Acanthamoeba Profilin Interacts with G-Actin To Increase the Rate of Exchange of Actin-Bound Adenosine 5′-Triphosphate†

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ABSTRACT: A sevenfold molar excess of *Acanthamoeba* profilin, a 12000-dalton protein that inhibits actin polymerization, increases the rate of exchange of ATP bound to G-actin with ATP in solution about 17-fold, i.e., from 7.7×10^{-4} to 1.3×10^{-2} s^{-1}, at 25 °C, 0.033 mM Ca^{2+}, and 0.1 mM ATP, pH 7.5. Detailed analysis of the equilibrium isotope-exchange data shows that profilin and actin form a 1:1 complex with $K_D = 4.7 \times 10^{-5}$ M and that the binding of profilin to actin is rapid and reversible. The actin–profilin complex binds 1 mol of ATP/mol, as does G-actin. Profilin does not interact with ATP or Ca^{2+}.

Approximately 50% of the cytoplasmic actin of nonmuscle cells remains unpolymerized, in the cell and in cell extracts, at concentrations up to 100-fold greater than the critical polymerization concentration of the purified actin (Bray & Thomas, 1976; Gordon et al., 1977). This apparent paradox was rationalized, at least in part, by the isolation from mammalian spleen, and then from platelets, of a 1:1 nonpolymerizable complex of G-actin with profilin, a protein with a molecular weight of ~16000 (Carlsson et al., 1976; Markey et al., 1978; Weeds & Harris, 1978). By amino acid sequence (Nystrom et al., 1979), calf spleen profilin has a molecular weight of 15220. When purified from the stable profilactin complex, profilin was shown to inhibit the polymerization of G-actin (Markey et al., 1978; Weeds & Harris, 1978; Carlsson et al., 1977). Subsequently, a similar, but not identical, protein was isolated in this laboratory from *Acanthamoeba castellanii* (Reichstein & Korn, 1979) and shown to inhibit actin polymerization by inhibiting the rate of nucleation, the first step in the polymerization process. *Acanthamoeba* profilin has a molecular weight of ~12000. From the ratio of profilin to actin required to inhibit actin polymerization, it was deduced that *Acanthamoeba* profilin also formed a 1:1 complex with monomeric actin.

Other than the resistance of the actin to polymerization, however, nothing is known about the properties of the profilin–actin complex. Monomeric G-actin contains 1 mol of tightly bound adenosine 5′-triphosphate (ATP) which protects G-actin from denaturation and is hydrolyzed to tightly bound adenosine 5′-diphosphate (ADP) when G-actin is polymerized to F-actin (Oosawa & Kasai, 1971; Engel et al., 1977). The ATP bound to G-actin freely exchanges with ATP in solution (Kuehl & Gergeley, 1969). In this paper, we report the effect of *Acanthamoeba* profilin on that exchange.

Experimental Procedures

Materials. Profilin was isolated from *A. castellanii* by the procedure of Reichstein & Korn (1979) with minor modifications; the concentration of potassium phosphate in the hydroxylapatite step was lowered from 5 to 1.5 mM, and 10 mM imidazole buffer was used for the Sephadex G-75 column. Profilin was stored in 10 mM imidazole, pH 7.5, 0.5 mM dithiothreitol, and 0.01% NaN_3. G-Actin was prepared from acetone powders of rabbit back and leg muscles by the method of Spudich & Watt (1971) as modified by Eisenberg & Kielley (1974).

For preparation of [γ-^{32}P]ATP-labeled G-actin, nonradioactive G-actin containing 1 mol of bound ATP (Oosawa & Kasai, 1971) was incubated for 36 h at 4 °C at a concentration of 1.5 mg/mL (35.7 μM) in 3 mM imidazole, pH 7.5, 0.1 mM $CaCl_2$, and 0.5 mM dithiothreitol containing 0.36 mM [γ-^{32}P]ATP (~13 μCi/μmol). The total radioactivity was measured on an aliquot of the solution, and then unbound ATP was removed by treatment with Dowex-1-8X (Mulhern et al., 1975). The protein concentration and radioactivity of the [γ-^{32}P]G-actin were then measured. The measured exchange was 0.96 ± 0.02 mol/mol of actin in three experiments taking into account the actin-bound as well as the free ATP. All of the experiments reported in this paper were carried out with one batch of actin. The specific activity of the actin-bound [γ-^{32}P]ATP was about 1.9×10^{11} cpm/mmol or 4.5×10^6 cpm/mg of actin.

Analytical Methods. The concentration of G-actin was determined spectrophotometrically by using an extinction coefficient at 290 nm of 0.62 mg^{-1} mL cm^{-1} (Gordon et al., 1976). Profilin concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Total calcium was determined by using a Perkin-Elmer atomic absorption spectrophotometer equipped with a graphite furnace.

Equilibrium Dialyses. Binding of Ca^{2+} to profilin was measured by dialyzing 0.5 mL of profilin solution (1 mg/mL) in 6.5 mM imidazole, pH 7.5, 0.1 mM ATP, and 0.5 mM dithiothreitol against 100 mL of the same buffer containing 0.1 mM $^{45}CaCl_2$ (9.6×10^5 cpm/mmol) at 25 °C for 15 h. A control dialysis bag containing only buffer was included to determine that the $^{45}Ca^{2+}$ had equilibrated on the two sides of the dialysis bag. Binding of ATP to profilin was measured with 0.1-mL dialysis cells and high-porosity membranes as described in detail elsewhere (Mockrin et al., 1975). Profilin, 2 mg/mL in 6.5 mM imidazole, pH 7.5, 0.1 mM $CaCl_2$, and 0.5 mM dithiothreitol, was dialyzed against the same buffer containing 0.2 mM [γ-^{32}P]ATP (13 μCi/μmol) for 4 h at 23 °C.

ATP Hydrolysis. [γ-^{32}P]ATP-labeled G-actin was diluted to 0.5 mg/mL in 6.5 mM imidazole, 0.03 mM $CaCl_2$, 0.5 mM dithiothreitol, and 0.12 mM [γ-^{32}P]ATP with and without profilin (1 mg/mL). The solutions were incubated at 25 °C, at various times aliquots of 50 μL were diluted into 0.5 mL of water, and the $^{32}P_i$ was measured as described by Pollard & Korn (1973).

Measurement of ATP Exchange. For determination of the rate of exchange of actin-bound ATP with ATP in solution, [γ-^{32}P]ATP-labeled G-actin was diluted to 0.5 mg/mL (~2.2

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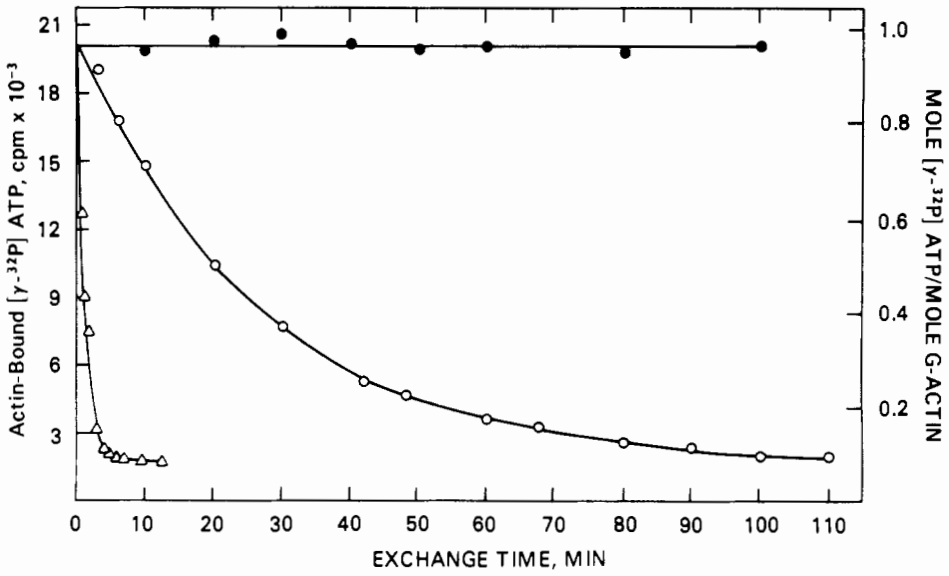


FIGURE 1: Exchange of G-actin bound [γ-^{32}P]ATP with nonradioactive ATP in solution, in the presence and absence of profilin as a function of time. Dowex-treated [γ-^{32}P]ATP-labeled G-actin (0.5 mg/mL) was incubated with and without profilin (1 mg/mL) at 25 °C in 6.5 mM imidazole, pH 7.5, 0.033 mM $CaCl_2$, 0.5 mM dithiothreitol, and 0.1 mM ATP. The exchange reaction was initiated by addition of the nonradioactive ATP after a 10-min incubation in its absence. Aliquots were removed at the indicated times and assayed for residual actin-bound [γ-^{32}P]ATP. Actin alone (○); actin plus profilin, 7:1 (△); actin plus profilin, 7:1, in the absence of nonradioactive ATP (●).

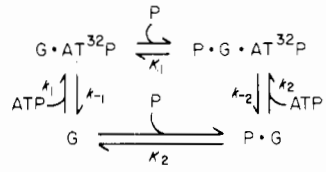
$\times 10^9$ cpm/mL) in 6.5 mM imidazole, pH 7.5, 0.033 mM $CaCl_2$, 0.5 mM dithiothreitol, and 0.1 mM nonradioactive ATP. The solution was incubated at 25 °C, and 100-μL aliquots were removed at various times and added to 20 μL of a 50% suspension of Dowex-1-8X. After 2 min with occasional agitation, the resin, which had bound all of the ATP not bound to the actin, was removed by centrifugation for 60–90 s at 3000g. The remaining actin-bound radioactive ATP was measured by scintillation counting of 10–20-μL aliquots of the supernatant solution.

Results

The loss of radioactivity from [γ-^{32}P]ATP-labeled G-actin in the presence and absence of *Acanthamoeba* profilin, at a profilin/actin ratio of 7:1, is shown in Figure 1. In both cases, 90% of the radioactivity was lost which represents 100% exchange since the concentration of nonradioactive ATP was ~9 times the concentration of [γ-^{32}P]ATP-labeled G-actin. That the loss of radioactivity was, in fact, due to exchange with nonradioactive ATP rather than to loss of [γ-^{32}P]ATP by denaturation of the actin was demonstrated in three ways. First, essentially no radioactive ATP was lost from the [γ-^{32}P]ATP-labeled G-actin when it was incubated in the same buffer, but without unlabeled ATP (Figure 1). Second, almost identical results were obtained in the reverse experiment starting with nonradioactive ATP-labeled G-actin and incubating it in the same buffer containing [γ-^{32}P]ATP. In fact, this is essentially identical with the way in which the [γ-^{32}P]ATP-labeled G-actin was obtained for the experiment shown in Figure 1. In the presence and absence of profilin (profilin/actin ratio of 7:1), the exchange of radioactive ATP for nonradioactive ATP was 0.95 mol/mol of G-actin. Third, all the preparations of actin polymerized normally when made 2 mM in $MgCl_2$.

Scheme I shows the loss of actin-bound ATP in the presence of profilin, where P = profilin and G = monomeric actin. This model assumes a 1:1 complex between profilin and actin and that their binding is rapid and reversible. When the experimental data are analyzed according to this model, it is further assumed that the k_1 and k_2 pathways can be ignored because the concentration of nonradioactive ATP in solution was ~9

Scheme I



times greater than the concentration of protein-bound ATP. Therefore, once a molecule of radioactive ATP dissociated from the actin, there was little chance that it would reassociate. This assumption introduces an error of <10%.

With these three assumptions, the loss of protein-bound radioactive ATP can be described by eq 1, where $C_b(t)$ is the cpm bound at any given time and K_1 is the dissociation constant for the profilin·G-actin·ATP complex.

$$-\frac{dC_b(t)}{dt} = \left(\frac{k_{-1} + k_{-2}[P]/K_1}{1 + [P]/K_1}\right)C_b = k_{obsd}C_b \quad (1)$$

Thus, for a fixed profilin concentration, a plot of ln $[C_b(t) - C_b(\infty)]$ vs. time [where $C_b(\infty)$ is the cpm after 9 half-lives] yields a straight line whose slope is the observed rate constant, k_{obsd}. Semilogarithmic plots of the data from 10 experiments such as that shown in Figure 1, with the ratio of profilin/actin varied between 0 and 7, are shown in Figure 2. A single exponential decay was observed at all concentrations of profilin.

Figure 3 shows the saturation curve that results when k_{obsd}, calculated from the curves in Figure 2, is plotted as a function of the concentration of profilin. A double-reciprocal plot of these data allows the calculation of K_1, the equilibrium constant for the interaction of profilin and G-actin·ATP, and k_{-2}, the rate constant for the dissociation of ATP from the profilin·G-actin·ATP complex. Equation 2, used for the double-reciprocal plot, was derived by setting k_{obsd} equal to the expression in parentheses in eq 1 and rearranging terms.

$$\frac{1}{k_{obsd} - k_{-1}} = \left(\frac{K_1}{(k_{-2} - k_{-1})}\right)\frac{1}{[P]} + \frac{1}{k_{-2} - k_{-1}} \quad (2)$$

Thus, a plot of $1/(k_{obsd} - k_{-1})$ vs. $1/[P]$ will be a straight line

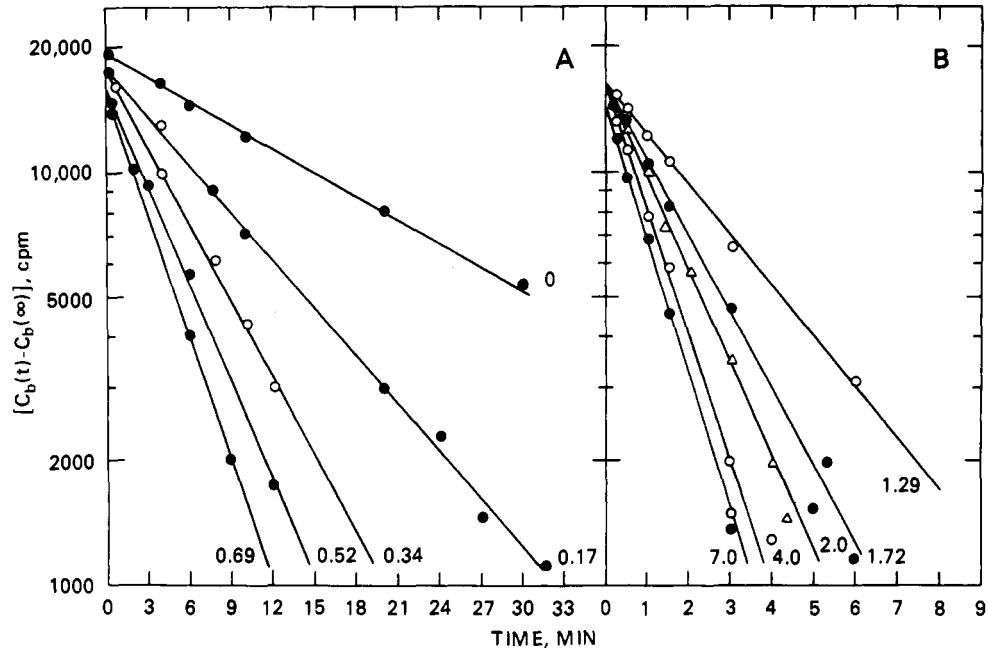


FIGURE 2: Semilogarithmic plots of the exchange of actin-bound [γ-^{32}P]ATP with nonradioactive ATP in solution as a function of time. Dowex-treated [γ-^{32}P]ATP-labeled G-actin was diluted and incubated with and without profilin for 10 min before adding nonradioactive ATP as in Figure 1. Residual bound [γ-^{32}P]ATP was measured at different times, and the data were plotted as in Figure 1. The radioactivity at $t = \infty$ was then subtracted from each time point, and the data were plotted. Each line represents a different profilin concentration between 0 and 1 mg/mL to give molar ratios of profilin/actin from 0 to 7, as indicated.

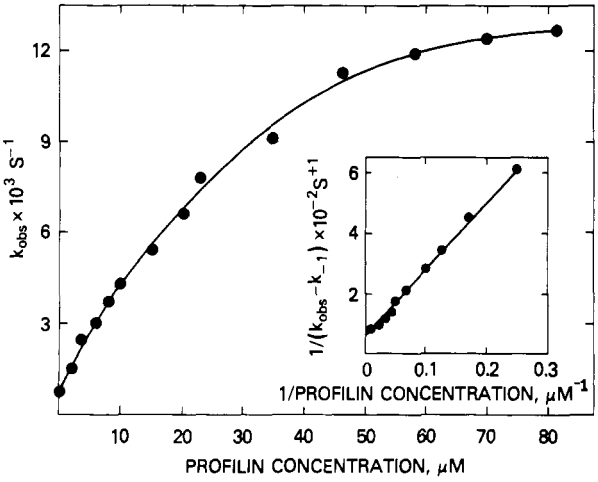


FIGURE 3: The observed ATP exchange rate as a function of profilin concentration. The rate constant for each profilin concentration was calculated from semilogarithmic plots (Figure 2) as described under Results. A double-reciprocal plot of these data, obtained by a least-squares analysis, is shown in the inset. The y-axis intercept is the reciprocal of $(k_{-2} - k_{-1})$, and the slope is $K_1/(k_{-2} - k_{-1})$; the values are 53.7 s (correlation coefficient = ±0.98) and 2.27×10^3 s·μM (correlation coefficient = ±0.98), respectively.

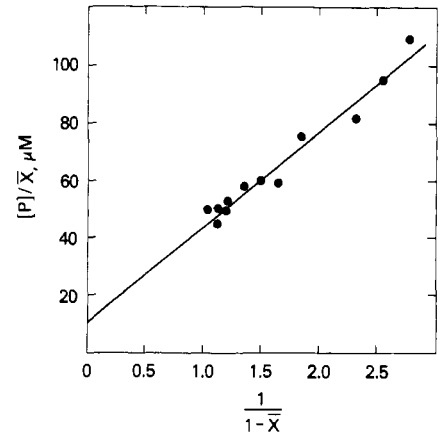


FIGURE 4: Scatchard plot analysis of the exchange rate data. [P] is the concentration of profilin and $\bar{x}$ is the fractional activity: $k_{obsd}^{P=P}/(k_{obsd}^{P=\infty} - k_{obsd}^{P=0})$. $k_{obsd}^{P=P}$ and $k^{P=0}$ were obtained from the plots in Figure 2, and $k_{obsd}^{P=\infty}$ was calculated from the y-axis intercept of the double-reciprocal plot shown in the inset of Figure 3.

whose y-axis intercept = $1/(k_{-2} - k_{-1})$ and whose slope = $K_1(k_{-2} - k_{-1})$, as shown in Figure 3, inset.

From the curve in Figure 2 for P = 0, the value for k_{-1}, the rate constant for the dissociation of ATP from G-actin·ATP, is 7.7×10^{-4} s^{-1} in 0.033 mM Ca^{2+} and 0.1 mM ATP. This value agrees reasonably well with the values obtained by Kuehl & Gergeley (1969), 4×10^{-4} s^{-1} in 0.03 mM Ca^{2+} and no ATP and 7×10^{-3} s^{-1} in 0.03 mM Ca^{2+} and 1 mM ATP, when one considers that the effect of ATP in solution is to lower the $[Ca^{2+}]$ which increases k_{-1}.

The value for k_{-2}, obtained by adding k_{-1} to the reciprocal of the y-axis intercept of Figure 3, inset, is 1.9×10^{-2} s^{-1}. The value for K_1, obtained by multiplying the slope of the curve in Figure 3, inset, by $(k_{-2} - k_{-1})$, is 4.2×10^5 M.

The fact that the experimental data fit the proposed scheme provides indirect evidence for the validity of the assumption that profilin and actin form a 1:1 complex. More direct evidence for this can be obtained, as shown by Timmons et al. (1974), by analyzing the data by a modified Scatchard plot, eq 3, where $[P_T]$ = total profilin concentration, $[G_T]$ = total actin concentration, K_D = dissociation constant, n = moles of profilin bound per mole of actin, and $\bar{x} = k_{obsd}^{P=P}/(k_{obsd}^{P=\infty} - k_{obsd}^{P=0})$ = fractional activity.

$$[P_T]/\bar{x} = n[G_T] + K_D[1/(1 - \bar{x})] \quad (3)$$

The value for $k_{obsd}^{P=\infty}$, 1.9×10^{-2} s^{-1}, was obtained from the y-axis intercept of the double-reciprocal plot in Figure 3, inset. Thus, in a plot of $[P]/\bar{x}$ vs. $1/(1 - \bar{x})$, as shown by Figure 4, K_D is the slope and nG_T is the intercept on the y axis. From the best straight line that fits the data, the slope = 33.2 μM (correlation coefficient = ±0.98) and the intercept = 11.1 μM

(correlation coefficient = ±0.98). Thus, $K_D = 3.3 \times 10^{-5}$ M and $n = 0.91$.

Because the exchange of ATP with G-actin·ATP is known to be strongly inhibited by Ca^{2+} (Kuehl & Gergeley, 1969), it was important to show that profilin did not affect the concentration of free Ca^{2+}. The concentrations of Ca^{2+} were measured in the samples containing 0, 1, 3, and 7 mol of profilin/mol of actin and were all 33 ± 2 μM. Equilibrium dialysis experiments with profilin (1 mg/mL) and $^{45}Ca^{2+}$ (0.1 mM; 9.6×10^5 cpm/mmol) in the same buffer used for the equilibrium isotope-exchange experiments showed no binding of Ca^{2+} to profilin (8800 and 9000 cpm in 100-μL aliquots from inside and outside the dialysis bag). In one set of experiments, the concentration of Ca^{2+} was raised from 33 to 150 μM, and the exchange rate was reduced, in agreement with the data of Kuehl & Gergeley (1969). For actin alone the rate was 2.4×10^{-4} s^{-1} and for profilin/actin (7:1) the rate was 5×10^{-3} s^{-1}, but the K_D and stoichiometry of binding were essentially unaffected, 3.7×10^{-5} M and 0.92 mol/mol, respectively.

In a final set of controls, it was determined that profilin neither hydrolyzed ATP, affected the rate of hydrolysis of ATP by G-actin, nor bound ATP (<0.01 mol of ATP/mol of profilin in equilibrium dialysis studies).

Discussion

The pronounced effect of *Acanthamoeba* profilin on the exchange rate of G-actin·ATP provides evidence for their interaction that is independent of the effect of profilin on actin polymerization, and analysis of the kinetic data provides additional evidence that *Acanthamoeba* profilin and actin form a 1:1 complex. Moreover, in these experiments, the binding of *Acanthamoeba* profilin to actin must have been rapid and reversible because the semilogarithmic plots of the exchange data (Figure 2) are strictly linear at concentrations of profilin well below the concentration necessary to saturate the actin. If binding were not rapid (relative to the exchange of ATP) and reversible, these plots would have been biphasic, due to a rapid exchange of ATP with the actin·ATP bound to profilin and a slower rate of exchange of ATP with the free actin·ATP.

The relatively weak binding of profilin to actin observed in these experiments may explain the difficulty in purifying a native profilin–actin complex, profilactin, from *Acanthamoeba* extracts (Reichstein & Korn, 1979) by procedures similar to those that have been used successfully to isolate profilactin from spleen (Carlsson et al., 1977) and platelets (Markey et al., 1978; Weeds & Harris, 1978). Alternatively, it is possible that the proper conditions for reconstituting stable profilactin have not yet been obtained, the profilin has been altered during its isolation, or other necessary components of the complete system have been removed.

Finally, it is apparent from the data in this paper that the actin in the reconstituted profilin–actin complex still retains 1 mol of ATP/mol of actin. Data are not available for isolated platelet profilactin, but isolated spleen profilactin apparently contains no more than 0.04 mol of ATP/mol of actin (Carlsson et al., 1977). It will be important to determine the ATP content of native profilactin in the cell and the influence of the bound ATP on the stability and properties of the complex.

Acknowledgments

We thank Dr. P. Boon Chock for his advice and assistance in the kinetic analysis of our data.

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