the N-terminal 20 residues of the tryptic 20K fragment participate in cross-linking with actin. It must be emphasized that the N-terminal 20 residues completely overlap with the 18K–20K heavy chain segment identified above as a segment participating in cross-linking with actin.

The cross-linker used here covalently cross-links amino groups to carboxyl groups only when they are in direct contact. It has been previously shown (Mornet et al., 1981b) that amino groups of S1 and carboxyl groups of actin are responsible for the covalent cross-linking of actin and S1 induced by EDC. In fact, the N-terminal segment of actin (residues 1–12) identified as the cross-linking site of both of the 20K and 50K fragments of the heavy chain (Sutoh, 1982a) contains a high proportion of acidic residues (Elzinga et al., 1973). The fact that the 18K–20K and 27K–35K heavy chain segments participate in cross-linking with this acidic segment of actin implies that some of the amino side chains in these segments are in direct contact with carboxyl groups in the N-terminal segment of actin. Therefore, it is likely that both of the 18K–20K and 27K–35K heavy chain segments bind to the N-terminal acidic segment of actin through ionic forces (Figure 7).

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