

Purification and Characterization of Two Isoforms of *Acanthamoeba* Profilin

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Abstract. *Acanthamoeba* profilin purified according to E. Reichstein and E. D. Korn (1979, *J. Biol. Chem.* 254:6174–6179) consists of two isoforms (profilin-I and -II) with approximately the same molecular weight and reactivity to a monoclonal antibody but different isoelectric points and different mobilities on carboxymethyl-agarose chromatography and reversed-phase high-performance liquid chromatography. The isoelectric points of profilin-I is ~5.5 and that of profi-

lin-II is ≥ 9.0 . Tryptic peptides from the two proteins are substantially different, which suggests that there are major differences in their sequences. At similar concentrations, both profilins prolong the lag phase at the outset of spontaneous polymerization and inhibit the extent of polymerization. Both forms also inhibit elongation weakly at the barbed end and strongly at the pointed end of actin filaments.

PROFILIN is a small protein that is thought to regulate actin polymerization in cells by forming a nonpolymerizable complex with actin monomers (Carlsson et al., 1976). Profilins have now been identified in many cell types (Ozaka and Hatano, 1984; Nishida et al., 1984; Dinubile and Southwick, 1985) including *Acanthamoeba* (Reichstein and Korn, 1979), where it is present in very high concentrations throughout the cytoplasm (Tseng et al., 1984). The effects of *Acanthamoeba* profilin on actin polymerization have been analyzed in detail (Tobacman and Korn, 1982; Tseng and Pollard, 1982; Tobacman et al. 1983; Pollard and Cooper, 1984). There is some disagreement as to whether a simple monomer sequestration or a more complex mechanism is necessary to explain the available data (see Pollard and Cooper, 1984 and Lal and Korn, 1985).

Acanthamoeba profilin consists of 125 amino acids and has partial sequence homology with vertebrate profilin (Ampe et al., 1985). Two different amino acids were found at five positions in the amino acid sequence, showing that there are at least two isoforms of *Acanthamoeba* profilin. We will refer to these proteins as profilin-IA and profilin-IB. All of these variable residues are uncharged, so it is not surprising that the isoforms of profilin-I co-purified and were not resolved by either gel electrophoresis in SDS or isoelectric focusing (Reichstein and Korn, 1979; Tseng et al., 1984). Both of these techniques also suggested that profilin prepared according to Reichstein and Korn (1979) is free of other components.

Here we report that the preparations of *Acanthamoeba* profilin used in previous studies consist not only of the two similar isoforms detected by amino acid sequencing (Ampe et al., 1985) but also another form (profilin-II) that can be separated from the major form (profilin-I) by cation exchange chromatography. Isoelectric focusing and tryptic peptide map-

ping both indicate major differences in the sequences of profilin-I and profilin-II. Nevertheless, both of these profilins react with a monoclonal antibody and inhibit actin polymerization in the same way.

Materials and Methods

Protein Purification

We purified profilin from sucrose extracts of *Acanthamoeba* by a modification (Tseng et al., 1984) of the method of Reichstein and Korn (1979) using chromatography on DEAE-cellulose, ammonium sulfate precipitation, chromatography on hydroxylapatite, and gel filtration on Sephadex G-75. Profilin-I and profilin-II were separated by isocratic cation exchange chromatography on a 1×51 cm column of carboxymethyl-agarose (Bio-Rad CM Biogel A, 100–200 mesh, Bio-Rad Laboratories, Richmond, CA) in 10 mM Pipes, pH 6.5. Actin was purified from *Acanthamoeba* (Pollard, 1984). A mouse monoclonal antibody to *Acanthamoeba* profilin was produced and characterized using the methods of Kiehart et al. (1984).

Biochemical Methods

Our methods for cell culture, gel electrophoresis, electrophoretic blotting, and antibody staining have been described by Tseng et al. (1984). We used an extinction coefficient of $1.2 \text{ cm}^{-2}\text{mg}^{-1}$ at 280 nm (Tseng et al., 1984) to determine the concentration of both profilin isoforms. This may slightly underestimate profilin-II since it binds ~20% less Coomassie Blue on SDS-gels. Nonequilibrium isoelectric focusing was carried out at 10°C with 5–10 W constant power with 0.5-mm-thick 1% agarose slab gels cast on Gel-Bond film (Marine Colloids Div., FMC Corp., Springfield, NJ) with 1% Pharmolyte ampholines, pH 3–9 (Sigma Chemical Co., St. Louis, MO) on an LKB Multiphor II electrophoresis unit (LKB Instruments Inc., Gaithersburg, MD) until current reached a minimum value (usually 3–9 mA).

Tryptic peptides were prepared by digestion of 2.15 mg/ml profilin-I or 0.69 mg/ml profilin-II in 2 M urea, 10 mM Pipes buffer (pH 6.5) with 1% (wt/wt) *N*-tosyl-L-phenylalanine chloromethyl ketone-trypsin (from bovine pancreas, 12,100 U/mg; Sigma Chemical Co.) for 18 h at 30°C, when an identical amount of fresh trypsin was added and the incubation continued for 8–20 h. The resulting peptides were analyzed by reversed-phase high-performance liquid

chromatography (HPLC)¹ on a Varian 5560 liquid chromatograph with a UV-200 variable wavelength detector, 8085 autosampler, and CDS-402 data station (Varian Associates, Inc., Palo Alto, CA). The stationary phase was a Vydac TP-C4 column (1.0 × 25 cm, The Separations Group, Hesperia, CA). The initial mobile phase consisted of 90% eluant A (0.1% aqueous trifluoroacetic acid) and 10% eluant B (acetonitrile) at 2 ml/min. 5 min after sample injection a linear gradient (1%/min) of eluant B was started and continued for 40 min. The column was washed with 65% eluant B before being returned to the initial conditions, and samples were injected every 82 min. Peak elution times between runs varied by <6 s.

Actin Polymerization Assays

Absolute rates of elongation at both ends of *Limulus* acrosomal processes were measured by electron microscopy (Pollard and Cooper, 1984). The time course of spontaneous polymerization was determined by 90° light scattering at 400 nm in a spectrofluorimeter. The extent of polymerization of actin was measured in two ways: (a) Viscosity of 0.75-ml samples was measured at 25°C in Cannon-Manning semi-microviscometers (size 150) from Cannon Instrument Co. (State College, PA). (b) 170- μ l samples were ultracentrifuged at 23 psi in a Beckman Airfuge (Beckman Instruments Inc., Palo Alto, CA) for 30 min at 22°C. Actin in the original sample and in 80 μ l of supernatant was measured by densitometry of SDS PAGE stained with Coomassie Blue. The stained actin obeyed Beer's Law in the range of 0 to 1.2 μ M, where we worked.

Results

Purification and Chemical Properties of Profilin-I and Profilin-II

Nonequilibrium isoelectric focusing (Fig. 1) separates our preparations of *Acanthamoeba* profilin into two different components, a major component with an isoelectric point of ~5.5 and a minor component that is much more basic. The basic component has an isoelectric point of ≥ 9 and apparently ran off the end of the focusing gels in the earlier work of Reichstein and Korn (1979) and Tseng et al. (1984). The ratio of the two components is about 4:1 in conventional preparations of *Acanthamoeba* profilin (Fig. 1, lanes A and F) and in crude extracts of the amoeba (Fig. 1, lane D).

We separated the two components in the profilin preparation by isocratic cation exchange chromatography on carboxymethyl-agarose (Fig. 2, A). Providing that the protein in the two peaks is chromatographed on Sephadex G-75 to remove large contaminants either before or after (Fig. 2, B) the cation exchange column step, the two components are pure by isoelectric focusing (Fig. 1, lanes B, C, G, and H), gel electrophoresis in SDS (Fig. 3, lanes E and F), and chromatography on the reversed-phase HPLC column (not shown). Under the HPLC conditions used for peptide mapping (see below), the neutral component and the basic component elute as single, well-separated peaks at 45.0 and 44.0 min, respectively. Treatment of the neutral component with alkaline phosphatase or neuraminidase did not affect its elution time on the reversed-phase column. The basic component has a slightly lower mobility on the SDS gels (Fig. 3). Since both components react with mouse monoclonal antibody AP-1 (Figs. 1 and 3), and they have very similar effects on actin polymerization (see below), we named the neutral component profilin-I and the basic component profilin-II.

Tryptic peptides of profilin-I and profilin-II show that the molecules have substantially different primary structures (Fig. 4). Tryptic peptides of profilin-I separated on reversed-phase HPLC into 12 major peaks (Fig. 4A) and profilin-II into nine major peaks (Fig. 4B). There are coincident peaks at 18.8,

¹ Abbreviation used in this paper: HPLC, high-performance liquid chromatography.

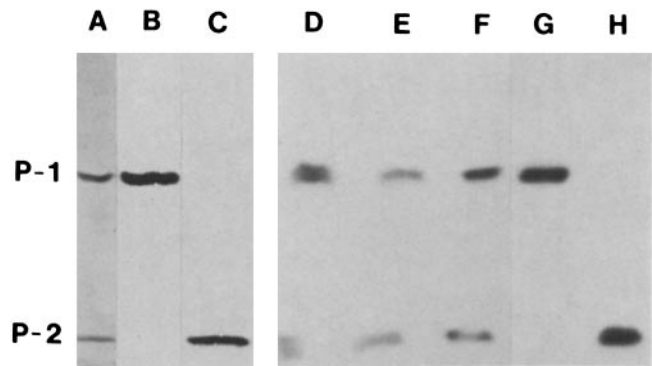


Figure 1. Isoelectric focusing of samples of *Acanthamoeba* profilins. P-1 and P-2, profilin-I and -II, respectively. Lanes A-C, stained with Coomassie Blue. Lane A, conventional profilin prepared according to Reichstein and Korn (1979). Lane B, purified profilin-I. Lane C, purified profilin-II. Lanes D-H, autoradiograms of immunoblots stained with monoclonal antibody AP-1. Lane D, crude extract. Lane E, DEAE fraction of profilin. Lane F, profilin purified according to Reichstein and Korn (1979). Lane G, purified profilin-I. Lane H, purified profilin-II.

19.4, and 19.6 min, but the 15 remaining peptides elute in different positions. These differences cannot be explained solely on the basis of posttranslational modifications of either protein unless one assumes complete derivatization of a given amino acid with the prosthetic group, an unlikely possibility. We conclude from these data that profilin-I and profilin-II have substantially different amino acid sequences.

The recently published sequence of *Acanthamoeba* profilin (presumably the sequence of profilin-I; Ampe et al., 1985) predicts that proteolysis by trypsin at the basic amino acids should produce eight peptides from both profilin-IA and -IB. If our peptide mapping procedure can discriminate between all tryptic peptides of profilin-I with two possible sequences (of which there are four), then the predicted number of profilin-I peptides increases to 12, the number observed in the present study. However, additional structural data will be required before these results can be interpreted as supporting the dual sequence described by Ampe et al. (1985). The extra peptides may also arise from incomplete cleavage at some basic residues.

Both isoforms of profilin have ultraviolet absorption spectra typical of proteins with absorption maxima at 278 nm, shoulders at 290 nm, and minima at 249 nm. The absorption ratio at 280/260 nm is 1.81 for profilin-I and 1.92 for profilin-II, so neither form, especially the more basic profilin-II, is likely to have a bound nucleotide.

Effects of Profilin Isoforms on Actin Polymerization

In spite of major differences in charge and primary structure, profilin-I and profilin-II have almost indistinguishable effects on the polymerization of *Acanthamoeba* actin (Figs. 5-7). These experiments were done with unlabeled *Acanthamoeba* actin because modification of cysteine-374 reduces the effect of profilin on polymerization (Malm, 1984; Lal and Korn, 1985).

Both profilin-I and profilin-II inhibit the rate and extent of spontaneous polymerization from monomers to the same degree (Fig. 5). As shown previously in detailed quantitative studies with mixtures of these isoforms (Tobacman et al., 1983; Pollard and Cooper, 1984), the prolonged lag phase is

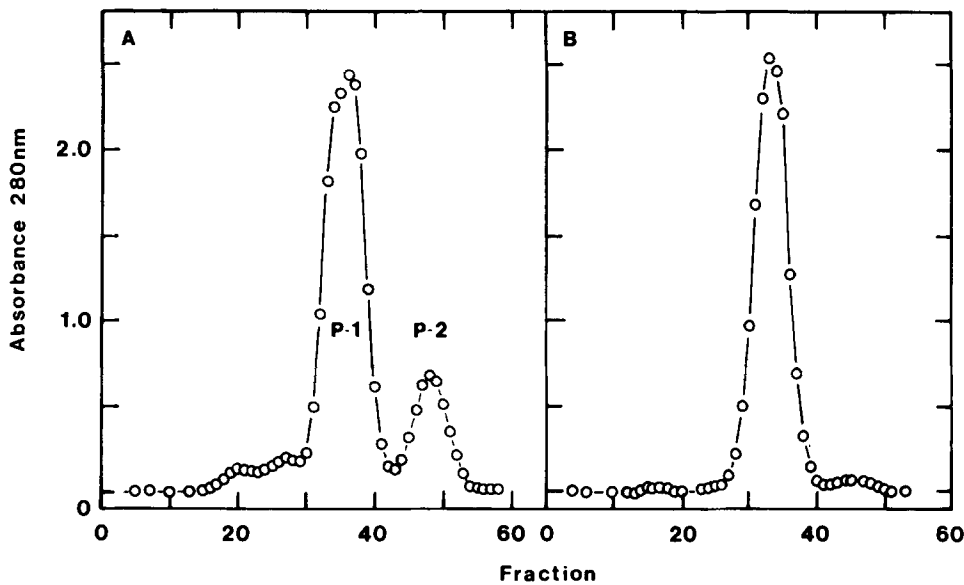


Figure 2. Chromatography of *Acanthamoeba* profilins. (A) Isocratic cation exchange chromatography on a 1×51 cm column of Bio-Rad carboxymethyl-agarose in 10 mM Pipes (pH 6.5) at 4°C . Sample: 1.5 ml containing ~ 26 mg of protein that bound to neither DEAE-cellulose or hydroxylapatite columns. These profilin-containing fractions were concentrated by precipitation with 2.4 M ammonium sulfate. Fraction size is 1.3 ml. The void volume is fraction 10. The total column volume is fraction 30, so both of the major peaks are retarded on the column. The two minor peaks of unbound material are minor contaminants that do not react with profilin antibodies. The major peak is profilin-I (P-1). The last peak is profilin-II (P-2). (B) Gel filtration of profilin-I from the carboxymethyl-agarose column on a 1×53 cm column of Sephadex G-75 in 10 mM Pipes (pH 6.5). Fraction size is 1.15 ml.

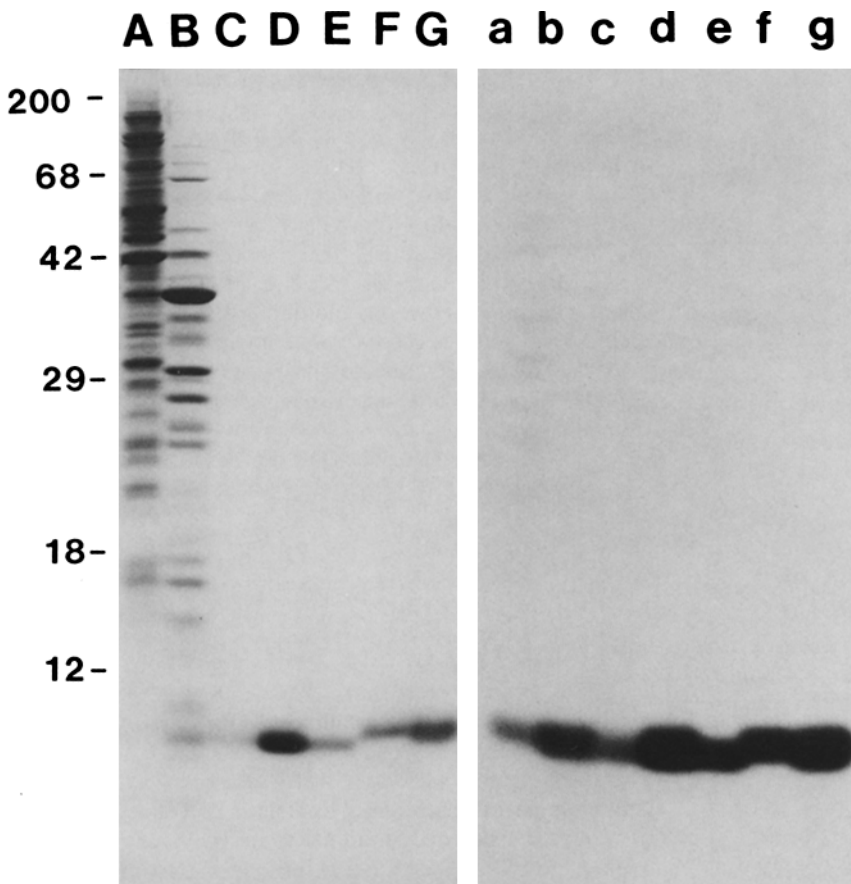


Figure 3. Gel electrophoresis in SDS of samples containing *Acanthamoeba* profilins. Lanes A-G, gel stained with Coomassie Blue. Lanes a-g, autoradiogram of an immunoblot stained with monoclonal antibody AP-1. Lanes A and a, crude extract. Lanes B and b, DEAE fraction. Lanes C and c, hydroxylapatite fraction. Lanes D and d, profilin purified according to Reichstein and Korn (1979). Lanes E and e, purified profilin-I. Lanes F and f, purified profilin-II. Lanes G and g, mixture of profilin-I and profilin-II.

attributable largely to inhibition of nucleation by profilin.

In steady state experiments using viscosity or pelleting of filaments to assess the extent of polymerization, profilin-I, profilin-II, and the natural mixture of the two isoforms all

inhibited polymerization to approximately the same extent (Fig. 6). Using the critical concentration of actin alone as the free monomer concentration in the presence of profilin and the shift in the critical concentration in the presence of profilin

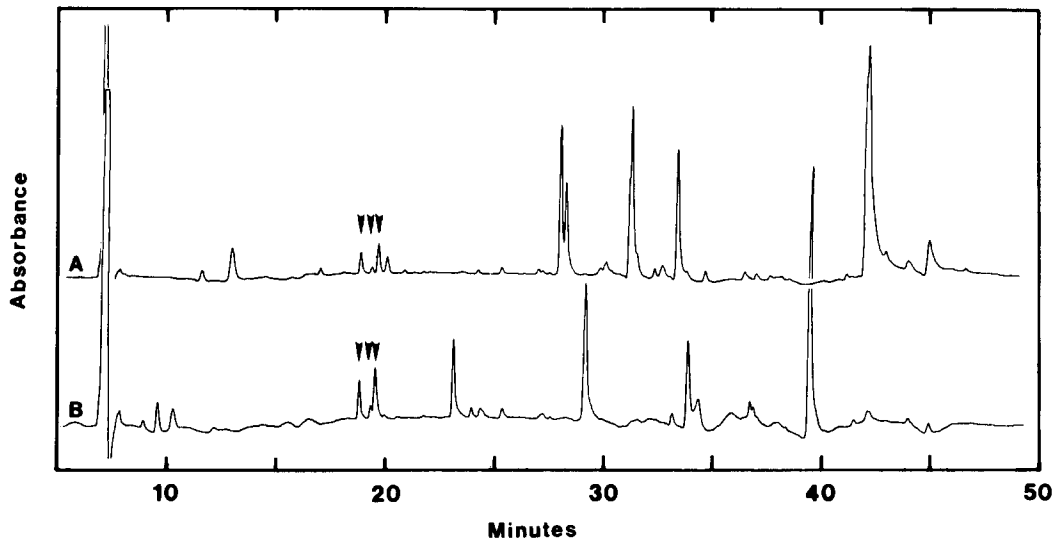


Figure 4. Analysis of tryptic peptides by HPLC. Trypsin digestion and chromatographic conditions are described in Materials and Methods. Peptides were detected by absorbance at 210 nm using a correction for absorbance by the mobile phase. Profile *A*, profilin-I (108 μg in 50 μl) plotted with 0.256 OD full scale. Profile *B*, profilin-II (34 μg in 50 μl) plotted with 0.128 OD full scale. Arrowheads denote coincident peaks.

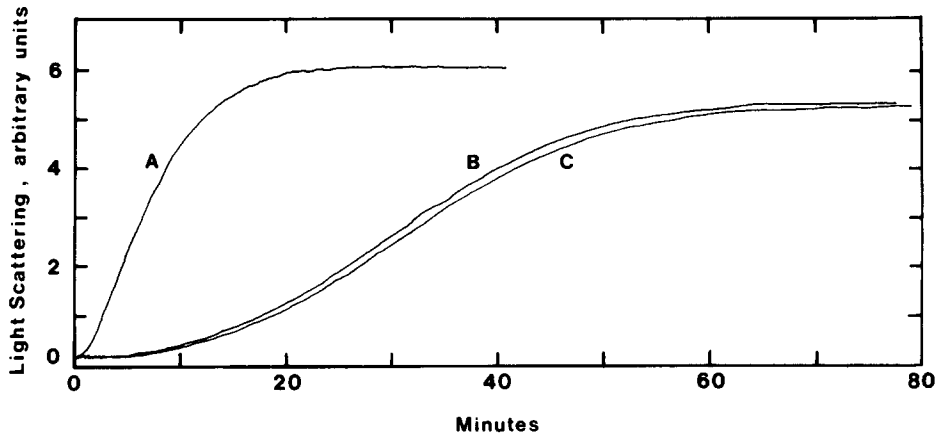


Figure 5. Effect of profilin isoforms on the time course of polymerization of *Acanthamoeba* actin. Polymer concentration was measured by 90° light scattering at 400 nm. Conditions: 25°C, 5 μM *Acanthamoeba* actin, 50 mM KCl, 1 mM MgCl_2 , 1 mM EGTA, 10 mM imidazole, 4 mM Pipes (final pH 7.1), 0.1 mM ATP, 0.25 mM dithiothreitol, 0.1 mM CaCl_2 . Curve *A*, no profilin. Curve *B*, 10 μM profilin-I. Curve *C*, 10 μM profilin-II.

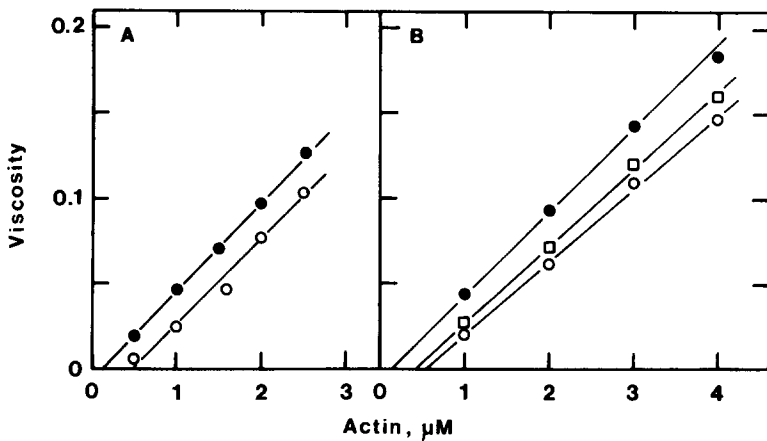


Figure 6. Effect of profilin isoforms on the steady state viscosity of *Acanthamoeba* actin. (*A*) ●, control. ○, 25 μM profilin purified according to Reichstein and Korn (1979) containing $\sim 20 \mu\text{M}$ profilin-I and $\sim 5 \mu\text{M}$ profilin-II. Conditions: 25°C, 50 mM KCl, 1 mM MgCl_2 , 13.5 mM imidazole (pH 7), 0.25 mM dithiothreitol, 0.1 mM ATP, 0.1 mM CaCl_2 . (*B*) ●, control. □, 20 μM profilin-I. ○, 20 μM profilin-II. Conditions: 25°C, 50 mM KCl, 1 mM MgCl_2 , 11.5 mM imidazole, 4.5 mM Pipes (final pH 7.1), 0.25 mM dithiothreitol, 0.1 mM ATP, 0.15 mM CaCl_2 . For both experiments polymerization was promoted by including 0.5 μM polymerized actin in each sample. The viscosities of the profilins alone were subtracted from the viscosities of the corresponding mixtures with actin.

as the concentration of the complex of actin-profilin (Tseng and Pollard, 1982; Tobacman and Korn, 1982), the data in Fig. 6 give dissociation constants of 10.0 μM for the mixture, 7.0 μM for profilin-I, and 9.7 μM for profilin-II. A parallel pelleting experiment gave K_d 's of 10 μM for both purified isoforms.

Like the mixture of isoforms (Fig. 7*A*), both profilin-I and profilin-II inhibit the rate of elongation at both ends of actin filaments (Fig. 7*B*). The experiment in Fig. 7*A* extends ex-

periments originally described by Pollard and Cooper (1984), in which the mixture of profilin isoforms inhibited elongation weakly at the barbed end and strongly at the pointed end. In Fig. 7*A* we show for the first time the effect of unfractionated profilin mixture on growth as a function of the concentration of actin. At all actin concentrations tested 50 μM profilin inhibits barbed end growth by $\sim 50\%$, so the apparent K_d is 50 μM if one assumes either simple or complex mechanisms (see Pollard and Cooper, 1984). At the pointed end there was

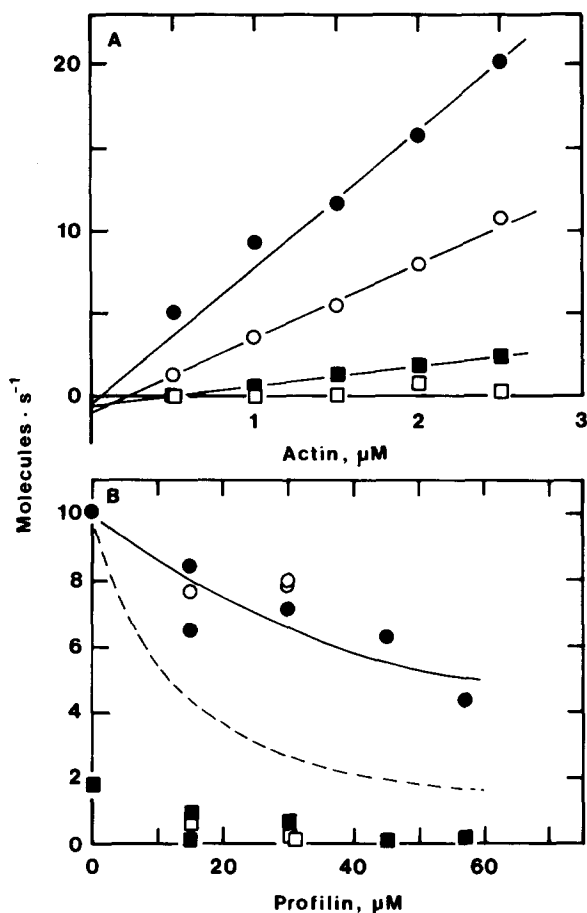


Figure 7. Effect of profilin isoforms on the rate of elongation of *Acanthamoeba* actin filaments from the ends of *Limulus* acrosomal processes. The rates were obtained from the mean lengths of filaments at either two (A) or three time points (B). (A) ● and ○, barbed end, ■ and □, pointed end. Filled symbols, controls. Open symbols, 50 μM profilin purified according to Reichstein and Korn (1979), containing a mixture of profilin-I and profilin-II. The line through the points for the barbed end without profilin (●) is drawn through a critical concentration of 0.1 μM actin, which was determined separately in steady state experiments like those in Fig. 6. Conditions: 22°C, 50 mM KCl, 1 mM MgCl_2 , 1 mM EGTA, 12.5 imidazole (pH 7), 0.12 mM dithiothreitol, 50 μM ATP, 50 μM CaCl_2 . (B) Dependence of the elongation rates on the concentration of purified profilin isoforms. ● and ○, barbed end. ■ and □, pointed end. Filled symbols profilin-I. Open symbols, profilin-II. The controls with zero profilin are the means from three separate experiments. The other points are individual determinations. Conditions: 22°C, 50 mM KCl, 1 mM MgCl_2 , 11 mM imidazole, 6.3 mM Pipes (final pH 7.1), 0.19 mM dithiothreitol, 75 μM CaCl_2 , 75 μM ATP. The smooth lines are theoretical curves for elongation rates assuming that only free actin adds to the barbed end and that the K_d for the actin-profilin complex is either 50 μM (continuous line) or 10 μM (dashed line).

no growth except at the highest concentrations of actin. At 2.5 μM actin <50% of the acrosomal processes grew any filaments at the pointed (slow) end, whereas 90% or more grew multiple filaments at the barbed (fast) end. Like the mixture of profilins (Fig. 7A), each of the isoforms also inhibits growth weakly at the barbed end (Fig. 7B). As shown by the theoretical curves, the data are compatible with a K_d of 50 μM and are inconsistent with a K_d of 10 μM . Both profilin-I and profilin-II inhibit growth at the pointed end more strongly than at the barbed end. Not only is the average rate slow, but the frequency of growth is depressed. For

example, in 30 μM profilin-II only 40% of the slow ends grew any filaments, and of those ends that did grow there were only 1 or 2 filaments, in contrast to the 10 in controls. At the barbed end 90 to 100% of the acrosomal processes grew filaments in controls and at all concentrations of profilin tested.

Discussion

Our data show that *Acanthamoeba* contains two forms of profilin that are both approximately the same size, react with a single monoclonal antibody, and inhibit actin polymerization in the same way, even though the two proteins have considerably different isoelectric points and almost unique tryptic peptides. These initial results suggest that the two forms of profilin have substantially different sequences, but this will have to be established directly. The microheterogeneity discovered when the mixture was originally sequenced (Ampe et al., 1985) cannot account for the major differences in the isoelectric points and tryptic peptides of the two purified isoforms, because those substitutions are largely isopolar and conservative. The minor basic isoform purified here was not detected in the sequencing studies. We conclude that there are probably two closely related variants of profilin-I (A and B) whose sequences were established by Ampe et al. (1985) and at least one considerably different form which we have named profilin-II. The observed differences in the charge of profilin-I and profilin-II are not due to phosphorylation since the major, more acidic species contains little or no phosphate (Tseng et al., 1984), and treatment with alkaline phosphatase did not alter its mobility on reversed-phase HPLC. Similarly, a bound nucleotide cannot account for the differences in isoelectric points since both isoforms have a high ratio of absorbance at 280/260 nm.

Work on vertebrate profilins suggests that there may also be neutral and basic isoforms of profilin in higher organisms. The original profilin isolated as a complex with actin from lymphoid organs (Carlsson et al., 1977) had an isoelectric point of from 9.2 to 9.4 (Nystrom et al., 1979). Profilin isolated from macrophages by a different method had an isoelectric point of 7.8 (Dinubile and Southwick, 1985).

The available functional experiments on purified profilin-I and profilin-II show that both inhibit actin polymerization in the same way, so previous more detailed work on mixtures of the isoforms (Tobacman, et al., 1983; Pollard and Cooper, 1984) has probably provided a good evaluation of their activities. This will, of course, have to be substantiated by future work with the individual components.

There is now agreement that the mixture of profilins as well as the two purified isoforms reduce the steady state extent of polymerization. Using the model of this process suggested by Tobacman and Korn (1982) and Tseng and Pollard (1982) where the shift in the critical concentration is the concentration of actin-profilin complex, we and Lal and Korn (1985) calculate an apparent K_d of 7 to 10 μM for the complex of actin with the mixture of profilins and each of the two purified isoforms. On the other hand, evaluation of growth by electron microscopy in this and previous papers (Tseng and Pollard, 1982; Tseng et al., 1984; Pollard and Cooper, 1984) always shows much weaker inhibition of elongation at the barbed end ($K_d \sim 50 \mu\text{M}$), as also observed with a profilin-like protein from *Thyone* (Tilney et al., 1983). Mixtures of profilin isoforms and the purified isoforms behave similarly in this assay. At the pointed end inhibition of the frequency and the rate

of elongation is stronger for all forms of profilin.

Although Lal and Korn (1985) found no reason to invoke complex mechanisms to explain their experiments with bulk samples of actin and profilin, our current work confirms and extends the previous electron microscopic studies of the effect of profilin on the elongation reaction. Thus, there remains a discrepancy between the weak inhibition by profilin of growth at the barbed end and its strong inhibition of nucleation, elongation at the pointed end, and the steady state extent of polymerization. One explanation that accounts for these findings is a complex model in which profilin and actin-profilin complexes can bind to and weakly cap the barbed end of filaments (Pollard and Cooper, 1984). This model and the simpler monomer sequestration model will have to be tested further with each of the purified profilin isoforms.

A final unanswered question is, Why does the cell produce three different profilins? The current evidence provides no clues, but the availability of two purified isoforms and selective antibodies will make it possible to look for the answer.

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