Improved Method for Mapping the Binding Site of an Actin-Binding Protein in the Actin Sequence. Use of a Site-Directed Antibody against the N-Terminal Region of Actin as a Probe of Its N-Terminus†

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ABSTRACT: An antibody was raised against the N-terminal 18 residues of rabbit skeletal muscle actin. By the use of this antibody as the N-terminal probe of actin and the fluorescent label at Cys-374 as its C-terminal probe, binding sites of depactin (an actin-depolymerizing protein from starfish oocytes) were identified in the actin sequence according to the method of Sutoh [Sutoh, K. (1982) *Biochemistry 21*, 3654–3661]. Cross-linking of the one-to-one complex of actin and depactin with 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) generated two types of cross-linked products with slightly different apparent molecular weights, denoted as 60KU and 60KL. By the use of the N-terminal probe, it was unequivocally revealed that the C-terminal actin segment of residues 357–375 participated in cross-linking with depactin to form 60KL. On the other hand, by the use of the C-terminal probe it was revealed that the N-terminal actin segment of residues 1–12 participated in cross-linking with depactin to form 60KU. Since EDC cross-links Lys residue with Asp or Glu residue only when they are in direct contact, the result indicates that some of the N-terminal residues 1–12 and the C-terminal residues 357–375 of actin participate in binding depactin. The introduction of the N-terminal probe (the antibody recognizing the actin N-terminus) has increased the flexibility of the mapping method for locating binding sites of actin-binding proteins in the actin sequence.

Actin is a major protein component of muscle and nonmuscle cells. It is associated with various types of proteins to form cytoskeletal and contractile structures. In muscle, actin forms a stable filamentous structure (F-actin) which is associated with several "regulatory" proteins such as tropomyosin and troponin. Cyclic association and dissociation of actin with myosin during ATP hydrolysis are most essential for muscle contraction. In nonmuscle cells, actin is involved in their cytoskeletal and motile structures and forms a variety of higher order structures, which are dynamically regulated by so-called "actin-binding proteins". These cells contain various types of actin-binding proteins: capping proteins cap an end of F-actin, gelation (bundling) proteins form F-actin networks (bundle), and depolymerizing proteins depolymerize F-actin into the monomeric form [see Stossel et al. (1985) as a review]. Myosin is also an important component of the motile apparatus of nonmuscle cells. These actin-binding proteins are recruited at specific times and special locations within a cell to form the specific type of cytoskeletal and motile structures needed there.

It is remarkable that a surprisingly large number of proteins bind to actin to exert significant effects on the organization of actin molecules. Since the surface area of the actin molecule is limited, especially when in the filamentous state, actin must have some unusual structural feature to accommodate such a large number of binding proteins. It is important to ask what the structural basis of the multiple protein–protein interactions between actin and actin-binding proteins is. Is there any specific binding site for a particular set of binding proteins? Do binding proteins with similar functions share the same binding site?

We have recently developed a simple method for locating actin segments that participate in binding various actin-binding proteins (Sutoh, 1982a, 1984; Sutoh & Mabuchi, 1984; Muneyuki et al., 1985; Sutoh & Hatano, 1986). Very interestingly, many actin-binding proteins with different functions use an N-terminal segment of actin as a part of their binding sites. They include myosin, depactin [an actin-depolymerizing protein isolated from starfish oocytes (Mabuchi, 1981, 1982, 1983)], fragmin [a barbed end capping protein from *Physarum polycephalum* (Hasegawa et al., 1980; Hissen, 1981a,b; Sugino & Hatano, 1982)], and cofilin [an actin-binding protein isolated from brain, which attaches to the side of F-actin (Nishida et al., 1984)]. In addition to the N-terminal segment, depactin seems to use a C-terminal segment of actin as well for its binding site (Sutoh & Mabuchi, 1984). Thus, the N-terminal and C-terminal segments of actin may be in proximity with each other to form a binding site to depactin, which is a small and globular protein (Mabuchi, 1983). Myosin heavy and light chains also use both the N-terminal and C-terminal segments of actin as a part of their binding sites (Sutoh, 1982a,b, 1983), supporting the notion that the N-terminal and C-terminal segments of actin are located close to each other to form a binding site for some of actin-binding proteins.

The published method for mapping the binding site of actin-binding proteins in the actin sequence relies on the C-terminal label of actin polypeptide (Sutoh, 1982a). Therefore, the binding site is mapped in the actin sequence from its C-terminus. Because of the possible importance of the N-terminal and C-terminal regions of actin for its interactions with actin-binding proteins, it is highly desirable to map the

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binding site *from both ends* of the actin polypeptide. In this paper, we describe an improved mapping method, which employs a site-directed antibody against the N-terminal segment of actin. The improved methodology has significantly increased the flexibility of the mapping technique. By using the technique, we have unequivocally shown that depactin, an actin-depolymerizing protein, binds to the N-terminal and C-terminal segments of actin.

Materials and Methods

Proteins and Reagents. Actin was prepared from rabbit skeletal muscle according to the method of Spudich and Watt (1971). Depactin was prepared from starfish oocytes as previously described (Mabuchi, 1983). Antibody against the N-terminal 18 residues of rabbit skeletal actin was raised according to the method of Bulinski et al. (1983). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC)[1] was purchased from Nakarai Chemical Co. (Tokyo). BNPS-skatole was from Pierce Chemical Co. (Rockford, IL). *N*-[7-(Dimethylamino)-4-methyl-5-coumarinyl]maleimide (DACM) was from Wako Chemical Co. (Tokyo). Vectastain and biotinylated anti-rabbit IgG antibody were purchased from Vector Laboratories (Burlingame, CA).

Cross-Linking of Actin–Depactin Complex with EDC. Cys-374 of rabbit skeletal G-actin was selectively labeled with DACM as described (Sutoh, 1982a). The DACM-labeled actin (0.5 mg/mL) was complexed with depactin in 0.1 M KCl and 10 mM MOPS (pH 7.4) at a weight ratio of 1:2 (depactin/actin). It was dialyzed against 0.1 M NaCl, 20 mM MOPS, and 2 mM $MgCl_2$ (pH 7.2). The cross-linking reaction was initiated by adding 0.1 volume of 0.2 M EDC. After 2 h at 25 °C, the reaction was quenched by the addition of Laemmli's sample buffer (Laemmli, 1970).

Chemical Cleavages of Peptides. A cross-linked sample was electrophoresed in the presence of $NaDodSO_4$ (Laemmli, 1970). After electrophoresis, gel strips were cut out. They were washed first with 50% ethanol and then with ethanol, and then the strips were dried in vacuo. These gel strips were treated with CNBr, BNPS-skatole, or hydroxylamine as previously described (Sutoh, 1982a,b, 1983, 1984; Sutoh & Mabuchi, 1984; Sutoh & Hatano, 1986). They were again washed with 50% ethanol and ethanol and dried in vacuo. The dried gels were soaked in Laemmli's sample buffer (Laemmli, 1970) and were directly layered on the second-dimension gel. After electrophoresis, fluorescent spots containing the C-terminus of actin were detected by illuminating the second-dimension gel with a UV lamp. These fluorescent spots were recorded by taking pictures.

Western Blotting of Peptides. Peptides mapped on the second-dimension gel were then transferred onto a Durapore membrane (type GV, Millipore) (Towbin et al., 1979). The membrane was washed with 0.15 M NaCl and 20 mM phosphate buffer (pH 7.0) containing 0.05% Tween 20 (denoted as TPBS) for 30 min and then was incubated with the antiserum against the actin N-terminal fragment (at a 200-fold dilution with TPBS) for 1 h. The membrane was again washed with TPBS three times for 5 min. It was then incubated with biotinylated secondary antibody (biotinylated anti-rabbit IgG antibody), which was diluted 200-fold with TPBS, for 1 h. The membrane was washed with TPBS as above. It was incubated with Vectastain, an avidin–peroxidase complex, for 1 h. After two washes with TPBS, it was washed with 0.15 M NaCl and 20 mM phosphate buffer (pH 7.0). Color development was carried out by the use of 4-chloronaphthol as a chromogen (Sutoh et al., 1984).

Peptides transferred onto the membrane were stained with gold colloid (15 nm) according to the method of Moeremans et al. (1985).

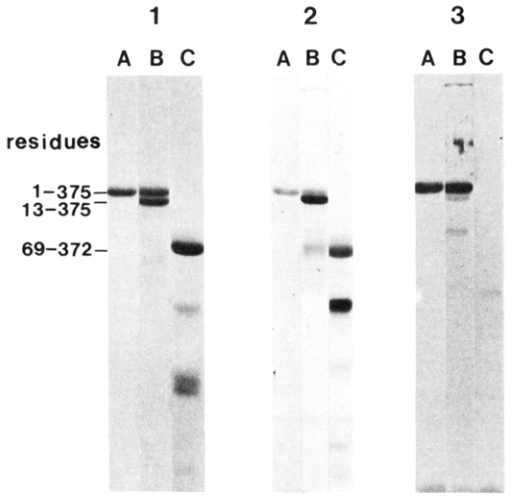

FIGURE 1: Characterization of an antibody raised against the N-terminal 18 residues of rabbit skeletal actin. Western blotting analysis of the intact actin and its digestion products. Lane A: intact actin. Lane B: actin treated with hydroxylamine to cleave the Asn-12–Gly-13 bond. The fragment of residues 13–375 is observed just below the actin band. Lane C: actin digested with trypsin. Trypsin-resistant core peptide (residues 69–372) is the major peptide observed on the gel. (1) Coomassie-stained acrylamide gels. (2) Peptides transferred onto a membrane were stained with gold colloid. (3) Peptides transferred onto a membrane were visualized by the antibody against the N-terminal 18 residues of actin. Notice that neither the fragment of residues 13–375 nor that of residues 69–372 was stained by the antibody, while the intact actin was heavily stained.

Results

Characterization of the Antibody against the N-Terminal Region of Actin. An antibody was raised against the N-terminal 18 residues of rabbit skeletal actin (Bulinski et al., 1983). Three injections of the N-terminal peptide coupled with hemocyanin were enough to produce antibody with high titer. Even after 10^5-fold dilution of the antiserum, ELISA on actin was still positive. The Western blotting assay (Towbin et al., 1979) revealed that in fact the antibody strongly reacted with actin (lane A in Figure 1), while it did not recognize the trypsin-resistant core of actin (residues 69–372) (Jacobson & Rosenbusch, 1976) (lane C in Figure 1). The antibody did not recognize the actin segment containing residues 13–375 which was generated by the hydroxylamine treatment (Sutoh, 1982a) (lane B in Figure 1). Thus epitope of the antibody seems to be in the N-terminal 12 residues. Actually, prediction of the antigenic site by the method of Hopp and Wood (1981) indicates that the first seven amino acids of actin are the site (Bulinski et al., 1983).

Cross-Linking of Actin and Depactin with EDC. When the one-to-one complex of actin and depactin was treated with a zero-length cross-linker EDC, covalent cross-links were introduced at the interface between two proteins. The $NaDodSO_4$ gel electrophoresis revealed that the EDC treatment generated cross-linked product with an apparent molecular weight of 60 000 (lane B in Figure 2), which was the covalent complex of one actin and one depactin (Mabuchi, 1983; Sutoh & Mabuchi, 1984). Although the 60K band seemed to be a

[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-methyl-3-bromoindolene; DACM, *N*-[7-(dimethylamino)-4-methyl-5-coumarinyl]maleimide; $NaDodSO_4$, sodium dodecyl sulfate; CNBr, cyanogen bromide; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

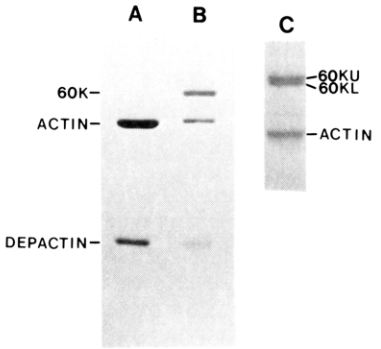

FIGURE 2: Cross-linking of the one-to-one complex of actin and depactin with EDC. Lane A: before the cross-linking. Lane B: after the cross-linking. Lane C: the same sample as in lane B was electrophoresed for a prolonged period to separate 60KU and 60KL, which migrated very closely in lane B. Cross-linking conditions: actin, 0.5 mg/mL; depactin, 0.25 mg/mL; solvent, 0.1 M NaCl, 20 mM MOPS, and 2 mM $MgCl_2$ (pH 7.2); cross-linker, 20 mM EDC; reaction time, 2 h; temperature, 25 °C.

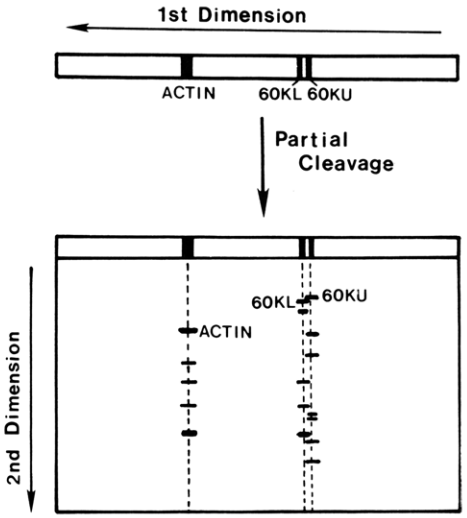

FIGURE 3: Schematic illustration of the mapping procedure. Cross-linked sample was electrophoresed in the first-dimension gel to separate 60KU and 60KL as much as possible. Peptides were then partially cleaved by a chemical such as CNBr in the gel. After the cleavage reaction, the first-dimension gel was layered on the second-dimension gel, and cleavage products were electrophoresed. Fragments separated on the gel were visualized either by the C-terminal probe or by the N-terminal probe.

single band at first sight (Mabuchi, 1983; Sutoh & Mabuchi, 1984), prolonged electrophoresis revealed that it actually was closely spaced doublet bands (lane C in Figure 2), indicating generation of two distinct types of cross-linked products. The upper band is denoted as 60KU and the lower as 60KL.

Mapping of Binding Sites of Depactin in the Actin Sequence with CNBr. Since the 60KU and 60KL bands were too close to each other to be purified separately, mapping of binding sites of depactin was carried out by employing the two-dimension gel as schematically shown in Figure 3. First, a cross-linked sample was electrophoresed in the first-dimension gel to separate 60KU and 60KL bands as much as possible. After cleavage of peptides by CNBr, BNPS-skatole, or hydroxylamine in the first-dimension gel, the gel was layered on the second-dimension gel. Cleavage products were electrophoresed on the second-dimension gel and visualized either by the N-terminal probe (the antibody against the N-terminal 18 residues of actin) or by the C-terminal probe (the DACM label at the Cys-374) of actin.

Figure 4 shows the second-dimension map of CNBr cleavage products. When the actin C-terminus was detected on the gel

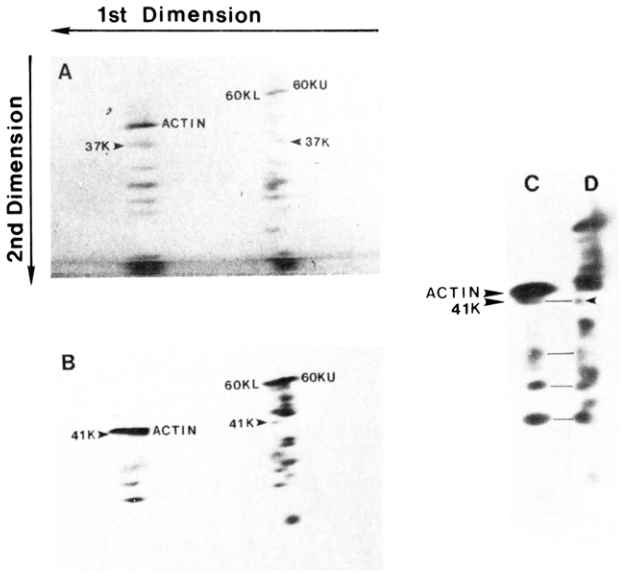

FIGURE 4: Mapping of the cross-linking sites of depactin in the actin sequence by the use of the CNBr cleavage. (A) Peptide fragments were visualized by illuminating the gel with a UV lamp. Only fragments containing the C-terminus of actin were detected (fluorescent spots are observed as dark spots in this map). The 37K fragment is indicated by an arrowhead. Note that band patterns generated from actin and from 60KU are completely in alignment with each other up to the 37K band, while no alignment is observed between band patterns from actin and from 60KL even in the low M_r region. (B) Peptide fragments were visualized by the use of the antibody against the actin N-terminus. The 41K fragment is indicated by an arrowhead. The 41K fragment released from actin is covered by the actin band in this map [see (C)]. Note that band patterns generated from actin and from 60KL are in alignment up to the 41K fragment [also see (C) and (D)], while no alignment is observed between band patterns generated from actin and from 60KU. (C and D) Fragments were separated and visualized as in B, but electrophoresis in the second dimension was carried out for a prolonged period for better separation of the 41K fragment and actin. Lanes corresponding to actin (C) and to 60KU plus 60KL (D) were cut out and taken together side by side for easier comparison. Bands in alignment are indicated by lines.

by the use of the fluorescent label at Cys-374 (Figure 4A), we observed ladderlike bands which were generated from non-cross-linked actin, 60KL and 60KU. Close examination of the map revealed that fluorescent bands generated from 60KU were in alignment with those from the non-cross-linked actin up to the 37K fragment (indicated by an arrowhead). In other words, partial cleavage of the 60KU actin–depactin complex released a series of free actin fragments containing its C-terminus up to the 37K-dalton fragment. Thus it seems that the cross-linking site of depactin in 60KU is outside the 37K segment of actin [residues 45–375 and/or 48–375 (Sutoh, 1982a, 1984)], i.e., within residues 1–44 or 1–47 of actin. On the other hand, fluorescent bands generated from 60KL were not in alignment with those from actin even in the low M_r region, indicating that the cross-linking site of depactin was close to the C-terminal region of actin.

In the above argument, we assumed that the fluorescent fragments from 60KU which were in alignment with those from non-cross-linked actin were free actin fragments containing its C-terminus. However, we have to consider the possibility that some of these fluorescent fragments are not free actin fragments but actually are actin fragments cross-linked with small depactin fragments. If this is the case, we would observed the complete alignment of fluorescent bands from actin and from the 60KU product up to the 42K actin band as discussed previously (Sutoh & Mabuchi, 1984; Sutoh & Hatano, 1986). As observed in Figure 4A, no fluorescent

band was detected at the 42K position in the ladderlike pattern generated from 60KU. Thus we conclude that fluorescent fragments from 60KU which are in alignment with those from actin are actually free actin fragments. Throughout this paper, the same argument is used to assure that particular fragments released from the cross-linked 60K products are free actin fragments with no attached depactin fragment.

Peptide fragments on the second-dimension gel were then electrophoretically transferred onto a membrane (Towbin et al., 1979). Fragments containing the N-terminus of actin were visualized by the use of the antibody against its N-terminal 18 residues. As shown in Figure 4B, ladderlike band patterns were generated from the non-cross-linked actin and from two cross-linked products. Unlike the band patterns shown in Figure 4A, no alignment was observed between bands released from actin and from 60KU even in the low M_r region. This result is consistent with the above conclusion that the cross-linking site of depactin in 60KU is in the N-terminal region of actin. On the other hand, bands released from 60KL were in alignment with those from actin up to the 41K fragment which migrated very closely to the intact actin band (indicated by an arrowhead). For better separation of the intact actin and the 41K fragment, electrophoresis was carried out for a prolonged period of time as shown in Figure 4C,D, in which the 41K fragment is indicated by an arrowhead. Considering the amino acid sequence of actin (Elzinga et al., 1973), we conclude that the 41K fragment released from actin must span residues 1–355 since the fragment necessarily contains the N-terminus of actin. The 41K fragment released from 60KL is also the actin fragment of residues 1–355, since no 42K band comigrating with actin is released from 60KL. Thus it seems that the cross-linking site of depactin in 60KL is outside residues 1–355, i.e., within residues 356–375. This conclusion is consistent with the above notion that the cross-linking site is close to the C-terminal region of actin.

Peptide Mapping by the BNPS-skatole Cleavage. A cross-linked sample was partially cleaved in the first-dimension gel by BNPS-skatole, which selectively digests polypeptide chain at tryptophan residues (Fontana, 1972). There are four tryptophan residues in actin (Trp-79, Trp-86, Trp-340, and Trp-356) (Elzinga et al., 1973). When fragments containing the C-terminus of actin were detected on the second-dimension gel by the use of the fluorescent label at Cys-374 (Figure 5A), a rather broad band with an apparent M_r of 35 000 was observed in lanes corresponding to the non-cross-linked actin and to the 60KU product. The 35K fragment generated from actin is residues 80–375 and/or 87–375 (Sutoh, 1984; Sutoh & Mabuchi, 1984; Sutoh & Hatano, 1986). Since the BNPS-skatole cleavage of 60KU and 60KL released fragments migrating close to the 42K actin band (Figure 5A), we cannot exclude the possibility that the 35K fragment released from 60KU contains small depactin fragments. However, considering the fact that the CNBr or the hydroxylamine cleavage of 60KU released actin C-terminal fragments larger than the 35K fragment, we tentatively identify the 35K fragment from 60KU as the free actin fragment of residues 80–375 and/or 87–375. Thus it is likely that the cross-linking site of depactin in 60KU is within the N-terminal residues 1–79 or 1–86 of actin.

The 35K fragment was not generated from 60KL by the BNPS-skatole cleavage (Figure 5A). Instead, the cleavage reaction generated peptides with smaller molecular weights, which must be cross-linked products of C-terminal fragments of actin (residues 341–375 and 357–375) and depactin fragments as shown below.

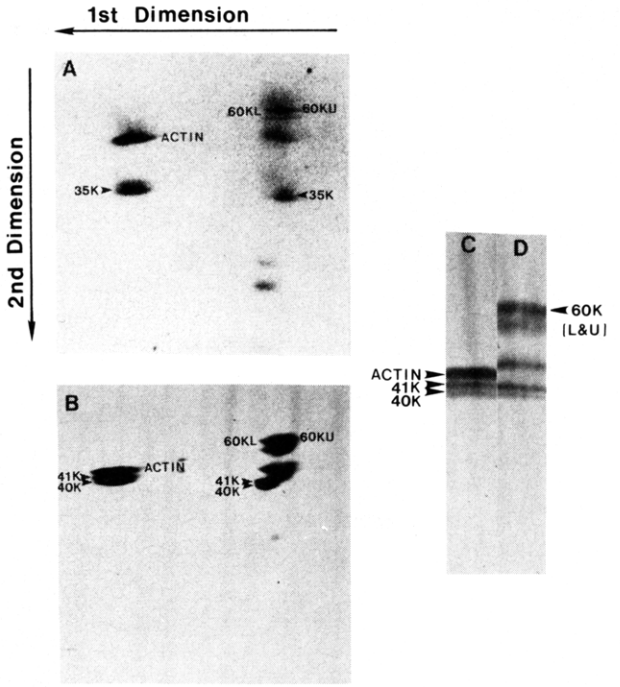

FIGURE 5: Mapping of cross-linking sites of depactin in the actin sequence by the use of the BNPS-skatole cleavage. (A) Peptide fragments were detected by illuminating the gel with a UV lamp. Note that the 35K fragment was released from actin and from 60KU, but not from 60KL. (B) Peptide fragments were visualized by the use of the antibody against the actin N-terminus. Note that the 41K and 40K fragments were released from actin and from 60KL, but not from 60KU. (C and D) Actin (C) and the 60K products (mixture of 60KU and 60KL) (D) were treated with BNPS-skatole. Cleavage products were electrophoresed side by side for easier comparison of fragments and were visualized by the antibody against the actin N-terminus.

Fragments on the second-dimension gel were then visualized by the use of the N-terminal probe (Figure 5B). From non-cross-linked actin, doublet bands with apparent molecular weights of 41 000 and 40 000 were generated. They are actin fragments containing residues 1–356 and 1–340, respectively. The same doublet bands were generated from 60KL but not from 60KU. Although both 60KU and 60KL released a fragment migrating close to actin, it was actually larger than that as revealed by electrophoresing cleavage products of actin and those of the 60K cross-linked complexes (mixture of 60KU and 60KL) side by side (Figure 5C,D). Thus the 41K and 40K doublet fragments released from 60KL must be free actin fragments of residues 1–356 and 1–340, respectively. In other words, the cross-linking site of depactin in 60KL is within the C-terminal segment of residues 357–375, consistent with the above results obtained by the use of the C-terminal probe.

Peptide Mapping by the Hydroxylamine Cleavage. The cross-linking site of depactin in the actin sequence was mapped by the use of the hydroxylamine cleavage (Bornstein & Balian, 1977), which digests the Asn-12–Gly-13 bond in actin (Sutoh, 1982a, 1984; Sutoh & Mabuchi, 1984; Sutoh & Hatano, 1986). When cleavage products were visualized by the C-terminal probe, it was observed that a fragment with an apparent M_r of 41 000 was released both from actin and from 60KU (Figure 6A). In addition to the parent peptides, the 41K fragment was the only spot visible on the map. The 41K fragment released from actin was residues 13–375 (Sutoh, 1982a). Since no 42K fragment was released from 60KU, the 41K fragment released from 60KU must be residues 13–375 of actin. Therefore, the cross-linking site of depactin in 60KU is outside residues 13–375, i.e., within residues 1–12 of actin. On the other hand, the cross-linking site of depactin in the

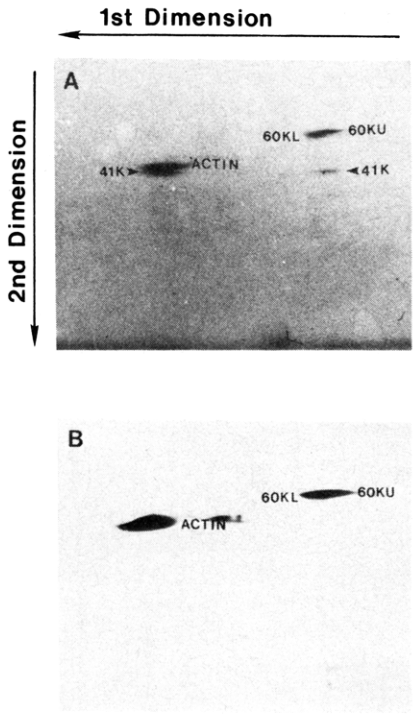

FIGURE 6: Mapping of cross-linking sites of depactin in the actin sequence by the use of the hydroxylamine cleavage. (A) Peptide fragments were detected by illuminating the gel with a UV lamp. The 41K fragment was released both from actin and from 60KU, but not from 60KL. (B) Peptide fragments were visualized by the antibody against the actin N-terminus.

60KL cross-linked product must be within residues 13–375 since the 41K fragment was not released from 60KL.

When the actin N-terminus was detected on the map, only the parent peptides (actin, 60KL, and 60KU) were visible (Figure 6B). The free N-terminal peptide of actin (residues 1–12) that was expected to be released from actin and 60KL was too small to be trapped on the membrane.

Mappings of cross-linked sites of depactin in actin by cleavages with three different chemicals are summarized in Figure 7. From these mappings, it is unequivocally concluded that residues 1–12 (open box) participate in cross-linking with depactin to form the 60KU product while residues 357–375 (crosshatched box) are involved in cross-linking with depactin to form the 60KL product.

Discussion

We have already shown that the binding site of an actin-binding protein in the actin sequence can be mapped very easily by cross-linking actin with the binding protein and then by partially cleaving the cross-linked product with various chemicals (Sutoh, 1982a, 1984; Sutoh & Mabuchi, 1984; Sutoh & Hatano, 1986). This method exploits the high reactivity of Cys-374 of actin toward various alkylating reagents. Since the C-terminus of actin is Phe-375 (Elzinga et al., 1973), modification of Cys-374 with a fluorescent dye provides a simple method for the C-terminus labeling of actin. After partial cleavages of the cross-linked product of actin and an actin-binding protein, the cross-linking site was identified just by visualizing fragments containing the actin C-terminus. By this technique, the cross-linking site was identified in the actin sequence *from its C-terminus*. The N-terminal segment of actin (residues 1–12) was identified as participating in binding myosin heavy chain (Sutoh, 1982a), depactin (Sutoh & Mabuchi, 1984), cofilin (Muneyuki et al., 1985), and fragmin (Sutoh & Hatano, 1986). The C-terminal segment of actin (residues 357–375) was also identified as the binding site for myosin light chain (Sutoh, 1982a) and depactin (Sutoh & Mabuchi, 1984).

Since mapping of the binding site was carried out from the C-terminus of actin by use of the C-terminal probe alone, identification of the site close to the C-terminal region was necessarily based on indirect argument, especially when there were more than two binding sites in the actin sequence (Sutoh & Mabuchi, 1984). To avoid difficulty in the mapping procedure, it is desirable to map the binding site both *from the C-terminus and from the N-terminus* of actin by both C-terminal and N-terminal probes. For the probe of the N-terminus of actin, we used an antibody raised against its N-terminal 18 residues (Bulinski et al., 1983). The epitope of the antibody is actually within its N-terminal 12 residues and possibly in the N-terminal 7 residues containing an acidic residue cluster (Hopp & Wood, 1981). Since all cleavage reactions used in this study (CNBr, BNPS-skatole, and hydroxylamine cleavages) digest actin peptide at positions closer to the C-terminus than the epitope, the antibody can be used as the N-terminal probe. By the use of the fluorescent label at Cys-374 as the C-terminal probe and the antibody as the N-terminal probe, now we can map the binding site of an actin-binding protein both from the C-terminus and from the N-terminus of actin.

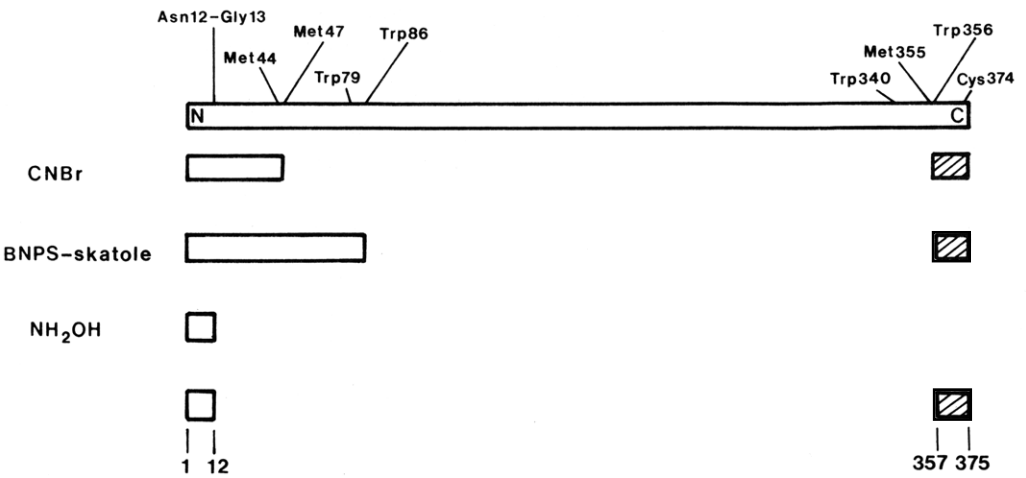

FIGURE 7: Mappings of cross-linking sites of depactin in the actin sequence by three different chemicals. The top bar represents an actin polypeptide chain in which some residues referred to in the text are indicated. Open bars under the actin polypeptide chain are actin segments that participate in cross-linking with depactin to form the 60KU product. Cross-hatched bars are actin segments that participate in cross-linking with depactin to form the 60KL product.

Depactin is a small actin-depolymerizing protein (M_r 17 000) isolated from starfish oocytes (Mabuchi, 1981, 1982, 1983). Previously we have shown that the N-terminal and C-terminal segments of actin participate in binding depactin by using the C-terminal probe alone and without separating the two types of cross-linked products (60KU and 60KL) (Sutoh & Mabuchi, 1984). In this paper, we reinvestigated the actin–depactin interaction to demonstrate the feasibility of the improved method.

The EDC cross-linking of the one-to-one actin–depactin complex generated two types of cross-linked products with slightly different apparent molecular weights (60KL and 60KU). The N-terminal probe unequivocally revealed a cross-linking site of depactin at the C-terminal segment of actin (residues 357–375), while the C-terminal probe revealed another site at its N-terminal segment (residues 1–12). Cross-linking of the N-terminal segment of actin with depactin generated one of the cross-linked products (60KU). Cross-linking of the C-terminal segment of actin with depactin generated the other product (60KL). Cross-linking at different points along actin and depactin polypeptides seems to generate two types of cross-linked products which are separable by gel electrophoresis. The same phenomenon was observed for the cross-linking of actin and myosin subfragment 1 (Sutoh, 1983). Since EDC cross-links Lys residue with Asp or Glu residue only when they are in direct contact, it is very likely that the cross-linking sites identified here are actually within the interface between actin and depactin. Thus we conclude that both termini of the actin polypeptide chain participate in binding depactin. Asp-1, Glu-2, Asp-3, Glu-4, and Asp-11 (Elzinga et al., 1973) in the N-terminal segment and Lys-359, Glu-361, Asp-363, Glu-364, and Lys-373 (Elzinga et al., 1973) in the C-terminal segment are candidates for residues in the binding site.

It seems that depolymerization of F-actin induced by depactin does not result from simple steric block of the actin–actin contact site by the binding protein. The N-terminal and C-terminal segments of actin identified as a part of the binding site of depactin also participate in binding myosin (Sutoh, 1982a,b, 1983). This is consistent with the previous finding that depactin competes for the binding to actin with myosin (Mabuchi, 1982). The binding site of myosin on F-actin seems to be located at the outer surface of the actin helix (Toyoshima & Wakabayashi, 1985a,b). Therefore, the bindng site of depactin identified here is also located at the outer surface of the F-actin helix, not at the actin–actin contact surface, though it is possible that the cross-linking reaction fails to detect another, if any, binding site of depactin located at the actin–actin contact surface. This notion is consistent with the finding that depactin binds to F-actin and actively sequesters actin subunit (Mabuchi, 1983).

It was previously shown that another actin-depolymerizing protein DNase I uses some residues in the CB 10 segment of actin (residues 48–82) as its binding site (Sutoh, 1984). It must be noted that the CB 10 segment contains Tyr-53 and Tyr-69, whose modifications results in the complete or partial loss of polymerizability of actin (Elzinga & Collins, 1972; Bender et al., 1976). Thus it is tempting to speculate that the actin segment recognized by DNase I participates in the actin–actin interaction and that DNase I inhibits the actin polymerization by a simple steric block mechanism. X-ray crystallography of the actin–DNase I complex has recently revealed that actin has two domains (Kabsch et al., 1985). A smaller domain contains Cys-10 as well as the major binding site for DNase I (Kabsch et al., 1985). The DNase I binding site on the domain is quite distant from Cys-10, which is close to or within the depactin-binding site. This is consistent with the above notion that depolymerization of F-actin by depactin is not induced by simple steric block of the actin–actin contact site.

In addition to the antibody against the N-terminal segment used here, we also have raised an antibody against the C-terminal region of actin by using a synthetic peptide corresponding to the C-terminal 10 residues of rabbit skeletal actin (K. Sutoh, unpublished result). Since the amino acid sequence of the C-terminal segment of actin is the same for almost all actins (Vandekerckhove & Weber, 1978a–c, 1984; Vandekerckhove et al., 1984), this antibody can be used as the C-terminal probe in place of the fluorescent label for almost all actins, though the fluorescent probe has its own merit, i.e., easier manipulation of cross-linked products. Unlike the antibody against the C-terminus, the antibody against the N-terminus used in the present study can recognize only the skeletal muscle α-actin because amino acid substitutions are most frequent in this N-terminal region [see Table I in Vandekerckhove and Weber (1984)]. However, it is possible to raise antibodies recognizing the N-terminal region of various actins by the use of synthetic peptides as antigens (Otey et al., 1986). These antibodies recognizing both termini of various actins would enable us to use crude preparations of actin-binding proteins or even in vivo systems for identifying binding sites of actin-binding proteins in the actin sequence. Thus the introduction of site-directed antibodies in the mapping procedure has significantly increased the flexibility of the technique for studying interactions between actin and actin-binding proteins.

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α-Helix-to-Random-Coil Transitions of Two-Chain, Coiled Coils: A Theoretical Model for the "Pretransition" in Cysteine-190-Cross-Linked Tropomyosin†

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ABSTRACT: The thermal unfolding curve for $\alpha\alpha$ tropomyosin in which the two chains are cross-linked at cysteine-190 shows two striking features that distinguish it from that of its counterpart for non-cross-linked molecules: (1) a "pretransition" at 25–50 °C and (2) a shift in the principal transition to higher temperature, but with the same steepness. Previously, the pretransition was explained by postulating that the cross-link produces local strains, yielding a pinched "bubble" of chain-separated random coil about C-190, whereas the rest of the coiled coil remains intact. Results from both enzymatic digestion kinetics and equilibrium calorimetric studies have been interpreted as consistent with the existence of such a bubble. To test this idea further, a theoretical model is devised whereby various physical features can be imposed and the resulting helix content and other properties calculated from the statistical mechanical theory of the helix–coil transition. Short-range interactions employed are the geometric mean values of those in α-tropomyosin. The helix–helix interaction free energy is also like that in α-tropomyosin, including its nonuniformity; i.e., it is made larger in the amino half of the molecule. Local strain is introduced by setting the helix–helix interaction to zero in a region about the cross-link. The results show that, alone, neither local strain nor nonuniformity serves to mimic the experiments. In concert, however, they reproduce all the main experimental features, if the strain is extensive ($\sim$29 residues) and somewhat dissymmetric. Theoretical helix probability profiles, however, show that no bubble of unfolded chains forms about the cross-link. Instead, in the pretransition, residues unfold from the weakly interacting end (residue 284) in to, but not through, the cross-link at C-190. The theory also indicates that the augmented stability for the principal transition occurs largely as a result of loop entropy. The same strain and nonuniformity are then employed to explore the effects of other possible cross-link positions. The thermal curves are shown to depend markedly on cross-link location. The curves are discussed in terms of loop entropy, which has drastic, long-range effects. Under appropriate circumstances it can produce, in the coiled-coil model, a thermal transition that is essentially all or none.

Two-chain, coiled-coil proteins have a strikingly simple molecular architecture. The two constitutent polypeptide chains are each wound in an α-helix, the helices are set side by side in parallel and in register, and the pair is given a slight supertwist (Fraser & MacRae, 1973). The structural integrity of such molecules has been the subject of a great many investigations (Cohen & Szent-Györgyi, 1957; Noelken, 1962; Noelken & Holtzer, 1964; Woods, 1969; Halsey & Harrington, 1973; Chao & Holtzer, 1975; Lehrer, 1978; Williams & Swenson, 1981; Potekhin & Privalov, 1982; Holtzer et al., 1983; Graceffa & Lehrer, 1984; Skolnick & Holtzer, 1985; Stafford, 1985). This interest arises partly because the simplicity of the structure makes it an attractive model system for elucidation of structure-stabilizing interactions in proteins but also partly because local helix-to-random-coil transitions have often been postulated as essential biochemical events in

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