Identification of Myosin-Binding Sites on the Actin Sequence†

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ABSTRACT: The rigor complex of actin and trypsin-treated myosin subfragment 1 (S1) whose heavy chain was cleaved into three fragments (20K, 25K, and 50K) was cross-linked with a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. The cross-linking reaction generated three types of cross-linked products with apparent molecular weights of 65K, 68K, and 95K. The 65K, 68K, and 95K products were covalently linked complexes of actin–20K fragment of the S1 heavy chain, actin–alkaline light chain 1, and actin–50K fragment of the S1 heavy chain, respectively. Cross-linking sites of S1 heavy and light chains on the actin sequence have been determined by digesting the cross-linked products with cyanogen bromide or with hydroxylamine and then mapping resulting peptides on sodium dodecyl sulfate gels. The result indicates that some of the N-terminal acidic residues of actin at positions 1, 2, 3, 4, and 11 are cross-linking sites of the 20K and 50K fragments of the S1 heavy chain while some of its C-terminal acidic residues at positions 360, 362, and 363 are cross-linking sites of the alkaline light chain 1.

Cyclic interaction of actin and myosin coupled with the hydrolysis of ATP is believed to be a basic process of muscle contraction. Among various intermediate complexes of actin and myosin postulated in the cyclic interaction [for example, see Lymn & Taylor (1971)], the "rigor" complex, which is formed when no nucleotide is bound on the ATPase active site of myosin, has been most extensively studied to elucidate the structural basis of the actin–myosin interaction.

The current structural model for the interaction is mainly based on the three-dimensional image reconstitution of electron micrographs of actin filaments decorated with isolated myosin heads [myosin subfragment 1 (S1)][1] obtained by Moore et al. (1970). They have shown that one S1 particle with an elongated "banana-like" shape tangentially attaches to one actin subunit at the outside radius of the actin filament through its distal end. Recent reexamination of the three-dimensional image reconstitution of decorated actin filaments, however, has presented different models of the actin–myosin interaction (Toyoshima & Wakabayashi, 1979; Wakabayashi, 1980; Wakabayashi & Toyoshima, 1981; Taylor & Amos, 1981). In their models, the S1 particle has a rather fat and irregular shape and attaches to the actin subunit through its distal end (Wakabayashi & Toyoshima, 1981; Taylor & Amos, 1981) or its side (Wakabayashi & Toyoshima, 1981). Unfortunately, however, the exact mode of attachment of S1 to actin still remains to be clarified, since unambiguous assignment of actin and S1 in the reconstituted image has not yet been achieved because of the limit of resolution.

Another complementary approach to elucidate the structure of the actin–myosin complex is to identify primary sequences involved in the interaction, employing chemical analysis. Mornet et al. (1981a,b) used a chemical cross-linking technique to determine actin-binding domains on S1. They showed, using trypsin-treated S1 whose heavy chain was cleaved into three fragments (20K, 25K, and 50K) (Mornet et al., 1979; Yamamoto & Sekine, 1979), that a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), generated cross-links between actin and the 20K fragment of the S1 heavy chain and also between actin and the 50K fragment of the S1 heavy chain. Thus, these fragments seem to contain actin-binding sites in their sequences.

In the present study, a similar cross-linking approach has been employed to determine myosin-binding sites on the primary sequence of actin. Selective labeling of cysteine-373 with the fluorescent dye *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) as well as one-dimensional peptide mapping of fluorescent products of partial chemical cleavage provided a simple method for identifying the sites on the actin sequence.

Materials and Methods

General Strategy for Identifying Cross-Linking Sites on the Actin Sequence. The strategy for determining locations of cross-links bridging actin subunits and S1 fragments (or S1 light chains) on the actin sequence is schematically shown in Figure 1. For the first step, fluorescent label is attached to the C terminus of actin. In the present experiment, cysteine-373 of actin was selectively labeled with the fluorescent dye DACM. Since cysteine-373 is next to the C-terminal residue (phenylalanine-374) (Elzinga et al., 1973), the DACM labeling of cysteine-373 works as the C-terminal labeling. The second step is to cross-link the DACM-labeled actin with S1. Partial cleavage of the cross-linked product as well as the control non-cross-linked actin by cyanogen bromide is the third step. In Figure 1, it is assumed, for purposes of simplicity, that there are three cleavage sites on the actin polypeptide chain [cleavage sites are denoted as (▼) in the figure], though actin actually has 16 methionine residues (Elzinga et al., 1973). Other chemical or enzymatic methods for digesting polypeptide chains are available as well. Finally, resulting cleavage products are electrophoresed on acrylamide gels in the presence of $NaDodSO_4$. Successive partial cleavage of DACM-labeled actin without cross-link would produce four fluorescent bands as shown on gel i in Figure 1, since the fluorescent dye DACM is covalently attached to the C terminus of actin. On the other hand, successive partial cleavage of a cross-linked product containing DACM-labeled actin would produce fluorescent peptides with higher molecular weights than the corresponding peptides of the control actin until the peptide containing the cross-link is removed, after which the fluorescence pattern

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[1] Abbreviations: S1, myosin subfragment 1; AL1, alkaline light chain 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.

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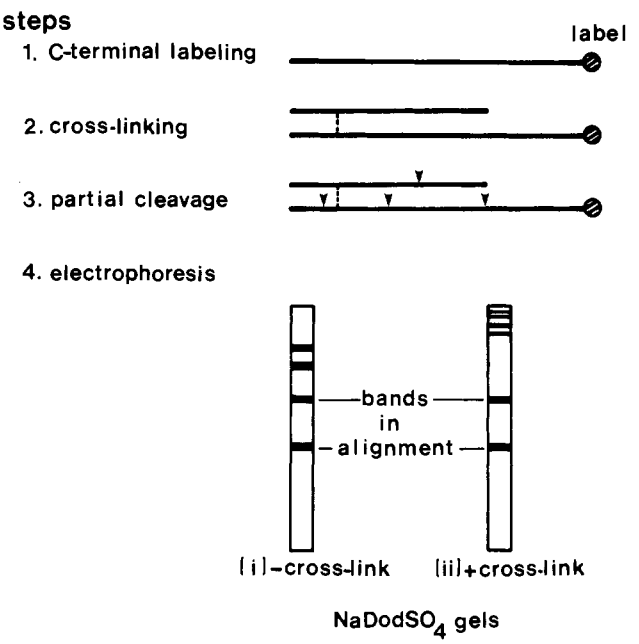

FIGURE 1: Schematic drawing of experiments for identifying the cross-linking site on the actin sequence. As the first step, actin is labeled with a fluorescent dye at its C terminus. The attached label is shown as a hatched circle. The second step is to introduce cross-link (---) between the labeled actin and the other peptide. The third step is to partially cleave the cross-linked material. Cleavage sites are shown as (▼). Cleavage products are then electrophoresed in the presence of $NaDodSO_4$. Fluorescent peptides are detected by illuminating gels with a UV lamp. Notice that two fluorescent bands in gel ii are in alignment with those in gel i, since the cross-link on actin is in the third segment from its labeled C terminus.

would come into alignment with that of the control actin as shown in gel ii in Figure 1. Thus, it is possible to locate the cross-linking site on the actin sequence by comparing the one-dimensional peptide map of the control actin and that of the cross-linked product.

In the above argument, it is assumed that the cross-linking site is in a single cyanogen bromide peptide. Can similar information be obtained from the one-dimensional peptide map, if several types of cross-links are distributed in several cyanogen bromide peptides? In this case, successive partial cleavage of a mixture of cross-linked products would produce fluorescent bands in alignment with those of the control actin once the cross-linking site nearest to the N terminus of actin is cleaved off. Fluorescent intensities of these bands would be, however, weaker than those of the corresponding bands of the control actin, since some of the cross-links are still on other segments of the actin peptide. When further successive cleavage removes all of the cross-links, the resulting fluorescence pattern would become the same as that of the control actin. Thus, close examination of intensities and positions of fluorescent bands on the one-dimensional peptide map of the control actin and those of the cross-linked material would reveal locations of cross-links on the actin sequence even when several cross-linking sites exist on actin.

Preparations of Proteins. Myosin subfragment 1 (S1) was prepared from rabbit skeletal muscle myosin according to Weeds & Taylor (1975). Actin was prepared following the method of Spudich & Watt (1971).

DACM Labeling of S1. S1 (1 mg/mL) in 30 mM KCl, 20 mM Tris-HCl, and 1 mM MgADP (pH 8.0) was reacted with 1.6 molar equiv of DACM for 3 min at 0 °C. The labeling reaction was terminated by the addition of 0.02 volume of 0.1 M *N*-acetylcysteine (pH 7.0). Under the reaction condition, two reactive thiols, SH_1 and SH_2, residing in the 20K segment of the S1 heavy chain are selectively labeled (Sutoh, 1981).

DACM Labeling of G-Actin. G-Actin (2 mg/mL) in 1 mM imidazole, 0.1 mM $CaCl_2$, and 0.1 mM ATP (pH 7.0) was reacted with 0.8 molar equiv of DACM for 1 min at 0 °C. Unreacted DACM was quenched by the addition of 0.02 volume of 0.1 M *N*-acetylcysteine. A portion of the resulting solution was exhaustively dialyzed against water and then lyophilized for the peptide mapping analysis. Another portion of G-actin was polymerized by the addition of NaCl and used for the cross-linking reaction.

DACM Labeling of Denatured Actin. Actin (2 mg/mL) in 6 M urea and 20 mM sodium acetate (pH 5.4) was reacted with a stoichiometric amount of DACM over cysteine residues for 3 min at 25 °C. Unreacted DACM was quenched by the addition of 0.05 volume of 0.1 M *N*-acetylcysteine. All five cysteine residues in the actin sequence (Elzinga et al., 1973) were accessible to the fluorescent dye under the reaction condition and, therefore, labeled with the reagent, though in varying amounts. For identification of a cyanogen bromide peptide containing cysteine-373, this particular cysteine residue was selectively alkylated with *N*-ethylmaleimide (Elzinga & Collins, 1975), and then other cysteine residues were labeled with DACM in denaturing solvent as above. These preparations of DACM-labeled actin were exhaustively dialyzed against water and then lyophilized.

Trypsin Treatment of S1. S1 (1 mg/mL) or DACM-labeled S1 (1 mg/mL) in 0.1 M NaCl and 10 mM imidazole (pH 7.0) was digested with trypsin (0.01 mg/mL) for 15 min at 25 °C. The reaction was terminated by the addition of excess trypsin inhibitor. Under the digestion condition, 20K, 25K, 50K, and 75K (25K + 50K) fragments were produced from the parent 95K heavy chain. Intact 95K heavy chain was not detected when checked by $NaDodSO_4$ gel electrophoresis.

Cross-Linking of F-Actin and S1. DACM-labeled F-actin (1 mg/mL) in 0.1 M NaCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0) was mixed with an equal volume of S1 (1 mg/mL) with or without trypsin treatment in the same solvent. To the mixture was added 0.05 volume of freshly prepared 0.1 M EDC in 0.1 M imidazole (pH 7.0). After the mixture was incubated for 1 h at 25 °C, the cross-linking reaction was terminated by addition of excess 2-mercaptoethanol.

Purification of Various Peptides from a Cross-Linked Mixture by Acrylamide Gel Electrophoresis. After DACM-labeled F-actin and trypsin-treated S1 were cross-linked, the reaction mixture was electrophoresed on acrylamide gels [10% acrylamide–0.3% bis(acrylamide)] in the presence of $NaDodSO_4$ (Laemmli, 1970). Fluorescent bands corresponding to cross-linked products (65K, 68K, and 95K) and actin were cut out from gels under illumination with a UV lamp. These peptides were eluted out of gels by electrophoresis and collected into dialysis tubes attached to bottoms of short acrylamide gels [10% acrylamide–0.3% bis(acrylamide)]. The collected peptides were again electrophoresed on acrylamide gels [10% acrylamide–0.3% bis(acrylamide)], and corresponding fluorescent bands were cut out. The gel slices were thoroughly washed with 10% methanol and then with 50% methanol. They were dried in vacuo and stored at 4 °C.

The 50K fragment of the S1 heavy chain was purified in the same way as above, except that the cross-linked mixture was further labeled with DACM in 6 M urea and 20 mM sodium acetate (pH 5.4) so that the 50K fragment might be readily detected on acrylamide gels.

Two-Dimensional Peptide Maps of Cyanogen Bromide Cleavage Products of DACM-Labeled Actin. Lyophilized

actin preparations labeled with DACM in various ways were dissolved in 70% formic acid and diluted with an equal volume of freshly prepared cyanogen bromide (1 M) in 70% formic acid. After the reaction mixture was incubated for 16 h at 25 °C, the solvent and reagent were removed under a stream of dry nitrogen.

Cleavage products were then electrophoresed on two-dimensional gels according to O'Farrell et al. (1977). Electrophoresis in the second dimension was run on acrylamide gels containing 15% acrylamide–0.45% bis(acrylamide) and 6 M urea (Sutoh, 1981), on which peptides with molecular weights of several thousands could be separated. After electrophoresis, fluorescent spots were detected under illumination with a UV lamp.

Two-Dimensional Peptide Maps for Identifying Constituents of the 95K Product. Actin, the 50K fragment of the S1 heavy chain, and the 95K product purified as described above were eluted from gel slices into dialysis tubes by electrophoresis. The collected peptides were dialyzed against 6 M urea and 1% acetic acid to remove $NaDodSO_4$ and then against water. The solvent was removed under a stream of dry nitrogen. The dried peptides were cleaved with cyanogen bromide, and cleavage products were electrophoresed on two-dimensional gels as above. The gels were stained with silver according to Oaklay et al. (1980). Although peptides in some spots with molecular weights of 2000–3000 were missed during the staining process, enough spots were detected on those gels to identify constituents of the 95K cross-linked product.

Partial Cyanogen Bromide Cleavage of Polypeptides and Electrophoresis of Cleavage Products. Dried gel slices containing the control non-cross-linked actin, the 65K product, the 68K product, or the 95K product obtained as above were soaked in freshly prepared 20 mM cyanogen bromide in 70% formic acid. The cleavage reaction was allowed to proceed for 1 h at 37 °C. The resulting gels were thoroughly washed with 20% methanol–1% 2-mercaptoethanol and then with 50% methanol. They were dried in vacuo.

The dried gels were soaked in 1% $NaDodSO_4$, 10 mM Tris-HCl, 10% glycerol, and 1% 2-mercaptoethanol (pH 8.0) for 5 h at 25 °C and then directly layered on a slab gel [15% acrylamide–0.45% bis(acrylamide)] with a stacking gel system (Laemmli, 1970). After electrophoresis, fluorescent bands on the gel were detected under illumination with a UV lamp.

Hydroxylamine Cleavage of Polypeptides and Electrophoresis of Cleavage Products. Dried gel slices containing the non-cross-linked actin, the 65K product, or the 95K product were soaked in freshly prepared 6 M guanidine hydrochloride–1 M hydroxylamine (pH 9.0) (Bornstein & Balian, 1977; Sutoh, 1981). The cleavage reaction was allowed to proceed for 4 h at 45 °C and then quenched by thoroughly washing gel slices with 20% methanol–1% 2-mercaptoethanol. They were finally washed with 50% methanol and dried in vacuo. Electrophoresis was carried out as above.

Results

Specificity of DACM Labeling of Actin. It is essential to exclusively label cysteine-373 of actin in order to identify cross-linking sites on the actin sequence, as shown under Materials and Methods. In the present experiment, a fluorescent dye (DACM) which has been shown to react selectively with cysteine residues (Yamamoto et al., 1978; Sutoh, 1981) was used for the labeling reaction. Specificity of the reaction was assessed by mapping fluorescent peptides on the two-dimensional gel (O'Farrell et al., 1977) after complete cyanogen bromide cleavage of the DACM-labeled actin.

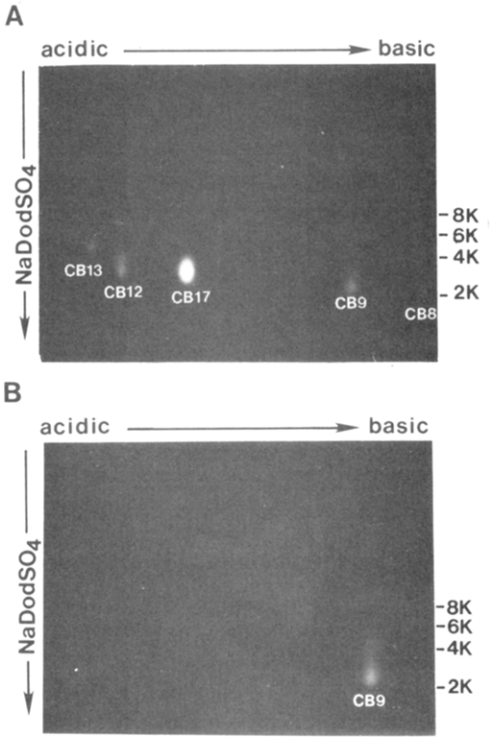

FIGURE 2: Peptide maps of products of complete cyanogen bromide cleavage of DACM-labeled actin preparations. (A) All-labeled actin; actin was labeled with DACM in denaturing solvent. (B) Actin was labeled in the G state. Electrophoresis in the second dimension was run on acrylamide gels containing 15% acrylamide–0.45% bis(acrylamide) and 6 M urea. After electrophoresis, peptides containing DACM fluorescence were detected by illumination with a UV lamp.

When actin was reacted with DACM in 6 M urea and 20 mM sodium acetate (pH 5.4), all five cysteine residues in the actin sequence (Elzinga et al., 1973; Elzinga & Collins, 1975) were labeled with the dye, though in varying extent. The all-labeled actin was then subjected to complete cyanogen bromide cleavage, and resulting peptides were electrophoresed on a two-dimensional gel following the method of O'Farrell et al. (1977). After electrophoresis, five major fluorescent spots with apparent molecular weights of 2000–5000 were detected on the gel as shown in Figure 2A, though a spot migrating at the most basic side showed extensive tailing. From the amino acid sequence of actin (Elzinga et al., 1973; Lu & Elzinga, 1977; Vandekerckhove & Weber, 1978a–c), molecular weights and isoelectric points of the five cysteine-containing peptides [CB-8, CB-9, CB-12, CB-13, and CB-17 (Elzinga et al., 1973; Elzinga & Collins, 1975)] were calculated and compared with the positions of those fluorescent spots on the gel, resulting in tentative assignment of the spots to these peptides as shown in Figure 2A.

Assignment of the CB-9 spot containing cysteine-373 was further supported by the following experiment. G-Actin was modified with the stoichiometric amount of *N*-ethylmaleimide under the condition where cysteine-373 was exclusively modified with the reagent (Elzinga & Collins, 1975). Unreacted actin thiols were then labeled with DACM under the denaturing condition as above. The peptide map of fluorescent products of complete cyanogen bromide cleavage of the doubly modified actin showed that the fluorescent spot assigned as the CB-9 peptide was totally missing, indicating that the spot really corresponds to the peptide containing cysteine-373.

When G-actin was modified with less than the stoichiometric amount of DACM for a short time (Materials and Methods), cysteine-373 was selectively labeled with the reagent, as evident

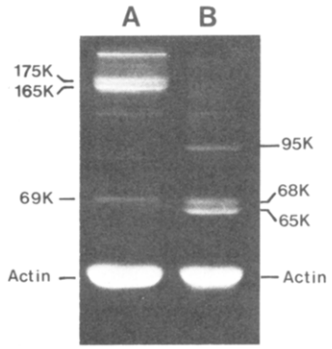

FIGURE 3: Cross-linking of DACM-labeled actin and S1. (A) Actin was cross-linked with S1. (B) Actin was cross-linked with trypsin-treated S1. Cross-linking conditions were the following: solvent, 0.1 M NaCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0) at 25 °C; concentration of proteins, 0.5 mg/mL F-actin and 0.5 mg/mL S1; concentration of EDC, 5 mM; reaction time, 1 h. Cross-linked products were electrophoresed on an acrylamide gel containing 10% acrylamide–0.15% bis(acrylamide). After electrophoresis, fluorescence of DACM covalently attached to actin was detected by illuminating the gel with a UV lamp.

from the peptide map of its cyanogen bromide cleavage products (Figure 2B). Thus, the DACM-labeled G-actin was polymerized by addition of NaCl and used for further experiments described below.

Cross-Linking of DACM-Labeled Actin and Trypsin-Treated S1. S1 produced by chymotryptic digestion of myosin consisted of heavy chains with a molecular weight of 95 000 and two types of light chains with molecular weights of 21 000 and 17 000 as noted by Frank & Weeds (1974) and Weeds & Taylor (1975). When the S1 preparation was further digested by trypsin, the 95K heavy chain generated three fragments with molecular weights of 20 000, 25 000, and 50 000 with a contaminating digestion intermediate with a molecular weight of 75 000, while the 21K light chain (alkaline light chain 1 or AL1) was transformed into a slightly shorter fragment and the 17K light (alkaline light chain 2 or AL2) was unchanged, consistent with previous observations (Mornet et al., 1979; Yamamoto & Sekine, 1979).

The trypsin-treated S1 was complexed with DACM-labeled F-actin, and then the cross-linking reaction was initiated by addition of the zero-length cross-linker EDC to the complex. Resulting cross-linked products were analyzed by acrylamide gel electrophoresis in the presence of $NaDodSO_4$. As shown in Figure 3, three major fluorescent bands with apparent molecular weights of 65 000, 68 000, and 95 000 appeared above the actin band upon the EDC treatment. Since only insignificant amounts of cross-linked materials were detected on an acrylamide gel when DACM-labeled F-actin alone was treated by EDC, these 65K, 68K, and 95K products seem to be generated from the cross-linking reaction between DACM-labeled actin subunits and various fragments of S1 as noted by Mornet et al. (1981a,b).

Among these fluorescent products, the 65K species was assigned as the cross-linked material between the DACM-labeled actin and the 20K fragment of the S1 heavy chain, since a fluorescent band with the same mobility was detected when the cross-linking reaction was carried out on a complex of F-actin and trypsin-treated S1 with its 20K fragment labeled with DACM as shown in Figure 4. A similar experiment led Mornet et al. (1981a,b) to the same conclusion.

When DACM-labeled F-actin and S1 without trypsin digestion were cross-linked with EDC, a fluorescent product with an apparent molecular weight of 69 000 was generated together with cross-linked products with apparent molecular weights

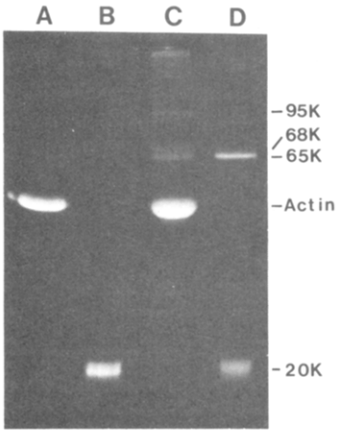

FIGURE 4: Identification of constituents of the 65K product. (A) DACM-labeled actin. (B) Trypsin-treated S1 with its 20K fragment labeled with DACM. (C) Cross-linked product of DACM-labeled F-actin and trypsin-treated S1. (D) Cross-linked product of F-actin and trypsin-treated S1 with its 20K fragment labeled with DACM. Electrophoresis was carried out on an acrylamide gel containing 10% acrylamide–0.3% bis(acrylamide). After electrophoresis, DACM fluorescence was detected by illuminating the gel with a UV lamp. Conditions for the cross-linking reaction were the same as those in Figure 3.

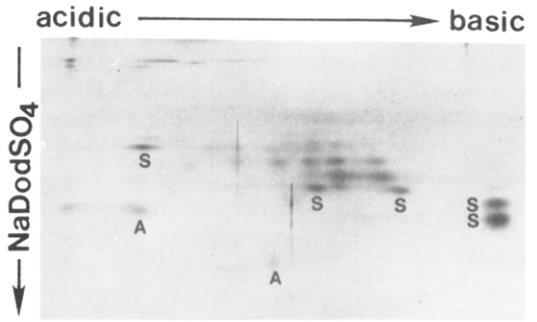

FIGURE 5: Peptide map of the product of complete cyanogen bromide cleavage of the 95K cross-linked material. Electrophoresis in the second dimension was run on an acrylamide gel containing 15% acrylamide–0.45% bis(acrylamide) and 6 M urea. Peptides were visualized by silver staining. Peptide spots marked as "A" originated from actin and those marked as "S" from the 50K fragment of the S1 heavy chain.

of 165 000 and 175 000 previously described by Mornet et al. (1981b) as shown in Figure 3 (lane A). Since the S1 preparation contained only AL1 and AL2 as low molecular weight materials, the 69K product detected in Figure 3 is most likely to be the cross-linked product of the DACM-labeled actin and AL1. Since trypsin digestion reduced the molecular weight of AL1 only slightly (Mornet et al., 1979; Yamamoto & Sekine, 1979), the 68K and 69K products seem to be the same cross-linked species of the DACM-labeled actin and AL1.

The 95K product was previously identified as the cross-linked product between actin and the 50K fragment of the S1 heavy chain, based on its apparent molecular weight (Mornet et al., 1981a,b). The assignment was confirmed by mapping cyanogen bromide peptides of the 95K product, the 50K fragment, or the DACM-labeled actin on a two-dimensional gel (O'Farrell et al., 1977). On the map of the 95K product shown in Figure 5, peptides originating from actin (labeled as "A") and those from the 50K fragment (labeled as "S") were detected, indicating that the 95K product contained both actin and the 50K fragment as its constituents.

Identification of Cross-Linking Sites on the Actin Sequence. Partial cyanogen bromide cleavage of the control non-cross-linked actin whose cysteine-373 was selectively labeled with

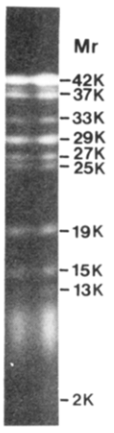

FIGURE 6: Fluorescent peptides produced by partial cyanogen bromide cleavage of the DACM-labeled actin. Electrophoresis was carried out on an acrylamide gel containing 15% acrylamide–0.45% bis(acrylamide). After electrophoresis, fluorescence of DACM covalently attached to cysteine-373 of actin was detected by illuminating the gel with a UV lamp.

Table I: Assignment of Fluorescent Peptides Generated by Partial Cleavage of the DACM-Labeled Actin

fluorescent peptides	corresponding residues	expected M_r[a]
42K[b]	1–374	42 000
37K	45–374 and 48–374	37 000 and 36 800
33K	83–374	32 800
29K	120–374 and 124–374	28 500 and 28 000
27K	133–374	27 000
25K	177–374	22 400
19K	191–374	20 800
15K	228–374	16 400
13K	269–374	12 000
broad bands	283–374	10 400
	299–374	8 500
	305–374	8 000
	313–374	7 000
	325–374	5 700
2K	355–374	2 300

[a]Molecular weight calculated from the amino acid sequence of actin (Elzinga et al., 1973). [b]Apparent molecular weight of the fluorescent peptide estimated from its mobility on an acrylamide gel.

DACM generated fluorescent peptides with various molecular weights as shown in Figure 6, in which at least nine discrete fluorescent bands could be detected as well as rather broad fluorescent bands appearing in the low molecular weight region. By comparing the molecular weights of these fluorescent peptides with those of peptides expected to be generated from actin by partial cyanogen bromide cleavage, it was possible to assign methionine residues whose cleavage resulted in production of these fluorescent peptides. Best agreement was obtained between molecular weights calculated from the amino acid sequence of actin (Elzinga et al., 1973; Lu & Elzinga, 1977; Vandekerckhove & Weber, 1978a–c) and those estimated from $NaDodSO_4$ gels, when the fluorescent peptides were assigned as in Table I. The agreement was fairly good, especially for peptides with higher molecular weights (27K–37K), though unambiguous assignment of peptides with lower molecular weights would require further chemical analysis.

The 65K cross-linked product purified to homogeneity was also subjected to partial cyanogen bromide cleavage, and resulting peptides were electrophoresed on an acrylamide gel in the presence of $NaDodSO_4$ as above. The fluorescence pattern on the gel is shown in Figure 7 (lane B) together with that of cleavage products of the control actin (lane A). Comparison

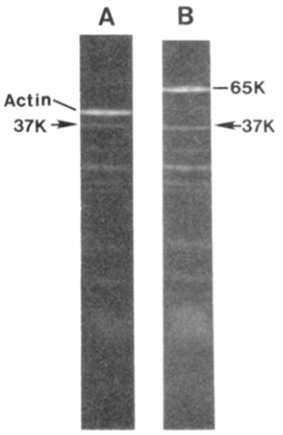

FIGURE 7: Fluorescent peptides produced by partial cyanogen bromide cleavage of the 65K cross-linked product. (A) Cleavage product of the control non-cross-linked actin. (B) Cleavage product of the 65K cross-linked material. Electrophoresis was carried out on an acrylamide gel containing 15% acrylamide–0.45% bis(acrylamide). After electrophoresis, fluorescence of DACM covalently attached to cysteine-373 of actin was detected by illuminating the gel with a UV lamp. Notice that fluorescent bands up to the 37K band are in alignment with each other in lanes A and B.

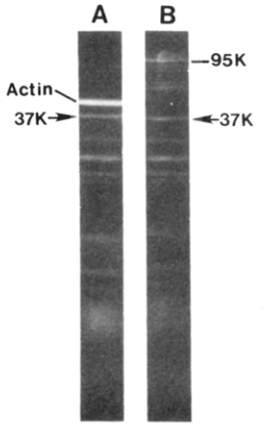

FIGURE 8: Fluorescent peptides produced by partial cyanogen bromide cleavage of the 95K cross-linked product. (A) Cleavage product of the control non-cross-linked actin. (B) Cleavage product of the 95K cross-linked material. Electrophoresis and detection of peptides were carried out as in Figure 7. Notice that fluorescent bands up to the 37K band are in alignment with each other in lanes A and B.

of these fluorescence patterns revealed that mobilities and relative intensities of fluorescent bands in lane B were the same as those in lane A up to the 37K band. The result implies that at least a major cross-linking site is located in the N-terminal segment of actin containing residues 1–44 or 1–47. Removal of either of the segments from the cross-linked product would generate the 37K fluorescent peptide. Since residues 26–47 of actin do not contain amino acids involved in the cross-linking reaction by EDC (lysine, aspartic acid, and glutamic acid) (Elzinga et al., 1973; Lu & Elzinga, 1977; Vandekerckhove & Weber, 1978a–c), it is most likely that the cross-linking site of the 20K fragment of the S1 heavy chain is located in the N-terminal 25 residues.

When the 95K cross-linked product purified to homogeneity was partially cleaved by cyanogen bromide, the resulting fluorescence pattern on an acrylamide gel was very similar to that of the 65K product as shown in Figure 8 (lane B). Again, the fluorescence pattern was in alignment with that of the partially cleaved actin up to the 37K band, and relative fluorescence intensities of aligned bands were very similar with each other, indicating that at least a major cross-linking site of the 50K fragment of the S1 heavy chain is also in the

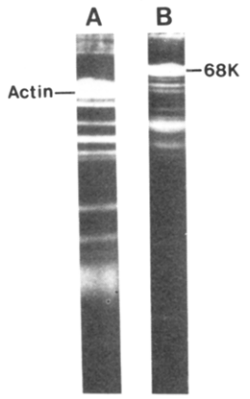

FIGURE 9: Fluorescent peptides produced by partial cyanogen bromide cleavage of the 68K cross-linked product. (A) Cleavage product of the control non-cross-linked actin. (B) Cleavage product of the 68K cross-linked material. Electrophoresis and detection of peptides were carried out as in Figure 7. Notice that none of the fluorescent bands in lanes A and B are in alignment.

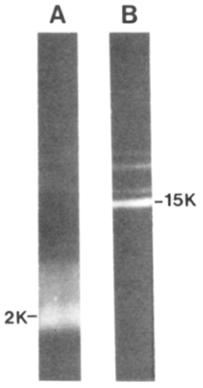

FIGURE 10: Fluorescent peptides produced by complete cyanogen bromide cleavage of the 68K cross-linked product. (A) Cleavage product of the control non-cross-linked actin. (B) Cleavage product of the 68K cross-linked material. Notice that the fluorescent 2K peptide is not detected in lane B.

N-terminal 25 residues of actin.

When the 68K cross-linked product purified to homogeneity was subjected to partial cyanogen bromide cleavage, the resulting fluorescence pattern was quite different from that of the control actin as shown in Figure 9. None of the fluorescent band generated by digesting the 68K product was in alignment with those of the control actin. When the 68K product was subjected to complete cyanogen bromide cleavage, a major fluorescent peptide released from the cross-linked product had an apparent molecular weight of 15 000 (lane B in Figure 10), while complete cyanogen bromide cleavage of the control actin generated a fluorescent peptide with an apparent molecular weight of 2000 as expected from its amino acid sequence (Elzinga et al., 1973; Lu & Elzinga, 1977; Vandekerckhove & Weber, 1978a–c) (lane A in Figure 10). These results indicate that the C-terminal segment of actin (residues 355–374) containing cysteine-373 has a cross-linking site to AL1.

Hydroxylamine Cleavage of Cross-Linked Products. In the N-terminal 25 residues of actin identified as the cross-linking site of S1 fragments there is one Asn–Gly bond (Asn-12–Gly-13), which is very susceptible to hydroxylamine cleavage (Bornstein & Balian, 1977). In order to obtain further information about the cross-linking site of S1 fragments on the actin sequence, we subjected the 65K and 95K cross-linked products as well as non-cross-linked actin to hydroxylamine cleavage (see Materials and Methods), and cleavage products were analyzed by $NaDodSO_4$ gel electrophoresis. As shown

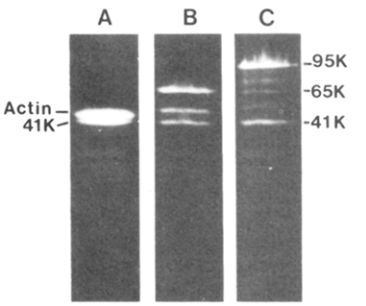

FIGURE 11: Fluorescent peptides produced by hydroxylamine cleavage of cross-linked products. (A) Cleavage product of the control non-cross-linked actin. (B) Cleavage product of the 65K cross-linked material. (C) Cleavage product of the 95K cross-linked material. Conditions for the cleavage reaction were as follows: solvent, 6 M guanidine hydrochloride–1 M hydroxylamine (pH 9.0) at 45 °C; reaction time, 4 h. Electrophoresis was carried out on an acrylamide gel containing 12% acrylamide and 0.36% bis(acrylamide) in the presence of $NaDodSO_4$. Fluorescence was detected by illuminating the gel with a UV lamp.

in Figure 11 (lane A), hydroxylamine treatment of the non-cross-linked actin generated a fluorescent peptide appearing just below the actin band. The apparent molecular weight of the fluorescent fragment was 41 000, consistent with that expected for a larger fragment generated by cleavage of actin at the Asn-12–Gly-13 bond. On the basis of fluorescence intensities detected on the actin and 41K bands, it is estimated that the yield of the cleavage reaction is about 20%.

As shown in lanes B and C in Figure 11, hydroxylamine treatment of the 65K and 95K cross-linked products again generated a fluorescent peptide with an apparent molecular weight of 41 000. These 41K fragments comigrated with the 41K fragment originating from the non-cross-linked actin when electrophoresed side by side on a slab $NaDodSO_4$ gel, indicating that the fluorescent 41K fragments originating from the cross-linked products are the actin peptide containing residues 13–374. Examination of fluorescence intensities in lanes B and C in Figure 11 revealed that the yield of the 41K fragments from the cross-linked products was similar to that from the control non-cross-linked actin. These results imply that at least a major cross-linking site of the 20K and 50K fragments of the S1 heavy chain on actin is located outside its 41K segment. Thus, it is concluded that these heavy chain fragments are cross-linked with the N-terminal segment of actin containing residues 1–12.

Discussion

Actin and trypsin-treated S1 were efficiently cross-linked by the zero-length cross-linker EDC to generate three types of cross-linked products with apparent molecular weights of 65K, 68K, and 95K. It was shown that the 65K, 68K, and 95K products were cross-linked complexes of actin–20K fragment, actin–AL1, and actin–50K fragment, respectively. Mornet et al. (1981a,b) previously detected the 65K and 95K products, by employing the same cross-linking reagent under slightly different conditions.

Partial cyanogen bromide cleavage of various cross-linked products and one-dimensional peptide mapping of resulting cleavage products revealed that both the 20K and 50K fragments of the S1 heavy chain were cross-linked with the N-terminal segment of actin spanning residues 1–25, while AL1 was cross-linked with its C-terminal segment spanning residues 355–374.

Further information about the cross-linking site of heavy chain fragments on the actin sequence was obtained, by employing another type of chemical cleavage of the polypeptide

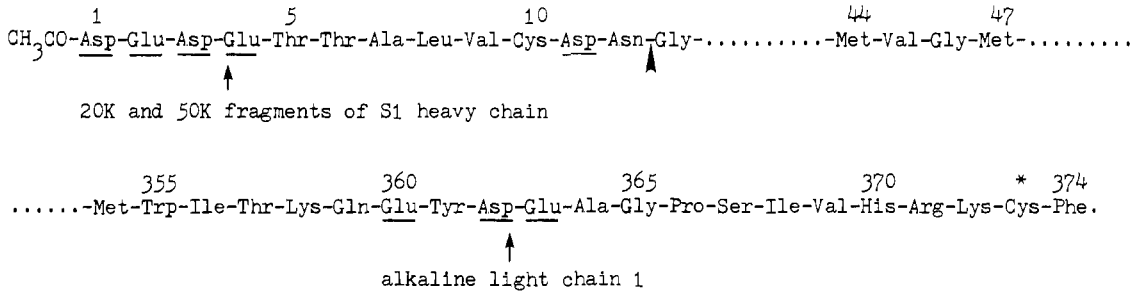

FIGURE 12: Cross-linker sites of S1 heavy and light chains on the actin sequence. Acidic residues expected to be cross-linking sites with S1 heavy and light chains are underlined. The Asn–Gly bond cleaved by hydroxylamine treatment is denoted as (▲). Cysteine-373 labeled with DACM is denoted with an asterisk. The amino acid sequence of actin is taken from Elzinga et al. (1973), Lu & Elzinga (1977) and Vandekerckhove & Weber (1978a–c).

chain. The 65K and 95K cross-linked products were subjected to hydroxylamine cleavage, to which the Asn–Gly bond in the actin sequence (Asn-12–Gly-13) was very susceptible (Bornstein & Balian, 1977). The hydroxylamine treatment of these cross-linked products generated an actin fragment of residues 13–374, suggesting that the cross-linking sites of the 20K and 50K fragments are located in the N-terminal 12 residues of actin.

Mornet et al. (1981b) previously showed that carboxyl groups of actin and amino groups of S1 were involved in the actin–S1 cross-linking induced by EDC. Examination of the amino acid sequence of actin (Elzinga et al., 1973; Lu & Elzinga, 1977; Vandekerckhove & Weber, 1978a–c) has revealed that its N-terminal 12 residues are in fact rich in acidic residues. These acidic residues are aspartic acids at positions 1, 3, and 11 and glutamic acids at positions 2 and 4. They are candidates for cross-linking sites of amino groups of the 20K and 50K fragments. At these sites, two polypeptides are in contact with each other, and, moreover, they interact with each other through ionic forces between amino and carboxyl groups. Therefore, it seems most likely that the 20K and 50K fragments of the S1 heavy chain really *bind* at and around some of these acidic chains in the N-terminal 12 residues of actin.

It has been shown that the N-terminal acidic segment of actin carries a disproportionately high number of amino acid exchanges when actins from various sources are compared, even though their amino acid sequences are highly conserved (Lu & Elzinga, 1977; Vandekerckhove & Weber, 1978a–c). The amino acid exchanges at the N-terminal segment of actin, however, have never destroyed its acidic nature (Vandekerckhove & Weber, 1978c), possibly because acidic residues in the segment are essential for the actin–myosin interaction, which plays vital roles in various living systems. Pressure during eukaryotic evolution to make the actin–myosin interaction most efficient might induce a high number of amino acid exchanges in the myosin-binding region of the actin sequence.

The C-terminal segment of actin spanning residues 355–374 has the binding site of AL1. The segment contains three acidic residues in cluster at positions 360, 362, 363 (Elzinga et al., 1973), which are candidates for cross-linking site(s) of AL1. Since no cross-linking could be detected between actin and AL2, it seems likely that the cross-linking site of actin in AL1 is in its N-terminal stretch of 41 residues, which are rich in lysine residues (Frank & Weeds, 1974). The notion is consistent with the finding that complete cyanogen bromide cleavage of the 68K product yielded the 15K fluorescent peptide. From the amino acid sequence of AL1 (Frank & Weeds, 1974), it is expected that cyanogen bromide cleavage of AL1 would produce a large N-terminal peptide (residues 1–99) with a molecular weight of 12 000. Therefore, cross-linking of the C-terminal segment of actin containing the DACM-labeled cysteine-373 with the N-terminal segment of AL1 would yield a fluorescent peptide with a molecular weight of 14 000 as a cyanogen bromide peptide, consistent with the observation. Thus, it is likely that the N-terminal segment of AL1 binds to actin at and around its acidic residues at positions 360, 362, and 363.

Consistent with the above result, affinity chromatography of the thrombic digest of AL1 demonstrated a direct interaction between a fragment of AL1 (residues 1–79) and actin (Winstanley & Trayer, 1979; Henry et al., 1980). NMR studies have also demonstrated that the N-terminal segment of AL1 (residues 1–41) interacts with actin in the acto–S1 rigor complex (Prince et al., 1981).

Although direct involvement of AL1 in the actin–myosin interaction has been shown as above, its physiological significance is unknown. A recent observation that the S1 heavy chain free from light chains exhibits the actin-activated ATPase comparable to the native S1 (Sivaramakrishnan & Burke, 1982) excludes the possibility that the AL1–actin interaction is essential for the actin-activated hydrolysis of ATP by myosin. The AL1 might play a regulatory role in the actin–myosin interaction, since it has been shown that the actin-activated ATPase activity of S1 isozymes is dependent on the types of alkaline light chains under certain conditions (Wagner & Weeds, 1977).

In summary, binding sites of S1 heavy and light chains are determined on the actin sequence as shown in Figure 12. Spacial locations of these binding sites on actin will be determined once X-ray diffraction studies of actin now in progress (Suck et al., 1981) finally show its three-dimensional structure at high resolution.

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Fluorescence Anisotropy of Labeled F-Actin: Influence of Divalent Cations on the Interaction between F-Actin and Myosin Heads†

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ABSTRACT: The interaction between F-actin and soluble proteolytic fragments of myosin, heavy meromyosin and myosin subfragment 1 without ATP, has been studied by measuring the static anisotropy and the transient anisotropy decay of the fluorescent chromophore *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine bound to F-actin. In the presence of Ca^{2+} ions, the mobility of the chromophore was strongly decreased by adding heavy meromyosin or myosin subfragment 1, and this conformation change of F-actin showed a strong cooperativity; that is, a very small amount of myosin heads induced the maximum anisotropy change. On the other hand, in the presence of Mg^{2+} ions, the addition of a small amount of myosin subfragment 1 or of heavy meromyosin increased the mobility of labeled F-actin that reached a maximum at a molar ratio of about 1/25 or 1/50, respectively. With further addition of myosin heads, the mobility of the labeled actin decreased. From these studies, one concludes that F-actin undergoes a conformation change by interacting with myosin heads, which depends on the nature of the divalent cations present in the solution.

The cyclic interaction of myosin and actin coupled with the ATP hydrolysis generates the mechanical force in the muscle contraction. During this process, the system passes through several states; one of these is thought to be identical with the stable complex formed by myosin and actin in the absence of ATP (the rigor complex). The presence of Mg^{2+} ions is required for the mechanochemical transduction of the ATP hydrolysis. The Mg–ATPase of myosin alone is small and is strongly enhanced by the myosin–actin interaction.

Until now it has not been established whether Mg^{2+} has a structural role in the actin–myosin interactions. The rigor complex has been studied by physical measurements in the presence and in the absence of Mg^{2+} (Thomas et al., 1979; Oosawa et al., 1973; Tawada, 1969; Fujime & Ishiwata, 1971; Los Calzo et al., 1975). But the influence of this ion on the conformation of the complex has not been systematically studied.

In the present work, we measured the average fluorescence anisotropy and the fluorescence anisotropy decay of F-actin labeled with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine, in solutions of which various amounts of the active subfragments of myosin, heavy meromyosin (HMM) and subfragment 1 (S_1), were added. These kinds of measurements brought information on the mobility of this fluorescent label in the time range between 1 ns and 1 μs (Wahl et al., 1975; Tawada et al., 1978; Ikkai et al., 1979). When the myosin subfragments were added to the F-actin solutions, we found that the label mobility changed in a different way according to whether Ca^{2+} or Mg^{2+} was present in the solution. These results suggested that the conformation of the rigor complex was dependent on the nature of the divalent cations that were present in the solution.

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