Physical, Immunochemical, and Functional Properties of *Acanthamoeba* Profilin

PETER C.-H. TSENG, MARSCHALL S. RUNGE, JOHN A. COOPER, ROBLEY C. WILLIAMS, JR.,* and THOMAS D. POLLARD
*Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and *Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235*

ABSTRACT *Acanthamoeba* profilin has a native molecular weight of 11,700 as measured by sedimentation equilibrium ultracentrifugation and an extinction coefficient at 280 nm of 1.4 $\times 10^4$ $M^{-1}cm^{-1}$. Rabbit antibodies against *Acanthamoeba* profilin react only with the 11,700 M_r polypeptide among all other ameba polypeptides separated by electrophoresis. These antibodies react with a 11,700 M_r polypeptide in *Physarum* but not with any proteins of *Dictyostelium* or *Naeglaria*. Antibody-binding assays indicate that ~2% of the ameba protein is profilin and that the concentration of profilin is ~100 μmol/liter cells. During ion exchange chromatography of soluble extracts of *Acanthamoeba* on DEAE-cellulose, the immunoreactive profilin splits into two fractions: an unbound fraction previously identified by Reichstein and Korn (1979, *J. Biol. Chem.*, 254:6174–6179) and a tightly bound fraction. Purified profilin from the two fractions is identical by all criteria tested. The tightly bound fraction is likely to be attached indirectly to the DEAE, perhaps by association with actin. By fluorescent antibody staining, profilin is distributed uniformly throughout the cytoplasmic matrix of *Acanthamoeba*. In 50 mM KCl, high concentrations of *Acanthamoeba* profilin inhibit the elongation rate of muscle actin filaments measured directly by electron microscopy, but the effect is minimal in KCl with 2 $MgCl_2$. By using the fluorescence change of pyrene-labeled *Acanthamoeba* actin to assay for polymerization, we confirmed our earlier observation (Tseng, P. C.-H., and T. D. Pollard, 1982, *J. Cell Biol.* 94:213–218) that *Acanthamoeba* profilin inhibits nucleation much more strongly than elongation under physiological conditions.

Profilin is a small protein, first isolated from mammalian lymphoid tissue (2), that has attracted considerable attention as a possible regulator of actin polymerization in nonmuscle cells. An initial study (2) suggested that mammalian profilin binds tightly to actin, completely blocking actin polymerization: spleen and thymus extracts yielded a 1:1 complex of profilin with actin that would not polymerize except under harsh conditions; and urea was required to dissociate the complex. Later, Blikstad et al. (1) found that a 2:1 molar excess of mammalian brain profilin completely prevented actin polymerization in 2 mM $CaCl_2$, 10 mM NaCl, but Grumet and Lin (6) found that mammalian profilin purified by another method inhibited, but did not prevent, the polymerization of muscle actin in $MgCl_2$. In recent experiments, Markey et al. (10) were able to polymerize the mammalian actin-profilin complex in $MgCl_2$. Several different types of nuclei stimulated polymerization.

Slightly smaller profilins with molecular weights of about 12,000 have been isolated from *Acanthamoeba* (16) and *Physarum* (13) without urea and found to inhibit the initial rate, but not the extent, of muscle actin polymerization in 2 mM $MgCl_2$ even at two- to threefold molar excess. More detailed studies by Tseng and Pollard (23) and Tobacman and Korn (21) established that *Acanthamoeba* profilin binds *Acanthamoeba* actin more strongly than muscle actin. In KCl and $CaCl_2$ the profilin inhibits the rate of elongation and therefore the extent of polymerization, as well as nucleation. These results could be explained by a simple mechanism. Profilin forms a 1:1 complex with actin that is inactive in all of the steps in actin polymerization. The two laboratories reported different results on the effect of profilin in the presence of $MgCl_2$: Tseng and Pollard (23) found that profilin strongly inhibits nucleation with only weak effects on the rate of elongation and the extent of polymerization; and Tobacman

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 JANUARY 1984 214–221

and Korn (21) reported that profilin inhibits both nucleation and elongation about equally. This difference has not yet been resolved. Ozaki et al. (13) obtained results with *Physarum* profilin similar to those of Tseng and Pollard with *Acanthamoeba* profilin. In 2 mM $MgCl_2$ *Physarum* profilin prolongs the lag phase but not the final extent of polymerization.

In addition to the ongoing biochemical analysis of the interaction of profilin with actin, there are important questions about the function of profilin in the cell that have not been approachable due to the lack of probes for the molecule. For example, there has been no assay for profilin to measure its cellular concentration, to follow its purification or to localize it in the cell. We report here the production and characterization of antibodies to profilin that fulfill these needs. We used profilin antibody binding assays to measure the profilin content of the cell and to identify two fractions of profilin separated by chromatography of ameba extracts on DEAE-cellulose. The first fraction, that does not bind to DEAE, was found previously and was used as the starting material for the original purification of *Acanthamoeba* profilin by Reichstein and Korn (16). The second fraction accounts for a large part of the total profilin that is lost during the Reichstein and Korn procedure. The profilin purified from this fraction is identical to the original profilin by all of the criteria tested. We also used the antibodies to localize profilin in the cell. Much of this work was presented at the 1981 and 1982 Annual Meetings of the American Society for Cell Biology (18, 24).

MATERIALS AND METHODS

Materials were purchased from the following sources: from Sigma Chemical Co., St. Louis, MO: Grade 1 ATP, grade 1 imidazole, EGTA, phenylmethylsulfonylfluoride, benzamidine, dithiothreitol, Triton X-100, and sodium azide; from Eastman Organic Chemicals, Rochester, NY: chloramine T; from Whatman Ltd., Springfield Mill, England: DE-52 (DEAE-cellulose); from Amersham Corp., Arlington Heights, IL: carrier-free ^{125}I sodium iodide. Difco Laboratories, Detroit, MI: complete and incomplete Freund's adjuvant. Aldrich Chemical Co., Milwaukee, WI: cyanogen bromide. Miles Laboratories, Elkhart, IN: "Pentex" sterile bovine serum albumin solution. LKB Instruments, Rockville, MD: ampholines. Pharmacia Fine Chemicals, Piscataway, NJ: Sephadex G-75, Sephadex G-150, Sepharose 4B, and Staphylococcal protein A. Schleicher and Schuell, Keene, NH: 0.45 μm BA-85 nitrocellulose paper. Schwarz-Mann, Orangeburg, NY: ultrapure ammonium sulfate. Dynatech Laboratories, Alexandria, VA: protein binding polystyrene microtiter wells. Bio-Rad Laboratories, Richmond, CA: hydroxylapatite. Cappel Laboratories, West Chester, PA: rhodamine labeled goat antibody to rabbit immunoglobulins (IgA, IgG, IgM heavy and light chains). Knox Gelatin, Inc., Engelwood Cliffs, NJ: gelatin.

Cell Culture

Acanthamoeba castellanii (Neff strain) was grown in aerated cultures of 25 ml to 16 liter in size according to Pollard and Korn (15), harvested by centrifugation in a 6-liter rotor and washed two times with 50 mM NaCl.

Protein Purification

PROFILIN: ~600 g of *Acanthamoeba* were lysed in 2 vol of ice cold 0.34 M sucrose, 1 mM EGTA, 1 mM ATP, 10 mM imidazole, pH 7.5, 1 mM phenylmethylsulfonylfluoride, 0.1 mM benzamidine using N_2 cavitation, and insoluble materials were pelleted by centrifugation in Beckman Ti45 rotors (Beckman Instruments, Inc., Palo Alto, CA) at 35,000 rpm for 75 min at 4°C. The supernatant was applied batch-wise [14] to a 900-ml column of DEAE-cellulose equilibrated with 10 mM imidazole pH 7.5, 0.6 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT. After washing the column with ~300 ml of column buffer, proteins were eluted with a 4-liter (0–400 mM) linear gradient of KCl in column buffer followed by 2 liter of 1 M KCl, 2 mM EDTA, 0.05% sodium azide. Fractions containing profilin were identified by solid phase antibody-binding assays described below. Profilin was purified from the unbound fraction (peak 1) by the method of Reichstein and Korn (16). The bound fraction of profilin coeluted with myosin-I, so that this fraction was first precipitated with 1.0 to 1.7 M ammonium sulfate (15) and then passed through an ADP-agarose column (11), two steps in the purification of myosin-I. Profilin that failed to bind to ADP-agarose was purified by the method of Reichstein and Korn (16) beginning with chromatography on a 1.5-×-60-cm column of DEAE cellulose equilibrated with 5 mM imidazole, pH 7.5, 0.5 mM DTT. The profilin did not bind to this second DEAE column.

ACTIN: *Acanthamoeba* actin was purified by the method of Gordon et al. (5) from the 0.21–0.30 M KCl fractions of the DEAE column used to prepare profilin. Rabbit skeletal muscle actin was purified by the method of MacLean-Fletcher and Pollard (9). The actins were modified on cys-373 with pyrene iodoacetamide by the Cooper et al. (3) modification of the method of Kouyama and Mihashi (7).

Biochemical Methods

PROTEIN CONCENTRATION: The concentration of *Acanthamoeba* profilin was measured by absorbance at 280 nm using $E = 1.2\ cm^2 \cdot mg^{-1}$ or $1.4 \times 10^4 M^{-1} cm^{-1}$. The extinction coefficient of *Acanthamoeba* profilin was determined by measuring the UV absorption spectrum and the refractive index of identical samples. The refractive index was measured by Ms. Trudy Carr in Dr. William Harrington's laboratory using Rayleigh interference optics on a Beckman Model E analytical ultracentrifuge. A specific refractive index of 0.25 mg/ml per fringe was assumed. Other extinction coefficients used were $0.62\ cm^2 \cdot mg^{-1}$ at 290 nm for actin and $1.4\ cm^{-2}\ mg^{-1}$ at 280 nm for immunoglobulin.

ANALYTICAL ULTRACENTRIFUGATION: The molecular weight of native profilin was determined by high speed analytical ultracentrifugation (26). A helium-neon laser light source (25) was employed, and Rayleigh interference patterns were recorded from cells of 30-mm optical path. Local apparent weight-average molecular weights, $M_{wa}(r)$, were calculated from microcomparator readings of the interference patterns by means of a computer program similar to those of Roark and Yphantis (17) and of Teller (20). Samples were observed at three different initial concentrations to test for possible heterogeneity of the protein [17, 20, 27]. The concentrations at the menisci of the solutions, although small, were not negligible. They were estimated by a successive-approximation technique (17). The partial specific volume of profilin was estimated, on the basis of the amino acid composition reported by Reichstein and Korn (16) and by the method of Lee and Timasheff (8), to be 0.73 ml/g.

ACTIN POLYMERIZATION ASSAYS: Viscosity was measured at 25°C with Ostwald capillary viscometers (Cannon Instruments, size 150) with samples of 0.6 ml. Absolute rates of elongation were measured by electron microscopy using glutaraldehyde-fixed myosin subfragment-I decorated actin filaments as nuclei (23). The polymerization of pyrene actin was measured by fluorescence using an excitation wave length of 365 nm and an emission wave length of 407 nm according to Cooper et al. (3).

GEL ELECTROPHORESIS: PAGE in SDS was carried out on 1.5-mm slab gels by a modification of the Laemmli method (14). Whole cell samples were prepared for gel electrophoresis as follows: *Acanthamoeba* were washed and resuspended in 2 vol of 10 mM Tris buffer, pH 7. The cells in this suspension were solubilized and proteins denatured by adding 10 vol of hot (100°C) dodecyl sulfate sample buffer and heating further at 100°C for 5 min. Lysis of the cells in sample buffer at room temperature results in extensive proteolysis. Whole cell samples or crude extracts of *Dictyostelium discoideum* ameba, *Physarum polycephalum* plasmodia and *Naeglaria gruberi* ameba were prepared for gel electrophoresis in similar ways.

ISOELECTRIC FOCUSING: Samples were dissolved in a buffer containing 8 M urea, 1% Triton X-100, and focused on 4% polyacrylamide gels with 2% ampholines (pH 3.5 to 10).

ORGANIC PHOSPHATE: Purified profilin was washed by trichloroacetic acid precipitation, extracted with TCA at 95°C, ashed, and assayed for phosphate according to Stull and Buss (19).

RADIOIODINATION OF PROTEINS: Staphylococcal protein A was labeled with ^{125}I using chloramine T and separated from the other reactants by gel filtration.

Immunological Methods

ANTIBODY TO PROFILIN: Two white New Zealand rabbits were immunized with 175 μg each of *Acanthamoeba* profilin emulsified in complete Freund's adjuvant and injected intradermally in about 20 separate locations lateral to the vertebral column. After 1 mo and in subsequent months each animal was boosted with 250 μg of profilin and bled two or more times 6–14 d following the boosts.

ANTIBODY PURIFICATION: Purified profilin (3.5 mg) was covalently attached to 1 ml of cyanogen bromide-activated Sepharose 4B (4). An immune immunoglobulin fraction was obtained from rabbit serum containing profilin antibodies by precipitation with 0 to 40% ammonium sulfate and allowed to react with the profilin-Sepharose overnight at 4°C. By solid phase binding assay, >95% of the profilin antibody bound to the column. After pouring into a small chromatographic column unbound material was eluted with 10 mM phosphate

buffer, pH 7.5, and bound antibody was released in two fractions with 2 M $MgCl_2$ followed by 4 M $MgCl_2$. The purified antibodies were dialyzed against 150 mM NaCl, 10 mM phosphate, pH 7.5.

SOLID PHASE ANTIBODY-BINDING ASSAY: To measure antibody concentration 0.5-μg aliquots of profilin in 50 μl of 5 mM imidazole, pH 7, 0.01% sodium azide were placed in the wells of microtiter plates and dried in a stream of warm air. The wells were washed once with 250 μl of TTX-BSA saline[1] (10 mM Tris-Cl, pH 7.8, 1 mg/ml Triton X-100, 1 mg/ml bovine serum albumin, 150 mM NaCl, 15 mM sodium azide). 50-μl aliquots of immune serum diluted in TTX-BSA saline were incubated in the wells for 2 h at room temperature. After removal of diluted serum the wells were washed three times with 250 μl of TTX-BSA saline for 5 min per wash. Antibody bound to the wells was detected by ^{125}I-Staphylococcal protein A, by using 50 μl containing ~10^5 cpm for 1 h at room temperature. After washing the wells three times with TTX-BSA saline, ^{125}I bound to the wells was counted with a gamma counter. The counts bound depended on the antibody concentration in the serum and gave sigmoid curves of counts bound vs. serum dilution.

The same assay was used to measure profilin concentrations. The profilin concentration in the sample was varied and assayed with a 1:100 dilution of antiprofilin serum and ^{125}I-protein A as above. In the range of 15 to 300 ng profilin there was a linear relation to ^{125}I bound to the well. This assay was used as a semiquantitative assay for profilin in crude samples containing other ameba proteins. A modification of this method applicable to very crude samples was to apply 5-μl samples containing 25–500 ng of profilin in SDS gel sample buffer to 1 × 1 cm squares on strips of nitrocellulose paper.

After washing with TTX-BSA saline, the strips were treated with 1 μg/ml purified profilin antibody in TTX-BSA for 1 h at 25°C, washed five times with TTX-BSA over 30 min, treated with 10^5 cpm/ml ^{125}I protein A in TTX-BSA for 60 min at 25°C, washed, and counted. The ^{125}I protein A bound to the paper was directly proportional to profilin in the range of 50 to 500 ng.

ANTIBODY STAINING OF ELECTROPHORETIC GELS: Polypeptides were transferred electrophoretically to nitrocellulose paper (22). The paper was washed with TTX-BSA saline with 0.01% SDS and then reacted with 1.1 μg/ml of purified profilin antibody in TTX-BSA saline for 2 h at 22°C. After washing with TTX-BSA saline for 30 min the paper was reacted with ^{125}I protein A (10^6 cpm/ml) for 2 h at 4°C. After washing three times for 30 min each with TTX-BSA saline, the paper was dried. Bound ^{125}I was detected by autoradiography using Kodak X-Omat AR film and a DuPont Lightening Plus intensifying screen at −70°C. The amount of ^{125}I protein A bound to profilin was quantitated by cutting out strips from the nitrocellulose paper and gamma counting. To calculate the amount of profilin in an unknown, we compared the counts with profilin standards run on the same gel and processed in parallel.

ANTIBODY STAINING OF CELLS: Coverslips were washed in detergent, rinsed with water, rinsed with absolute ethanol, and stored in ethanol. Subsequently, they were coated with 0.1% polylysine, rinsed with water, and dried. The polylysine-coated coverslips were reacted with 100 mM glutaraldehyde for 30 min and rinsed briefly before applying amebas (from a low density culture) in 1.5% mM glucose, 50 mM NaCl, 20 mM sodium phosphate, pH 7. After allowing 1 h for the cells to attach, adherent cells were fixed for 15 min with 2% formaldehyde (made fresh from paraformaldehyde) in the glucose-NaCl-phosphate buffer. After a brief rinse with buffer the cells were treated with acetone at −20°C for 30s and rehydrated in gelatin-PBS (2 mg/ml gelatin, 150 mM NaCl, 10 mM sodium phosphate, pH 7.5) containing 1 mM ethanolamine for 5 min. The cells were washed twice with gelatin-PBS (phosphate-buffered saline) for 20 min and reacted with 1.25 to 10 μg/ml of purified antibody in gelatin-PBS with 1 mg/ml nonimmune goat Ig for 1 h at 22°C. After washing once with gelatin-PBS, the cells were reacted for 1 h at 22°C with a 1:100 dilution of rhodamine-labeled goat-anti-rabbit Ig in gelatin-PBS. The rhodamine-goat anti-rabbit Ig was previously absorbed with formaldehyde-fixed, acetone-extracted amebas. Unbound antibody was washed off with three changes of gelatin-PBS over 1 h and the coverslips were mounted for fluorescence microscopy. Controls included preimmune Ig, adsorbed immune Ig and second antibody alone. Cells stained with specific antibody and controls were photographed with identical exposures.

RESULTS

Separation of Two Profilin Fractions by Ion Exchange

When sucrose extracts of *Acanthamoeba* are fractionated on DEAE-cellulose, immunoreactive profilin elutes in two peaks (Fig. 1). The peaks are approximately equal in size by the solid phase binding assay, but due to interference by the many other components in these crude fractions, the assay is not necessarily quantitative. In addition to these two distinct peaks, essentially every fraction between them contains a low concentration of profilin which can be detected by using less dilute samples in the solid phase antibody-binding assays. The unbound fraction was identified previously by Reichstein and Korn (16). This peak was pooled and used as the starting material for the purification of the profilin used in most of the experiments reported here. The second peak of immunoreactive profilin elutes between KCl concentrations of 120 and 170 mM. This zone also contains myosin-I and is well separated from myosin-II, capping protein, actin, and gelation protein which elute at higher concentrations of KCl (Fig. 1). The profilin from this second peak can be purified using the same steps described by Reichstein and Korn (16) for the unbound fraction beginning with an additional DEAE cellulose column. The second peak profilin does not bind to the second DEAE column.

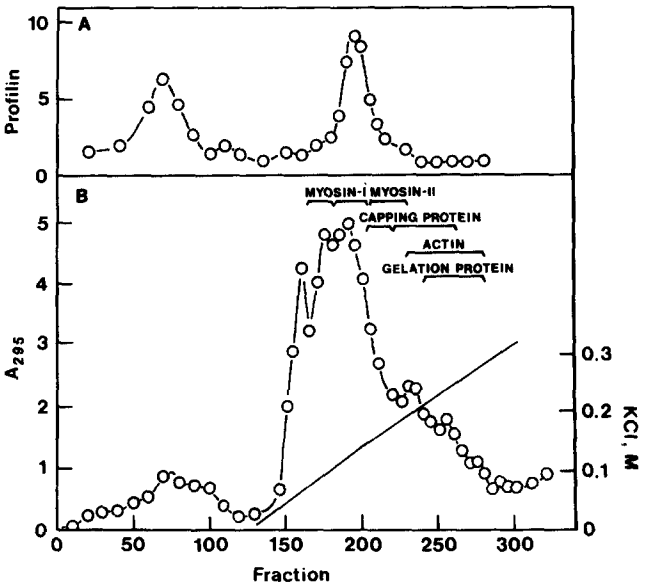

FIGURE 1 DEAE-cellulose chromatography of 1,200 ml of cold, 0.34 M sucrose extract of *Acanthamoeba* on a 5-x-46-cm column equilibrated with 10 mM imidazole (pH 7.5), 0.5 mM ATP, 0.2 mM $CaCl_2$, and 0.5 mM dithiothreitol and eluted with a 0 to 0.4 M KCl gradient. Fractions were 18 ml. (*A*) Solid phase antibody binding assay for profilin in units of ^{125}I-protein A counts per 10^{-3} min. (*B*) Absorbance at 295 nm (○), KCl gradient (—), and the elution positions of other proteins (brackets).

The profilin purified from the two peaks is identical in every way that we have tested. This includes chromatography (not shown) on hydroxylapatite, DEAE-cellulose and Sephadex G-75, gel electrophoresis in SDS (Fig. 2), isoelectric focusing in urea (Fig. 2), and effects on muscle actin polymerization by the viscometric assays described by Tseng and Pollard (23). Profilin isolated from DEAE peak 1 contains no detectable organic phosphate by direct chemical analysis, so the bound fraction (with the same isoelectric point) must also be free of phosphate.

Physical Properties of Acanthamoeba Profilin

Acanthamoeba profilin consists of a single low molecular weight polypeptide as shown previously by Reichstein and Korn (16). On gel electrophoresis in SDS ameba profilin has

[1] *Abbreviations used in this paper:* $M_{wa}(r)$, weight-average molecular weight; PBS, phosphate-buffered saline; TTX-BSA, 10 mM Tris-Cl, pH 7.8, 1 mg/ml Triton X-100, 1 mg/ml bovine serum albumin, 150 mM NaCl, 15 mM sodium azide.

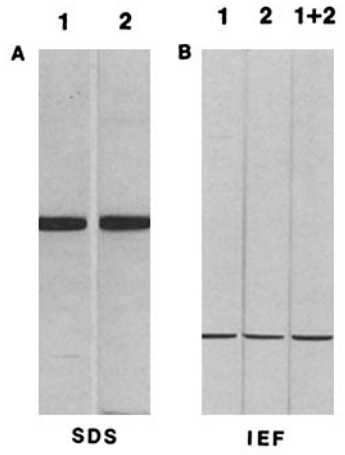

FIGURE 2 Comparison of profilin purified from peaks 1 and 2 of the DEAE column. (*A*) Polyacrylamide gel (12.5%) electrophoresis in SDS. (*B*) Isoelectric focusing in urea.

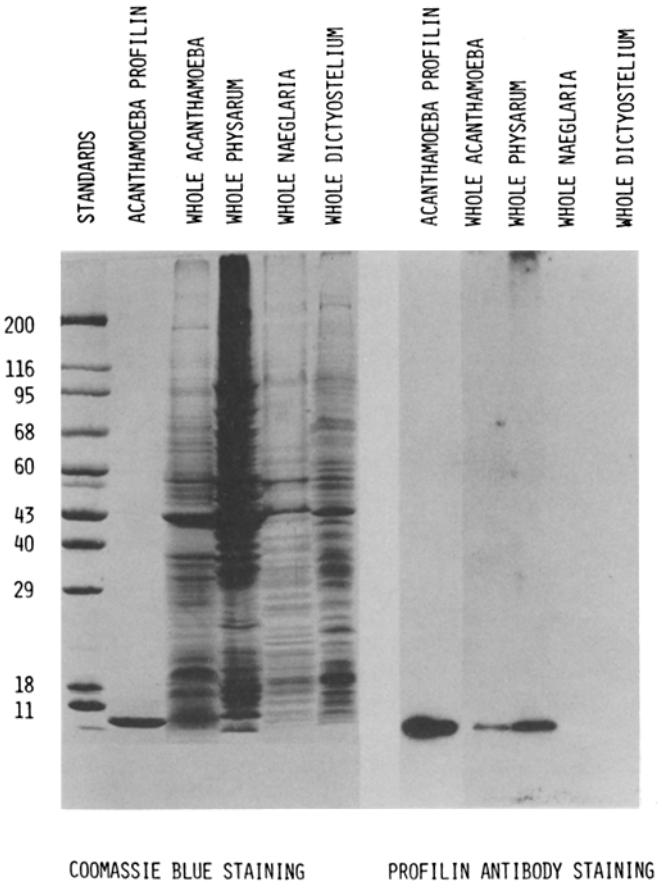

FIGURE 3 Gel electrophoresis and antibody staining of profilin and polypeptides from several cell types. The samples on the left are stained with Coomassie Brilliant Blue. The samples on the right were transferred electrophoretically to nitrocellulose paper and stained with affinity purified rabbit antibodies to *Acanthamoeba* profilin. The location of antibodies bound to the paper was identified by reaction with radioiodinated protein A and autoradiography.

a slightly higher mobility than cytochrome *c* (10,700 mol wt) as shown in Fig. 3 and in two other buffer systems. Gel electrophoresis of several standards in this range (insulin, glucagon, cytochrome *c*, ribonuclease) failed to give linear plots of log molecular weight vs. mobility, so that the electrophoretic mobility of ameba profilin in SDS is not a reliable measure of its molecular weight. Therefore we determined the molecular weight by high speed sedimentation equilibrium ultracentrifugation (Fig. 4). The $M_{wa}(r)$ increases slightly

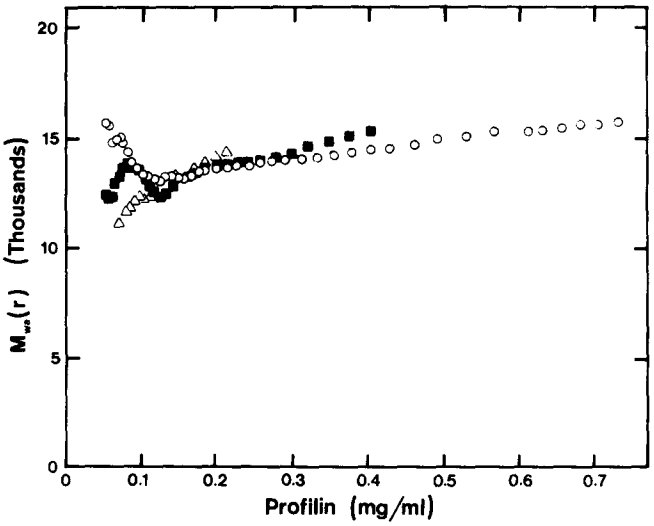

FIGURE 4 Weight-average molecular weights, $M_{wa}(r)$, of *Acanthamoeba* profilin as a function of local concentration in the ultracentrifuge cell. Measurements were made at sedimentation equilibrium after 28 h at 4°C and 36,000 rpm in an An-E rotor. Initial concentrations = 0.64 mg/ml (○); 0.32 mg/ml (■); 0.16 mg/ml (△).

with increasing concentration, indicating reversible self-association of the protein. These oligomers can also be detected by chemical cross-linking (T. D. Pollard, unpublished observation). For this reason, the data were fit to a monomer-dimer relationship: $2\ P_1 \rightleftharpoons P_2$, so that, $M_{wa}(r) = (M_1/2Kc)\,(1 + 4Kc(1 + 4Kc)^{1/2})$, where P_1 represents the monomer and P_2 the dimer; K is the association constant, C is the local concentration in the ultracentrifuge cell in mg/ml, and M_1 is the molecular weight of the monomer. The best-fitting value of the monomer molecular weight is 11,700 ± 800. The slight nonoverlap of the data from solutions with different initial concentrations indicates a slight heterogeneity of the solute (27), perhaps due to the presence of a small amount of material of high molecular weight. The monomer molecular weight obtained is unlikely to be seriously perturbed by this fact.

Immunological Studies

Both of the rabbits immunized with *Acanthamoeba* profilin produced antibodies. Purified antiprofilin reacts with SDS denatured profilin after it is transferred to nitrocellulose paper (Fig. 3). It also reacts exclusively with a polypeptide of the same electrophoretic mobility in crude extracts of *Acanthamoeba* and *Physarum* (Fig. 3), but not with any polypeptides in *Dictyostelium* or *Naeglaria*. This demonstrates the specificity of the antibody and that neither *Acanthamoeba* nor *Physarum* contain a stable (under denaturing conditions) form of profilin larger than the 11,700-mol-wt polypeptide isolated by the method of Reichstein and Korn (16).

Profilin Concentration in the Cell

Profilin concentration in whole cells and crude extracts was estimated in two ways. In both cases the samples were solubilized in SDS. The polypeptides were either applied directly to nitrocellulose paper or first separated by gel electrophoresis before transfer to nitrocellulose. The amount of profilin on the paper was measured by quantitating the amount of profilin antibody that bound to standards and crude mixtures of polypeptides (Table I). Autoradiography of the samples trans-

TABLE I

Profilin Concentration in Cellular Fractions Estimated by Antibody Binding

Method	Profilin		
	(% total protein)	*(mg/ml packed cells)*	*μmol/liter packed cells*
Spot assay on nitrocellulose paper*			
Whole cells	2.5	1.2 (SD 0.1)	106
Homogenate	2.2	1.1 (SD 0.1)	95
140,000-*g* supernatant	4.1	1.1 (SD 0.1)	103
140,000-*g* supernatant	3.7	1.1 (SD 0.1)	91
Electrophoretic transfer from SDS gel‡			
Whole cell	1.4	0.7	60
Homogenate	1.6	0.8	68
140,000-*g* supernatant	2.1	0.6	51

* Values are the mean of five or six samples varying in concentration by a factor of 4. The homogenates and supernatant were prepared in sucrose buffer as described for profilin extraction. Two samples of supernatant were tested in this experiment. The total protein is 50 mg/ml packed cells; 58% of this total protein was recovered in the 140,000-*g* supernatant.

‡ Values are the mean of two or three samples varying in concentration by a factor of 4.

ferred from the electrophoretic gel established that the antibodies bound only to polypeptides with the same size as the purified profilin (Fig. 3). The two methods gave similar results (Table I). Both assays also indicate that essentially all of the profilin is recovered in the 140,000-*g* supernatant during extraction of the cells showing that it is freely soluble in the homogenate.

Fluorescent Antibody Staining

The purified antibodies to profilin were used to localize profilin in the ameba using the indirect fluorescent antibody method (Fig. 5). There is uniform staining throughout the cytoplasmic matrix so the fluorescence intensity is determined primarily by the cell thickness. The contractile vacuole, digestive vacuoles, and other unidentified vacuoles do not stain, but the nucleus stains with the same intensity as the cytoplasm. The peripheral hyaline cytoplasm has a low intensity (Fig. 5, *c–f*) in comparison with the thicker endoplasm, but even these thin regions can be stained. Fig. 5, *g* and *h*, shows an example of a cell where the basal plasma membrane remained attached to the cover slip but most of the cytoplasm was removed during preparation. Here the faint staining that extends to the plasma membrane in filopodia as well as all other parts of the cortex could be photographed without interference from the brightly stained endoplasm.

Effects of Acanthamoeba Profilin on Actin Polymerization

Electron microscopic measurement of elongation rates: as we showed earlier for *Acanthamoeba* actin (23), *Acanthamoeba* profilin inhibits the rate of muscle actin filament elongation in 50 mM KCl (Fig. 6). Ten times more profilin is required for a given effect on muscle actin than *Acanthamoeba* actin, explaining why we observed little effect on muscle actin elongation previously (23). The K_d calculated from this data by the method of Tseng and Pollard (23) is 40 μM. This agrees reasonably well with published values (20, 22) determined by steady state measurements. In 50 mM KCl, 1 mM $MgCl_2$, very high concentrations of profilin inhibit the elongation rate of muscle actin filaments only a small amount. Similar experiments with *Acanthamoeba* actin (23) showed that *Acanthamoeba* profilin also has a weak effect on the elongation rate in 50 mM KCl with 1 mM $MgCl_2$.

Comparison of the effects of profilin on elongation and nucleation: Using a fluorometric assay for *Acanthamoeba* actin polymerization that is superior to the viscometric assay we used earlier (23), we confirmed that profilin inhibits nucleation much more strongly than elongation in the presence of $MgCl_2$ (Fig. 7). When actin filament nuclei are present, 10 μM profilin reduces the initial rate of elongation about 10% (Fig. 7*a*). Without added nuclei, 10 μM profilin prolongs the lag phase substantially (Fig. 7*b*). Since the elongation rate with 10 μM profilin is only 10% lower than the control, the prolonged lag must be due to a lower nucleation rate. Note also that 10 μM profilin does not reduce the steady state polymer concentration in this KCl $MgCl_2$ buffer, further evidence that the effect on elongation is minimal. Similar effects of profilin were obtained in KCl-$MgCl_2$ with 0.1 mM $CaCl_2$ or 1 mM EGTA, indicating that Ca^{++} does not affect the action of profilin in these assays.

DISCUSSION

Acanthamoeba profilin is a small protein that binds weakly to actin monomers (12) and inhibits the formation of actin polymer nuclei (16, 21, 23). In our hands profilin inhibits nucleation much more strongly than elongation under physiological conditions (23; present report), suggesting that the detailed mechanism of action may be complex. *Physarum* profilin appears to behave in a similar fashion (13). On the other hand, under similar conditions *Acanthamoeba* profilin prepared by Tobacman and Korn (21) inhibits both elongation and nucleation. Both laboratories have continued detailed studies of the mechanism of action of *Acanthamoeba* profilin that will hopefully resolve these differences and explain exactly how actin polymerization is modulated by profilin.

Here we report some of the physical properties of *Acanthamoeba* profilin that are required to interpret the on-going quantitative studies of the mechanism of action. We have also determined the concentration and distribution of profilin in the cell, information that is necessary to understand its physiological functions.

Profilin Content of Acanthamoeba

One must know the intracellular concentration of profilin and actin to interpret the physiological significance of biochemical experiments with these proteins. From densitometry electrophoresis gels, Reichstein and Korn (16) estimated that profilin is 2–3% of the total *Acanthamoeba* protein. This is, of course, an upper limit, because any number of other proteins could have the same motility. Using specific antibodies we found that profilin is ~2% of the total protein and has a concentration of ~1.1 mg/g packed cells. This is 100 μM profilin in the cell pellet. Taking into account medium trapped between the cells in the pellet and the fraction of the cell inaccessible to profilin (vacuoles), we estimate that the concentration of profilin in the cytoplasm may be as high as 200 μM. The actin content has never been measured by a specific assay, but by densitometry of electrophoresis gels, actin may constitute as much as 14% of the total protein (5). This is ~170 μM in the cell pellet and up to 350 μM in the

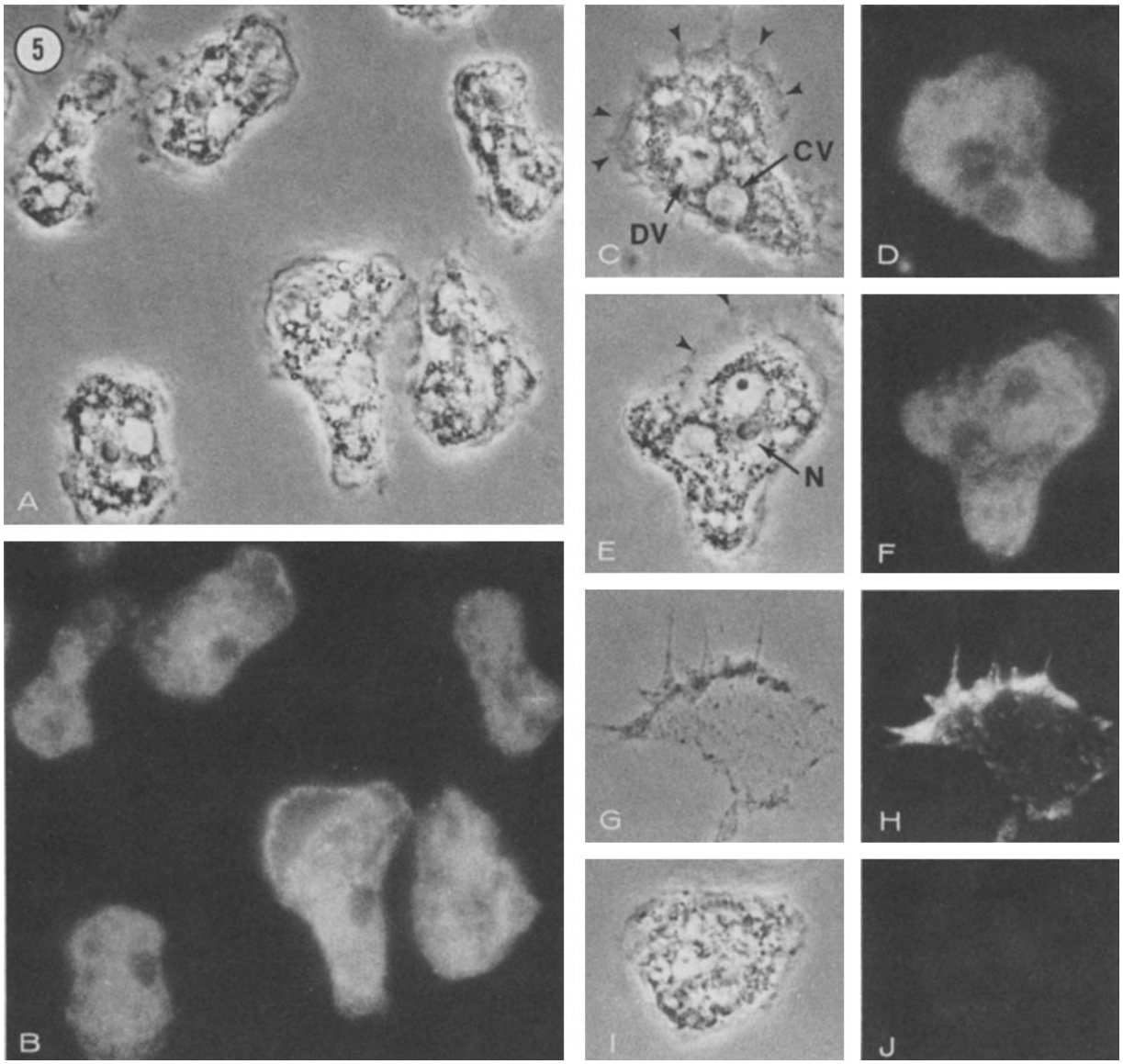

FIGURE 5 Phase-contrast and fluorescence micrographs of *Acanthamoeba* stained indirectly with purified antibodies to profilin. Concentrations of antiprofilin: (*A* and *B*) 5 μg/ml; (*C–H*) 1.25 μg/ml. *I* and *J* are controls with 5 μg/ml preimmune Ig. Nucleus (*N*), contractile vacuole (*CV*), digestive vacuole (*DV*), hyaline ectoplasm (arrowheads). The bulk of the cell in *G* and *H* was lost during preparation, making it possible to photograph the basal plasma membrane and part of the cortical hyaline ectoplasm without interference. All micrographs are at the same magnification.

cytoplasm. Unless this estimate of the actin content is way off, there is an excess of actin over profilin in the cell. However, the available evidence (16, 23) indicates that profilin does not bind to actin filaments and about half of the actin in the cell is polymerized (5). Therefore there is probably an excess of profilin (~200 μM) over actin monomers (~150 μM) in the cytoplasm. At these concentrations, most of the actin monomers would be bound to profilin in the cell, but there would still be ~10 μM free actin monomers if the K_d in the cell were 5 μM. Thus, profilin could buffer the free actin monomer concentration in the cell, but the available data suggest that there should be a substantial concentration of free monomers.

Distribution of Profilin in the Cell

The fluorescent antibody staining shows that profilin is distributed relatively uniformly throughout the cytoplasmic matrix, similar to a soluble protein, rhodamine-labeled ovalbumin, microinjected into living *Acanthamoeba* (D. P. Kiehart and T. D. Pollard, unpublished observation). The profilin is present, but clearly not concentrated, in the cortex where most of the actin filaments and capping protein are found (24). This distribution of profilin is consistent with its size, solubility, and inability to bind to actin filaments. The presence of profilin in the nucleus is at present unexplained, but not surprising considering its small size.

When the cell is homogenized in a cold sucrose buffer, virtually all of the profilin is soluble and recovered in the 140,000-*g* supernatant, further evidence that it is not bound to actin filaments or other large cellular components. When the sucrose extract is fractionated by DEAE-cellulose chromatography the profilin is recovered in two peaks of about the same size that account for most of the profilin in the cell. The first pool fails to bind to DEAE and was originally recognized by Reichstein and Korn (16). The second pool

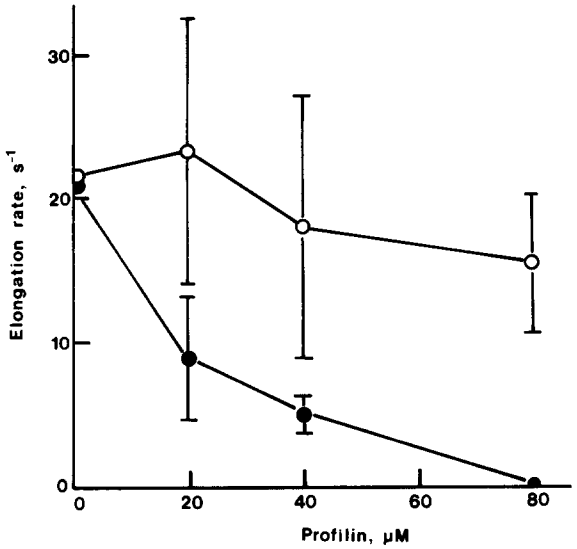

FIGURE 6 Barbed end elongation rate of muscle actin filaments as a function of *Acanthamoeba* profilin concentration. Conditions: actin monomer 4 μM, 50 mM KCl, 10 mM imidazole (pH 7), 22°C. (●) KCl. (○) KCl with 2 mM $MgCl_2$. Values plotted are mean lengths in molecules divided by time in seconds ± standard deviation.

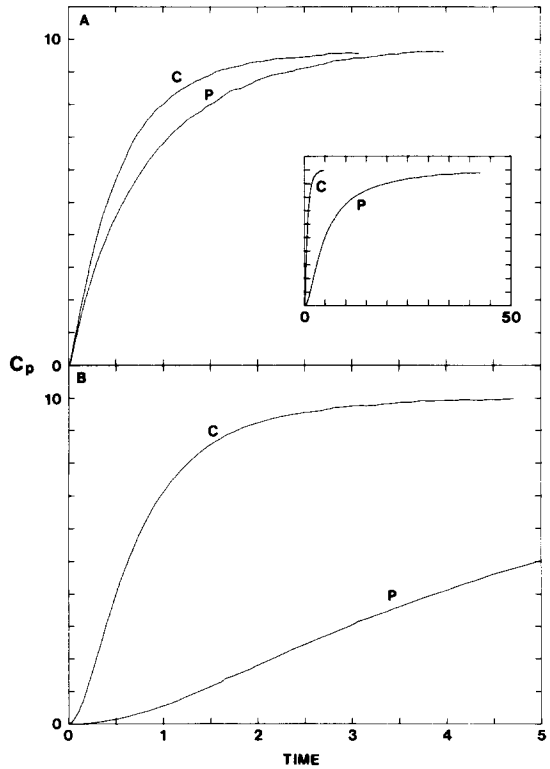

FIGURE 7 Time course of 5 μM *Acanthamoeba* actin polymerization in the presence (*P*) and absence (C) of 10 μM *Acanthamoeba* profilin. 5% of the actin was labeled with pyrene. The polymer concentration (C_p) is given in arbitrary fluorescence units. The time is in thousands of seconds. Conditions: 50 mM KCl, 10 mM imidazole, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP, 25°C. (*A*) Polymerization nucleated by 0.5 μM unlabeled actin filaments. (*B*) No nuclei added. This time course is shown on an expanded scale in the *inset* to *A*.

binds to DEAE and elutes with other proteins in a salt gradient. When purified, the second pool profilin appears to be identical to the first pool profilin. We conclude that there is only one species of profilin in *Acanthamoeba* and that it is split into two fractions when the extract is chromatographed on DEAE. One possible explanation is that part of the profilin in the extract is free and part is bound to another component, such as actin. Although free profilin does not bind to DEAE, the fraction of the profilin complexed with the second component may bind indirectly to DEAE if the second component has a high affinity for DEAE. This bound profilin fraction elutes in low concentrations as the column is washed with the equilibration buffer and the early part of the salt gradient. The bulk of the bound fraction of profilin is eluted when the salt concentration reaches 120 to 170 mM KCl. The failure of this bound profilin to bind to DEAE when applied to a second column is strong evidence that it is bound indirectly to the first DEAE column.

Proteins Related to Acanthamoeba Profilin in Other Cells

We used antibodies to profilin to look for proteins cross reactive with the 11,700-mol-wt profilin in *Acanthamoeba* and some other cells. Our results show that *Acanthamoeba* probably contains only one form of profilin and that the 11,700-mol-wt profilin is not a degradation product of a larger protein. We can conclude that the native profilin molecule in *Acanthamoeba* is ~15% smaller than its vertebrate counterpart. It also differs in amino acid composition and isoelectric point (16). *Physarum* contains a profilin (13) of the same molecular weight as *Acanthamoeba* profilin that cross-reacts with our profilin antibodies, but *Dictyostelium* and *Naeglaria* do not. This indicates that the antibodies have limited cross-reactivity between species or, less likely, that neither *Dictyostelium* nor *Naeglaria* have profilin or an analogous protein.

This work was supported by research grant GM-26338 from the National Institutes of Health.

Received for publication 5 April 1983, and in revised form 25 August 1983.

REFERENCES

1 Blikstad, I., I. Sundkvist, and S. Erickson. 1980. Isolation and characterization of profilactin and profilin from calf thymus and brain. *Eur. J. Biochem.* 105:425–433.
2. Carlsson, L., L. E. Nystrom, I. 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.
3. Cooper, J. A., S. B. Walker, and T. D. Pollard. 1983. Pyrene actin: documentation of the validity of a sensitive assay for actin polymerization. *J. Muscle Res. Cell Motility.* 4:253–262.
4. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. *J. Biol. Chem.* 245:3059–3065.
5. Gordon, D. J., E. Eisenberg, and E. D. Korn. 1976. Characterization of cytoplasmic actin isolated from *Acanthamoeba castellanii* by a new method. *J. Biol. Chem.* 251:4778–4786.
6. Grumet, M., and S. Lin. 1980. Reversal of profilin inhibition of actin polymerization in vitro by erythrocyte cytochalasin-binding complexes and cross-linked actin nuclei. *Biochem. Biophys. Res. Commun.* 92:1324–1334.
7. Kouyama, T., and K. Mihashi. 1981. Fluorimetry study of N-(1-pyrenyl)-iodoacetamide-labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. *Eur. J. Biochem.* 114:33–38.
8. Lee, J. C., and S. N. Timasheff. 1974. The calculation of partial specific volumes of proteins in guanidine hydrochloride. *Arch. Biochem. Biophys.* 165:268–273.
9. MacLean-Fletcher, S., and T. D. Pollard. 1980. Identification of a factor in conventional muscle actin preparation which inhibits actin filament self-association. *Biochem. Biophys. Res. Commun.* 96:18–27.
10. Markey, F., H. Larsson, K. Weber, and U. Lindberg. 1982. Nucleation of actin polymerization from profilactin opposite effects of different nuclei. *Biochem. Biophys. Acta.* 704:43–51.
11. Maruta, H., H. Gadasi, J. H. Collins, and E. D. Korn. 1979. Multiple forms of *Acanthamoeba* myosin-I. *J. Biol. Chem.* 254:3624–3630.
12. Mockrin, S. C., and E. D. Korn. 1980. *Acanthamoeba* profilin interacts with G-actin to increase the rate of exchange of actin-bound ATP. *Biochemistry.* 19:5359–5362.
13. Ozaki, K., H. Sugino, T. Hasegawa, S. Takahashi, and S. Hatano. 1983. Isolation and characterization of *Physarum* profilin. *J. Biochem. (Tokyo).* 93:295–298.
14. Pollard, T. D. 1982. Myosin purification and characterization. *In* Methods and Perspectives in Cell Biology. L. Wilson, editor. Academic Press, Inc., New York. 24:301–311.
15. Pollard, T. D., and E. D. Korn. 1973. *Acanthamoeba* myosin. I. Isolation from

Acanthamoeba castellanii of an enzyme similar to muscle myosin. *J. Biol. Chem.* 248:4682–4690.
16. Reichstein, E., and E. D. Korn. 1979. *Acanthamoeba* profilin—protein of low-molecular weight from *Acanthamoeba-castellanii* that inhibits actin nucleation. *J. Biol. Chem.* 254:6174–6179.
17. Roark, D. E., and D. A. Yphantis. 1968. Studies of self-associating systems by equilibrium ultracentrifugation. *Ann. NY Acad. Sci.* 164:245–278.
18. Runge, M. S., P. C. Tseng, R. C. Williams, J. A. Cooper, and T. D. Pollard. 1981. Characterization of profilin and profilin-actin interactions. *J. Cell Biol.* 91(2, Pt. 2):300*a*. (Abstr.)
19. Stull, J. T., and J. E. Buss. 1977. Phosphorylation of cardiac troponin by cAMP-dependent protein kinase. *J. Biol. Chem.* 252:851–857.
20. Teller, D. C. 1973. Characterization of proteins by sedimentation equilibrium in the analytical ultracentrifuge. *Methods Enzymol.* 346–441.
21. Tobacman, L. S., and E. D. Korn. 1982. The regulation of actin polymerization and the inhibition of monomeric actin ATPase activity by *Acanthamoeba* profilin. *J. Biol. Chem.* 257:4166–4170.
22. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
23. Tseng, P., and T. D. Pollard. 1982. Mechanism of action of *Acanthamoeba* profilin. Demonstration of actin species specificity and regulation by micromolar concentrations of $MgCl_2$. *J. Cell Biol.* 94:213–218.
24. Tseng, P., J. A. Cooper, and T. D. Pollard. 1982. Localization of actin, profilin, capping protein, and gelatin protein in *Acanthamoeba* by fluorescence microscopy. *J. Cell Biol.* 95(2, Pt. 2):326*a*. (Abstr.)
25. Williams, R. C., Jr. 1978. Continuous laser optics in the ultracentrifuge. *Methods Enzymol.* 48:195–191.
26. Yphantis, D. A. 1964. Equilibrium ultracentrifugation of dilute solutions. *Biochemistry.* 3:297–317.
27. Yphantis, D. A., J. J. Correia, M. L. Johnson, and G.-M. Wu. 1978. Detection of heterogeneity in self-associating systems. *In* Physical Aspects of Protein Interactions. N. Catsimpoolas, editor. Elsevier/North-Holland, New York. 275–303.