# *Acanthamoeba* Actin and Profilin Can Be Cross-linked between Glutamic Acid 364 of Actin and Lysine 115 of Profilin

**Joel S. Vandekerckhove,* Donald A. Kaiser,‡ and Thomas D. Pollard‡**

*Laboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Gent, Belgium; ‡Department of Cell Biology and Anatomy, Johns Hopkins Medical School, Baltimore, Maryland 21025

***Abstract.*** *Acanthamoeba* profilin was cross-linked to actin via a zero-length isopeptide bond using carbodiimide. The covalently linked 1:1 complex was purified and treated with cyanogen bromide. This cleaves actin into small cyanogen bromide (CNBr) peptides and leaves the profilin intact owing to its lack of methionine. Profilin with one covalently attached actin CNBr peptide was purified by gel filtration followed by gel electrophoresis and electroblotting on polybase-coated glass-fiber membranes. Since the $NH_2$ terminus of profilin is blocked, Edman degradation gave only the sequence of the conjugated actin CNBr fragment beginning with Trp-356. The profilin-actin CNBr peptide conjugate was digested further with trypsin and the cross-linked peptide identified by comparison with the tryptic peptide pattern obtained from carbodiimide-treated profilin. Amino-acid sequence analysis of the cross-linked tryptic peptides produced two residues at each cycle. Their order corresponds to actin starting at Trp-356 and profilin starting at Ala-94. From the absence of the phenylthiohydantoin-amino acid residues in specific cycles, we conclude that actin Glu-364 is linked to Lys-115 in profilin. Experiments with the isoforms of profilin I and profilin II gave identical results. The cross-linked region in profilin is homologous with sequences in the larger actin filament capping proteins fragmin and gelsolin.

PROFILINS are small actin-binding proteins expressed in high concentrations in the cytoplasm of nonmuscle cells (Carlsson et al., 1977; Reichstein and Korn, 1979; for reviews, see Stossel et al., 1985; Pollard and Cooper, 1986). Actin bound to profilin does not assemble normally into filaments, and it is therefore generally believed that profilin acts as an actin monomer buffer accounting for much of the unpolymerized actin in the cytoplasm. The mechanism of action is complicated and a full understanding will ultimately require knowledge of the structures of both profilin and actin and in particular the contact regions between these molecules.

In this paper, we describe biochemical experiments that establish at the level of the specific amino acids one contact site between the two proteins that can be cross-linked by a carbodiimide zero-length crosslinker. These experiments were carried out with profilins and actin isolated from *Acanthamoeba castellanii.* This organism contains one major actin type (Vandekerckhove et al., 1984) and at least three isoforms of profilin called profilin IA, profilin IB, and profilin II. Profilins IA and IB differ in sequence at only 5 of the 125 residues (Ampe et al., 1985). The basic isoform, profilin II can be separated from profilin I (Kaiser et al., 1986) and differs from profilin I at a minimum of 19 different residues (Ampe et al., 1987; Pollard and Rimm, manuscript submitted for publication). Most of these differences involve conserved residues and are concentrated in the middle third part of the molecule.

In spite of these structural differences, both profilins are indistinguishable in functional assays (Kaiser et al., 1986), suggesting that their actin-binding sites form strictly conserved regions. Both isoforms of *Acanthamoeba* profilin were chemically cross-linked to actin by a zero-length isopeptide bond. The cross-linked residues and their surrounding sequences in both profilin and actin were identified by sequencing purified cross-linked peptides. The cross-linking site in actin is glutamic acid -364, located near the COOH terminus. Lysine -115 of profilin is the other side of the cross-link. This residue is surrounded by sequences similar to those present in other actin-binding proteins (Ampe and Vandekerckhove, 1987).

## Materials and Methods

### Reagents

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)[1] and *N*-hydroxylsulfosuccinimide (NHS) were purchased from Pierce Chemicals (Rockford, IL). Both these reagents were made up at concentrations of 50 mM just before use in 2 mM potassium phosphate buffer and the pH adjusted to 7.5. Cyanogen bromide (CNBr) came from Fisher Chemicals (Pittsburgh, PA). Trypsin, glycine, 2-(*N*-morpholino)ethanesulfonic acid (MES), iodoacetamide, polyproline, 2-mercaptoethanol, DTT, and imidazole were purchased

1. *Abbreviations used in this paper*: DEAE, diethylaminoethyl; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid; NHS, *N*-hydroxylsulfosuccinimide; P-A, profilin-actin; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

from Sigma Chemical Co. (St. Louis, MO). Guanidine-HCl and ammonium sulfate were from Schwarz-Mann Biotech (Cleveland, OH). Solvents used for HPLC were obtained from Carlo Erba (Milano, Italy). Other chemicals were reagent grade. The polyproline agarose column was prepared by the method of Tuderman et al. (1975) as modified by Tanaka and Shibata (1985).

### *Protein Purification*

Profilin I and profilin II were purified from *Acanthamoeba castellanii* according to Kaiser et al. (1986) except that the final column was 1 × 60-cm bed of CM cation exchange resin (Amicon Corp., Danvers, MA) equilibrated with MES (pH 6.0). Profilin I does not bind to the column. Profilin II was eluted with a 0–1.2 M gradient of KCl in 10 mM MES. Actin was purified from *Acanthamoeba* by the method of Pollard (1984).

### *Chemical Cross-linking*

Actin and profilin were dialyzed at 4°C versus 2 mM potassium phosphate (pH 7.5) and combined at a 1:1 molar ratio in concentrations of 20–25 μM. For example, large preparations consisted of 1 μmol of each protein in a total volume of 45 ml. Samples were warmed to 20°C and EDC and NHS were added to concentrations of 1 mM. After 10 min, EDC and NHS were added again to raise their concentrations to 2 mM. After a further 20 min, 200 mM glycine was added to a final concentration of 10 mM to quench the reaction.

### *Amino-acid Sequencing*

The amino-acid sequence was determined by gas-phase sequence analysis using a type 470A sequenator equipped with an on-line phenylthiohydantoin amino-acid analyzer type 120A (Applied Biosystems Inc., Foster City, CA). The sequenator was run according to manufacturer's instructions.

Sequences of the profilin-actin (P-A) 14-kD and P-A 16-kD complexes were determined from material electroblotted on glass-fiber membranes (Bauw et al., 1987). Briefly, sheets of glass-fiber paper GF/C (Whatman Inc., Clifton, NJ) were cut to the size of the gel and immersed for 5 min in an aqueous solution containing 300 mg poly(4-vinyl-*N*-methylpyridinium iodide) and 10 mg of DTT/100 ml. Before blotting, sheets were washed in 100 ml distilled water to remove excess of unbound polybase. Electroblotting was carried out essentially as described for immunoblotting using 50 mM Tris, 50 mM boric acid as transfer buffer. The immobilized protein was detected by a dilute fluorescamine stain (1 mg/liter acetone) and visualized under UV light. Protein spots were excised and mounted in the reaction chamber of the gas-phase sequenator.

## *Results*

For orientation, the overall strategy and results of our experiments are represented schematically in Fig. 1. Actin and profilin are chemically cross-linked and the conjugated complex separated from free monomers by passage over a DEAE and polyproline column (Fig. 1, *A–D*). CNBr cleaves the cross-linked actin at multiple sites but leaves the profilin intact because of the absence of methionine, the result of which is a conjugate between profilin and the linked actin CNBr fragment. This conjugate can be separated from the smaller actin fragments because of its larger size (Fig. 1, *D–F*). Amino-acid sequence analysis on the total conjugate or on the cross-linked tryptic peptide identifies the amino acids participating in the covalent bond between the two protein molecules.

### *Cross-linking of Actin and Profilin*

When treated with EDC and NHS, *Acanthamoeba* actin and profilin are covalently cross-linked to each other with a yield of ~20% (Fig. 2, lane *B*). The electrophoretic band with an apparent molecular mass of 55 kD consists of a 1:1 complex of actin and profilin, given its size and the fact that it reacts on immuno-blots with antibodies to both actin and profilin (data not shown). We will refer to this 55-kD conjugate as the 55-kD A-P complex throughout the paper.

We have examined the cross-linking conditions in some detail. Both EDC and NHS are required for a good yield of cross-linked proteins. NHS alone does not promote cross-linking, but improves the yield obtained with EDC alone. Reagent concentrations of 1–2 mM were found to be optimal by testing a wide range of concentrations. The acylisourea formed by the reaction of EDC with protein carboxyl groups is unstable in water. NHS reacts with this intermediate to form a reactive ester that is stable in water but reacts readily with protein amino groups to form a stable amide cross-link (Staros et al., 1986). Dialysis of the proteins versus 2 mM phosphate buffer for as little as 2 h improves the yield of cross-linked protein compared with the usual actin monomer buffers containing ATP, imidazol, and DTT. This effect of phosphate has not been investigated in detail. The cross-linking yield is also dependent on the concentration of both proteins, particularly that of actin.

### *Purification of the Cross-linked Complex of Actin and Profilin*

The A-P complex was first subjected to DEAE chromatography, which removes all of the free profilin and partially separates the A-P complex from free actin (Fig. 2, lane *C*, and Fig. 3 *A*). The free profilin obtained at this point was used as the source of profilin treated with the same cross-linking chemicals as the 55-kD A-P complex and served as the control in comparing tryptic peptide patterns (see below; Fig. 7). Affinity chromatography on polyproline agarose removes essentially all of the free actin, yielding highly purified 55-kD A-P complex (Fig. 2, lane *D*, and Fig. 3 *B*). Both free profilin from the DEAE column and the A-P complex bound quantitatively to polyproline agarose, showing that the cross-linking reaction did not affect the polyproline binding activity. After elution of the A-P complex from polyproline with 5 M guanidine, the actin cysteines were reduced to reverse spurious disulfides formed during previous manipulations and alkylated with iodoacetamide (Craven et al., 1965). Finally, the samples were dialyzed exhaustively against water and then lyophilized.

### *Cleavage at Methionine and Purification of the Products*

Since *Acanthamoeba* profilins have no methionine, it was possible to cleave the *Acanthamoeba* actin in the A-P complex at its 14 methionine residues (Vandekerckhove et al., 1984) with cyanogen bromide leaving the profilin intact. The cyanogen bromide cleavage was carried out in the dark in 4 ml of 70% formic acid with 380 mM cyanogen bromide (Steers et al., 1965). After 20 h at room temperature, the sample was diluted with 5 vol of water and lyophilized. The peptides were resuspended in water and relyophilized. In addition to smaller actin peptides at the bottom of the gel, the main product had a molecular mass of ~14 kD (Fig. 2, lane *E*). It corresponded to intact profilin with a covalently bound actin peptide of ~20 residues. There was also a small amount of a 16-kD polypeptide (marked with brackets in Fig. 2, lane *E*) that also reacted with antibodies to profilin.

The CNBr-digest was fractionated by gel filtration on a P-60

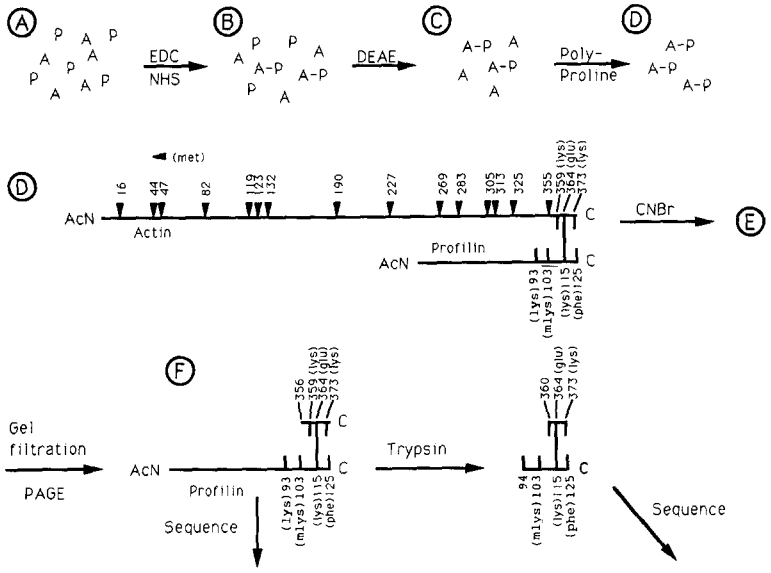



*Figure 1.* Schematic showing the overall experimental design and results.

column (Bio Gel; Bio-Rad Laboratories, Cambridge, MA) (Fig. 4). Two major protein peaks elute close to the exclusion volume and contain profilin as assayed by ELISA. Analysis on a 20% SDS-polyacrylamide gel of aliquots of the pooled fractions revealed equal amounts of the 14- and 16-kD complex, in the first peak and a major 14-kD band present in the second peak (Fig. 5). Each protein band was quantitated by in situ hydrolysis of the immobilized proteins followed by amino acid analysis (Vandekerckhove et al., 1985). Taking into account the amounts applied to the gels, the yield of the 16-kD complex is <10% of the 14-kD complex, so that the 16-kD complex could not be characterized in detail at this time (see also below).

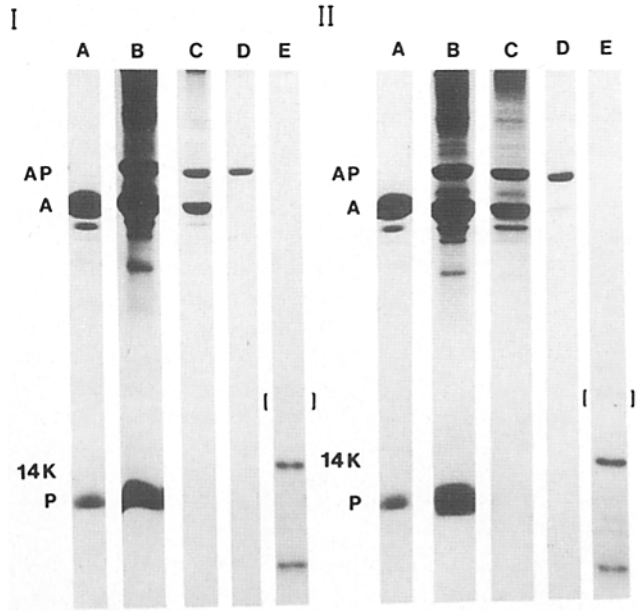



*Figure 2.* Analysis of actin-profilin cross-linking and isolation of cross-linked peptides by PAGE in sodium dodecyl sulfate. *I* is a preparation with profilin I. *II* is a preparation with profilin II. Proteins were separated by electrophoresis in 20% gels. *A*, actin; *P*, profilin; *AP*, cross-linked actin-profilin complex. Lanes *A* show actin and profilin standards. The weaker band below actin in lanes *A* represents a proteolytic degradation product of actin (the actin core). Lanes *B* show the products formed by reacting a 1:1 mixture of actin and profilin with EDC and NHS as described in Materials and Methods. Lanes *C* show the retained peak from the DEAE-cellulose column (see Fig. 3 *a*). Lanes *D* show the fraction bound to polyproline agarose and eluted with 5 M guanidine-HCl (see Fig. 3 *B*). Lanes *E* show the products of cyanogen bromide digestion of the 55-kD actin-profilin complex. Brackets highlight the faint 16-kD band.

### *Sequence Analysis of the A-P Complex*

The gel-separated A-P complexes were electroblotted on glass-fiber membranes and stained with fluorescamine as described by Bauw et al. (1987).

Each of the blots was subjected to gas-phase sequencing. The results are illustrated in Fig. 6, showing the successive HPLC-chromatograms of the on-line PTH(phenylthiohydantoin)-amino acid analyses of cycles 1 through 10 of the 14-kD A-P complex (Fig. 5, lane *C*). The deduced amino-acid sequence (W-I-S-K-E-E-Y-D) corresponds with a cleavage at the most COOH-terminal methionine residue of the actin molecule and indicates that at least one of the 20 COOH-terminal residues of actin was cross-linked to profilin. Note that no sequence was obtained from the conjugated profilin which has a blocked $NH_2$ terminus and no methionine (Fig. 1 *F*). The same $NH_2$ terminal sequence was also observed in the 14-kD A-P band and in the 16-kD A-P band derived from the second gel filtration peak (Fig. 5, lane *B*). We were surprised that the 16-kD A-P band gave only a single amino acid at each cycle, since we expected a second sequence from the additional 2-kD peptide accounting for the molecular mass difference between the 14-kD and 16-kD bands. This result may be because of an $NH_2$ terminal terminally blocked second actin fragment but also to secondary cross-links or other modifications that may have shifted the mobility of a fraction of the 14-kD molecules.

### *Identification and Sequence Analysis of the Cross-linked Tryptic Peptide*

Another aliquot (50 $\mu$g) of the peak containing the major 14-

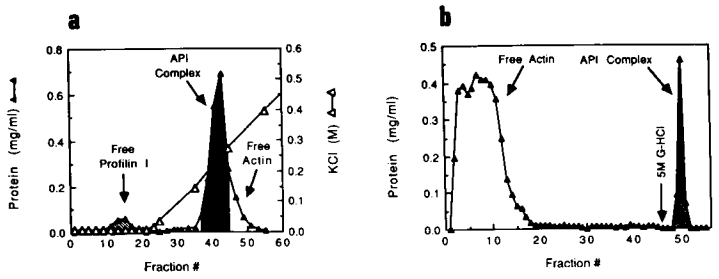



*Figure 3.* Purification of the cross-linked profilin I actin complex. *a*, a 1.5 × 30 cm column of DEAE-cellulose was equilibrated at 2 m hydrostatic pressure with 5 mM imidazole (pH 7.5), 0.1 mM $CaCl_2$, 2 mM DTT at 4°C. The sample consisted of 48 ml of the cross-linking reaction dialyzed overnight versus the column buffer. The sample was washed on with 20 ml of column buffer and bound proteins eluted with a 300-ml gradient of 0–0.6 M NaCl in column buffer. Fractions of 6 ml were collected and analyzed for protein by the Bradford (1976) method and by gel electrophoresis. The voided fractions contained all of the free profilin. The bound fractions all consisted of mixtures of 55-kD actin-profilin complex and free actin, with the complex enriched on the leading edge of the main peak and actin enriched on the trailing side of the peak. The shaded region of the eluted peak indicate the fractions pooled (16 ml) and carried on to the polyproline agarose column. The unbound fractions (*lightly shaded*) were used as the profilin control for analyzing tryptic peptides of the actin-profilin complex by sequencing. *B*, a 1.5 × 11.0 cm column of polyproline agarose (Fig. 3 *b*) was equilibrated with 0.1 M NaCl, 0.1 mM DTT, 0.1 M glycine, 10 mm Tris-HCl (pH 8.0). The sample was washed on with 400 ml of column buffer and the bound fractions eluted with 5 M guanidine HCl, 200 mM ammonium bicarbonate (pH 8.1), 50 mM DTT. Fractions of 7.5 ml were collected and analyzed for protein and by gel electrophoresis. The peak of 55-kD actin-profilin complex that was bound to the column (Fig. 2, lane *D*) was then reduced and alkylated with iodoacetamide before cleavage with cyanogen bromide.

kD complex (Fig. 4, peak 2) was digested with trypsin and the cross-linked tryptic peptide purified. The yield from tryptic digestion was unusually low (~10%) which may be explained by extensive modification of the lysine side chains during the carbodiimide treatment. The resulting peptides were separated by reversed-phase HPLC (Fig. 7 *A*). This separation pattern was compared with a similar chromatogram obtained from a tryptic digest of EDC-treated profilin used as blank (see above; Figs. 2 and 3 *A*). The chromatograms differ in a few minor peptides and in three major peptides (Fig. 7, *arrows*). The minor peptides were not further investigated. The 14-kD A-P-specific major peptide shows, as expected for a cross-linked peptide, a double sequence: one corresponding with the previously recognized actin COOH-terminal sequence starting at Trp-356 and a second equally intensive sequence that by difference was found compatible with the COOH-terminal tryptic peptide starting at residue 94 of profilin (Fig. 8). This cross-linked peptide was sequenced over 25 cycles confirming the expected residues in each of the two individual strands except for cycles 9 and 22, where the predicted glutamic acid (in actin) and lysine (in profilin) were not found. These data indicate that the two peptides are linked with each other via the actin glutamic acid-364 and the profilin lysine-115, in agreement with the

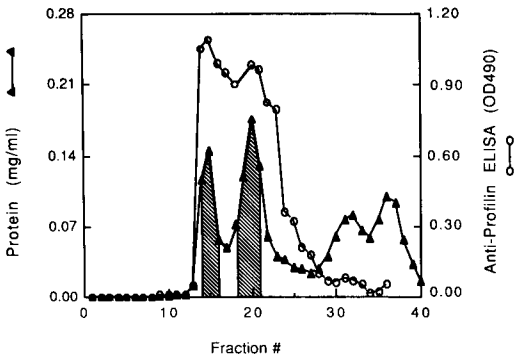



*Figure 4.* Gel filtration of the cyanogen bromide digest of purified 55-kD A-PI complex. The lyophilized digest was resuspended in 0.4 ml 70% formic acid and sonicated 3 times for 30 s each to disperse the precipitate. Then 0.4 ml of 70% formic acid and 0.8 ml 25% acetic acid were added before centrifuging for 5 min in a microfuge (Eppendorf made by Brinkmann Instruments Inc., Westbury, NY) to pellet a small amount of insoluble material. The supernatant was applied to a 1.5 × 50 cm P-60 column (Bio Gel; Bio-Rad Laboratories) equilibrated and eluted with 25% acetic acid at a hydrostatic pressure of 50 cm. Fractions of 1.1 ml were collected and analyzed for protein and antiprofilin immunoreactivity by ELISA assay with a monoclonal antibody (Kaiser et al., 1986). The details of this method have been submitted for publication (Kaiser, D. A., B. Levin, P. Goldschmidt-Clermont, and T. D. Pollard, 1989). Two pools were made (*shaded regions*) and used for sequence analysis.


*Figure 5.* Glass-fiber electroblotting of the gel-purified 14-kD and 16-kD A-PI complexes. Aliquots of the CNBr fragments (pooled peak fractions shown in Fig. 4) were further purified by SDS-PAGE in a 20% gel. The separated spots were electroblotted on poly-(4-vinyl-*N*-methylpyridinium iodide)-coated glass-fiber sheets and stained with fluorescamine. The major band was excised and mounted on the gas-phase sequenator. Lane *A* shows the reference mixture (Bio-Rad Laboratories) with (*from top to bottom*): BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme. Lanes *B* and *C* show the fragments present in the first and second peak respectively of Fig. 4; lane *D*, *Acanthamoeba* profilin (Fig. 3 *A*).

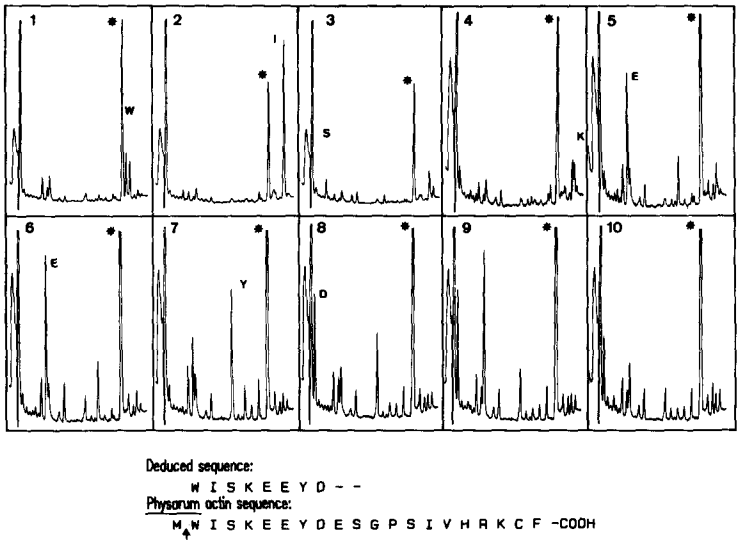



*Figure 6.* Amino-acid sequence analysis of the electroblotted 14-kD profilin I actin CNBr fragment. The stepwise liberated phenylthiohydantoin (PTH) amino acids were identified by an on-line PTH analyzer (Applied Biosystems Inc.) operated according to the manufacturer's instructions. The HPLC chromatograms of cycles 1–10 are shown. The identified residues are indicated by the one-letter notation. Capital letters indicate the allocated residues at each cycle. The asterisk indicates the position of the reaction by-product diphenylthiourea.

known cross-linking specificity of carbodiimides. The absence of tryptic cleavage at the peptide bond 359–360 is because of the two acidic residues following the potential cleavage site COOH terminal of Lys-359 and was previously also noticed during structural analysis of the intact actin molecule (Vandekerckhove et al., 1984). The length of the COOH-terminal profilin peptide is explained by the lack of cleavage at the trimethyllysine residue at position 103 and at the cross-linked lysine at site 115. The cross-linked peptide accounts for the sum of the two major peptides specific for the profilin chromatogram (Fig. 7 *B arrows*). These two peaks correspond with peptides 94–115 and 116–125. The other peptides in the profilin chromatogram are derived from different parts of the profilin molecule (results not shown). This indicates that the very limited digestion was not the result of insolubility of a part of the molecule but rather of a general protease resistance of the protein resulting from the EDC modification side reactions. This excludes the possibility that specific cross-linked peptides would have gone undetected in the insoluble core.

A similar analysis was also carried out with profilin II. No differences were noticed with the cross-linking results of profilin I.

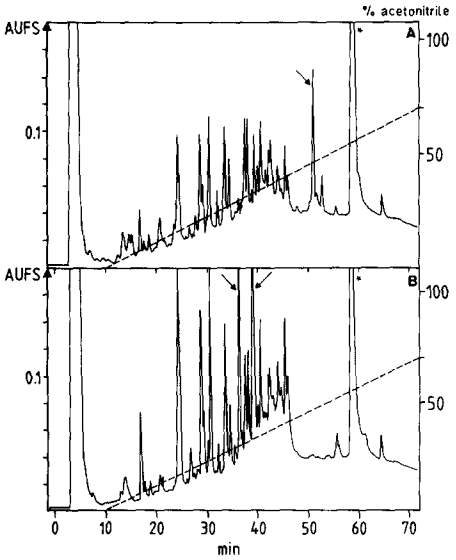
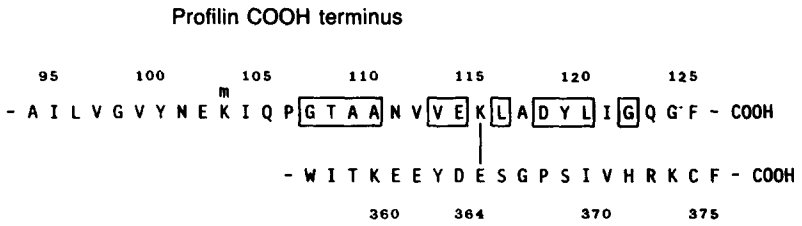



*Figure 7.* HPLC patterns of tryptic peptides of the 14-kD A-PI complex (*A*) and EDC-treated profilin I (*B*). The cross-linked proteins dissolved in 25% acetic acid (see Fig. 4) were dialyzed against water and the solution was made 0.5% in $NH_4HCO_3$ with a 5% solution. Trypsin was added at an enzyme to substrate ratio of 1–20 (by weight) and the digestion continued for 4 h at 37°C and finished by acidification with trifluoroacetic acid (TFA) (final concentration 1%). The solution was centrifuged for 5 min in a tube (Eppendorf made by Brinkmann Instruments, Inc.) to remove insoluble material and the supernatant was loaded on a C4 (0.46 × 25 cm) reversed-phase column (Vydac, Separations Group, Hesperia, CA) equilibrated in 0.1% TFA (solvent A). Peptides were eluted with a linear gradient of solvent B (0.1% TFA, 70% acetonitrile) applied over a 60-min period and started after 10 min. These experiments were carried out with an HPLC apparatus (Waters Associates, Milford, MA) consisting of a gradient controller model 680, two pumps (model 510), and a lambda max 481 detector measuring the eluate absorbancy at 215 nm (absorption units full scale). Note the presence of a major difference peptide eluting at a retention time of 51 min (*A*).

## Discussion

We have covalently linked *Acanthamoeba* profilin and actin using a water-soluble carbodiimide and *N*-hydroxylsulfosuccinimide, reagents that specifically link carboxyl groups to amino groups through isopeptidyl linkages. Since the cross-link is formed from the amino-acid side chains themselves, it is referred to as "zero-length" and requires close contact between the side chains in the complex of the two proteins. Thus, the high yield of cross-link between Glu-364 of actin and Lys-115 of profilin is strong evidence that these two side chains form an ionic bond when the two proteins bind together. It is important to stress that this contact must be only one of many that bind the two proteins together, although it is probably the only such contact where an amino group and carboxyl group are in close proximity. Since there are a large number of glutamates, aspartates, and lysines in the two proteins, the occurrence of one main cross-link is strong evidence that the coupling reaction has identified a specific contact site to the nearly total exclusion of spurious cross-links. A second specific cross-linked peptide is also produced, but




| Cycle No. | Profilin | | Actin | |
|---|---|---|---|---|
| | Amino acid | pmol | Amino acid | pmol |
| 1 | Ala | 210 | Trp | 100 |
| 2 | Ile | 370 | Ile | 370 |
| 3 | Leu | 195 | Thr | 50 |
| 4 | Val | 170 | Lys | 85 |
| 5 | Gly | 175 | Glu | 180 |
| 6 | Val | 160 | Glu | 175 |
| 7 | Tyr | 180 | Tyr | 180 |
| 8 | Asn | 140 | Asp | 100 |
| 9* | Glu | 130 | Glu | 130 |
| 10 | Lys (Me) | – | Ser | 40 |
| 11 | Ile | 120 | Gly | 120 |
| 12 | Gln | 140 | Pro | 100 |
| 13 | Pro | 140 | Ser | 30 |
| 14 | Gly | 160 | Ile | 100 |
| 15 | Thr | 80 | Val | 70 |
| 16 | Ala | 120 | His | 30 |
| 17 | Ala | 110 | Arg | 20 |
| 18 | Asn | 90 | Cys | – |
| 19 | Val | 70 | Phe | 10 |
| 20 | Val | 60 | | |
| 21 | Glu | 50 | | |
| 22* | Lys | – | | |
| 23 | Leu | 40 | | |
| 24 | Ala | 45 | | |
| 25 | Asp | 35 | | |

*Figure 8.* Summary of the sequence analysis of the cross-linked tryptic peptide. The analysis covers the sequence of the cross-linked tryptic peptide shown in Fig. 7 *A*. Amino acids are numbered according to their positions in the respective proteins. The isopeptide bond formed by EDC between lys 115 of profilin and glu 364 of actin is shown by a vertical line. Residues in the profilin sequence that are conserved in the homologous region of fragmin (Ampe and Vandekerckhove, 1987) are shown in boxes. The yields of the liberated PTH amino acids are listed in the accompanying table. The sequences starting from profilin 94 and actin 354, respectively, are listed. Cycles in which the two sequences are expected to generate the same residue are underlined. The asterisks indicate the cross-linked residues. Lys (Me) or $^{m}K$ stand for trimethyllysine.

its occurrence in low yields has prevented us from identifying the involved residues.

The participation of profilin Lys-115 in the covalent cross-link establishes that actin binds near the C terminus of profilin. This is in accord with the sequence homology found in the COOH-terminal halves of yeast (Oechsner et al., 1987) and *Acanthamoeba* (Ampe et al., 1988) profilins. Both proteins are 50% identical in the last 50 residues. With the addition of the sequence data of the vertebrate profilins (Nyström et al., 1979; Ampe et al., 1988; Kwiatkowski and Bruns, 1988), it is now obvious from the pattern of conservative substitutions that all the proteins named profilin had a common ancestor. Interestingly, none of the other profilins that have been sequenced have a lysine at the position equivalent to lysine -115 of the *Acanthamoeba* profilins. Although the absence of this residue may account for the differences in the properties of various profilins, lysine -115 cannot be considered for holding the two proteins together. More likely, the binding of profilin and actin involves a number of residues in the general region of lysine -115. In this complex, the Σ amino group of lys -115 may be the only amino group in close proximity to a carboxyl group.

It is even more intriguing to compare the COOH terminus of *Acanthamoeba* profilin with other actin-binding proteins. As noted by Ampe and Vandekerckhove (1987), there is a region of ~30 residues flanking Lys-115 of the *Acanthamoeba* profilins that is homologous with sequences from both the 42-kD actin filament severing protein from *Physarum* called fragmin (or Cap42[a]) and the 90-kD severing protein from vertebrates called gelsolin. *Dictyostelium* severin, a relative of fragmin, also has such a homologous sequence (André et al., 1988). In each case, the homologous sequences are located ~100 residues from the $NH_2$ terminus near the end of a 14-kD domain that is repeated 3 or 6 times in these larger proteins (Kwiatkowski et al., 1986). In fact, this 14-kD $NH_2$ terminal domain of gelsolin can be isolated by restricted proteolytic cleavage and retains the ability to bind to actin (Yin et al., 1988). Although the residue corresponding to Lys-115 is absent from these homologous proteins, we think that it is possible that they all have the same common ancestor that gave rise to the profilins and by gene duplication to the larger actin-binding proteins. This hypothesis predicts that the region corresponding to the *Acanthamoeba* profilin residues 95–125 represents an ancient actin-binding site.

Our evidence for contact between the COOH termini of

actin and profilin confirms indirect evidence based on effects on the stability of the A-P complex produced by proteolytic removal or chemical modification of the extreme COOH-terminal residues of actin (Malm et al., 1983; Malm, 1984). Further, binding of *Acanthamoeba* profilin enhances the fluorescence of either pyrene or rhodamine bound to the cys-374 of muscle or *Acanthamoeba* actin (Lee et al., 1988). Both of these effects could have been mediated by conformational changes in the actin some distance from the actual binding site, but the cross-linking results strongly suggest that the profilin makes direct contact with the COOH terminus of actin.

The identification of the profilin binding site on actin provides some clues about the differences in affinity that profilins have for muscle and cytoplasmic actins. Most investigators (Tobacman and Korn, 1981; Tseng and Pollard, 1982; Larsson and Lindberg, 1988) have found that profilins bind to cytoplasmic actin more strongly than muscle actin under conditions where actin polymerizes (but for an exception, see Lee et al., 1988). The COOH terminal region (last 20 residues) of actin is not absolutely conserved and has stepwise accumulated differences during the evolution from primitive eukaryotes to the muscles of vertebrates (Table I). One of the three known changes (Ser → Ala, 365) is adjacent to Glu-364 that forms the ion bridge to profilin Lys-115. These differences may well contribute to the lower affinity of muscle actin for profilin.

Like gelsolin and severin, *Acanthamoeba* profilin binds to the barbed end of actin filaments (Pollard and Cooper, 1984), so the cross-linking results suggest that the COOH terminus of actin is located at the barbed end of the filament. First, it has been clear for several years that actin Cys-374 contacts another actin subunit in filaments, since it can be cross-linked to Lys-191 of an adjacent actin molecule (Elzinga and Phelan, 1984). Second, Cys-374 interacts with tropomyosin (Moir and Levine, 1986), a protein that binds to the surface of the actin filaments, so one can conclude that the COOH terminus of actin is located on the side of the filament rather than along its axis. This has also been suggested by cross-linking of myosin light chains (Sutoh, 1982), depactin (Sutoh and Mabuchi, 1984), and fragmin (Sutoh and Hatano, 1986) to the COOH terminal region of actin (Sutoh and Mabuchi, 1986) and by experiments with antibodies to the COOH terminus of actin (Boyer et al., 1987). Third, the interpretation of low resolution electron density maps of the actin-DNase complex (Kabsch et al., 1985) is consistent with the COOH terminus of actin being located in the larger of the two domains of the molecule and Millonig et al. (manuscript in preparation) have evidence that the larger domain is located at the barbed end of the filament. Thus, all of the available evidence is in agreement that the COOH terminus of actin is located near the surface at the barbed end of filaments, but this hypothesis needs further testing since none of the evidence is really definitive.

In conclusion, we have shown that *Acanthamoeba* profilin and actin have at least one contact site near their COOH termini involving profilin *Lys*-115 and actin *Glu*-364. Together with our current efforts to determine the three-dimensional structure of profilin (Magnus et al., 1988), this provides the first detailed description of an actin-binding site on a cytoplasmic protein. Sequence homology in this region in other actin-binding proteins (Ampe and Vandekerckhove, 1987) provides evidence that this is a primitive actin-binding site that is widely distributed in nature.

*Table I. The Contact Sites of Actin with Acanthamoeba Profilin: an Amino-Acid Sequence Comparison in the Different Isoforms*

| Actins | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | * | | | | | | | | | | | |
| I | S | K | E | E | Y | D | E | S | G | P | S | I | V | H | R | K | C | F (1) |
| I | S | K | Q | E | Y | D | E | S | G | P | S | I | V | H | R | K | C | F (2) |
| I | S | K | Q | E | Y | D | E | A | G | P | S | I | V | H | R | K | C | F (3) |
| I | T | K | Q | E | Y | D | E | A | G | P | S | I | V | H | R | K | C | F (4) |

The COOH-terminal sequences (residues 357–375) of *Acanthamoeba* (1), vertebrate cytoplasmic (2), smooth muscle (3), and skeletal muscle (4) actins are shown. The asterisks indicate the position of the glutamic acid, cross-linked to Acanthamoeba profilin. Boxes highlight the stepwise accumulated substitutions during evolution from lower eukaryotes to higher eukaryotes around the cross-linked glutamic acid.

We gratefully thank Dr. M. De Cock for her help in the preparation of this manuscript, J. Van Damme and M. Puype for assistance in the determination of the amino-acid sequences, and Professor M. Van Montagu for his support. J. Vandekerckhove is Research Associate of the Belgian National Fund for Scientific Research (N.F.W.O.).

This investigation was supported by grants from the Belgian N.F.W.O., the Commission of European Communities, and a National Institutes of Health Research Grant GM-26338 to T. D. Pollard.

Received for publication 21 December 1988 and in revised form 29 March 1989.

### *References*

Ampe, C., and J. Vandekerckhove. 1987. The F-actin capping proteins of *Physarum polycephalum*: cap42(a) is very similar, if not identical, to fragmin and is structurally and functionally very homologous to gelsolin; cap42(b) is *Physarum* actin. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:4149–4157.

Ampe, C., J. Vandekerckhove, S. L. Brenner, L. Tobacman, and E. D. Korn. 1985. The amino acid sequence of *Acanthamoeba* profilin. *J. Biol. Chem.* 260:834–840.

Ampe, C., M. Sato, T. D. Pollard, and J. Vandekerckhove. 1987. The primary structure of the basic isoform of *Acanthamoeba* profilin. *Eur. J. Biochem.* 170:597–601.

Ampe, C., F. Markey, U. Lindberg, and J. Vandekerckhove. 1988. The primary structure of human platelet profilin: reinvestigation of the calf spleen profilin sequence. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 228:17–21.

André, E., F. Lottspeich, M. Schleicher, and A. Noegel. 1988. Severin, gelsolin, and villin share a homologous sequence in regions presumed to contain F-actin severing domains. *J. Biol. Chem.* 263:722–727.

Bauw, G., M. De Loose, D. Inzé, M. Van Montagu, and J. Vandekerckhove. 1987. Alterations in the phenotype of plant cells studied by $NH_2$-terminal amino-acid sequence analysis of proteins electroblotted from two-dimensional gel-separated total extracts. *Proc. Natl. Acad. Sci. USA.* 84:4806–4810.

Boyer, M., J. Feinberg, H. K. Hue, J. P. Capony, Y. Benyamin, and C. Routstan. 1987. Antigenic probes locate a serum-gelsolin-interaction site on the C-terminal part of actin. *Biochem. J.* 248:359–364.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Carlsson, L., L.-E. Nystrom, F. Sundkvist, F. Markey, and U. Lindberg. 1977. Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* 115:465–483.

Craven, G. R., E. Steers, and C. B. Anfinsen. 1965. Purification, composition, and molecular weight of the β-galactosidase of *Escherichia coli* K12. *J. Biol. Chem.* 240:2468–2477.

Elzinga, M., and J. J. Phelan. 1984. F-actin is intermolecularly cross linked by N,N′-p-phenylene-dimaleimide through lysine-191 and cysteine-374. *Proc. Natl. Acad. Sci. USA.* 81:6599–6602.

Kabsch, W., G. Mannherz, and D. Suck. 1985. Three-dimensional structure of the complex of actin and DNase I at 4.5 Å resolution. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2113–2118.

Kaiser, D. A., M. Sato, R. Ebert, and T. D. Pollard. 1986. Purification and characterization of two isoforms of *Acanthamoeba* profilin. *J. Cell Biol.*

102:221–226.
Kwiatkowski, D. J., and G. A. P. Bruns. 1988. Human profilin. Molecular cloning, sequence comparison and chromosomal analysis. *J. Biol. Chem.* 263:5910–5915.
Kwiatkowski, D. J., T. P. Stossel, S. H. Orkin, J. E. Mole, H. R. Colten, and H. L. Yin. 1986. Plasma and cytoplasmic gelsolins are encoded by a single gene and contain a duplicated actin-binding domain. *Nature (Lond.).* 323:455–458.
Larsson, H., and U. Lindberg. 1988. The effect of divalent cations on the interaction between calf spleen profilin and different actins. *Biochim. Biophys. Acta.* 953:95–105.
Lee, S., M. Li, and T. Pollard. 1988. Evaluation of the binding of *Acanthamoeba* profilin to pyrene-labeled actin by fluorescence enhancement. *Anal. Biochem.* 168:148–155.
Malm, B. 1984. Chemical modification of *cys*-374 of actin interferes with the formation of the profilactin complex. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 173:399–402.
Malm, B., H. Larsson, and U. Lindberg. 1983. The profilin actin complex further characterization of profilin and studies on the stability of the complex. *J. Muscle Res. Cell Motil.* 4:569–588.
Moir, A. J. G., and B. A. Levine. 1986. Protein cognitive sites on the surface of actin: a proton NMR study. *J. Inorg. Biochem.* 28:271–278.
Nyström, L.-E., U. Lindberg, J. Kendrick-Jones, and R. Jakes. 1979. The amino acid sequence of profilin from calf spleen. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 101:161–165.
Oechsner, U., V. Magdolen, and W. Bandlow. 1987. The cDNA and deduced amino-acid sequence of profilin from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 15:9078.
Pollard, T. D. 1984. Polymerization of ADP-actin. *J. Cell Biol.* 99:769–777.
Pollard, T. D., and J. A. Cooper, 1984. Quantitative analysis of the effect of *Acanthamoeba* profilin on actin filament nucleation and elongation. *Biochemistry.* 23:6631–6641.
Pollard, T. D., and J. A. Cooper. 1986. Actin and actin-binding proteins. *Annu. Rev. Biochem.* 55:987–1035.
Reichstein, E., and E. D. Korn. 1979. *Acanthamoeba* profilin, a protein of low molecular weight from *Acanthamoeba castellani* that inhibits actin nucleation. *J. Biol. Chem.* 254:6174–6179.
Staros, J. V., R. W. Wright, and D. M. Swingle. 1986. Enhancement by *N*-hydroxylsulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Anal. Biochem.* 156:220–222.
Steers, E., G. R. Craven, C. B. Anfinsen, and J. L. Bethune. 1965. Evidence for nonidentical chains in the β-galactosidase of *Escherichia coli* K12. *J. Biol. Chem.* 240:2478–2484.
Stossel, T. P., C. Chaponnier, R. Ezzell, J. H. Hartwig, P. Janmey, D. Kwiatkowski, S. Lind, D. Smith, R. S. Southwick, H. L. Yin, and K. S. Zaner. 1985. Nonmuscle actin binding proteins. *Annu. Rev. Cell. Biol.* 1:353–402.
Sutoh, K. 1982. Identification of myosin-binding sites on the actin sequence. *Biochemistry.* 21:3654–3661.
Sutoh, K., and I. Mabuchi. 1984. N-terminal and C-terminal segments of actin participate in binding depactin, an actin-depolymerizing protein from starfish oocytes. *Biochemistry.* 23:6757–6761.
Sutoh, K., and S. Hatano. 1986. Actin-fragmin interactions as revealed by chemical cross-linking. *Biochemistry.* 25:435–440.
Sutoh, K., and I. Mabuchi. 1986. 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. *Biochemistry.* 25:6186–6192.
Tanaka, M., and H. Shibata. 1985. Poly(L-proline)-binding proteins from chick embryos are profilin and profilactin. *Eur. J. Biochem.* 151:291–297.
Tobacman, L., and E. D. Korn. 1982. The regulation of actin polymerization and the inhibition of monomeric actin ATPase activity by *Acanthamoeba castellanii* profilin. *J. Biol. Chem.* 257:4166–4170.
Tseng, P., and T. Pollard. 1982. Mechanism of actin of *Acanthamoeba* profilin: demonstration of actin species specificity and regulation by micromolar concentrations of $MgCl_2$. *J. Cell Biol.* 94:213–218.
Tuderman, L., E. Kuutti, and K. Kivirikko. 1975. An affinity-column procedure using poly(L-proline) for the purification of prolyl hydroxylase. *Eur. J. Biochem.* 52:9–16.
Vandekerckhove, J., A. A. Lal, and E. D. Korn. 1984. Amino-acid sequence of *Acanthamoeba* actin. *J. Mol. Biol.* 172:141–147.
Vandekerckhove, J., G. Bauw, M. Puype, J. Van Damme, and M. Van Montagu. 1985. Protein-blotting on polybrene-coated glass-fiber sheets. A basis for acid hydrolysis and gas-phase sequencing of picomole quantities of protein previously separated on sodium dodecyl sulfate/polyacrylamide gel. *Eur. J. Biochem.* 152:9–19.
Yin, H. L., K. Iida, and P. A. Janmey. 1988. Identification of a polyphosphoinositide-modulated domain in gelsolin which binds to the sides of actin filaments. *J. Cell Biol.* 106:805–812.