Fragmin: A Calcium Ion Sensitive Regulatory Factor on the Formation of Actin Filaments†

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ABSTRACT: *Physarum* actinin previously isolated [Hatano, S., & Owaribe, K. (1976) in *Cell Motility* (Goldman, R., Pollard, T., & Rosenbaum, J., Eds.) Vol. 3, Book B, p 499, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY] was found to be a 1:1 complex of actin and fragmin which is a regulatory factor in the formation of actin filaments. Since fragmin did not contain a cysteine residue, it was purified from the complex by the selective cleavage of actin with 2-nitro-5-thiocyanobenzoic acid, followed by column chromatography. Fragmin had nearly the same molecular weight as actin, but had a quite different amino acid composition. When added to G-actin before polymerization, fragmin accelerated the initial viscosity increase of actin solutions induced by salts, but kept the final viscosity much lower than normal F-actin. When added to F-actin after polymerization, fragmin drastically reduced the viscosity of actin solutions. In both cases, the final products of reaction of fragmin with actin were short F-actin filaments. The number average length of the filaments decreased with the increasing molar ratio of fragmin to actin, and the length distribution was always exponential. Fragmin required for its activity a concentration of free Ca^{2+} higher than 10^{-5} M. When the concentration of free Ca^{2+} was lower than 10^{-7} M, fragmin affected neither actin polymerization nor F-actin. The regulation by Ca^{2+} was reversible.

In most of the nonmuscle cells, the motile system composed of actin and myosin does not make any permanent structure but repeats assembly and disassembly. Actin in these cells is found in both filamentous (Ishikawa et al., 1969; Lazarides & Weber, 1974) and nonfilamentous or dispersed states (Abramowitz et al., 1975; Bray & Thomas, 1976; Merriam & Clark, 1978). However, highly purified actin from nonmuscle cells has the same polymerizability as actin from muscle (Hatano & Owaribe, 1976; Gordon et al., 1977). Therefore, nonmuscle cells must have regulators for the formation of actin filaments. Actually, several proteins which interact with actin and regulate its polymerization, α-actinin (Ebashi et al., 1964), β-actinin (Maruyama, 1965), DNase I (Hitchcock et al., 1976), profilin (Carlsson et al., 1977), gelsolin (Yin & Stossel, 1979), etc., have been found from various cells.

Actin isolated by Hatano & Oosawa (1966) from *Physarum* plasmodium formed F-actin upon the addition of salts, but, in the presence of Mg^{2+}, it formed another polymer which had low viscosity and remarkable ATPase activity. Kamiya et al. (1972) demonstrated that muscle actin could form a similar polymer if polymerized with β-actinin. Later, the original preparation of *Physarum* actin was found to contain a protein similar to β-actinin in its properties (Maruyama et al., 1976). Hatano & Owaribe (1976) purified this protein and named it *Physarum* actinin. In $NaDodSO_4$[1] gel electrophoresis, *Physarum* actinin showed a single band, the mobility of which was the same as actin. Therefore, some doubt was left as to whether or not *Physarum* actinin is really a new protein different from actin.

In this work, we have undertaken further purification of *Physarum* actinin. In the course of the experiment, *Physarum* actinin has been shown to be a 1:1 complex of actin and the other protein. This protein, after being freed from actin, has approximately the same molecular weight as actin, but has a quite different amino acid composition. It reacts with both G- and F-actin and makes actin polymers very short. This reaction requires Ca^{2+}. We call the new protein fragmin.

Materials and Methods

Preparation of Physarum Actinin. Plasmodia of the myxomycete, *Physarum polycephalum*, were cultured by using the method of Camp (1936) with some modification (Hatano & Oosawa, 1966).

In the previous method (Hatano & Owaribe, 1976), muscle myosin was used in the process of purification of actin and actinin from *Physarum*, but here we do not use it. Fresh plasmodia (240 g) were homogenized in an equal volume of an extraction medium containing 10 mM EDTA, 0.4 mM DTT, and 20 mM Tris-HCl buffer of pH 8.2 with a Potter-Elvehjem glass homogenizer and then centrifuged at 80000*g* for 90 min at 4 °C. (All subsequent procedures were carried out at 0–4 °C.) The supernatant, after the pH was adjusted to 8.0, was fractionated by a DEAE-cellulose column (first) (Figure 1). The effect of each fraction on the polymerization of muscle G-actin was examined as follows. After the addition of a part of each fraction (0.2 mL) to 0.2 mL of muscle G-actin solution, the actin (final concentration 0.9 mg/mL) was polymerized in the presence of 0.1 M KCl, 2 mM $MgCl_2$, 1 mM ATP, 20 mM imidazole-HCl buffer of pH 7.0, and 1 mM Ca-EGTA buffer of pCa 5 at 22 °C for 60 min. The fractions (shaded area in Figure 1) which reduced the flow birefringence of the actin solution were found to contain *Physarum* actinin. To those fractions, ammonium sulfate was added to 65% saturation, and precipitates were collected by centrifugation at 16000*g* for 20 min. They were dissolved in a solution containing 50 mM KCl, 0.2 mM DTT, and 10 mM Tris-HCl buffer of pH 8.0 and dialyzed against the same solution. The dialysate was subjected to gel filtration on a Sephadex G-200 column (Figure 2). To the fractions containing actinin, ammonium sulfate was added to 65% saturation, and precipitates were collected by centrifugation and

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; TNB-CN, 2-nitro-5-thiocyanobenzoic acid; HMM, heavy meromyosin.

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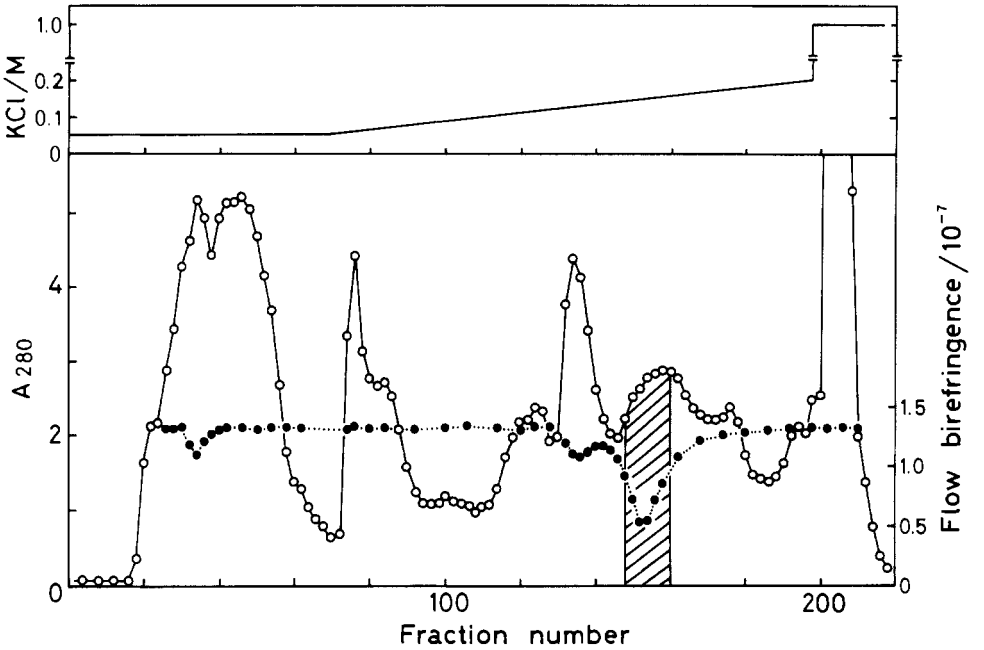

FIGURE 1: Ion exchange chromatography of *Physarum* extract. The extract was applied to a DEAE-cellulose column (first) (3.2 × 60 cm) equilibrated with 50 mM KCl, 0.2 mM DTT, and 10 mM Tris-HCl buffer of pH 8.0. The column was washed with 1 L of the same solution and eluted with a linear gradient of 50–200 mM KCl in 0.2 mM DTT and 10 mM Tris-HCl buffer of pH 8.0. The elution was performed with a flow rate of 64 mL/h. Fractions of 15 mL were collected and assayed for absorbance at 280 nm (○). Flow birefringence (●) of solutions of muscle actin which was polymerized after the addition of a part of each fraction was measured as described under Materials and Methods.

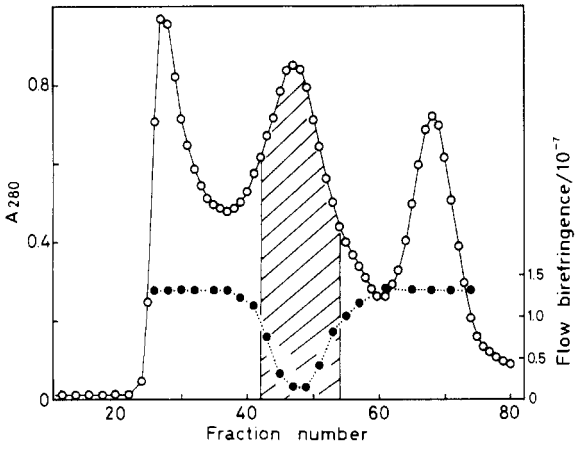

FIGURE 2: Gel filtration of the actinin fraction from the DEAE-cellulose column (Figure 1). The actinin fraction was applied to a Sephadex G-200 column (2.6 × 105 cm) equilibrated with 50 mM KCl, 0.2 mM DTT, and 10 mM Tris-HCl buffer at pH 8.0. The column was eluted by the same solution with a flow rate of 16 mL/h. Fractions of 5.9 mL were collected and assayed for absorbance at 280 nm (○). Flow birefringence (●) was measured as in Figure 1.

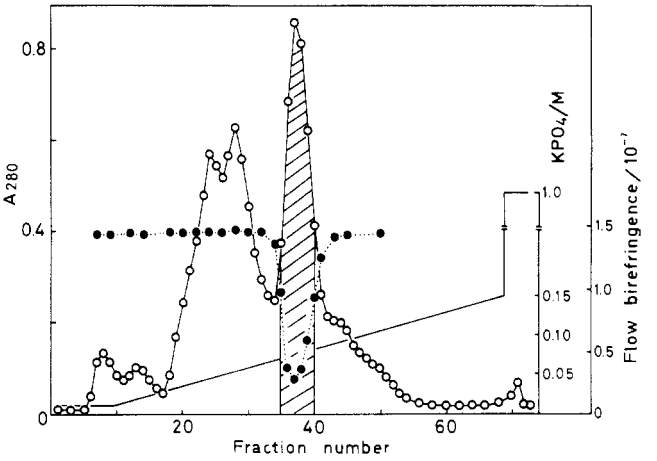

FIGURE 3: Hydroxylapatite chromatography of the actinin fraction from the Sephadex G-200 column (Figure 2). The actinin fraction containing 0.6 M KCl was applied to hydroxylapatite column (first) (1.2 × 30 cm) equilibrated with 0.6 M KCl, 0.2 mM DTT, and 10 mM potassium phosphate buffer of pH 6.8. The column was eluted with a linear gradient of 10–150 mM potassium phosphate buffer of pH 6.8 in 0.6 M KCl and 0.2 mM DTT. The elution was performed with a flow rate of 19 mL/h. Fractions of 3.2 mL were collected and assayed for absorbance at 280 nm (○). Flow birefringence (●) was measured as in Figure 1.

dissolved into a solution of 0.2 mM DTT and 10 mM potassium phosphate buffer at pH 6.8. After we added 0.25 volume of 3 M KCl, the solution was fractionated by a hydroxylapatite column (first) (Figure 3). The actinin fractions were dialyzed against a solution of 0.1 mM DTT and 10 mM Tris-HCl buffer of pH 8.2. The dialysate was concentrated by a Dia-Flow apparatus with a UM 10 membrane and finally centrifuged at 105000*g* for 30 min. In $NaDodSO_4$ gel electrophoresis, the supernatant gave a single band which was equivalent to *Physarum* actinin purified by Hatano & Owaribe (1976). This supernatant was found to consist of two proteins, actin and fragmin. Further purification of fragmin is described under Results.

Assay of Fragmin Activity. At each step of purification, the activity of fragmin was examined in the following way. After the addition of various amounts of sample protein to a G-actin solution of a constant concentration, [A], G-actin was polymerized in 0.1 M KCl, 2 mM $MgCl_2$, 1 mM ATP, and 1 mM Ca-EGTA buffer of pCa 5 and 20 mM imidazole-HCl buffer of pH 7.0, at 22 °C for 60 min. Flow birefringence (Δn) of the polymerized actin solution was measured as described earlier (Hatano & Owaribe, 1976). The concentration of the sample protein, [F], which was needed for 50% suppression of Δn of the polymerized actin, was estimated. The ratio [A]/[F] was defined as G activity.

Similarly, various amounts of sample protein were added to an F-actin solution of a constant concentration, [A], and, after incubation of 60 min, the flow birefringence Δn was measured. The concentration of sample protein, [F'], which

was needed for 50% reduction of Δn was estimated. The ratio [A]/[F'] was defined as F activity.

In the assay, actin from rabbit muscle was used.

Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was carried out according to the method of Weber & Osborn (1969) using 7.5% polyacrylamide slab gels. For urea–$NaDodSO_4$ gel electrophoresis, sample solutions were dialyzed against a solution containing 1% $NaDodSO_4$, 1% β-mercaptoethanol, 25% glycerol, 6 M urea, and 10 mM sodium phosphate buffer of pH 7.2 and then boiled for 30 min. Gels and running buffer contained 6 and 4 M urea, respectively. Gel pieces after staining with Coomassie Brilliant Blue were scanned at 570 nm in a Gilford spectrophotometer, Model 250, equipped with a Gilford linear transport, Model 2410-S.

Amino Acid Analysis. The protein sample was hydrolyzed in 6 N HCl at 110 °C in evacuated sealed tubes. Hydrolysis times were varied as 15, 24, 64, and 72 h. Amino acid analysis was carried out on a homemade analyzer based on a JEOL JLC-3 liquid chromatograph separation system and a JLC-BL2 optical detection system.

Electron Microscopy. Samples were mounted on grids covered with carbon-coated collodion film and stained with 1% uranyl acetate. Specimens were observed with a JEM 110-C electron microscope at an accelerating voltage of 80 kV.

Other Procedures and Materials. Viscosity was measured in Ostwald type viscometers. Flow birefringence was measured in a homemade concentric cylinder apparatus.

Protein concentrations were determined by the biuret method (Gornall et al., 1949) in which absorbance at 540 nm was assumed to be 0.070 at 1 mg/mL fragmin or actin in a light path of 1 cm. Free sulfhydryl groups were assayed with *p*-chloromercuribenzoate as previously described (Boyer, 1954). ATPase activity was measured by the method of Murphy & Riley (1962). Concentrations of free Ca^{2+} (pCa 5–8) and free Mg^{2+} (pMg 5–7) were calculated by assuming the apparent dissociation constants of Ca-EGTA and Mg-EDTA at pH 7.0 to be 4.8×10^{-6} and 2.4×10^{-5} M, respectively (Amos et al., 1976).

Muscle actin was prepared from rabbit skeletal muscle as previously described (Ebashi & Ebashi, 1964). Muscle HMM was prepared according to the method of Szent-Györgyi (1953). *Physarum* actin was prepared according to the method of Hatano & Owaribe (1977). Block copolymers of F-actin and the complex of F-actin with HMM were prepared by the method of Kondo & Ishiwata (1976).

TNB-CN was the generous gift of Dr. H. Ueno of Kyoto University. Hydroxylapatite was purchased from Seikagaku Kogyo, Sephadex from Pharmacia Fine Chemicals, and DEAE-cellulose (DE-52) from Whatman Ltd.

Results

Purification of Fragmin. As described under Materials and Methods, after the hydroxylapatite column chromatography (first), the fraction of *Physarum* actinin gave a single band in $NaDodSO_4$ gel electrophoresis. However, in urea–$NaDodSO_4$ gel electrophoresis, it separated into two bands, as shown in Figure 4D. The two bands had the same area in the densitometer, and the lower band corresponded to actin. That is, our preparation of *Physarum* actinin contained nearly equal amounts of two proteins–actin and another which we call fragmin.

The cysteine content of *Physarum* actinin was estimated to be about 1.4 mol/42 000 g of protein by titration with *p*-chloromercuribenzoate, while that of *Physarum* actin has been reported to be 4 mol/42 000 g (Vandekerkhove & Weber, 1978). Fragmin might not have cysteine residues. Therefore, actinin was treated with TNB-CN, which could cut the peptide bond at the N-terminal side of the cysteine residues (Jacobson et al., 1973). The protein was first treated with 0.1 mM DTT, 6 M urea, and 0.1 M Tris-HCl buffer of pH 8.2 at 25 °C for 30 min, and then, after the addition of 0.7 mM TNB-CN, it was further incubated for 30 min. The cleavage reaction was started by raising the pH to 9.2 at 35 °C and continued for 18 h.

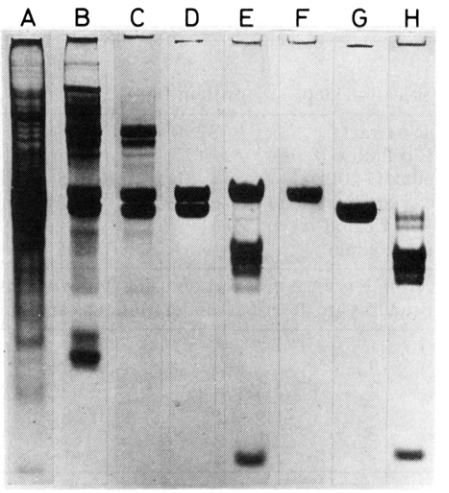

FIGURE 4: Urea–$NaDodSO_4$ gel electrophoresis of fractions obtained during the purification of fragmin. Electrophoresis was performed as described under Materials and Methods. The run length of bromophenol blue was 10.5 cm: (A) *Physarum* extract; (B) DEAE-cellulose (first) fraction; (C) Sephadex G-200 fraction; (D) hydroxylapatite (first) fraction; (E) hydroxylapatite (first) fraction treated with TNB-CN; (F) purified fragmin; (G) *Physarum* actin; (H) *Physarum* actin treated with TNB-CN.

Urea–$NaDodSO_4$ gel electrophoresis (Figure 4E) showed that the protein of the upper band was not cleaved, while that of the lower band was cleaved into several fragments, the electrophoretic pattern of which was similar to that of *Physarum* actin treated with TNB-CN.

After 5 mM β-mercaptoethanol was added, the protein solution was dialyzed against a solvent containing 0.6 M KCl and 20 mM potassium phosphate buffer of pH 6.8. The dialysate that contained fragmin and actin fragments was applied to a hydroxylapatite column (second) (1 × 25 cm), which had been equilibrated with the same solvent as that for the dialysis. After elution with a linear gradient of 20–110 mM potassium phosphate buffer in 0.6 M KCl, the fraction containing fragmin was identified by its ability to reduce the flow birefringence of polymerized actin. The fraction was dialyzed against a solvent containing 30 mM KCl, 6 M urea, and 10 mM Tris-HCl buffer of pH 8.0. The dialysate was applied to a DEAE-cellulose column (second) (0.9 × 18 cm) equilibrated with the same solvent as used for dialysis. The column was eluted with a linear gradient of 30–120 mM KCl in 6 M urea and 10 mM Tris-HCl buffer of pH 8.0. The main peak containing fragmin was dialyzed against a solution containing 20 mM imidazole-HCl buffer of pH 7.0 and concentrated with a Dia-Flow apparatus equipped with a UM-10 membrane. Finally, the solution was centrifuged at 105000*g* for 30 min. In urea–$NaDodSO_4$ gel electrophoresis, the supernatant gave a single band as shown in Figure 4F. From 240 g of plasmodia, 1.2 mg of fragmin was obtained.

In Table I, the activity of fragmin preparation is compared during the process of purification.

Reconstitution of the Equimolar Complex of Actin and Fragmin. An equimolar association was observed when actin

Table I: Summary of the Purification[a]

purification step	protein (mg)	fragmin act.[b] G	F
soluble extract	3290	0.5	
DEAE-cellulose (first)	127	3.2	
Sephadex G-200	36	10.5	0.7
hydroxylapatite (first)	9.5	33.3	2.3
TNB–CN treated		29.4	16.7
purified fragmin	1.2	62.5	55.6

[a] Results from a typical preparation using 240 g of plasmodium. [b] Assay procedures are described under Materials and Methods.

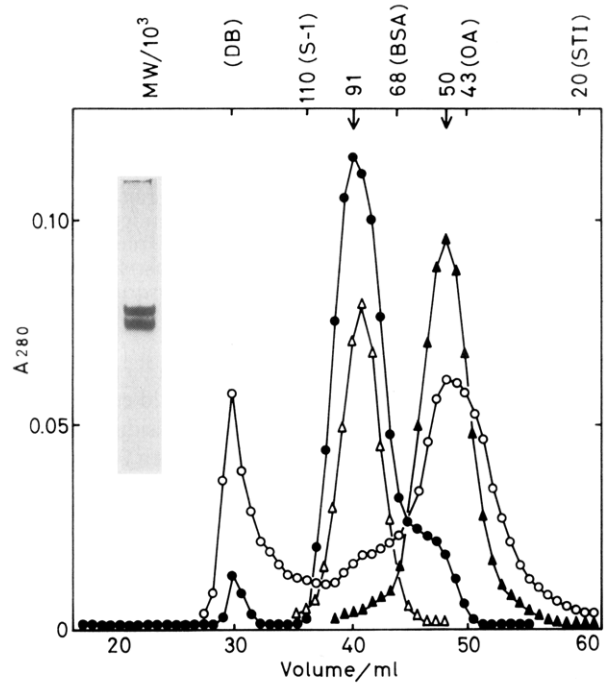

FIGURE 5: Molecular weight estimation of the reconstituted actin–fragmin complex by gel filtration on Sephadex G-100. *Physarum* actin (0.5 mg) and fragmin (0.5 mg) were mixed in the medium as described in the text and incubated at 4 °C for 4 h. The mixture of actin and fragmin incubated in the presence of Ca^{2+} (●) or EGTA (○), fragmin (▲), and *Physarum* actinin (△) was subjected to gel filtration on a Sephadex G-100 column (1 × 110 cm). Molecular weights of the proteins were estimated by using dextran blue (DB), myosin subfragment 1 (S-1), bovine serum albumin (BSA), ovalbumin (OA), and soybean trypsin inhibitor (STI) as references. The inset shows the urea–$NaDodSO_4$ gel electrophoretic pattern of the reconstituted complex eluted at 40 mL.

was mixed with the same amount of fragmin in the medium containing 50 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.1 mM DTT, and 5 mM imidazole-HCl buffer of pH 7.0. Figure 5 shows Sephadex G-100 chromatography of fragmin and the reconstituted complex of actin and fragmin. Molecular weights of the complex and fragmin were estimated as 91 000 and 50 000, respectively. The elution volume and the urea–$NaDodSO_4$ gel electrophoretic pattern of the reconstituted complex were the same as those of our preparation of *Physarum* actinin. That is, *Physarum* actinin is a 1:1 complex of actin and fragmin. Fragmin did not bind to G-actin when they were incubated in a medium containing 0.2 mM EGTA instead of $CaCl_2$.

Amino Acid Composition of Fragmin. In Table II, the amino acid composition of fragmin is compared with that of *Physarum* actin. Significant differences in composition were found in the amounts of methionine, isoleucine, leucine, lysine, N^{τ}-methylhistidine, and cysteine. Neither cysteine residues

Table II: Amino Acid Compositions of Fragmin and *Physarum* Actin[a]

amino acid	fragmin	actin
Asx	36	31
Thr	19	27
Ser	17	25
Glx	50	42
Pro	14	19
Gly	38	32
Ala	37	29
$^1/_2$-Cys	0[b]	3
Val	20	22
Met	3	15
Ile	15	25
Leu	36	27
Tyr	14	14
Phe	19	13
Lys	29	19
His	10	8
N^{τ}-Me-His	0	1
Arg	13	18
total	370	370

[a] Amino acid compositions of fragmin and actin were normalized to total amino acid residues of 370 for both proteins. Tryptophan was not determined. [b] This value was estimated by the TNB-CN treatment experiment, as described in the text.

nor disulfide bridges were detected in fragmin by the TNB-CN treatment after preincubation in 0.1 M DTT, 6 M urea, and 0.1 M Tris-HCl buffer of pH 8.0 at 35 °C for 2 h.

Effect of Fragmin on F-Actin and Actin Polymerization. The action of fragmin on F-actin is most remarkable. *Physarum* actinin had only a small effect on F-actin if it was added after polymerization of actin. However, after separation from *Physarum* actin by treatment with TNB-CN, fragmin was found to greatly reduce the flow birefringence of an F-actin solution, even if it was added after the polymerization of actin (Table I). This fragmin effect was enhanced threefold by purification with column chromatography.

Various amounts of fragmin were added to an F-actin solution, which contained 0.1 M KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, and 20 mM imidazole-HCl buffer of pH 7.0. Then the reaction was initiated by adjusting the free Ca^{2+} concentration at pCa 5 with 2 mM Ca-EGTA buffer. As shown in Figure 6A, the viscosity of the solution decreased within 30 s, and the final viscosity decreased with increasing amounts of added fragmin. At a molar ratio of fragmin to actin of 1:5, the viscosity dropped to a level near that of a G-actin solution.

When G-actin was polymerized by the addition of 0.1 M KCl, 2 mM $MgCl_2$, 1 mM ATP, and 20 mM imidazole-HCl buffer of pH 7.0 in the presence of fragmin and 1 mM Ca-EGTA of pCa 5, the viscosity rapidly increased, but the final value of the viscosity was much smaller than that in the absence of fragmin (Figure 6B). The final values of viscosity of actin solutions were nearly equal when equal amounts of fragmin were added before and after polymerization of actin (Figure 7).

As shown in the electron micrograph of Figure 8B, the final products from the reaction of fragmin with F-actin were short F-actin fragments. F-Actins polymerized in the presence of various amounts of fragmin are also shown in Figure 8C–E. Shorter filaments were formed when larger amounts of fragmin were added. The length distribution of these F-actin filaments gave nearly an exponential distribution, as shown in Figure 9A, which would be expected if a dynamic equilibrium existed between monomer and polymer (Oosawa & Kasai, 1962; Oosawa, 1970).

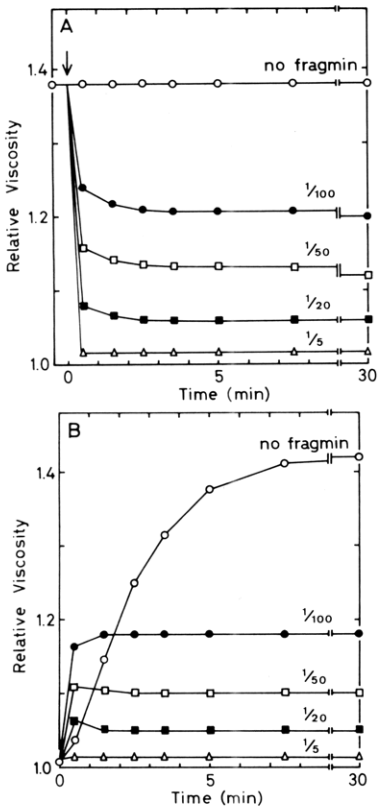

FIGURE 6: (A) Effect of fragmin on F-actin. Various amounts of fragmin were added to *Physarum* F-actin solutions. Solvent conditions are described in the text. After free Ca^{2+} concentrations at pCa 5 were adjusted with 2 mM Ca-EGTA buffer (as shown by the arrow), viscosity changes were monitored at 22 °C. (B) Effect of fragmin on actin polymerization. After various amounts of fragmin were added, *Physarum* G-actin was polymerized as described in the text. As a control, actin was polymerized in the absence of fragmin. Polymerization was monitored by viscometry at 22 °C. The concentration of actin was 0.48 mg/mL. Molar ratios of fragmin to actin are shown in the figures.

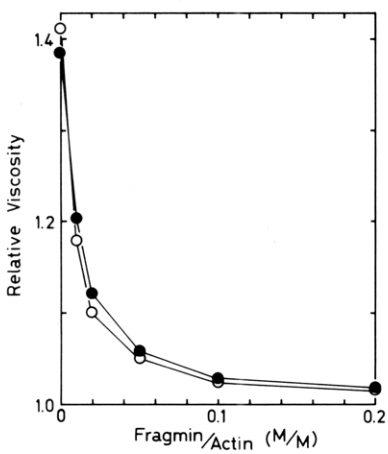

FIGURE 7: Correlation between the final viscosity of actin solution and the amount of added fragmin. The final viscosity values shown in Figure 6, A and B, are plotted as a function of the molar ratio of fragmin to actin. Symbols: (●) from Figure 6A; (○) from Figure 6B.

The length distribution of F-actin fragments obtained by adding fragmin to F-actin also gave an exponential distribution, as shown in Figure 9B, suggesting random breaking of F-actin. In both cases, Figure 9, A and B, the number average length agreed well with that calculated, assuming that almost all actins were in the polymer state and each F-actin filament had one monomer of fragmin. The length distribution of intact

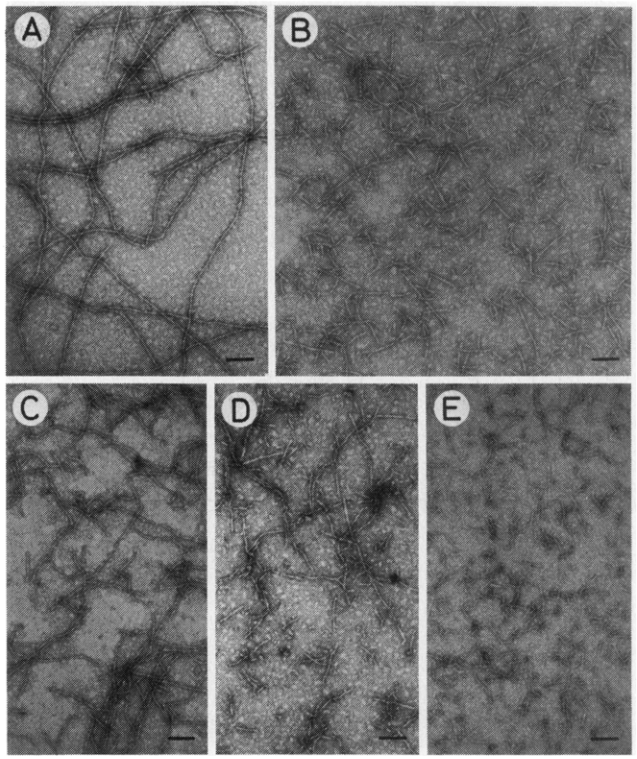

FIGURE 8: Electron micrographs of *Physarum* actin filaments: (A) F-actin; (B) F-actin fragments induced by addition of fragmin to F-actin solution (1:5 in molar ratio); (C–E) actin filaments polymerized in the presence of various amounts of fragmin. Molar ratios of fragmin to actin were 1:50 (C), 1:20 (D), and 1:5 (E). Scale bars in all photographs represent 0.1 μm.

F-actin filaments is exponential and the number average length is about 1 μm (Kawamura & Maruyama, 1970), which is much longer than those in Figure 9, A and B.

Fragmin could not react with F-actin that had been fully decorated with muscle HMM. If HMM were dissociated by ATP, fragmin could react with F-actin. To confirm the direct ability of fragmin to cut F-actin into fragments, we used block polymers of F-actin and the complex of F-actin with HMM. F-Actin and the complex of F-actin with HMM were separately prepared and then mixed. About 20% of the polymers were found to consist of three blocks, such as complex–F-actin–complex. When fragmin was added at a molar ratio of 1:5, three-block polymers of the above type were all eliminated. Fragmin reacted with F-actin in the middle block directly.

When fragmin was added to G- and F-actin solutions in a molar ratio of 1:10 in the presence of 0.1 M KCl, 2 mM $MgCl_2$, 0.5 mM ATP, and 20 mM imidazole-HCl buffer of pH 7.0 and 0.5 mM Ca-EGTA buffer of pCa 5 at 23 °C, the shortened actin filaments had ATPase activities of 2.5 and 2.3 nmol of P_i per mg of actin per min, respectively. These activities were nearly the same as that reported earlier, i.e., 2.2 nmol of P_i per mg of actin per min, which was observed when *Physarum* actinin was added to G-actin in a weight ratio of 1:5 (Hatano & Owaribe, 1979). F-Actin had an ATPase activity of 0.16 nmol of P_i per mg of actin per min in the absence of fragmin. Fragmin did not split ATP at all.

Ca^{2+} Sensitivity. The ability of fragmin to produce short F-actin filaments was found to be regulated by free Ca^{2+}. All experiments in the above section were performed in the presence of free Ca^{2+} of pCa 5. Figure 10A shows the effect of fragmin on actin polmerization at various concentrations of free Ca^{2+}. When the concentration of free Ca^{2+} was lower than 1×10^{-7} M, fragmin had no effect on actin polymeri-

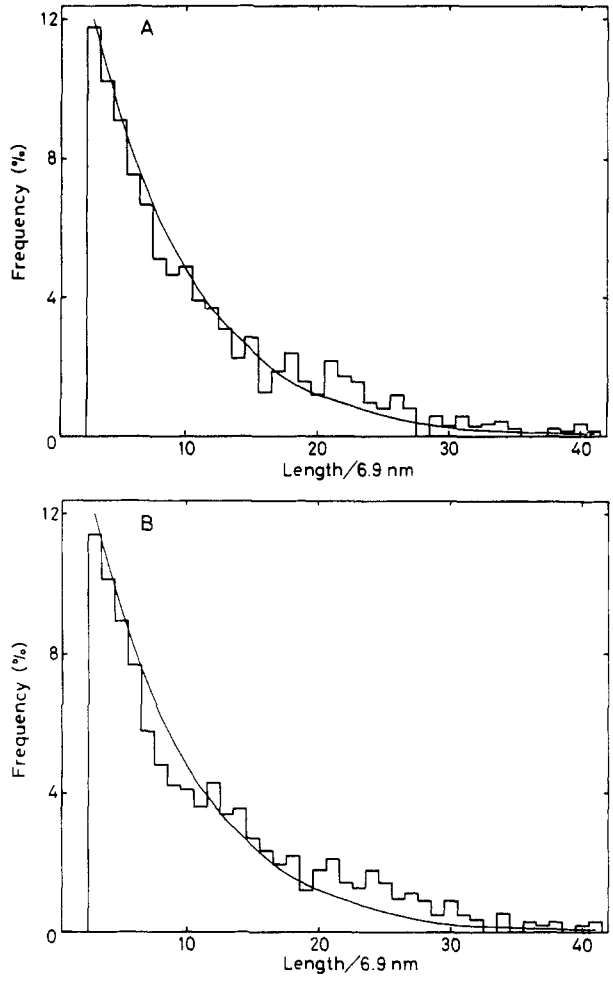

FIGURE 9: (A) Length distribution of actin filaments polymerized in the presence of fragmin. The molar ratio of fragmin to actin was 1:20. Actin filaments shorter than 14 nm were not able to be detected by electron microscopy. The total number of filaments was 512. The number average length was 72.2 nm. The theoretical curve was obtained by assuming that almost all actin was in the polymer state, a dynamic equilibrium existed between monomer and polymer, and each F-actin filament had one monomer of fragmin. The number average length from the theoretical curve was 64.2 nm. (B) The length distribution of F-actin fragments induced by the addition of fragmin to F-actin. The molar ratio of fragmin to actin was 1:20. The total number of filaments was 687. The number average length was 76.7 nm. The theoretical curve, which is the same as that of A, was obtained by assuming that fragmin cut F-actin at random positions.

zation. The effect appeared around 1×10^{-6} M Ca^{2+} and seemed to be saturated at higher concentrations of Ca^{2+}. Any concentrations of free Mg^{2+} from 1×10^{-7} to 1×10^{-3} M did not activate the action of fragmin on actin polymerization.

Similarly, the action of fragmin on F-actin depended on the concentration of free Ca^{2+} (Figure 10B). Fragmentation of F-actin by fragmin was observed only above 1×10^{-6} M free Ca^{2+}. Ca^{2+} could not be replaced by Mg^{2+}.

The reverse process from short actin filaments to long filaments was observed when the concentration of free Ca^{2+} was lowered (H. Sugino and T. Hasegawa, unpublished results). After short F-actin filaments were formed in the presence of fragmin at pCa 5, the pCa was increased to 8. Then the viscosity began to increase very slowly. It took about 20 h to recover half of the viscosity of the F-actin solution without fragmin.

Physarum actinin isolated by the method of Hatano & Owaribe (1976) showed Ca^{2+} sensitivity similar to that of fragmin under conditions where free Ca^{2+} concentrations were

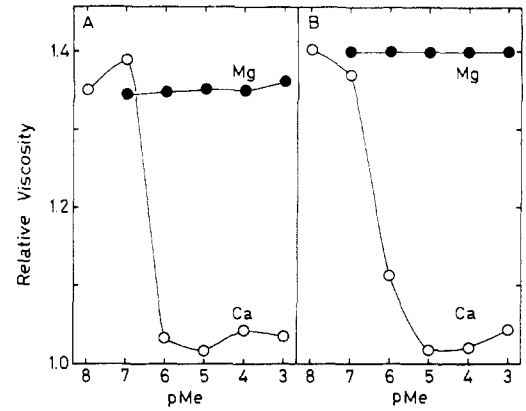

FIGURE 10: Effect of Ca^{2+} and Mg^{2+} on the action of fragmin. (A) After the addition of fragmin, *Physarum* G-actin was polymerized in the presence of 0.1 M KCl, 1 mM ATP, and 20 mM imidazole-HCl buffer of pH 7.0 and various concentrations of free Ca^{2+} and free Mg^{2+}. Viscosity values 30 min after the initiation of polymerization are plotted as a function of the divalent cation concentration. (B) Fragmin was added to *Physarum* F-actin solutions containing 0.1 M KCl, 1 mM ATP, and 20 mM imidazole-HCl buffer of pH 7.0 and various concentrations of free Ca^{2+} and free Mg^{2+}. Viscosity values 30 min after the addition of fragmin are plotted as a function of the divalent cation concentration. The concentrations of actin and fragmin were 0.5 and 0.035 mg/mL, respectively. The concentrations of free Ca^{2+} and free Mg^{2+} were buffered with 1 mM Ca-EGTA and 1 mM Mg-EDTA, respectively. The Mg^{2+} concentrations of pMg 3 and 4 were produced by addition of 0.1 mM $MgCl_2$ plus 0.01 mM EGTA and 1 mM $MgCl_2$ plus 0.1 mM EGTA, respectively.

controlled by Ca-EGTA buffer. Mg^{2+} had no effect on the action of *Physarum* actinin.

Discussion

We have found that *Physarum* actinin previously isolated by Hatano & Owaribe (1976) was a 1:1 complex of actin and fragmin. The complex was dissociated by urea–NaDodSO_4 gel electrophoresis, and the difference between the two proteins was confirmed in the amino acid composition. Since fragmin had no cysteine residues, the selective chemical cleavage of actin with TNB-CN was effective for purification of fragmin from the complex. As shown in Table I, the activity of fragmin, particularly its F-actin action, was increased during purification. Although fragmin was treated with 6 M urea during purification, its activity was regenerated after dialysis.

Fragmin shortened the F-actin filaments. The length distribution of shortened fragments was found to be nearly exponential, suggesting that fragmin cut the filaments into fragments at random positions. The experiment using F-actin with blocks decorated with HMM supported this interpretation. The number average length of shortened filaments was nearly equal to that expected when each filament had one fragmin monomer, probably at its end.

When fragmin was added to G-actin before polymerization, the initial rate of polymerization was increased, but short filaments were finally formed. Fragmin made a complex with actin and promoted the formation of a nucleus for polymerization of actin. Fragmin increased the number of F-actin filaments and decreased the average length of the filaments. The length distribution was exponential, and the number average length was nearly equal to that obtained by adding fragmin to preformed F-actin at the same molar ratio. The ATPase activity of F-actin was increased by the addition of fragmin. The increase of the ATPase activity can be explained by the increase in the number of polymer ends. It is very likely that fragmin was found to the initiation end of F-actin and the polymerization–depolymerization equilibrium was estab-

lished at the other end, that is, the growing end. Fragmin seemed to have little effect on the concentration of G-actin coexisting with F-actin.

The number average length obtained from Figure 8B was 62 nm, which was 2.2× as long as that calculated, i.e., 28 nm. The origin of the disagreement is not clear.

Previously, *Physarum* actinin was found to form actin polymers of a different shape from F-actin, flexible and sometimes globular (Fujime & Hatano, 1972). After purified fragmin was added, however, the electron micrograph gave pictures of actin filaments, the local structure of which was the same as F-actin. The action of fragmin was mostly to shorten actin filaments, although the formation of actin polymers different from F-actin could not be completely excluded.

We have found that the action of fragmin on actin is regulated by micromolar concentrations of free Ca^{2+}. It is suggested that fragmin has a specific site to bind Ca^{2+}; only when fragmin has bound Ca^{2+} does it react with actin to cut F-actin filaments or form an initiation end of the filaments.

The regulation of Ca^{2+} was reversible. However, once short actin filaments were formed by fragmin in the presence of Ca^{2+}, the recovery of long filaments took a long time after the removal of free Ca^{2+} from the solvent. Ca^{2+} bound to fragmin would be masked by the interaction of fragmin with actin.

Physarum actinin isolated by the method of Hatano & Owaribe (1976) showed Ca^{2+} sensitivity. It is probable that *Physarum* actinin was activated not by Mg^{2+} but by traces of Ca^{2+} in the previous experiments (Hatano & Owaribe, 1976, 1979). Muscle β-actinin exhibited a function similar to that of fragmin, in the sense that both proteins keep G-actin in a state of low viscosity even in the presence of salts (Kamiya et al., 1972). Muscle β-actinin, however, did not cut preformed F-actin, but only prevented fragmented F-actin from reassociating (Maruyama, 1966). It did not require Ca^{2+} for its activity. Therefore, fragmin and β-actinin are different proteins.

Fragmin was extracted from plasmodium as a complex with actin. This does not necessarily mean that they are a complex in vivo. The location and the state of fragmin in living cells will be the subject of future investigations.

Hinssen (1979) has isolated a Ca^{2+}-sensitive *Physarum* protein which inhibits the polymerization of actin but has only a small effect on the viscosity of F-actin solution. The molecular weight of the protein is 44 000 using $NaDodSO_4$ gel electrophoresis and 88 000 using gel filtration. It is very likely that the protein isolated by Hinssen is a complex of actin and fragmin. Yin & Stossel (1979) have isolated a protein, which is called gelsolin, from macrophage. The molecular weight of gelsolin has been estimated to be 91 000 by $NaDodSO_4$ gel electrophoresis and 160 000 by gel filtration. The effect of gelsolin on F-actin and its Ca^{2+} sensitivity are similar to those of fragmin. Fragmin and gelsolin may play a similar role in cell motility, that is, regulation of gel–sol transformation.

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References

Abramowitz, J. W., Stracher, A., & Detwiler, T. C. (1975) *Arch. Biochem. Biophys. 167*, 230.

Amos, W. B., Routledge, L. M., Weis-Fogh, T., & Yew, F. F. (1976) in *Calcium in Biological Systems* (Duncan, C. J., Ed.) pp 273–301, Cambridge University Press, London.

Boyer, P. D. (1954) *J. Am. Chem. Soc. 76*, 4331.

Bray, D., & Thomas, C. (1976) *J. Mol. Biol. 105*, 527.

Camp, W. G. (1936) *Bull. Torrey Bot. Club 63*, 205.

Carlsson, L., Nyström, L.-E., Sundkvist, I., & Markey, F. (1977) *J. Mol. Biol. 115*, 465.

Ebashi, S., & Ebashi, F. (1964) *J. Biochem. (Tokyo) 55*, 604.

Ebashi, S., Ebashi, F., & Maruyama, K. (1964) *Nature (London) 203*, 645.

Fujime, S., & Hatano, S. (1972) *J. Mechanochem. Cell Motil. 1*, 81.

Gordon, D. J., Boyer, J. L., & Korn, E. D. (1977) *J. Biol. Chem. 252*, 8300.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751.

Hatano, S., & Oosawa, F. (1966) *Biochim. Biophys. Acta 127*, 488.

Hatano, S., & Owaribe, K. (1976) in *Cell Motility* (Goldman, R., Pollard, T., & Rosenbaum, J., Eds.) Vol. 3, Book B, pp 499–511, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hatano, S., & Owaribe, K. (1977) *J. Biochem. (Tokyo) 82*, 201.

Hatano, S., & Owaribe, K. (1979) *Biochim. Biophys. Acta 579*, 200.

Hinssen, H. (1979) in *Cell Motility: Molecules and Organization* (Hatano, S., Ishikawa, H., & Sato, H., Eds.) pp 59–85, University of Tokyo Press, Tokyo.

Hitchcock, S. E., Carlsson, L., & Lindberg, U. (1976) *Cell 7*, 531.

Ishikawa, H., Bischoff, R., & Holtzer, H. (1969) *J. Cell Biol. 43*, 312.

Jacobson, G. R., Schaffer, M. H., Stark, G. R., & Vanaman, T. C. (1973) *J. Biol. Chem. 248*, 6583.

Kamiya, R., Maruyama, K., Kuroda, M., Kawamura, M., & Kikuchi, M. (1972) *Biochim. Biophys. Acta 256*, 120.

Kawamura, M., & Maruyama, K. (1970) *J. Biochem. (Tokyo) 67*, 437.

Kondo, H., & Ishiwata, S. (1976) *J. Biochem. (Tokyo) 79*, 159.

Lazarides, E., & Weber, K. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2268.

Maruyama, K. (1965) *Biochim. Biophys. Acta 102*, 542.

Maruyama, K. (1966) *Biochim. Biophys. Acta 126*, 389.

Maruyama, K., Kamiya, R., Kimura, S., & Hatano, S. (1976) *J. Biochem. (Tokyo) 79*, 709.

Merriam, R. W., & Clark, T. G. (1978) *J. Cell Biol. 77*, 439.

Murphy, J., & Riley, J. P. (1962) *Anal. Chim. Acta 27*, 31.

Oosawa, F. (1970) *J. Theor. Biol. 27*, 69.

Oosawa, F., & Kasai, M. (1962) *J. Mol. Biol. 4*, 10.

Szent-Györgyi, A. G. (1953) *Arch. Biochem. Biophys. 42*, 305.

Vandekerkhove, J., & Wever, K. (1978) *Nature (London) 276*, 720.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Yin, H. L., & Stossel, T. P. (1979) *Nature (London) 281*, 583.