

Evaluation of the Binding of *Acanthamoeba* Profilin to Pyrene-Labeled Actin by Fluorescence Enhancement

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We have used a fluorescence assay to measure the binding of *Acanthamoeba* profilin to monomeric *Acanthamoeba* and rabbit skeletal muscle actin labeled on cysteine-374 with pyrene iodacetamide. The wavelengths of the pyrene excitation and emission maxima are constant at 346 and 386 nm, but the fluorescence is enhanced up to 50% by profilin. The higher fluorescence is largely due to higher absorbance in the presence of profilin. The fluorescence enhancement has a hyperbolic dependence on the concentration of profilin, suggesting a single class of binding sites. Linear Scatchard plots yield an estimate of the dissociation constant, K_d , of the complex of profilin with pyrenyl-actin. In low-ionic-strength buffers with 2 to 6 mM imidazole (pH 7.0) and 0.1 mM CaCl_2 the K_d is 9 μM for both muscle and *Acanthamoeba* actin. In 50 mM KCl the K_d for the complex with *Acanthamoeba* actin is 16 μM , while the K_d for the complex with muscle actin is greater than 50 μM . © 1988 Academic Press, Inc.

KEY WORDS: *Acanthamoeba*; profilin; pyrene-labeled actin; fluorescence enhancement; actin-profilin complex.

Profilin is the prototype of a class of proteins that are thought to regulate actin polymerization inside cells by binding to actin monomers (1,2). It is generally believed that the actin-profilin complex cannot polymerize into actin filaments and that polymerization is regulated by sequestering a fraction of the actin monomers in these complexes (3,4). In this way, profilin could act as an actin monomer buffer in the cell. In the case of *Acanthamoeba*, that is a reasonable suggestion, because the profilin concentration is so high—over 100 μM (2,5). Another function may involve the weak binding of profilin to the barbed end of actin filaments with consequent inhibition of elongation (6,7).

Since the main influence of profilin on actin depends on its binding actin monomers, the major questions regarding this mechanism are the stoichiometry, affinity, and kinetics of the binding reaction under various conditions. Although considerable

effort has gone into studying these matters, the information is still fragmentary and not completely consistent, even for *Acanthamoeba* profilin, the most thoroughly investigated representative of the class of proteins (Table 1).

In the present work, we demonstrate that binding of *Acanthamoeba* profilin enhances the fluorescence of pyrenyl-actin monomers from both *Acanthamoeba* and rabbit skeletal muscle. This provides a rapid, direct assay for the concentration of the actin-profilin complex under conditions where actin does not polymerize and we use the method to reevaluate the dissociation constant (K_d) of the complex. The results differ in several important ways from previous conclusions, particularly those based on polymerization assays. Specifically, the affinity of *Acanthamoeba* profilin is the same for amoeba and muscle actins and is not strongly influenced by the pyrene on Cys-374. A preliminary ac-

TABLE 1

DISSOCIATION CONSTANTS FOR THE COMPLEX OF *Acanthamoeba* PROFILIN WITH ACTIN (μM)

Assay method and conditions	<i>Acanthamoeba</i> actin		Muscle actin		Ref.
	Native	Pyrene	Native	Pyrene	
Depolymerizing buffers					
Nucleotide exchange	—	—	47	—	(8)
Dialysis rate	—	—	10	—	(9)
Actin ATPase (0.35 mM MgCl ₂)	6	—	70	—	(10)
Pyrene fluorescence	—	9	—	9	Present report
20 to 100 mM KCl					
Critical concentration	1-8	4	>50	>60	(3, 4, 10)
Elongation rate					
EM (barbed end)	4	—	40	—	(4, 5)
Fluorescence	—	6	—	>50	(10)
Pyrene fluorescence	—	16	—	>50	Present report
1 mM MgCl ₂ ± 50 mM KCl					
Critical concentration					
Viscosity	7-10	—	—	—	(12)
Pelleting	10	—	—	—	(12)
Light scattering	5-10	40	—	—	(11)
Elongation rate					
EM (barbed end)	50	—	>50	—	(4, 5, 7, 12)
EM (pointed end)	2-5	—	—	—	(7, 12)
Fluorescence	—	100	—	—	(7)
Light scattering	5-10	—	—	—	(11)
Pyrene fluorescence	—	12	—	—	Present report

count of some of this work was presented at the 1982 annual meeting of the American Society for Cell Biology (15).

MATERIALS AND METHODS

Acanthamoeba profilin and actin and rabbit muscle actin were purified as described by Tseng and Pollard (4). This preparation of profilin is a mixture of three isoforms, profilin-IA, -IB, and -II (12). The mixture of profilin-IA and -IB has the same effect on actin polymerization as profilin-II (12). Most of the experiments were done with a mixture of the three isoforms, but a few experiments were done with profilin-I and profilin-II purified by cation-exchange chromatography (12). Protein concentrations were measured

by uv absorption assuming an extinction coefficient at 280 nm of $1.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for all isoforms (5). The actins were labeled on Cys-374 with *N*-(1-pyrenyl)-iodoacetamide (Molecular Probes, Junction City, OR) by a modification (18) of the method of Kouyama and Mihashi (19). This resulted in incorporation of approximately one pyrene group per actin molecule specifically on Cys-374 (19). The extent of incorporation was measured by absorbance (Cary Model 119 spectrophotometer) using extinction coefficients of $2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 344 nm for bound pyrene (19) and $2.66 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 290 nm for actin (corrected for absorbance of the dye) (18). Actin was also labeled on Cys-374 with iodoacetamidotetramethylrhodamine (Molecular Probes) (20).

The fluorescence enhancement assay for profilin binding to pyrene actin was carried out at 25°C using a Perkin-Elmer Model 650-10S spectrofluorometer in the ratio mode with excitation at 342 or 346 nm and emission at 385 or 388 nm. The quantitative changes in fluorescence given were corrected for buffer fluorescence.

RESULTS

Effect of Acanthamoeba Profilin on the Fluorescence of Pyrenyl-Actin

Acanthamoeba profilin enhanced the absorbance (Fig. 1A) and the fluorescence of pyrenyl-actin from either *Acanthamoeba* (Figs. 1B, 2, and 3) or rabbit muscle (Fig. 3B, inset). The increase in absorbance accounted for 70 to 80% of the fluorescence enhancement. Profilin itself made an insignificant contribution to the absorbance (Fig. 1A) or the fluorescence (Fig. 2A) of the mixtures at wavelengths above 310 nm. The maxima of the absorption spectrum and the fluorescence excitation and emission spectra of pyrenyl-actin were the same as those in the presence and absence of profilin. The fluorescence enhancement occurred during the time required to mix the samples (about 10 s) and was stable for more than 1 day. Most of the experiments reported here were done with a 4:1 mixture of profilin-I and profilin-II (12), but both purified isoforms enhance the fluorescence of pyrene-actin. A given concentration of profilin-II enhanced the fluorescence slightly more than that concentration of profilin-I.

The fluorescence of mixtures of pyrenyl-actin and profilin depended on the concentration of both proteins. Two different experiments showed that the fluorescence was directly proportional to the concentration of pyrenyl-actin. First, the extrapolated maximum fluorescence enhancement obtained from the X intercept of Scatchard plots (Fig. 3B) was directly proportional to the concentration of pyrene-labeled *Acanthamoeba* and

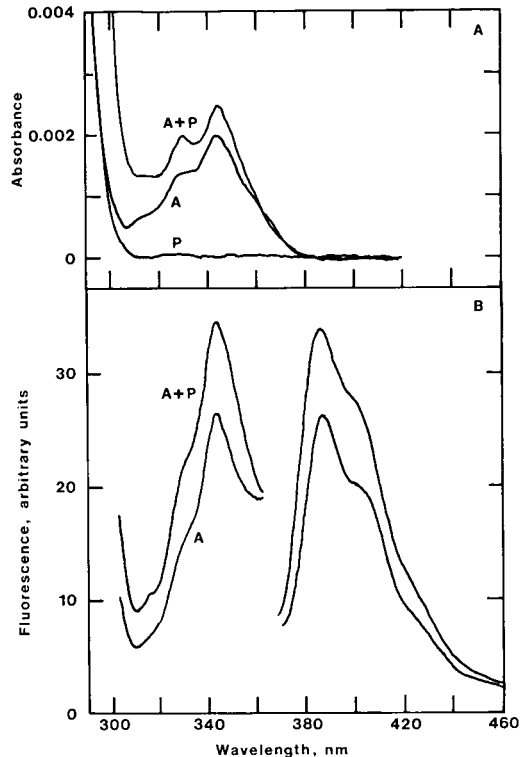


FIG. 1. Ultraviolet absorption and fluorescence spectra of pyrenyl-actin and *Acanthamoeba* profilin-I. Conditions: 3.5 mM imidazole (pH 7.5), 0.05 mM CaCl_2 , 0.1 mM ATP, 0.25 mM dithiothreitol, 0.5 mM sodium azide, 2 μM actin (40% pyrenyl labeled), and 32 μM profilin-I, 25°C. (A) Ultraviolet absorption spectra of profilin (P), actin (A), and the mixture (A + P). (B) Fluorescence excitation and emission spectra: excitation at 343 nm; emission at 385 nm.

muscle actin (Fig. 3C). Second, in mixtures of pyrenyl- and native actins with a fixed total concentration, the fluorescence enhancement was directly proportional to the fraction of pyrenyl-actin (data not shown).

The enhancement of the fluorescence of pyrenyl-actin had a hyperbolic dependence on the concentration of profilin (Figs. 2A and 3A). The results were the same with individual samples (Fig. 2A) and with titration of a single sample (Fig. 3A). In the titration experiments, small volumes of a concentrated profilin stock were added sequentially to a

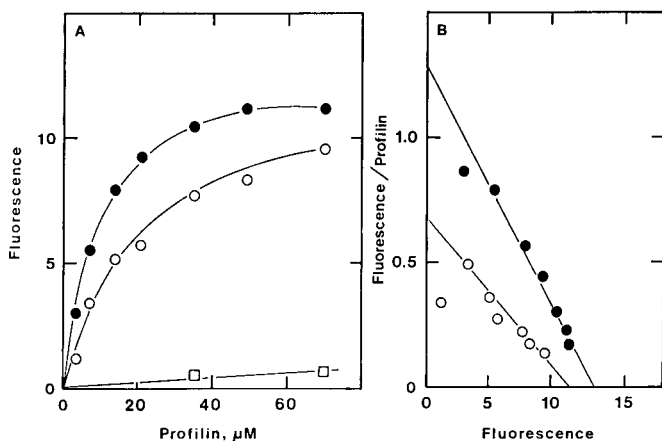


FIG. 2. Dependence of fluorescence enhancement of pyrene-labeled actin on the concentration of profilin. Conditions: $1 \mu\text{M}$ 60% pyrenyl-actin, variable concentrations of mixed profilin isoforms, 6 mM imidazole (pH 7.0), 0.1 mM CaCl_2 , 0.1 mM ATP, and 0.25 mM dithiothreitol, 25°C . Excitation at 342 nm; emission at 388 nm. The values are corrected for the fluorescence contributed by the buffer and the profilin alone (□). (A) Fluorescence enhancement above that of actin alone in arbitrary units vs concentration of profilin. (●) Actin plus profilin in low salt buffer. (○) Actin plus profilin in buffer with 50 mM KCl. (B) Scatchard plots of the data in (A); same symbols. The fluorescence enhancement was taken as the concentration of the actin-profilin complex. Since the actin concentration was low and the binding was weak, essentially all of the profilin was free, so the total concentration was taken as the concentration of free profilin. The slopes give the dissociation constants.

single sample of pyrenyl-actin and a correction factor was used to account for the dilution of the actin. Using this approach we could collect a considerable body of data with small amounts of protein. For example, a complete titration curve can be obtained with 40 μg of actin and 600 μg of profilin.

Evaluation of Binding Constants

Using the fluorescence enhancement as a measure of the concentration of actin complexed with profilin, Scatchard plots (21) of the dependence of the fluorescence enhancement on the concentration of profilin were linear (Figs. 2B and 3B). Thus the fluorescence change followed a simple binding isotherm and the binding affinity could be calculated from the slopes of these plots. The method gave consistent results with a mean K_d for 100% pyrene-labeled *Acanthamoeba* actin of $9.0 \mu\text{M}$ (SD, $3.7 \mu\text{M}$) in 2 mM imidazole

(pH 7.0), 0.1 mM CaCl_2 . The experiment in Fig. 2 gave a similar K_d of $11.4 \mu\text{M}$ for 60% labeled actin in 6 mM imidazole (pH 7.0), 0.1 mM ATP, 0.1 mM CaCl_2 , 0.25 mM DTT.¹

The effect of *Acanthamoeba* profilin on the fluorescence of pyrene-labeled muscle actin was nearly identical to its effect on *Acanthamoeba* actin. The fluorescence spectra and Scatchard plots (not shown) were the same as those in Figs. 1, 2, and 3. The extrapolated maxima from the Scatchard plots depended on the concentration of muscle actin just like the amoeba actin (Fig. 3C). In 2 mM imidazole (pH 7.0), 0.1 mM CaCl_2 , the average K_d for muscle actin was $9.4 \mu\text{M}$ (SD, $2.2 \mu\text{M}$).

Since the Scatchard plots of the data were linear, the fluorescence enhancement is in-

¹ Abbreviations used: DTT, dithiothreitol; EGTA, ethylene glycol bis(β -aminoethyl ether) N,N' -tetraacetic acid.

terpreted most simply as the binding of profilin to a single site on the actin. While the data are not consistent with multiple sites with substantially different affinities, they do not provide any direct evidence regarding the stoichiometry of the complex of actin and profilin.

Effect of Salt on the Fluorescence Enhancement

For pyrene-labeled *Acanthamoeba* actin monomers, the fluorescence enhancement brought about by profilin is similar in low salt buffer (Fig. 1A) and in 50 mM KCl. At actin concentrations below the critical concentration (1.5 μM), the excitation and emission spectra of pyrenyl-actin were identical with and without 50 mM KCl, except that the amplitude of the fluorescence was about 10% lower in KCl. This change occurred during the time required for mixing KCl with the

actin and was stable for more than 30 min. In KCl profilin enhanced the fluorescence to a maximum of about 40% (Fig. 2A). Scatchard plots were linear (Fig. 2B) and the K_d was slightly higher than in the absence of KCl. In six experiments in 50 mM KCl the mean K_d was 15.7 μM with a SD of 2.0 μM . In a single early experiment we obtained a much higher value of 55 μM , for reasons that we do not understand.

In 50 mM KCl, profilin caused much smaller changes in the fluorescence of pyrene-labeled muscle actin, so we could not calculate an accurate K_d . We estimate that it was greater than 50 μM .

In experiments with 50 mM KCl, 1 mM $\text{MgCl}_2 \pm 0.5$ mM EGTA, we used 0.2 μM pyrene-labeled *Acanthamoeba* actin to evaluate the effect of profilin on the fluorescence. The critical concentration for polymerization was 0.4 μM without EGTA and 0.15 μM with EGTA, so there was no polymerization

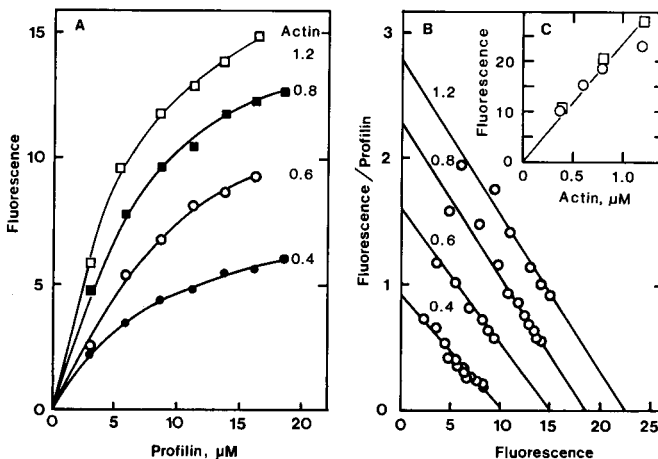


FIG. 3. Dependence of fluorescence enhancement on the concentrations of profilin and 100% pyrene-labeled *Acanthamoeba* actin. Conditions: 2 mM imidazole, pH 7.0, 0.1 mM CaCl_2 . Single samples of pyrene-labeled actin were titrated by adding sequentially small (1% total volume) aliquots of profilin. A correction was made for the dilution of the actin. (A) Fluorescence enhancement vs concentration of profilin: (●) 0.4 μM actin; (○) 0.6 μM actin; (■) 0.8 μM actin; (□) 1.2 μM actin. (B) Scatchard plots of the data in (A) calculated as in Fig. 2B. The micromolar actin concentrations are given to the right of each plot. (C) Dependence of the extrapolated maximum fluorescence (from Scatchard plots) on the concentration of pyrene-labeled actin: (○) *Acanthamoeba* actin from the X intercepts in Fig. 3B; (□) muscle actin from an experiment similar to that in Figs. 3A and 3B.

during the time required to collect the data. In 50 mM KCl, 1 mM $\text{MgCl}_2 \pm \text{EGTA}$, the fluorescence of actin alone was slightly lower than in 50 mM KCl. The effect of profilin on the fluorescence was similar to that under the conditions described above. Scatchard plots of the data gave K_d 's of about 11 μM in 50 mM KCl, 1 mM MgCl_2 , 0.1 mM CaCl_2 , and 12 μM in this buffer with 0.5 mM EGTA. The extrapolated maximum fluorescence was about the same in KCl-Mg-EGTA and low salt buffer.

Effect of Profilin on the Fluorescence of Rhodamine-Labeled Actin

In the low salt buffer, *Acanthamoeba* profilin enhanced the fluorescence of 1 μM rhodamine-labeled muscle actin. At a profilin concentration of 20 μM , the enhancement was 66% as much as the enhancement of pyrenyl-actin. Profilin-II gave a larger enhancement than profilin-I, similar to the results with pyrenyl-actin.

DISCUSSION

Characterization of the Fluorescence Assay for the Actin-Profilin Complex

The results of this study are all consistent with the conclusion that the complex of pyrenyl-actin with *Acanthamoeba* profilin has a higher fluorescence than the pyrenyl-actin alone. The enhancement of the fluorescence has a hyperbolic dependence on the concentration of profilin and the maximum fluorescence is directly proportional to the concentration of pyrenyl-actin. The presence of low concentrations of unlabeled, native actin monomers has no detectable effect on the fluorescence of the pyrenyl-actin. The fluorescence enhancement is largely due to an increase in absorption the pyrenyl group, most likely because the chromophore has a different environment in the complex than on the free actin. The change in absorption due to profilin may be useful for some ex-

periments, but the fluorescence signal is much stronger at the low concentrations of pyrenyl-actin employed in most of our work.

The fluorescence of pyrenyl-actin is sensitive to both polymerization and binding to other proteins, including gelsolin (16), vitamin D-binding protein (17), and profilin. The spectroscopic results show that the mechanism of fluorescence enhancement differs substantially at least for polymerization and binding to profilin. When pyrenyl-actin polymerizes, there is a 25-fold increase in fluorescence due to a 3-fold increase in absorption at 365 nm and an 8-fold increase in fluorescence emission at 386 and 407 that is largely attributable to a larger quantum yield (19). Since these effects of polymerization cannot be explained simply by a change in the polarity of the environment, Kouyama and Mihashi (19) suggested that there must be a change in the interaction of the chromophore with an amino acid side chain in the actin molecule. In contrast, the fluorescence change upon binding profilin is much smaller, is accounted for mostly by increased absorption, and does not involve the appearance of any new absorption or emission peaks. Consequently, any conformational changes and local environment near Cys-374 must be substantially different in actin filaments and in the actin-profilin complex.

The fact that profilin enhances the fluorescence of pyrene bound to the penultimate residue of actin (cysteine-374) provides new evidence that profilin binds near the C-terminus of the actin molecule or that binding is associated with a conformational change in the C-terminal region. This was suggested previously, when it was found that vertebrate profilin inhibits the polymerization of native actin more strongly than actin with the C-terminal phenylalanine removed by carboxypeptidase (24) or with cysteine-374 modified with pyrenyl-iodoacetamide or another alkylating agent (13). In polymerization assays, *Acanthamoeba* profilin also had a lower affinity for pyrene-labeled *Acanthamoeba*

actin than for unlabeled actin (11). We have now shown directly that *Acanthamoeba* profilin binds near the C-terminus of actin by chemically crosslinking it to glutamic acid 364 of actin (T. D. Pollard, D. A. Kaiser, and J. Vandekerckhove, manuscript in preparation).

This fluorescence method has a number of advantages compared with other methods used to measure interactions between actin and profilin (Table 1). It is fast, sensitive, and requires only small amounts of protein. Multiple rapid measurements can be made on a single sample of actin by titration with profilin. In principle, the method should be usable for rapid kinetic analysis to measure the rate constants for binding and dissociation. Perhaps most importantly, the method is direct and involves (so far as we know) only three species—actin monomers, profilin monomers, and the actin–profilin complex. Consequently it can be used in a wide variety of buffer conditions without having to make any assumptions about the effect of the conditions on other reactions such as polymerization, nucleotide exchange, or nucleotide hydrolysis. When one relies on a second reaction, such as polymerization, to measure the concentration of a complex, one must assume that the polymerization reaction has no influence on the binding reaction. This assumption has generally been implied but never proven to be true.

The fluorescence method has one particular disadvantage, the possibility that modification of Cys-374 with pyrenyl-iodoacetamide alters the affinity of the actin for profilin. Judging from polymerization assays the affinity of both *Acanthamoeba* (11) and vertebrate (13) profilins is lower for pyrenyl-actin than for native actin. On the other hand, Dinubile and Southwick (14) found no such effect using a polymerization assay with macrophage profilin, and our fluorescence assay with pyrenyl-actin gave K_d 's similar to those obtained with unlabeled actin by other methods that do not depend on polymeriza-

tion. These differences suggest that it might be worth testing whether the modification of Cys-374 alters the interaction of the actin–profilin complex with filaments rather than inhibiting the interaction of profilin with actin monomers.

Characterization of the Binding of Profilin to Actin Monomers

Previous studies (Table 1) led to the conclusion that *Acanthamoeba* profilin binds to native amoeba actin with higher affinity than either native muscle actin or pyrenyl-amoeba actin, but our new results show that it binds equally well to all three, at least at low salt concentrations. The K_d is about 10 μM for amoeba actin and the concentrations of KCl and MgCl_2 that are commonly used to polymerize actin have only a minor influence on the affinity. On the other hand, salt greatly weakens the binding of the amoeba profilin to muscle actin. This is responsible, in part, for the earlier conclusion that binding to muscle actin is weak (3,4). The presence of 0.35 mM MgCl_2 may have contributed to the weak binding measured in the ATPase assay of Tobacman and Korn (3). We have no explanation for the very high K_d measured in low salt with a nucleotide-exchange assay (8), although it is possible that binding of profilin to a secondary, weak site may influence both nucleotide exchange and hydrolysis.

The situation in KCl– MgCl_2 is less clear and the new data do not resolve the present disagreements regarding the mechanism of *Acanthamoeba* profilin. The problem is that various types of assays have given different results with *Acanthamoeba* actin (Table 1), and more work will be required to understand why electron microscopic assays for growth at the barbed end of filaments (4,5,7,12) show that the affinity is low ($K_d = 50$ to 100 μM), while parallel experiments on the polymerization of bulk samples of native actin using light scattering (11) and the

present analysis by fluorescence enhancement all indicate that the K_d is 10 μM or perhaps even less. One complex mechanism consistent with most of the data, including those in this report, has been proposed (7).

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