Mechanism of Regulation of Actin Polymerization by Physarum Profilin

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ABSTRACT
Physarum profilin reduces the rates of nucleation and elongation of F-actin and also reduces the extent of polymerization of actin at the steady state in a concentration-dependent fashion. The apparent critical concentration for polymerization of actin is increased by the addition of profilin. These results can be explained by the idea that Physarum profilin forms a 1:1 complex with G-actin and decreases the concentration of actin available for polymerization. The dissociation constant for binding of profilin to G-actin is estimated from the kinetics of polymerization of G-actin and elongation of F-actin nuclei and from the increase of apparent critical concentration in the presence of profilin. The dissociation constants for binding of Physarum profilin to Physarum and muscle actins under physiological ionic conditions are in the ranges of 1.4–3.7 μM and 11.3–28.5 μM, respectively. When profilin is added to an F-actin solution, profilin binds to G-actin which co-exists with F-actin, and then G-actin is dissociated from F-actin to compensate for the decrease of the concentration of free G-actin and to keep it constant at the critical concentration. At the steady state, free G-actin of the critical concentration is in equilibrium not only with F-actin but also with profilin–G-actin complex. The stoichiometry of 1:1 for the formation of complex between profilin and G-actin is directly shown by means of chemical cross-linking.

Actin is a ubiquitous protein in eucaryotic cells and takes two forms, the unpolymerized and the polymerized forms. In nonmuscle cells the amount of unpolymerized form of actin exceeds 50% of the cytoplasmic actin even though ionic conditions in these cells favor complete polymerization (1). The cells have been expected to contain some regulatory systems to keep actin in the unpolymerized form. Recently, several actin-binding proteins that regulate the conversion between the unpolymerized and the polymerized forms of actin have been found in many nonmuscle cells (7, 21).

An unpolymerized form of actin was first isolated from calf spleen as a 1:1 complex with another protein termed profilin (6). Many mammalian tissues (2, 19, 24), Acanthamoeba (30), and sea urchin egg (23) contain similar proteins. Profilins have a molecular weight of 12,000–16,000 daltons and reduce the rate of polymerization of actin in vitro. Tobacman and Korn (34) and Tseng and Pollard (36) have reported that Acanthamoeba profilin decreases the elongation rate of actin filaments and increases the apparent critical concentration for polymerization of actin, and have explained these effects of profilin on the basis of the formation of a 1:1 complex with actin.

Physarum plasmodia show cytoplasmic shuttle streaming based on an actin-associating motile system (12, 15). The shuttle streaming, which is caused by a rhythmical contraction–relaxation cycle of cytoplasmic actomyosin biochemically, is associated morphologically with the sol–gel transformation of cytoplasm. Isenberg and Wohlfarth-Bottermann (17) showed that a considerable amount of actin is in the unpolymerized form within flowing endoplasm, and proposed the idea that the cyclic sol–gel transformation of the cytoplasm is based on G–F transformation of actin. Actin-binding proteins may play key roles in such a cytoplasmic sol–gel transformation. Physarum profilin isolated from plasmodia is one of the actin-binding proteins that modulate the equilibrium between G- and F-actin. As shown in a previous paper, Physarum profilin was similar to profilins isolated from other sources in its molecular weight (13,000) and its effect on polymerization of actin according to the preliminary kinetic analysis, although the amino acid composition of the profilin was notably different from other profilins (29).

In this article, to understand the mechanism of regulation of polymerization of actin by Physarum profilin, we have examined the effects of the profilin on the rates of polymerization of actin, elongation of F-actin nuclei, and depolymerization of F-actin, as well as on the extents of polymerization...
and depolymerization of actin at steady states. All of the results obtained can be explained by a 1:1 binding of the profilin to G-actin. The dissociation constant for binding of Physarum profilin to Physarum actin (or muscle actin) was estimated as on the order of 10^{-6} M (or 10^{-5} M) under physiological ionic conditions.

MATERIALS AND METHODS

Plasmodia of Physarum polycephalum were cultured by the method of Camp (5) with slight modifications described earlier (12).

Physarum profilin was prepared by the procedure of Ozaki et al. (29). Fragmin was purified by the procedure of Hasegawa et al. (11). Physarum actin was purified by gel filtration through Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the method of Hatano and Owariue (14).

Muscle actin was extracted from acetone-dried rabbit skeletal muscle (9) and was purified by the method of Kondo and Ishiwata (20) with gel filtration through Sephardex G-100 as the final step.

Profiles concentration was determined by the absorbance at 280 nm using 
\[ \varepsilon = 2.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \] (29). Physarum actin, muscle actin, and fragmin concentrations were determined by the biuret method (10) using \( A_{660}^{biuret} = 0.20 \) (13). The molecular weight of Physarum profilin was determined to be 13,000 by the analysis of the amino acid composition (29). Molecular weights of actin and fragmin were both taken to be 43,000.

Polymerization of actin was monitored with conventional semi-micro Ostwald viscometers. The flow times for a buffer solution were 13 s. The extent of polymerization of actin at steady states was determined by the flow birefringence, which was measured with a homemade concentric cylinder apparatus (13). For the experiments shown in Figs. 3-7, actin was polymerized in a standard buffer solution consisting of 0.4 mM dithiothreitol (DTT), 0.1 mM CaCl\(_2\), 1 mM ATP, 1.5 mM NaN\(_3\), 5 mM imidazole-HCl, pH 7.0, and 50 mM KCl (or 50 mM KCl plus 1 mM MgCl\(_2\)). F-actin passed through a 25-gauge syringe needle four times as used as nuclei for the elongation experiments. The initial rate of polymerization of actin was determined directly from the slope of the viscosity vs. time curves extrapolated to time zero.

The chemical cross-linking of Physarum actin to Physarum profilin was done by the use of cross-linking reagents, dimethyl suberimidate (DMS) (8) and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) (32). Actin (0.26 mg/ml) and profilin (0.32 mg/ml) in 0.1 M triethanolamine hydrochloride, pH 8.5, 0.1 mM ATP, 0.2 mM DTT, and 50 mM KCl were mixed with DMS (3.7 mM) which was dissolved in triethanolamine hydrochloride, pH 8.5, immediately before use. In another experiment, actin and profilin in 10 mM imidazole-HCl, pH 7.0, 0.1 mM ATP, 0.2 mM DTT, and 50 mM KCl were mixed with EDC dissolved in imidazole-HCl, pH 7.0. The concentrations of actin, profilin, and EDC were 0.42 mg/ml, 0.50 mg/ml, and 15 mM, respectively, for the experiments shown in Fig. 1, and 0.26 mg/ml, 0.32 mg/ml, and 5 mM, respectively, for the experiments shown in Fig. 2. The reaction mixtures were left at 20°C for 2 h, and then polypeptide components in the samples were analyzed by PAGE in the presence of SDS.

SDS PAGE was carried out by the method of Hubbard and Lazarides (16) which was a slight modification of the method of Laemmli (22). Gels were stained with Coomassie Brilliant Blue (Sigma Chemical Co., St. Louis, MO) and scanned at 570 nm in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). A silver stain was also used for detection of the components in the samples (25).

RESULTS

Binding of Physarum Profilin to Physarum Actin

When Physarum profilin was applied to a DEAE-cellulose (DE-52, Whatman, England) column equilibrated with a buffer solution containing 50 mM KCl, 0.2 mM DTT, 0.1 mM ATP, and 10 mM Tris-HCl, pH 8.2, at 4°C, profilin passed through the column. However, when a mixture of profilin and Physarum actin was applied to the same column, passed through the column. However, when a mixture of

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**Figure 1** Ion-exchange column chromatography of the mixture of Physarum actin and profilin. Fractions were analyzed by 13% SDS-PAGE and by protein-dye binding assay according to the method of Bradly (4) in C; gels were stained with silver after electrophoresis. (A) Actin (0.42 mg/ml) and profilin (0.50 mg/ml) in 50 mM KCl, 0.1 mM ATP, 0.2 mM DTT, and 10 mM Tris-HCl, pH 8.2, (3 ml) were applied to a DEAE-cellulose column (1.2 x 5 cm) equilibrated with the same buffer solution. A linear gradient of KCl from 50 to 250 mM (50 ml) was applied, and then 2-ml fractions were collected. Numbers at the top of the lanes on SDS PAGE correspond to the fraction number of the column chromatography. std., molecular weight standards; A, Physarum actin (43,000 daltons) and P, Physarum profilin (13,000 daltons). (B) Actin (0.42 mg/ml) and profilin (0.50 mg/ml) in 10 mM imidazole-HCl, pH 7.0, 0.1 mM ATP, 0.2 mM DTT, and 50 mM KCl (3 ml) were incubated with 15 mM EATC at 20°C for 2 h, and then the reaction was quenched by the addition of excess 2-mercaptoethanol. The sample was dialyzed against 10 mM Tris-HCl, pH 8.2, 0.1 mM ATP, 0.2 mM DTT, and 50 mM KCl at 4°C for 20 h and applied to a DEAE-cellulose column under the same conditions as described in A. Numbers at the top of the lanes on SDS PAGE correspond to the fraction number of

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1. **Abbreviations used in this paper:** DMS, dimethyl suberimidate; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide.
sample was -0.7, which was estimated by scanning gels of SDS PAGE stained with Coomassie Brilliant Blue. When a mixture of profilin and F-actin in 0.1 M KCl was centrifuged at 100,000 g for 90 min at 15°C, profilin remained in the supernatant. These results suggested that Physarum profilin interacted with G-actin at a molar ratio of 1:1 but did not apparently interact with F-actin.

To show that profilin did, in fact, form a 1:1 complex with G-actin, proteins were treated with a chemical cross-linking reagent, EDC, which forms zero-length covalent bonds between carboxyl and amino groups in the contact area. When a mixture of profilin and actin treated with EDC was applied to the same column as described above, a 50,000-dalton peptide and actin were observed as major bands in the fractions coming off the column after applying a gradient of KCl, but cross-linked actin oligomers were not observed (Fig. 1 B). When the fractions 31–34 of Fig. 1A which contained both actin and profilin were collected and treated with EDC, the 50,000-dalton peptide was detected on SDS PAGE (data not shown). Fig. 2 shows that if actin or profilin alone were treated with EDC, cross-linked products were not formed appreciably. However, a major new band at a molecular weight of 50,000 appeared when a mixture of profilin and actin was treated with EDC (Fig. 2). These results show that profilin strongly interacted with G-actin and the new 50,000-dalton peptide was a cross-linked 1:1 complex between profilin and G-actin, although its apparent molecular weight was a little lower than the expected value (56,000) on SDS PAGE. Thus, the formation of a 1:1 complex between profilin and G-actin expected in the results shown in Fig. 1A was proved.

Another cross-linking reagent, dimethyl suberimidate (DMS), which forms 1.1-nm-long covalent bonds between amino groups, was tested (Fig. 2, lanes d-f). In this case, two major new bands appeared; apparent molecular weights were 50,000 and 52,000. We supposed that these new polypeptides were the cross-linked products of profilin–G-actin complex, and the variation of motility of the cross-linked products on SDS PAGE was caused by conformational modification due to intramolecular cross-linking between the different sites.

**Initial Rate of Polymerization**

The effect of Physarum profilin on polymerization of Physarum and muscle actins was investigated by viscometry (Fig. 3). Profilin strongly inhibited the polymerization of Physarum actin in a solution containing 2 mM MgCl₂, 0.2 mM DTT, 0.1 mM ATP, and 20 mM imidazole-HCl, pH 7.0 (Fig. 3A). The initial rate of polymerization of 10 μM Physarum actin was reduced to 1/15 by 6.4 μM profilin and to 1/300 by 13 μM profilin. Profilin also inhibited the polymerization of muscle actin (Fig. 3B), although the effect was weaker than on Physarum actin. The initial rate of polymerization of 7.4 μM muscle actin was reduced to 1/18 by 15 μM profilin.

**Initial Rate of Elongation**

Polymerization of actin consists of at least two steps, nucleation and elongation, and nucleation is a rate-limiting step.
in the process (18, 28). It is important to investigate the effect of profilin on the nucleation step and the elongation step separately. The rate of elongation was determined by the addition of fragmented F-actin as a nucleus to a G-actin solution containing profilin.

Equal amounts of F-actin nuclei were added to solutions of G-actin containing 50 mM KCl and 0.1 mM CaCl₂ at various concentrations of Physarum profilin (Fig. 4). In this experimental condition the polymerization of G-actin was extremely slow without F-actin fragments. Fragmented F-actin markedly increased the rate of polymerization of Physarum actin even in the presence of profilin. The rate of elongation decreased with increasing concentration of profilin added. Profilin of 5, 10, and 20 μM reduced the initial rate of polymerization of 10 μM Physarum G-actin to 2/3, 1/3, and 1/6, respectively.

Extent of Polymerization

The extent of polymerization or depolymerization of actin in the presence and absence of Physarum profilin at steady states was investigated by starting from G- or F-actin as shown in Figs. 5 and 6. Actins were incubated with profilin for 22-24 h at 22°C in the standard buffer solution containing 50 mM KCl (or 50 mM KCl plus 1 mM MgCl₂), and then the flow birefringence was measured.

Fig. 5 shows the degrees of flow birefringence of Physarum actin solutions, one of which was polymerized from 19 μM G-actin and the other was depolymerized from 19 μM F-actin at the same concentration of profilin. In these two solutions, polymerization of actin which started from G- and F-actin reached the same extent. The reduction of the degree of flow birefringence by profilin was linearly proportional to the concentration of profilin.

This reduction is attributable to the increase of the apparent critical concentration in the presence of profilin. In Fig. 6, the relationship between the extent of polymerization and the total concentration of actin in the presence of profilin was exactly parallel to that in the absence of profilin. The critical concentration for Physarum actin was increased from 0.6 to 3.0 μM by the addition of 10 μM profilin both in 50 mM KCl and 50 mM KCl plus 1 mM MgCl₂ (Fig. 6A) and that for muscle actin was increased from 1.5 to 2.5 μM by 20 μM profilin in 50 mM KCl (Fig. 6B).

The decrement in the extent of polymerization, which is the increment of the apparent critical concentration, was linearly proportional to the concentration of profilin. As the concentration of free G-actin in equilibrium with F-actin (actual critical concentration) is independent of the concentration of profilin at any concentration of actin, the increment of apparent critical concentration corresponds to the amount of complex formed. Thus, free G-actin has to be in equilib-
rium not only with F-actin but also with complex of profilin-G-actin at the steady state. Profilin reduces the concentration of F-actin unaffected the length distribution of filaments unlike the effects of end-blocking proteins; for example, fragmentin, villin, and severin sever F-actin filaments and block the association of the fragments by capping their ends (3, 11, 37).

Calculation of the Dissociation Constant for Binding of Physarum Profilin to G-actin

As described in the previous sections, our results strongly suggest that profilin binds to actin monomer at a molar ratio of 1:1 and the concentration of actin available for polymerization is reduced because the complex cannot polymerize. According to this scheme, we can calculate the dissociation constants for binding of Physarum profilin to Physarum and muscle actins.

(a) The dissociation constant was calculated from the reduction of the degree of flow birefringence at steady states (Figs. 5 and 6). As summarized in Table I, the dissociation constants for binding of profilin to Physarum and muscle actins are 1.9 μM in 50 mM KCl or 50 mM plus 1 mM MgCl₂ and 28.5 μM in 50 mM KCl, respectively. In Fig. 5, the concentration of profilin given by extrapolating the values of flow birefringence to zero is ~80 μM. This value is the minimal concentration required for complete depolymerization of Physarum F-actin at a concentration of 19 μM. The dissociation constant calculated from these values is 2.0 μM as the critical concentration is 0.6 μM in this condition.

(b) The dissociation constant was calculated from the initial rate of polymerization (Fig. 3). The initial rate of polymerization depends on the third or fourth power of the concentration of actin (18, 28, 35). If the initial rate of polymerization is proportional to the power of the concentration of G-actin, the initial concentration of free G-actin in the presence of profilin can be estimated from the initial rate of polymerization. For example, 6.4 μM profilin reduced the initial concentration of Physarum G-actin from 10 to 5 μM; the concentration of complex, therefore, was 5 μM (Table II). The dissociation constants for binding of profilin to Physarum and muscle actins in 2 mM MgCl₂ range from 1.4 to 3.7 μM and 11.3 to 16.2 μM, respectively (Table II).

(c) The dissociation constant was calculated from the rate of elongation (Fig. 4). We can estimate the decrease in the concentration of free G-actin due to the formation of profilin-G-actin complex from the initial rate of nucleated polymerization, as the initial rate of elongation is directly proportional to the concentration of G-actin above the critical concentration; (k⁺C - k⁻) = k* (C - k⁻/k⁺), where k⁺ and k⁻ are the rate constants of association of monomers to F-actin and dissociation of monomers from F-actin, respectively, C is the concentration of G-actin, and k⁻/k⁺ is defined as the critical concentration (28). For example, profilin at 10 μM reduced the initial rate of elongation of F-actin at 10 μM of Physarum G-actin to 1/3 of that in the absence of profilin. As the critical concentration is 0.6 μM in this condition (Fig. 6A), the initial concentration of free G-actin is estimated as 3.7 μM. Therefore, the equivalent of 6.3 μM G-actin must be in the form of the complex. The dissociation constant for binding of profilin to Physarum actin is 2.2 μM in this example. Thus, the values of the dissociation constant for formation of profilin-G-actin complex estimated by different methods are in reasonable agreement (Tables I and II).

Rate of Depolymerization

When various concentrations of Physarum profilin were added to Physarum F-actin in the standard buffer solution containing 50 mM KCl, the viscosity of each sample decreased to new levels at the steady state (Fig. 7). It took 1–4 h to reach the steady-state levels, where the viscosity depended on the concentration of profilin added. However, the initial rate of decrease in viscosity was independent of the concentration of profilin >10 μM. For 50% of the reduction in viscosity, 5–25 min was required.

When profilin is added to an F-actin solution, profilin rapidly binds to G-actin which co-exists with F-actin in the solution. Profilin reduces the rate of association of monomer to polymer of actin by reducing the concentration of free G-actin. The initial rate of decrease in viscosity is independent of the concentration of profilin because the dissociation of monomer from polymer of actin is rate limiting in this

### Table I

| Total profilin | Critical concentration | Complex | Free profilin | K₀ |
|---------------|------------------------|---------|---------------|----|
| μM            | μM                     | μM      | μM            | μM |
| **Physarum**  |                        |         |               |    |
| 10.0          | 0                      | 0.6     | —             | —  |
| 10.0          | 10.0                   | 3.0     | 2.4           | 7.6| 1.9|
| 10.0          | 20.0                   | 1.5     | —             | —  |
| 10.0          | 20.0                   | 2.5     | 1.0           | 19.0| 28.5|

The data for the critical concentrations were obtained from Fig. 6. The concentration of complex is calculated as the difference between the critical concentrations in the presence and absence of profilin. The critical concentration in the absence of profilin corresponds to the concentration of free G-actin in the presence of profilin. The dissociation constant (K₀) is calculated by the relation of [free G-actin] [free profilin]/[complex], assuming stoichiometry of 1:1.

### Table II

| Total profilin | Initial rate | Free G-actin | Complex | Free profilin | K₀ |
|---------------|-------------|--------------|---------|---------------|----|
| μM            | μM/min⁻¹    | μM           | μM      | μM            | μM |
| **Physarum**  |             |              |         |               |    |
| 10.0          | 0           | 0.3          | 10.0    | —             | —  |
| 6.4           | 0.11        | 7.7          | 2.3     | 1.1           | 3.7|
| 6.4           | 0.02        | 5.0          | 5.0     | 1.4           | 1.4|
| 10.0          | 0.004       | 3.4          | 6.6     | 3.4           | 1.8|
| 13.0          | 0.001       | 2.4          | 7.6     | 5.4           | 1.7|
| **Muscle**    |             |              |         |               |    |
| 7.4           | 0           | 0.07         | —       | —             | —  |
| 1.9           | 0.05        | 6.7          | 0.7     | 1.2           | 11.5|
| 3.7           | 0.04        | 6.2          | 1.2     | 2.5           | 12.9|
| 7.5           | 0.02        | 5.3          | 2.1     | 5.4           | 13.6|
| 15.0          | 0.004       | 3.7          | 3.7     | 11.3          | 11.3|
| 23.0          | 0.003       | 3.4          | 4.0     | 19.0          | 16.2|

The data shown in the first through the third columns were obtained from Fig. 3. Initial rate of polymerization of actin was directly measured on the graphs. The rate is the increment in specific viscosity per one minute. The concentration of free G-actin is calculated as follows: ([free G-actin]/[free G-actin in the absence of profilin]):/(initial rate in the presence of profilin)/initial rate in the absence of profilin). The concentration of complex is the difference between the total and free G-actin concentrations. The concentration of free profilin is the difference between the total profilin and complex concentrations. The dissociation constant (K₀) is calculated by the relation of [free G-actin] [free profilin]/[complex], assuming stoichiometry of 1:1.

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The addition of 1 mM MgCl₂ increased the initial rate of polymerization of Physarum actin both in the presence and absence of Physarum profilin (Fig. 8). The extent of polymerization of Physarum actin was unaffected by the additional MgCl₂, however, and the extent of polymerization depended on the concentration of profilin in the same way both in the presence and absence of MgCl₂ (Fig. 5).

**Effect of Fragmin**

The initial rate of polymerization of Physarum actin in the presence of Physarum profilin was markedly accelerated by fragmin in the presence of CaCl₂ (Fig. 9) but it was not affected in the absence of CaCl₂ (data not shown). It has been reported that fragmin accelerates the initial stage of polymerization of actin in the presence of micromolar concentrations of CaCl₂, and keeps the length of actin filaments short (11, 31). Our results show that fragmin interacts with free G-actin to form nuclei and increases the initial rate of polymerization of actin although the final viscosity is reduced in the presence of profilin. Profilin does not interfere directly with the interaction between fragmin and actin.

**DISCUSSION**

Polymerization of actin consists of nucleation and the growth process (18, 28). Each of the processes is closely related to the concentration of G-actin. Analysis of the effect of Physarum profilin on these two processes that occur during polymerization gave us insight into the stoichiometric binding of actin to profilin. Profilin sequesters G-actin, so that the rates of actin polymerization and F-actin elongation decreases and the apparent critical concentration increases.

When G-actin and profilin are mixed, G-actin binds to profilin with a dissociation constant of 1.4–3.7 μM (for Physarum actin) or 11.3–16.2 μM (for muscle actin) (Table II). Upon the addition of salts, only the remaining free G-actin can form nuclei and associate to F-actin. When the polymerization proceeds in the mixture, the dissociation of complex continues until the final steady state is reached. When profilin is added to an F-actin solution, G-actin which co-exists with F-actin (at the critical concentration) binds to profilin, and then G-actin is dissociated from F-actin to compensate for the decrease of the concentration of free G-actin and to keep the concentration of free G-actin constant. At the steady state, an equilibrium is established among the following states:

F-actin ⇄ G-actin (of the critical concentration)

⇌ profilin–G-actin complex.

Such a mechanism of regulation of polymerization of actin by Physarum profilin is identical to that reported in the case of Acanthamoeba profilin (34, 36).

The results from the kinetic analysis of polymerization of actin in the presence of profilin were consistently explained by the idea that profilin forms a 1:1 complex with G-actin. We showed directly the stoichiometry of 1:1 for the formation of complex between profilin and G-actin by means of chemical cross-linking (Figs. 1 and 2). It has been reported that mammalian profilactin is a 1:1 complex of actin and profilin (6), and the stoichiometry for binding of Acanthamoeba profilin to muscle actin was calculated to be 1:1 (26).

The effect of Physarum profilin on muscle actin is weaker than that on Physarum actin (Figs. 3 and 6). The dissociation constant for binding of Physarum profilin to muscle actin is
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