Isolation and Characterization of a High Molecular Weight Actin-binding Protein from *Physarum polycephalum* Plasmodia

KAZUO SUTOH, MAKOTO IWANE, FUMIO MATSUZAKI, MASAKO KIKUCHI, and ATSUSHI IKAI
Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

ABSTRACT A high molecular weight actin-binding protein was isolated from the *Physarum polycephalum* plasmodia. The protein (HMWP) shares many properties with other high molecular weight actin-binding proteins such as spectrin, actin-binding protein from macrophages, and filamin. It has a potent activity to cross-link F-actin into a gel-like structure. Its cross-linking activity does not depend on calcium concentrations. Hydrodynamic studies have revealed that the protein is in the monomeric state of a polypeptide chain with molecular weight of ~230,000 in a high ionic strength solvent, while it self-associates into a dimer under physiological ionic conditions. Electron microscopic examinations of HMWP have shown that the monomer particle observed in a high ionic strength solvent is rod shaped with the two-stranded morphology very similar to that of spectrin. On the other hand, under physiological ionic conditions, the HMWP dimer shows the dumb-bell shape with two globular domains connected with a thin flexible strand.

Many high molecular weight actin-binding proteins have been isolated from various tissues and cells. These proteins are grouped into two classes according to their immunological properties and their morphologies (1, 2). One class includes actin-binding proteins isolated from rabbit alveolar macrophages (ABPs)[1] (3), from human platelets (4), from HeLa cells (5), and from baby hamster kidney cells (6). Filamins isolated from smooth muscle (7, 8) and from guinea pig vas deferens (9) also belong to this class. All these proteins are homodimers of a single type of polypeptide chains with apparent molecular weight of ~270,000. The other class includes spectrin from erythrocytes (10), fodrin from brain (11–14), and TW-260/240 from brush border of epithelial cells (1). These proteins are tetramers of two types of polypeptide chains with slightly different molecular weights. For example, spectrin consists of two α-chains ($M_r = 240,000$) and two β-chains ($M_r = 220,000$). The spectrin tetramer is in equilibrium with heter-odimer of α- and β-chains (15). Fodrin is also in the dimer-tetramer equilibrium (12).

Morphologies of these actin-binding proteins have been examined by the rotary shadowing technique (16). Proteins belonging to the former class (ABP-type) are observed as flexible strands with contour length of ~1600 Å. Two 270,000-mol-wt polypeptides seem to be joined end-to-end to generate the single-stranded morphology characteristic of the ABP-type proteins (17, 18). Actin-binding proteins belonging to the latter class (spectrin-type) are observed as flexible rods (1, 16, 18). Unlike the ABP-type proteins, the flexible rod consists of two thinner strands. The two strands, each of which consists of a single polypeptide chain, intertwine to form a rod-like particle with length of ~1,000 Å. These two-stranded dimers then self-associate to form tetramers ~2,000 Å long. The two-stranded morphology is characteristic of the spectrin-type proteins.

Immunochemical studies have revealed differences between the ABP-type and spectrin-type proteins (1, 2). Antibody raised against a spectrin-type protein did not cross-react with ABP-type proteins and vice versa.

All these actin-binding proteins are capable of cross-linking

---

F-actin into a gel-like structure. ABP-type proteins, especially ABP isolated from macrophages, have much higher activity than spectrin-type proteins (13, 14, 19, 20). Although it is generally assumed that the cross-linking of F-actin with these actin-binding proteins has functional importance in vivo, exact functional roles of these proteins still remain to be elucidated. Most of our knowledge of functional roles of these actin-binding proteins has come from detailed studies on spectrin (10). Spectrin molecules form cytoskeletal networks with actin and other proteins. The cytoskeleton is closely associated with erythrocyte membrane and serves as a structure maintaining the characteristic shape of erythrocytes. Other high molecular weight actin-binding proteins also have been identified in the cortical cytoplasm by immunochemical techniques, implying that these proteins have functional roles similar to those of spectrin (21).

In this paper, we present data showing that *Physarum polycephalum* plasmodia contain a high molecular weight actin-binding protein, which shares many properties with other high molecular weight actin-binding proteins described above.

MATERIALS AND METHODS

Proteins: Crude actomyosin preparations were obtained from *Physarum polycephalum* plasmodia by the method of Hatano and Tazawa (22) with slight modifications. Imidazole buffer (20 mM, pH 7.0), dithiothreitol (DTT) (0.2 mM), and leupeptin (10 μg/ml) were included in all solutions used for the preparation. Phenylmethylsulfonyl fluoride (1 mM) was also included in a solution used for extracting proteins from plasmodia. Without protease inhibitors (leupeptin and phenylmethylsulfonyl fluoride), the *Physarum* actin-binding protein was readily cleaved by endogeneous proteases.

Muscle actin was prepared from an acetone powder by the method of Spudich and Watt (23). It was further purified by the column chromatography on Sephadex G150.

Ultracentrifugal Analysis of the Purified HMWP: The purified actin-binding protein (HMWP) was subjected to sedimentation equilibrium and sedimentation velocity runs by an analytical ultracentrifuge (Beckman model E, Beckman Instruments, Inc., Palo Alto, CA) with scanner equipment. Sedimentation equilibrium experiments were carried out on HMWP (0.15 and 0.3 mg/ml) in 0.5 M or 50 mM KCl containing 20 mM imidazole (pH 7.0) at 5,213 rpm. HMWP (<0.3 mg/ml) in 0.5 M or 50 mM KCl containing 20 mM imidazole (pH 7.0) was also subjected to sedimentation velocity measurements at 36,000 rpm.

HPLC Analysis of the Purified HMWP: The purified HMWP (0.1 mg/ml) was dialyzed against 0.5 M KCl and 20 mM imidazole (pH 7.0) or against 50 mM KCl and 20 mM imidazole (pH 7.0). The resulting HMWP solution (100 μl) was loaded on a high performance liquid chromatography (HPLC) column (TSK G4000SW) equilibrated either with 0.5 M KCl and 20 mM imidazole (pH 7.0) or with 50 mM KCl and 20 mM imidazole (pH 7.0). Proteins were monitored by the absorbance at 280 nm.

Stokes radius of HMWP was determined from its elution volume, by using standard proteins with known Stokes radii. Standard proteins were myosin, apoferritin (monomer and dimer), thyroglobulin, catalase, and lactoglobulin.

Cross-linking of F-Actin with HMWP: F-actin (0.3 mg/ml) in 50 mM KCl and 20 mM imidazole (pH 7.0) was mixed with various amount of the *Physarum* HMWP (0.6 mg/ml) in the same solvent. After the mixture was incubated at 4°C for 16 h in a 2-mm-diam glass tube, its low shear viscosity was measured by the falling ball method (24). Incubation of the mixture at 25°C for 30 min gave similar results.

Sedimentation analysis of the F-actin-HMWP mixture was carried out by an analytical ultracentrifuge (Beckman model E) with Schlieren optics. Higher concentration of F-actin (1 mg/ml) was used to detect the F-actin peak. The mixture was incubated at 25°C for 30 min before being loaded in the centrifuge cell.

Molecular Morphology of HMWP: The purified HMWP (0.1 mg/ml) was dialyzed against 0.5 M or 50 mM ammonium acetate (pH 7.2). The solution was then diluted with 9 vol of 0.5 M or 50 mM ammonium acetate containing 50% glycerol (16). It was then sprayed on freshly cleaved mica. The mica was dried under vacuum at room temperature, and then rotary shadowed with platinum/carbon on an uncooled rotary-shadowing stage at an

angle of 6° with revolution of 35 rpm. The replica was examined in an electron microscope (Hitachi model H300). As a control, spectrin dimers prepared from bovine erythrocytes were examined under the same conditions.

Rotary-shadowed particles were photographed in the electron microscope at a magnification of 40,000 × and photographically enlarged to 240,000 × on photography papers. Contour length and diameter of HMWP particles were measured by Numonics model 1250 (Numonics Corp., Lansdale, PA).

Gel Electrophoresis of Whole Homogenate of the Physarum plasmodia: The *Physarum* plasmodia were homogenized in 3 vol of 0.5 M NaCl, 20 mM imidazole, and 30 mM EDTA (pH 7.0). Just before homogenizing the plasmodia, we added phenylmethylsulfonyl fluoride (1 mM) and leupeptin (30 μg/ml) to prevent cleavages of proteins with endogeneous proteases. The whole homogenate was then treated with DNase I (50 μg/ml) for 30 min at 0°C. Solid urea was added to the DNase-treated homogenate to make its final concentration 8 M. It was incubated for 1 h at 37°C, and then mixed with 0.1 vol of 10% SDS and 10% 2-mercaptoethanol. It was further incubated for 3 h at 37°C or boiled for 5 min. When the whole homogenate was directly denatured in SDS without pretreatment by 8 M urea, protein aggregates were observed on the top of the SDS/acrylamide gel.

Other Analytical Methods: Amino acid composition of HMWP was determined by an amino acid analyzer (Hitachi model 835). Apparent molecular weight of HMWP was estimated by the SDS/acrylamide gel electrophoresis (25) by using spectrin α-chain (M_r = 240,000), spectrin β-chain (M_r = 220,000), and myosin heavy chain prepared from rabbit skeletal muscle (M_r = 200,000) as standards.

The SDS/acrylamide gel electrophoresis was carried out according to the method of Laemmli (25), and the gels were scanned by a conventional gel scanner (Fuji-Riken model FD IV; Fuji-Riken, Tokyo, Japan). Protein concentrations were determined by the method of Lowry et al. (26).

RESULTS

Preparation of a High Molecular Weight Actin-binding Protein

When whole homogenate of the *Physarum* plasmodia was electrophoresed in the presence of SDS, actin and myosin (heavy chain) were detected on the gel as major components (Fig. 1, lane *A*). Actin and myosin accounted for 20% and 3% of total peptides on the gel, respectively. Besides these contractile proteins, another polypeptide with its apparent molecular weight of 270,000 was present in an appreciable amount on the gel. The 270,000-mol-wt polypeptide accounted for 0.6% of total peptides on the gel.

Extraction of the homogenate with a high ionic strength solvent and subsequent precipitation of actomyosin in a low ionic strength solvent enriched actin and myosin (22) as shown in Fig. 1 (lane *B*). This crude actomyosin preparation contained the 270,000-mol-wt polypeptide. Repeated washes of the actomyosin preparation with a low ionic strength solvent never removed this polypeptide from actomyosin, indicating that this might be another member of a family of high molecular weight actin-binding proteins. We therefore tried to isolate and characterize this protein (which we call "HMWP," an abbreviation of high molecular weight protein).

Crude actomyosin was dissolved in 0.5 M KCl, 20 mM Tris-HCl, and 0.2 mM DTT (pH 8.0) and then dialyzed against the same solvent for 16 h. It was centrifuged at 100,000 *g* for 1 h to precipitate actin and myosin. HMWP was enriched in the supernatant (Fig. 1, lane *C*). Protein in the supernatant was precipitated by addition of ammonium sulfate (60% saturation) and the precipitate was collected by centrifugation. It was dissolved in a small volume of 0.5 M KCl, 20 mM imidazole, and 0.2 mM DTT (pH 7.0), and then dialyzed against the same solvent. The resulting crude HMWP preparation (Fig. 1, lane *D*) was treated with 0.6 M KI to depolymerize F-actin according to the method of Nachmias (27). We used 4 mM MgATP in place of the 2.5 mM Mg-pyro-
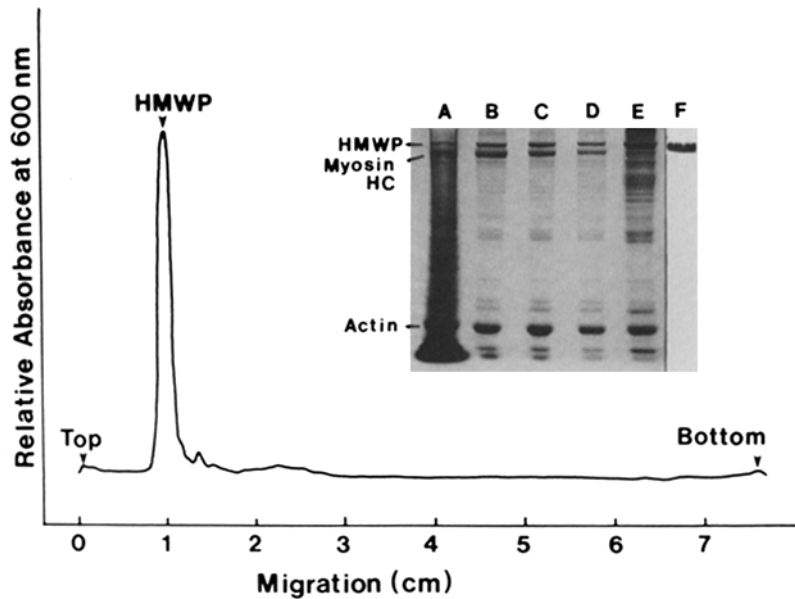
FIGURE 1 Purification of the *Physarum* HMWP. SDS/acrylamide gels. Lane *A*, whole homogenate of the *Physarum* plasmodia; lane *B*, crude actomyosin; lane *C*, supernatant of the crude actomyosin after high speed centrifugation in a high ionic strength solvent; lane *D*, crude HMWP; lane *E*, pooled fraction from the CL6B column; lane *F*, purified HMWP. A densitometric trace of gel *F* is shown. Notice that HMWP is the predominant polypeptide detected on the gel. *HC*, heavy chain.

phosphate in the original procedure. After the incubation of the crude HMWP preparation with 0.6 M KI for 30 min at 4°C, insoluble materials were removed by centrifugation at 100,000 *g* for 30 min. The clear supernatant was loaded on a Sepharose CL6B column (3 × 80 cm) which was pre-equilibrated with 0.5 M KCl, 20 mM imidazole, and 0.1 mM DTT (pH 7.0) and then washed with 20 ml of 0.6 M KI, 20 mM imidazole, and 1 mM MgATP (pH 7.0) just before loading the KI-treated solution. The elution profile from the column is shown in Fig. 2. Fractions containing HMWP were identified by the SDS/acrylamide gel electrophoresis, and then pooled. The pooled fractions (Fig. 1, lane *E*) were directly loaded on a hydroxyapatite column (2 × 5 cm) pre-equilibrated with 0.5 M KCl and 5 mM KH_2PO_4-K_2HPO_4 (pH 7.0). Proteins were eluted with 400 ml of a gradient from 5 mM to 1 M of the phosphate buffer containing 0.5 M KCl. The SDS/acrylamide gel electrophoresis revealed that HMWP was eluted in the peak between 0.4 and 0.5 M phosphate buffer (Fig. 3). Fractions containing HMWP were pooled and concentrated by Ficoll 400. The resulting HMWP was dialyzed against 50 mM KCl and 20 mM imidazole (pH 7.0) and then centrifuged at 100,000 *g* for 2 h. By the last step of the purification procedure, separation of the third and fourth peaks was very often rather poor. In those instances, fractions of these peaks were pooled, dialyzed against 0.5 M KCl and 0.2 M KH_2PO_4-K_2HPO_4 (pH 7.0), and then loaded on the hydroxyapatite column again. Proteins were eluted with 400 ml of a gradient from 0.2 to 1 M of the phosphate buffer containing 0.5 M KCl. HMWP was finally purified to 90% homogeneity as judged from the SDS/acrylamide gel electrophoresis (Fig. 1, lane *F*). Starting from 300 g of wet plasmodia, 1–2 mg HMWP was routinely obtained.

Interaction of HMWP with F-Actin

F-actin in 50 mM KCl and 20 mM imidazole (pH 7.0) was mixed with various amount of HMWP dissolved in the same solvent. As the molar ratio of HMWP to actin increased, low
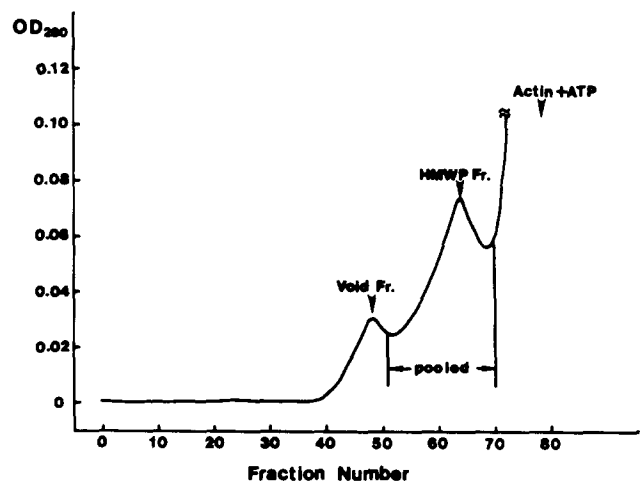

FIGURE 2 Fractionation of crude HMWP on a Sepharose CL6B column. After the KI treatment, crude HMWP was loaded on a CL6B column (3 × 80 cm). Proteins were eluted with 0.5 M KCl and 20 mM imidazole (pH 7.0) and monitored by the absorbance at 280 nm. Flow rate was 30 ml/h and fractions of 3 ml were collected. Fractions (*Fr.*) indicated as "pooled" were those pooled for further purification.

shear viscosity (24) of the mixture increased as shown in Fig. 4. When the molar ratio reached 1:350 (HMWP monomer/actin monomer), the mixture formed a gel-like structure and, therefore, its viscosity could not be measured any more. Consistent with the result, ultracentrifugal analysis by the use of a Beckman model E centrifuge revealed that the mixture of HMWP and F-actin in a molar ratio of 1:350 (HMWP monomer/actin monomer) formed a huge complex which sedimented at an early stage of centrifugation and that no free F-actin remained in the supernatant after spinning down the complex. These results clearly indicate that HMWP is capable of cross-linking F-actin.

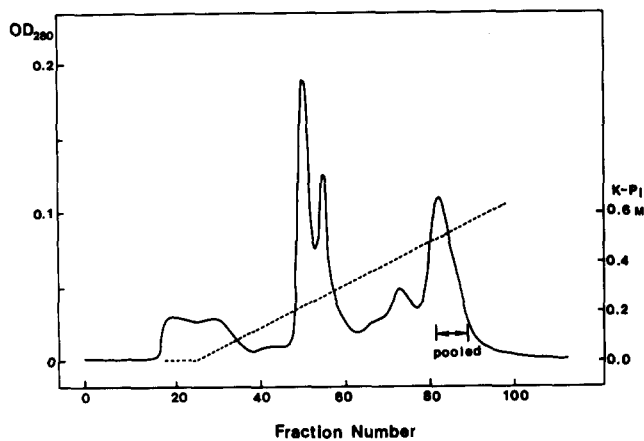Addition of 0.1 mM $CaCl_2$ or 1 mM EGTA to the mixture

FIGURE 3 Fractionation of CL6B-purified fractions on a hydroxy-apatite column. The "pooled" fractions from the CL6B column were directly loaded on a hydroxyapatite column (2 × 5 cm) equilibrated with 0.5 M KCl and 5 mM KH_2PO_4-K_2HPO_4 (pH 7.0). Proteins were eluted with a gradient of the phosphate buffer (5 mM to 1 M). Flow rate was 20 ml/h and fractions of 3 ml were collected. Proteins were monitored by the absorbance at 280 nm.
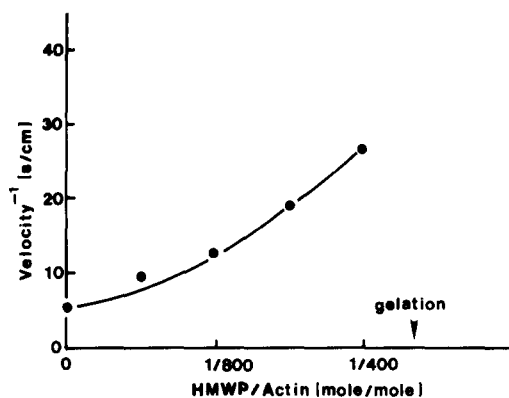

FIGURE 4 Low shear viscosity of the mixture of HMWP and F-actin with various molar ratio of HMWP to actin. Velocity of a ball falling in a tube filled with the mixture of HMWP and F-actin in 50 mM KCl and 20 mM imidazole (pH 7.0) was measured at 25°C. Inverse of the velocity was plotted against the molar ratio of HMWP to actin monomer. At the 1:350 molar ratio, F-actin formed a gel-like structure in which the ball did not move.

of HMWP and F-actin did not affect the gelation activity of the *Physarum* HMWP.

Physicochemical Properties of HMWP

Purified HMWP was subjected to sedimentation equilibrium run in 0.5 M KCl and 20 mM imidazole (pH 7.0). The resulting lnC vs. r^2 plot gave a straight line, indicating that HMWP had a well defined size in this solvent. Its molecular weight was calculated to be 230,000, assuming $\bar{v}$ as 0.724 (calculated from the amino acid composition of HMWP [see Table II]). Since the apparent molecular weight of a single HMWP polypeptide chain was estimated to be 270,000 by the SDS/acrylamide gel electrophoresis, it is most likely that HMWP is in its monomeric state in the high ionic strength solvent.

When HMWP was subjected to the sedimentation equilibrium run in 50 mM KCl and 20 mM imidazole (pH 7.0), it was found that HMWP had a tendency to self-associate at the bottom of the centrifuge cell where protein concentration

became very high. Therefore, it was impossible to carry out the experiment in the low ionic strength solvent.

Sedimentation coefficients of HMWP were determined in a high or low ionic strength solvent. In 0.5 M KCl and 20 mM imidazole (pH 7.0), HMWP sedimented as a component with its sedimentation coefficient of 7.4S (Fig. 5, *a* and *c*). As shown later, it seems that a small amount of HMWP dimer was present (<10%) in a high ionic strength solvent, though the major component was HMWP monomer. It would be difficult, however, to detect the dimeric species from sedimentation boundaries.

When sedimentation velocity experiments were carried out on HMWP in 50 mM KCl and 20 mM imidazole (pH 7.0), it was found that HMWP sedimented as a component with its sedimentation coefficient of 11.5S (Fig. 5, *b* and *d*). Although HMWP has a tendency to self-associate in a low ionic strength solvent as shown above, its boundary shape (Fig. 5*b*) suggests that HMWP in 50 mM KCl and 20 mM imidazole (pH 7.0) is fairly homogeneous in its size at least when its concentration is low (<~0.3 mg/ml).

Stokes radius of HMWP in a low or high ionic strength solvent was determined by the use of HPLC (TSK G4000SW column). When HMWP in 0.5 M or 50 mM KCl containing 20 mM imidazole (pH 7.0) was loaded on the column and then eluted with 0.5 M KCl or 50 mM KCl solvent, a single peak was observed in the elution profile (Fig. 6), consistent with the above notion that HMWP is homogeneous in size both in low and high ionic strength solvents. Stokes radius of HMWP was estimated to be 78 Å in the high ionic strength solvent and 90 Å in the low ionic strength solvent by the use of standard proteins with known Stokes radii. By combining the Stokes radii and sedimentation coefficients, molecular weights of HMWP in low and high ionic strength solvents were calculated to be 420,000 and 240,000, respectively. The
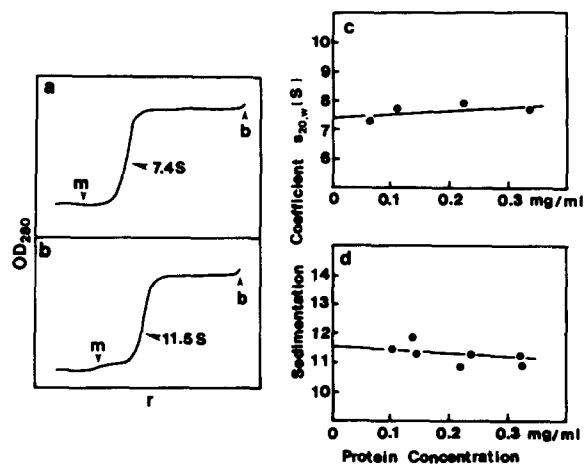

FIGURE 5 Sedimentation velocity analysis of the purified HMWP. The purified HMWP was sedimented at 36,000 rpm in a high or low ionic strength solvent. (a) A scanner trace of the sedimentation boundary of the purified HMWP in 0.5 M KCl and 20 mM imidazole (pH 7.0); (b) a scanner trace of the sedimentation boundary of the purified HMWP in 50 mM KCl and 20 mM imidazole (pH 7.0). m and b indicate positions of meniscus and bottom of the centrifuge cell, respectively. r is the distance from the center of revolution. These sedimentation boundaries were traced at 1 h after reaching 36,000 rpm. (c) Concentration dependence of sedimentation coefficients ($s_{20,w}$) of HMWP in 0.5 M KCl and 20 mM imidazole (pH 7.0); (d) concentration dependence of sedimentation coefficients ($s_{20,w}$) of HMWP in 50 mM KCl and 20 mM imidazole (pH 7.0).
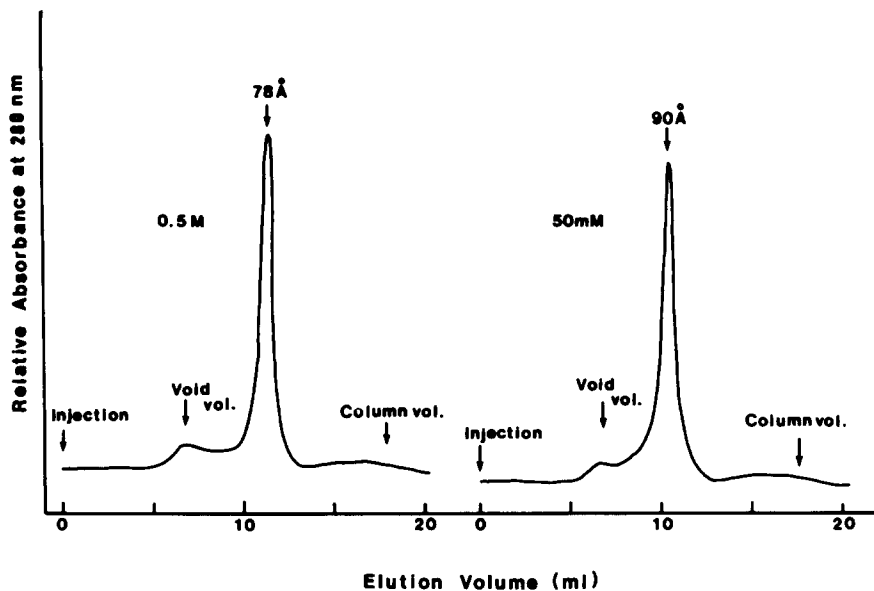
Relative Absorbance at 280 nm

Elution Volume (ml)

latter value is consistent with the molecular weight determined by sedimentation equilibrium within limits of experimental errors (230,000 vs. 240,000). All hydrodynamic data are listed in Table I.

The above studies clearly indicate that the *Physarum* HMWP is in the monomeric state in a high ionic strength solvent while it self-associates to generate dimer in a low ionic strength solvent.

Molecular Morphology of HMWP

Purified HMWP was examined by rotary shadowing of dilute solution in 0.5 M ammonium acetate-50% glycerol or 50 mM ammonium acetate-50% glycerol (16). Fig. 7A shows a general view of HMWP in the high ionic strength solvent. A gallery of individual particles is shown in Fig. 8. Most HMWP particles were observed as flexible rods (Fig. 8, A–H). Since HMWP is in the monomeric state under the conditions used for electron microscopic examinations, these rod-like particles must be HMWP monomers. The contour length of these particles was estimated to be 460 Å ± 50 Å (average of 101 rod-like particles).

Besides the rod-like particles, Y-shaped particles (Fig. 8, *I* and *J*) and particles with a loop (Fig. 8, *K* and *L*) were also observed, though in smaller amounts. The results imply that HMWP might take the two-stranded morphology similar to that of spectrin. When two thinner strands loosely interwine, HMWP would be observed as a Y-shaped particle or a particle with a loop (1, 16, 18). On the other hand, tight association of two strands would generate a rod-like particle. The two-stranded character of HMWP is further supported by its mass to length ratio. The ratio of HMWP (230,000/460 Å) is very close to that of spectrin (460,000/1,000 Å), which shows the two-stranded morphology (16, 18). The mass to length ratio of ABP-type proteins, however, is smaller (540,000/1,800 Å) (17).

There exists a small amount of end-to-end HMWP dimers in electron micrographs (indicated by an arrow in Fig. 7A and in Fig. 8, *M–P*). Contour length of the dimer was 880 Å ± 90 Å (average of 12 particles). Size and shape of these dimeric particles are strikingly similar to those of spectrin dimers seen under the same conditions (Fig. 8, *Q–S*). The structural similarity between spectrin dimer and HMWP di-

TABLE I

Physical Properties of HMWP in a Low or High Ionic Strength Solvent

Parameters	0.5 M KCl	50 mM KCl
Sedimentation coefficient ($s_{20,w}$)	7.4S	11.5S
Stokes radius	78 Å	90 Å
Molecular weight by $s_{20,w}$ and Stokes radius	240,000	420,000
Molecular weight by sedimentation equilibrium	230,000	—

mer (in a high ionic strength solvent) fortifies our notion that HMWP takes the two-stranded morphology similar to that of spectrin in a high ionic strength solvent. It must be noted here that HMWP dimer is a end-to-end dimer while spectrin dimer is a side-by-side dimer.

When HMWP in 50 mM ammonium acetate-50% glycerol was examined by the rotary shadowing, it was found that its morphology was very different from that observed in 0.5 M ammonium acetate-50% glycerol (Figs. 7B and 9). In these electron micrographs, HMWP was seen to have two globular domains joined by a thin, flexible strand. Length between two distal ends of the particle along its long axis was 600 Å ± 55 Å (average of 71 particles). The globular domain had diameter of 140 Å ± 13 Å at its widest point.

As revealed by hydrodynamic studies, these dumb-bell shaped particles are dimers of HMWP. As can be seen by comparing HMWP dimers observed in low or high ionic strength solvent (Fig. 9, A–L vs. Fig. 8, M–P), the dumb-bell shaped dimer cannot be generated by end-to-end association of rod-like monomers. Thus, it seems likely that shift of ionic strength induces changes in polypeptide folding of HMWP such that the rod-like structure is further folded into the globular domain.

Amino acid Composition of HMWP

Table II shows amino acid composition of the purified HMWP. For comparison, amino acid compositions of filamin (8), spectrin (28), and macrophage ABP (29) are shown. It seems that the amino acid composition of HMWP is very similar to those of other actin-binding proteins. Similarity of
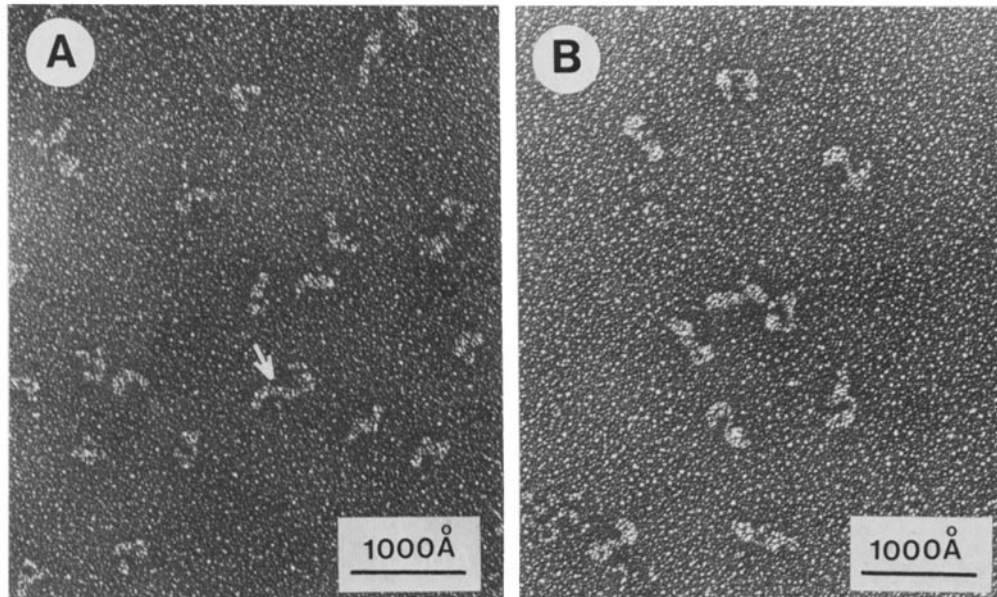
Figure 7.   Survey electron micrographs of HMWP rotary shadowed with platinum/carbon. (A) HMWP dried from 0.5 M ammonium acetate-50% glycerol. (B) HMWP dried from 50 mM ammonium acetate-50% glycerol. × 150,000.
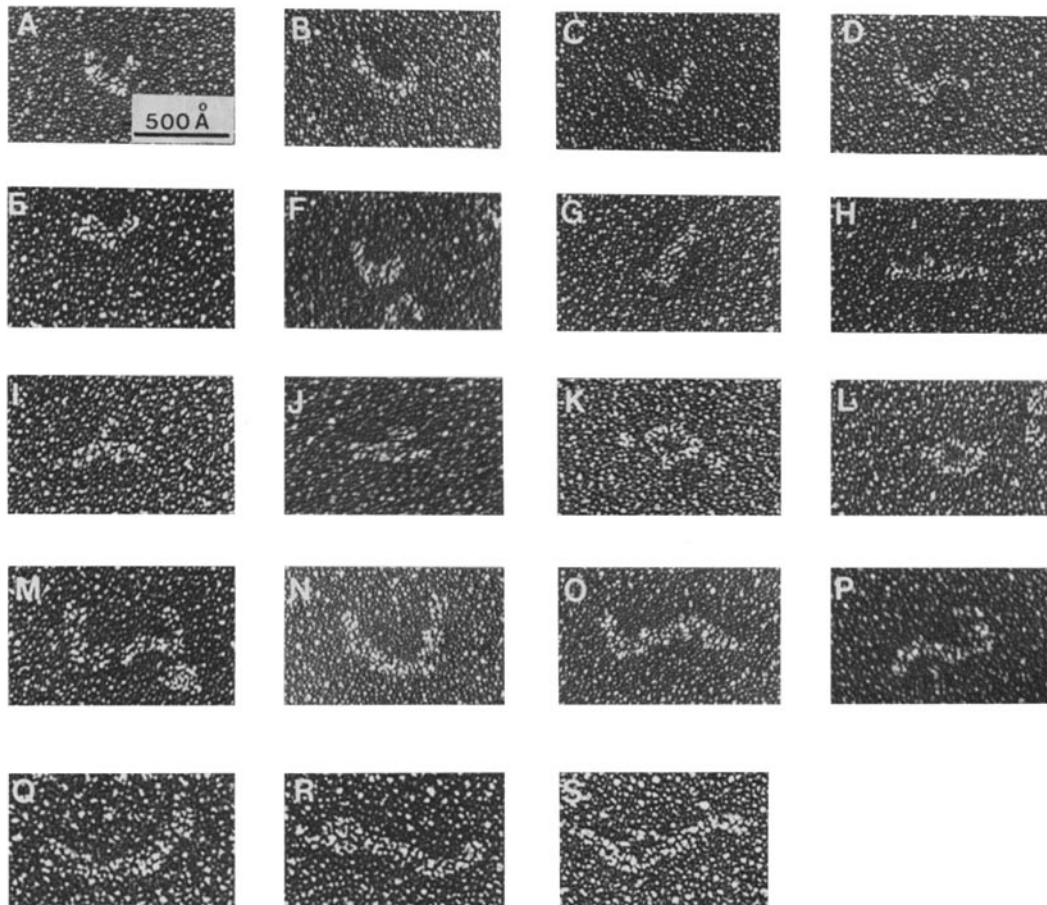

Figure 8   Representative molecules of HMWP in a high ionic strength solvent. HMWP was dried from 0.5 M ammonium acetate-50% glycerol and rotary shadowed with platinum/carbon. (A–H) Rod-like HMWP particles; (I and J) Y-shaped HMWP particles; (K and L) HMWP particles with a loop; (M–P) HMWP dimers joined end-to-end; (Q–S) spectrin dimers as controls. × 240,000.
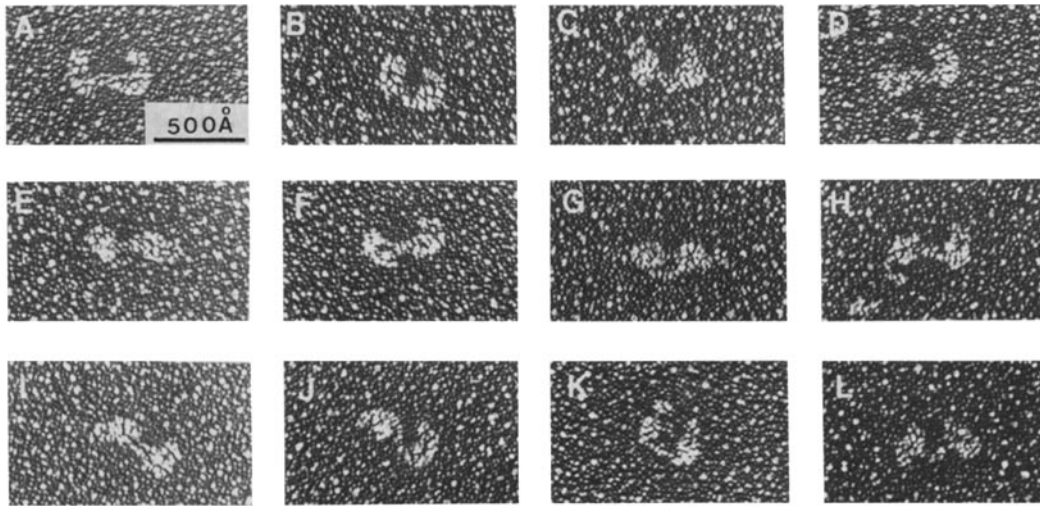
FIGURE 9 Representative molecules of HMWP in a low ionic strength solvent. HMWP was dried from 50 mM ammonium acetate-50% glycerol and rotary shadowed with platinum/carbon. × 240,000.

TABLE II

Amino Acid Composition of HMWP and Comparison with Other High Molecular Weight Actin-binding Proteins

Amino Acid	HMWP	Spectrin*	Filamin[‡]	ABP[§]
Asp	14.3[‖]	10.9	7.6	8.7
Thr	5.4	3.6	5.3	6.2
Ser	6.9	4.1	6.8	6.8
Glu	10.4	20.5	9.5	11.4
Pro	10.2	2.4	8.0	7.1
Gly	11.1	4.9	12.5	11.8
Ala	9.0	9.1	9.3	7.4
Cys	—	1.1	2.0	0.4
Val	6.2	4.7	10.0	8.5
Met	0.2	1.7	0.8	1.3
Ile	1.9	4.0	3.3	4.4
Leu	7.7	12.4	6.0	6.2
Tyr	2.4	2.0	2.6	3.1
Phe	3.1	3.0	2.9	3.2
Lys	6.0	6.7	4.8	6.0
His	2.9	2.6	2.3	2.2
Arg	2.2	5.8	5.5	4.1

* Marchesi et al. (30).
[‡] Wang (8).
[§] Stossel and Hartwig (31).
[‖] mol/100 mol.

the amino acid composition is more striking between HMWP and filamin or ABP than between HMWP and spectrin.

DISCUSSION

We have purified a high molecular weight actin-binding protein (HMWP) from the Physarum polycephalum plasmodia. The protein shares many properties with other high molecular weight actin-binding proteins such as spectrin, filamin, and ABP. Subunit molecular weight of the Physarum HMWP was estimated to be 270,000 from the SDS/acrylamide gel electrophoresis or 230,000 from hydrodynamic measurements. The molecular weight is very close to those of other high molecular weight actin-binding proteins. Furthermore, the Physarum HMWP can cross-link F-actin into a gel-like structure as other proteins do.

High molecular weight actin-binding proteins isolated so far are grouped into two classes according to their molecular morphologies as well as their antigenicities (11). Spectrin-like

proteins are rod-like molecules with two-stranded morphology while ABP-type proteins are single-stranded (1, 16–18) (Fig. 10). Examination of molecular morphology of the Physarum HMWP has revealed that HMWP in a high ionic strength solvent is a rod-like flexible particle with a contour length of 460 Å and that the particle shows the two-stranded morphology similar to that of spectrin (Fig. 10). Since the Physarum HMWP is in the monomeric state in the high ionic strength solvent as revealed by hydrodynamic studies, two thinner strands in the rod-like particle would consist of a single polypeptide chain, while each thinner strand of spectrin dimer consists of an α- or β-chain (16, 18). This notion is consistent with the observation that contour length of the Physarum HMWP in the high ionic strength solvent is half of that of spectrin dimer (460 Å vs. 1,000 Å) while its width is similar to that of spectrin dimer. Actually, the end-to-end dimer of HMWP observed in the high ionic strength solvent, though scarce, shows remarkable resemblance to spectrin dimer, in which two polypeptide chains run side-by-side.

When the Physarum HMWP in a low ionic strength solvent was examined by rotary shadowing, its molecular morphology was very different from that observed in a high ionic strength
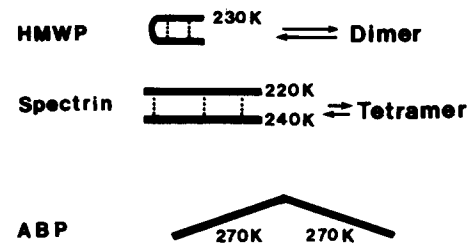

FIGURE 10 Schematic representation of molecular morphologies of HMWP, spectrin, and ABP. Solid lines represent polypeptide strands. An HMWP polypeptide (~230,000 mol wt) is folded in a two-stranded rod, while two strands of spectrin dimer are composed of one α-chain (240,000 mol wt) and one β-chain (220,000 mol wt). The two-stranded structure may be stabilized by noncovalent forces (dotted lines). Two ABP polypeptides (270,000 mol wt) are joined end-to-end to form a single-stranded rod. HMWP monomer is in equilibrium with dimer, depending on ionic conditions. Spectrin dimer is also in equilibrium with tetramer, depending on ionic conditions. On the other hand, ABP homodimer is stable in various ionic conditions. (K, × 10³).

solvent. It was observed that HMWP had two globular domains connected with a thin, flexible joint and were dumb-bell shaped. It is tempting to speculate that two globular domains correspond to two actin-binding sites necessary for cross-linking F-actin and that each domain consists of a single polypeptide chain. Examinations of molecular morphologies of HMWP in a low or high ionic strength solvent indicate that simple end-to-end association of rod-like monomers of HMWP observed in a high ionic strength solvent cannot generate the dumb-bell shaped HMWP dimer observed in a low ionic strength solvent. The two strands in the rod-like structure of HMWP might be folded further into a globular domain by the shift of ionic strength. The notion is consistent with the observation that the length between two distal ends along the dumb-bell shaped dimer is shorter than the contour length of the end-to-end dimer in the high ionic strength solvent (600 vs. 880 Å).

It must be noted here that the ionic strength in the *Physarum* plasmodia is close to that of the low ionic strength solvents used in this study (50 mM KCl-20 mM imidazole or 50 mM ammonium acetate-50% glycerol). Thus, it seems that the dumb-bell shaped HMWP dimer is the molecule acting as a cross-linker of F-actin in the *Physarum* plasmodia. In this context, flexibility of the thin strand connecting two globular domains of the dimer particle seems to be functionally important. If the two globules correspond to two actin-binding sites, the flexible joint would give these sites enough freedom to attach to neighboring F-actin filaments (17).

The striking difference in molecular morphologies of the dumb-bell shaped HMWP dimer and the rod-shaped actin-binding proteins such as spectrin and ABP might lead one to think that the treatment of HMWP with KI, which is a chaotropic reagent, during its purification induced changes in its structure. However, we think this is unlikely for the following reasons. (*a*) Brain fodrin and TW-260/240 prepared by procedures that include the KI treatment showed the two-stranded morphology characteristic of the spectrin-type molecule (1, 2). (*b*) The purified HMWP was capable of cross-linking F-actin very efficiently. (*c*) The purified HMWP had well-defined size and shape both in low and high ionic strength solvents. (*d*) Our preliminary experiments showed that HMWP purified by hydroxyapatite and DEAE cellulose columns without the KI treatment had the same Stokes radii as HMWP purified here, both in low and high ionic strength solvents.

Thus, we conclude that morphology of the *Physarum* HMWP is quite distinct from that of other high molecular weight actin-binding proteins, though its basic architecture is very similar to that of spectrin-type molecules. It will be interesting, therefore, to explore structural relationships between the *Physarum* HMWP and other high molecular weight actin-binding proteins by using immunochemical techniques.

REFERENCES

1. Glenny, J. R., P. Glenny, M. Osborn, and K. Weber. 1982. An F-actin and calmodulin-binding protein from isolated intestinal brush boders has a morphology related to spectrin. *Cell.* 28:843–854.
2. Glenny, J. R., P. Glenny, and K. Weber. 1982. Erythroid spectrin, brain fodrin, and intestinal brush boder proteins (TW-260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type-specific subunit. *Proc. Natl. Acad. Sci. USA.* 79:4002–4005.
3. Stossel, T. P., and J. H. Hartwig. 1975. Interactions between actin, myosin and an actin-binding protein from rabbit alveolar macrophages. *J. Biol. Chem.* 250:5706–5712.
4. Rosenberg, S., A. Stracher, and R. C. Lucas. 1981. Isolation and characterization of actin and actin-binding protein from human platelets. *J. Cell. Biol.* 91:201–211.
5. Weihing, R. R. 1983. Purification of a HeLa cell high molecular weight actin binding protein and its identification in HeLa cell plasma membrane ghosts and intact HeLa cells. *Biochemistry.* 22:1839–1847.
6. Scloss, J. A., and R. D. Goldman. 1979. Isolation of a high molecular weight actin-binding protein from baby hamster kidney (BHK-21) cells. *Proc. Natl. Acad. Sci. USA.* 76:4484–4488.
7. Wang, K., J. F. Ash, and S. J. Singer. 1975. Filamin, a new high-molecular-weight protein found in smooth muscle and non-muscle cells. *Proc. Natl. Acad. Sci. USA.* 72:4483–4486.
8. Wang, K. 1977. Filamin, a new high-molecular-weight protein found in smooth muscle and nonmuscle cells. Purification and properties of chicken gizzard filamin. *Biochemistry.* 16:1857–1865.
9. Wallach, D., P. J. A. Davies, and I. Pastan. 1978. Purification of mammalian filamin. *J. Biol. Chem.* 253:3328–3335.
10. Branton, D., C. M. Cohen, and J. Tyler. 1981. Interaction of cytoskeletal proteins of human erythrocyte membrane. *Cell.* 24:24–32.
11. Levine, J., and M. Willard. 1981. Fodrin: axonally transported polypeptides associated with the internal periphery of many cells. *J. Cell Biol.* 90:631–643.
12. Kakiuchi, S., K. Sobue, K. Kanda, K. Morimoto, S. Tsukita, S. Tsukita, H. Ishikawa, and M. Kurokawa. 1982. Correlative biochemical and morphological studies of brain calspectin: a spectrin-like calmodulin-binding protein. *Biomed. Res.* 3:400–410.
13. Bennett, V., J. Davis, and W. E. Fowler. 1982. Brain spectrin, a membrane-associated protein related in structure and function to erythrocyte spectrin. *Nature (Lond.).* 299:126–131.
14. Burridge, K., T. Kelly, and P. Mangeat. 1982. Nonerythrocyte spectrins: actin-membrane attachment proteins occurring in many cell types. *J. Cell Biol.* 95:478–486.
15. Ungewickell, E., and W. Gratzer. 1978. Self-association of human spectrin. A thermodynamic and kinetic study. *Eur. J. Biochem.* 88:379–385.
16. Shotton, D. M., B. E. Burke, and D. Branton. 1979. Molecular structure of human erythrocyte spectrin: biophysical and electron microscopic studies. *J. Mol. Biol.* 131:303–329.
17. Hartwig, J. H., and T. P. Stossel. 1981. Structure of macrophage actin-binding protein molecules in solution and interacting with actin filaments. *J. Mol. Biol.* 145:563–581.
18. Tyler, J. M., J. M. Anderson, and D. Branton. 1980. Structural comparison of several actin-binding macromolecules. *J. Cell Biol.* 85:489–495.
19. Brotschi, E. A., J. H. Hartwig, and T. P. Stossel. 1978. The gelation of actin by actin-binding protein. *J. Biol. Chem.* 253:8988–8993.
20. Brenner, S. L., and E. D. Korn. 1979. Spectrin-actin interaction. *J. Biol. Chem.* 254:8620–8627.
21. Stossel, T. P., J. H. Hartwig, H. L. Yin, K. S. Zaner, and O. I. Stendahl. 1982. Actin gelation and the structure of cortical cytoplasm. *Cold Spring Harbor Symp. Quant. Biol.* 47:569–578.
22. Hatano, S., and M. Tazawa. 1968. Isolation, purification and characterization of myosin B from myxomycete plasmodia. *Biochim. Biophys. Acta.* 154:507–519.
23. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
24. MacLean-Fletcher, S. D., and T. D. Pollard. 1980. Viscometric analysis of the gelation of *Acanthamoeba* extract and purification of two gelation factors. *J. Cell Biol.* 85:414–428.
25. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
26. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
27. Nachmias, V. T. 1974. Properties of *Physarum* myosin purified by a potassium iodide procedure. *J. Cell Biol.* 62:54–65.
28. Marchesi, S. L., E. Steer, V. T. Marchesi, and T. W. Tillack. 1970. Physical and chemical properties of a protein from red cell membranes. *Biochemistry.* 9:50–57.
29. Stossel, T. P., and J. H. Hartwig. 1976. Interaction of actin, myosin, and a new actin-binding protein of rabbit pulmonary macrophages. *J. Cell Biol.* 68:602–619.