ACANTHAMOEBA PROFILIN BINDING TO FLUORESCEIN-LABELED ACTINS

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ABSTRACT The binding constants of *Acanthamoeba* profilin to fluorescein-labeled actin from *Acanthamoeba* and from rabbit skeletal muscle have been determined by measuring the reduction in the actin tracer diffusion coefficients, determined by fluorescence photobleaching recovery, as a function of added profilin concentration. Data were analyzed using a two-parameter nonlinear regression analysis to determine the profilin–actin dissociation constant K_d and the profilactin diffusion coefficient, D_{PA}. For fluorescein-labeled *Acanthamoeba* actin, the least-squares estimates for K_d and D_{PA}, along with approximate single standard deviation confidence intervals, are K_d = 48 (36, 63) μM and D_{PA} = 6.72 (6.62, 6.81) $\times 10^{-7}$ cm^{2}s^{-1}. For fluorescein-labeled skeletal muscle actin, the corresponding values are K_d = 147 (94, 225) μM and D_{PA} = 6.7 (6.3, 7.0) $\times 10^{-7}$ cm^{2}s^{-1}. These dissociation constants are the first to be determined from direct physical measurement; they are in agreement with values inferred from earlier studies on the effect of profilin on the assembly of actin that had been fluorescently labeled or otherwise modified at Cys 374. These results place important restrictions on the interpretation of experiments in which fluorescently labeled actin is used as a probe of living cytoplasm or cytoplasmic extracts that include profilin.

INTRODUCTION

The dynamic microstructure of the cytoskeletal contractile apparatus is determined by the reversible self-assembly of certain cytoplasmic proteins, of which actin is generally the most abundant. Globular actin (G-actin) is stable under conditions of low salt, but spontaneously assembles to form filaments (F-actin) when salts are added to the solution. Under physiological conditions the steady-state ratio lies far in the direction of filaments. Hence the cell requires a regulatory mechanism to favor G-actin if a pool of monomers is to be maintained so that new filaments can be constructed when and where they are required. A general mechanism for this type of regulation is the binding of G-actin by a small protein called profilin to form a 1:1 complex, called profilactin, which stabilizes the actin monomer from assembly.

Profilin was first isolated from mammalian cells (Carlsson et al., 1977) and has subsequently been found in a number of cell types, including the protozoan *Acanthamoeba castellanii* (Reichstein and Korn, 1979). Although profilin was first assumed to act only through its ability to bind G-actin, recent evidence has been suggested that profilin also binds to one or both ends of actin filaments (Tilney et al., 1983; Pollard and Cooper, 1984; Kaiser et al., 1986). In either model the dissociation constant of the profilactin complex is an essential characteristic parameter of the mechanism. The dissociation constant of profilactin has been determined primarily by examination of the concentration dependence of the effect of profilin on actin assembly. Most of the quantitative determinations of the dissociation constant of profilactin have been performed using *Acanthamoeba* profilin and actin, and the reported values range from 1 to 11 μM (Tseng and Pollard, 1982; Tobacman and Korn, 1982; Tobacman et al., 1983; Tseng et al., 1984; Pollard and Cooper, 1984; Lal and Korn, 1985; Kaiser et al., 1986). Similar values of K_d (1–10 μM) have been determined for mammalian profilactin (Tilney et al., 1983; Nishida, 1985; DiNubile and Southwick, 1985), but interspecies binding between mammalian skeletal muscle and *Acanthamoeba* profilin is about an order of magnitude weaker (Mockrin and Korn, 1980; Tobacman and Korn, 1982; Tseng and Pollard, 1982). Although there has been one report of regulation of profilactin dissociation by micromolar concentrations of $MgCl_2$ (Tseng and Pollard, 1982), most investigators have found the binding to be remarkably insensitive to solution conditions.

Chemical modification of actin to prepare a fluorescent conjugate has proved to be a valuable approach both for the observation of actin assembly and dynamics in vivo (e.g., Taylor and Wang, 1980) and for the study of actin assembly in vitro. In the latter category are included techniques that rely on fluorescence quantum yield changes (Kouyama and Mihashi, 1981), fluorescence energy transfer (Wang and Taylor, 1981), and fluorescence photo-bleaching recovery (FPR) (Lanni et al.,

1981). The site of attachment to actin is generally the reactive sulfhydryl group of Cys 374, most commonly with an iodoacetamide linkage to the dye. A large body of literature supports the usual assumption that labeling actin at this site does not perceptibly affect its self-assembly behavior. However, the putative effect of fluorophore labeling upon the interaction of actin with various regulatory proteins is more controversial.

Malm (1984) reported that chemical modification of Cys 374 of skeletal muscle actin, either with *N*-ethylmaleimide or with *N*-(1-pyrenyl)iodoacetamide, effectively counteracted the inhibiting effect of calf spleen profilin on actin polymerization. Lal and Korn (1985) reported similar observations for *Acanthamoeba* profilin and *Acanthamoeba* actin; addition of the pyrenyl group to Cys 374 of the actin raised the dissociation constant from 5 to 40 μM, as determined by actin assembly assays. However, DiNubile and Southwick (1985) did not observe an effect of modification of Cys 374 with macrophage profilin.

We report here the first physical measurements of actin–profilin binding and the profilactin dissociation constant for *Acanthamoeba* profilin interacting with fluorescein-labeled actin from either *Acanthamoeba* or rabbit skeletal muscle. The binding has been detected through the reduction in the translational diffusion coefficient, as measured by FPR (Peters et al., 1974; Axelrod et al., 1976; Edidin et al., 1976; Jacobson et al., 1976; Ware, 1984). Our results support much of the previous literature to constitute additional evidence that particular caution must be taken in the design of certain experiments involving fluorescently labeled actin.

EXPERIMENTAL PROCEDURES

Acanthamoeba castellanii, kindly supplied by Dr. E. D. Korn, was grown in 15-liter aerated carboys (Pollard and Korn, 1973). *Acanthamoeba* profilin was isolated by the method of Reichstein and Korn (1979) with minor modifications. The procedure used does not separate isoforms of profilin. The buffer for the separation of profilin on Sephadex G-75 and in which profilin was stored (at 0°C) was 5 mM imidazole, 3 mM NaN_3, 0.75 mM β-mercaptoethanol, pH 7.5. Profilin concentration was measured by absorbance at 280 nm using $\epsilon = 1.4 \times 10^4\ cm^{-1}M^{-1}$ (Tseng et al., 1984). The ability of the prepared profilin to interact with actin was verified by assays of actin assembly in the presence of varied concentration of profilin, using FPR with trace fluorescein-labeled *Acanthamoeba* actin as an assembly assay (Lanni et al., 1981).

Acanthamoeba actin was isolated by a slight modification of the method of Gordon et al. (1976) from the 0.19–0.30 M KCl fractions of the DEAE-cellulose column used to prepare profilin. Rabbit skeletal muscle actin was purified from commercial acetone powder according to the method described by Pardee and Spudich (1982) with gel filtration on Sephadex G-150 (MacLean-Fletcher and Pollard, 1980). Purified actin was stored for several days in pellet form at 4°C. In preparation for labeling and experimentation, homogenized actin was depolymerized by dialysis against 5 mM imidazole, 0.5 mM ATP, 0.1 mM $CaCl_2$, 3 mM NaN_3, 0.75 mM β-mercaptoethanol, pH 7.5, followed by clarification by centrifugation. Actin concentrations were measured by absorbance at 290 nm using $\epsilon = 2.7 \times 10^4\ cm^{-1}M^{-1}$.

Actin was labeled by reaction of actin filaments with 5-(iodoacetamido)-fluorescein (Molecular Probes, Inc., Junction City, OR) by a modification of the method of Wang and Taylor (1980). Unreacted label was separated from protein by gel filtration through Sephadex G-25-150. Extent of labeling was assessed by absorbance at 495 nm using an extinction coefficient of $6 \times 10^4\ cm^{-1}M^{-1}$. The concentration of labeled actin was determined by the Bradford (1976) assay using unmodified G-actin as standard.

Translational diffusion coefficients of labeled actin and its complex with profilin were determined using the modulation detection method of FPR (Lanni and Ware, 1982). In this approach a square-wave pattern is photobleached in the sample, and a monitoring beam passing through a translated grating produces a modulation of the fluorescence. As the pattern contrast decays due to translational diffusion of the labeled species, the detected modulation envelope decays in proportion. The data form for a single labeled diffusing species is an exponential with time constant given by $(DK^2)^{-1}$, where D is the tracer translational diffusion coefficient of the labeled species and K is the wave vector of the photobleached pattern, given by $2\pi/L$, where L is the pattern spacing. All FPR measurements for this report were made at 20°C using a K vector of $841\ cm^{-1}$.

FPR data for a sample containing labeled actin and unlabeled profilin are expected to contain two exponential decays: one due to actin alone and the second due to the profilactin complex. From the relative molecular weights (actin, 42,000; profilin, 12,000), we anticipate that these exponentials will differ only slightly in time constant and thus will not be resolved. For *Acanthamoeba* actin, the data were well fit by a second order cumulant expansion (Koppel, 1972) using a nonlinear least-squares procedure (Bevington, 1969). For each sample the least-squares estimates were averaged over five to twenty replicate measurements. The data from muscle actin were not well fit by a second- or third-order cumulant expansion. Double exponential fits to those data revealed the presence of small portions of an aggregate labeled species of constant size and proportions. The exponential with the shorter time constant gave the proper value for actin diffusion and followed the binding of profilin, so the diffusion coefficient from that term is used in determining the binding constant.

The diffusion coefficients for labeled actin were measured at constant actin concentration (16 μM) as a function of the molar ratio of profilin to actin. If X is the mole fraction of actin bound to profilin, we can represent the measured diffusion coefficient as

$$\tilde{D} = (1 - X)\, D_A + X D_{PA}, \tag{1}$$

where D_A, D_{PA} are the diffusion coefficients of actin monomer and profilactin complex, respectively. The dissociation constant of the actin–profilin equilibrium can be expressed as

$$K_d = C_A\,[(C_P/C_A) - X](1 - X)/X, \tag{2}$$

where C_A is the total actin concentration and C_P is the total profilin concentration. Following Zero et al. (1983) we solve these equations for X and perform a two-parameter nonlinear regression analysis to estimate K_d and D_{PA}. The analyses could be refined further to account for the presence of unlabeled actin in the sample. Since unlabeled actin binds profilin much more tightly than labeled actin, the net effect of the unlabeled actin would be to reduce the concentration of free profilin that can interact with the labeled actin. However, since experiments were performed under conditions of excess profilin, and since for all samples the labeled actin was 50% or more of the total actin, these corrections turn out to be less than the reported experimental error.

RESULTS

FPR traces for samples of *Acanthamoeba* G-actin with *Acanthamoeba* profilin were well fit by the cumulant expansion. The first cumulant was used to calculate the weighted average diffusion coefficient, and the second cumulant provided a measure of the variance. Mean values of the normalized second cumulant were always <0.05 and

fluctuated ~0.02–0.03, showing no evidence of a trend with increasing ratio of profilin concentration to actin concentration. Such small values for the variance would not be achieved for a cumulant fit to a sum of two exponentials whose time constants differ by the amounts observed for D_A and D_{PA}. Hence we conclude that during the time period of an FPR measurement (seconds), free and bound profilin interchange rapidly to provide a single weighted average diffusion coefficient in the measurement.

The diffusion coefficients for labeled *Acanthamoeba* actin as a function of the ratio of profilin concentration to actin concentration are shown in Fig. 1. Error bars for each point represent standard deviations among repeated measurements on the same sample. The line in Fig. 1 represents the binding isotherm calculated using Eqs. 1 and 2 with $D_A = 7.86 \times 10^{-7}$ cm^{2}s^{-1}. The least-square estimates for K_d and D_{PA}, along with approximate single standard deviation confidence intervals, are K_d = 48 (36, 63) μM and $D_{PA} = 6.72$ (6.62, 6.81) $\times 10^{-7}$ cm^{2}s^{-1}. The confidence intervals are defined by an F statistic and found by a search over the sum of squares surface (Draper and Smith, 1966; Johnson, 1983).

Fig. 2 shows the dependence of the measured diffusion coefficient of the fast component of a two-exponential fit for labeled skeletal muscle actin interacting with *Acanthamoeba* profilin. The uncertainties in these points and in the corresponding isotherm are relatively greater. The least-squares estimates for the dissociation constant and the profilactin diffusion coefficient are, respectively, K_d = 147 (94, 225) μM and $D_{PA} = 6.7$ (6.3, 7.0) $\times 10^{-7}$ cm^{2}s^{-1}, where again we have indicated approximate single standard deviation confidence intervals for each parameter.

The data of Figs. 1 and 2 can be re-analyzed to quantify the possible effect of unlabeled actin in the samples. The most extreme effect would result if $K_d = 0$ for unlabeled

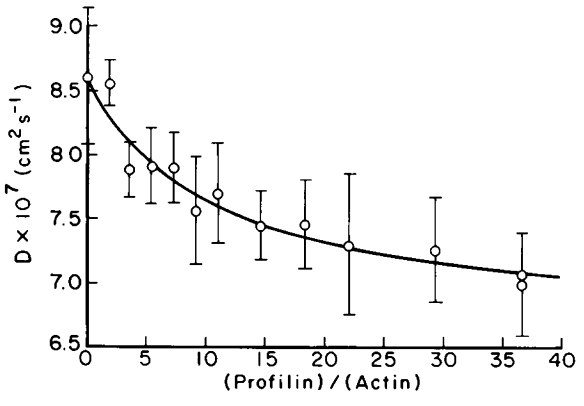

FIGURE 1 Plot of the average diffusion coefficient (at 20°C) of fluorescein-labeled *Acanthamoeba* actin as a function of the molar ratio of *Acanthamoeba* profilin to actin. Profilin was added to a constant actin concentration of 16 μM. Standard deviations from at least five replicate measurements are shown for each point. The isotherm drawn through the points corresponds to a K_d of 48 μM with $D_A = 7.86 \times 10^{-7}$ cm^{2}s^{-1} and $D_{PA} = 6.67 \times 10^{-7}$ cm^{2}s^{-1}.

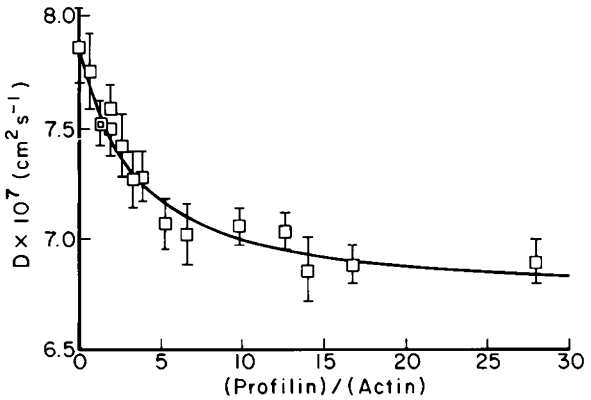

FIGURE 2 Plot of an experiment conducted as described for Fig. 1, except that the labeled species was skeletal muscle actin. The isotherm drawn corresponds to K_d = 147 μM, $D_A = 8.6 \times 10^{-7}$ cm^{2}s^{-1}, and $D_{PA} = 6.7 \times 10^{-7}$ cm^{2}s^{-1}. As described in the Experimental Procedures section, the model for fitting the FPR traces in this set of measurements was a double exponential, rather than the cumulant method used for the data of Fig. 1, so the slight differences for D_A are probably not significant.

actin (i.e., unlabeled actin binds profilin much stronger than does labeled actin). Numerical analysis for that case gives K_d = 37 μM and $D_{PA} = 6.77 \times 10^{-7}$ cm^{2}s^{-1} for labeled *Acanthamoeba* actin, and K_d = 132 μM and $D_{PA} = 6.7 \times 10^{-7}$ cm^{2}s^{-1} for labeled muscle actin. Thus in both cases the effect of unlabeled actin in the samples was less than experimental uncertainty.

DISCUSSION

These measurements represent the first direct physical determination of a profilin–actin binding constant. The only measurement in the literature to which we can compare directly is the report by Lal and Korn (1985), from actin assembly assays, that the apparent binding constant of *Acanthamoeba* profilin to pyrene-labeled *Acanthamoeba* actin is 40 μM. Our result for fluorescein-labeled *Acanthamoeba* actin is consistent with their value to within experimental error. The most important point to emphasize is that the measured value of K_d for labeled actin with profilin is an order of magnitude greater than the reports for unlabeled actin with profilin (Tseng and Pollard, 1982; Tobacman and Korn, 1982; Tobacman et al., 1983; Tseng et al., 1984; Pollard and Cooper, 1984; Lal and Korn, 1985; Kaiser et al., 1986). It is evident that the presence of the dye at Cys 374 greatly reduces the actin–profilin affinity. This observation caused us to speculate that profilin binding to labeled actin should be accompanied by a change in the fluorescence yield and/or spectrum. We have searched for these effects but have not been able to confirm them, although there is one preliminary report of such an effect using pyrene-labeled actin (Lee et al., 1982).

The significance of the reduced actin–profilin affinity upon labeling the actin relates to the interpretation of experiments in which fluorescently labeled actin is added

to living cytoplasm, cytoplasmic extracts, or other solutions that contain profilin (for reviews see Kreis and Birchmeier, 1982; Taylor et al., 1986). Often the desired assumption is that the labeled actin distributes itself in the same manner as unlabeled actin. As Taylor et al. (1986) have pointed out, that assumption is subject to several restrictions, not the least of which is that labeled actin is much less susceptible to regulation by binding with profilin.

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