Kinetics of Gelsolin Interaction with Phalloidin-Stabilized F-Actin. Rate Constants for Binding and Severing[†]

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ABSTRACT: The kinetics of gelsolin interaction with actin filaments have been investigated using two fluorescent probes, tetramethylrhodamine isothiocyanate-labeled phalloidin bound to F-actin and *N*-(1-pyrenyl)iodoacetamide-labeled actin. We have also analyzed the F-actin severing by gelsolin using an assay for actin filaments which measures the polymerization rate of monomeric actin added to the gelsolin-severed filaments. Phalloidin-stabilized actin filaments were used in order to minimize the depolymerization reaction and thus simplify the kinetic analysis. Because gelsolin activity is Ca^{2+}-activated, experiments were conducted in the presence of 0.5 mM $CaCl_2$ to ensure maximal activity. We show that the interaction of gelsolin with F-actin may be separated into two distinct kinetic phases which correspond to binding and severing events. Using a two-step model of gelsolin activity, we have determined that gelsolin binds to F-actin with an association rate constant of 2×10^7 M^{-1} s^{-1}, dissociates with a rate constant in the range 0.4−1.2 s^{-1}, and subsequently severs phalloidin-stabilized F-actin with a first-order rate constant of 0.25 s^{-1}. Characterization of the binding and severing reactions will facilitate further investigation of gelsolin activity and its regulation.

Gelsolin is an actin binding protein which severs actin filaments by breaking the noncovalent bonds between actin subunits within a filament, and subsequently remains bound to the barbed end of the actin filament (Harris & Weeds, 1984). Gelsolin is composed of six repeating segments, termed S1−S6 (Kwiatkowski et al., 1986), and has been shown to have three actin binding sites which are located in the S1, S2, and S4 regions (Bryan, 1988; Weeds & Maciver, 1993; Pope et al., 1993). The S2 site has been identified as the site which binds F-actin, and the S1 site is thought to be the site primarily responsible for the severing reaction (Chaponnier et al. 1986; Yin et al., 1988; McLaughlin et al., 1993). Gelsolin is expressed as a plasma form and an intracellular form (Yin et al., 1984). Plasma gelsolin is believed to be important in clearance of actin filaments from the circulation subsequent to cellular disruption (Lind et al., 1986; Lee & Galbraith, 1992). Intracellular gelsolin is thought to affect the Ca^{2+}-regulated remodeling of the cortical cytoskeletal network (Stossel, 1994); small substoichiometric amounts of gelsolin have been shown to induce gel to sol transitions in cross-linked actin networks (Yin et al., 1979).

As a step toward understanding the machinery of cell motility, it is necessary to know the kinetics of the proteins involved (Pollard, 1992). Analysis of the kinetics of actin filament severing by gelsolin has been problematic. Techniques such as viscosity, flow birefringence, light scattering, and electron microscopy have been used to analyze the steady-state degree of actin severing by gelsolin (Yin et al., 1980; Harris & Weeds, 1983; Janmey et al., 1983), but these techniques have not been employed with time resolution adequate to describe the kinetics of severing. Pyrenyl-actin[1] has been used to indirectly determine the actin severing activity of gelsolin by monitoring the depolymerization rate of severed actin filaments (Harris & Weeds, 1983; Coué & Korn, 1985; Bryan & Coluccio, 1985). Interestingly, it had been noted that upon addition of gelsolin to pyrene-labeled F-actin, a two-phase decrease in fluorescence occurred (Harris & Weeds, 1983; Coué & Korn, 1985); however, it was not clear what kinetic event each phase represented. The data in this report demonstrate that the two phases represent gelsolin binding to F-actin and F-actin depolymerization. Furthermore, by using phalloidin to prevent depolymerization, the binding kinetics can be studied independently.

A large range of values for the rate constant for association of gelsolin to F-actin (k_+) have been published (Allen & Janmey, 1994; Ditsch & Wegner, 1994; Schoepper & Wegner, 1992); these were measured using different techniques and conditions. Schoepper and Wegner (1992) determined $k_+ = 1.5 \times 10^4$ M^{-1} s^{-1} using gelsolin labeled with NBD. Ditsch and Wegner (1994) reported a value of 3×10^6 M^{-1} s^{-1}, which was determined indirectly by analyzing the time course of actin polymerization nucleated by gelsolin. Allen and Janmey (1994) used TRITC-phalloidin bound to F-actin as a fluorescent probe to measure the severing activity of gelsolin and found k_+ to be highly dependent upon free [Ca^{2+}], reporting a maximal value for $k_+ = 1.5 \times 10^6$ M^{-1} s^{-1}. They suggested that the actual

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[1] Abbreviations: TRITC, tetramethylrhodamine isothiocyanate; pyrenyl-actin, actin labeled with *N*-(1-pyrenyl)iodoacetamide; NBD, 4-chloro-7-nitro-2,1,3-benzoxadiazole.

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rate constant for gelsolin association to F-actin approached the diffusion limit for gelsolin and that their determination for k_+ was limited by how fast the reaction could be driven in their experimental system. All of these previous studies included a rate constant for gelsolin association to F-actin in their kinetic models but no rate constant for severing, or else they assumed the rate of severing to be very rapid compared to the rate of association. Therefore, a rate constant for severing of F-actin by gelsolin has not been previously reported.

Here, we report studies of F-actin severing by gelsolin employing rapid stopped-flow measurements of two fluorescent probes, TRITC-phalloidin and pyrenyl-F-actin. Both fluorescent probes indicate that the reaction of gelsolin with phalloidin-stabilized F-actin in the presence of Ca^{2+} results in a decrease in fluorescence intensity with a second-order rate constant of $k_+ = 2 \times 10^7$ M^{-1} s^{-1}. Kinetic analysis of F-actin labeled with TRITC-phalloidin also indicates a second phase of fluorescence intensity decrease due to the severing of filaments with a rate constant of 0.25 s^{-1}. The severing rate is corroborated independently using the rate of monomeric actin add-on as a measure of the number of new actin filaments produced by gelsolin's severing activity.

MATERIALS AND METHODS

Chemicals. All reagents were analytical grade. ATP and TRITC-phalloidin were purchased from Sigma Chemical Co., phalloidin was from Boehringer, and *N*-(1-pyrenyl)iodoacetamide (pyrene) was from Molecular Probes.

Protein Preparation. Actin was extracted from rabbit skeletal muscle acetone powder (Szent-Gyorgyi, 1951) and purified by previously published procedures (Selden et al., 1986). Before use, 10 mL of ~100 μM monomeric actin was passed through a 2.5 cm × 100 cm Sephacryl S-300 gel filtration column and eluted with 5 mM HEPES, 0.2 mM ATP, 0.02 mM $CaCl_2$, and 0.01% NaN_3, pH 7.0 (G-buffer). Actin prepared by these procedures was converted to Mg-actin by a 10 min incubation in G-buffer containing 0.1 mM EGTA and 0.1 mM $MgCl_2$.

Gelsolin was purified from human plasma by the method of Kurokawa et al. (1990). Aliquots of purified gelsolin (4−8 μM) were frozen in liquid nitrogen and stored at −80 °C in 5 mM HEPES, 0.02 mM KCl, and 0.5 mM EGTA, pH 7.0. The gelsolin preparation was 95% pure by SDS−polyacrylamide gel electrophoresis.

Protein concentrations were calculated based on molar extinction coefficients of 27 400 M^{-1} cm^{-1} at 290 nm for actin and 117 230 M^{-1} cm^{-1} at 280 nm for gelsolin.

Fluorescent Labeling of Actin. Pyrenyl-actin was prepared by a modification of the method of Kouyama and Mihashi (1981). Mg-Actin, 25 μM, was polymerized by the addition of 2 mM $MgCl_2$ and 0.1 M KCl (F-buffer) at room temperature. A 3−5-fold molar excess of *N*-pyrenyliodoacetamide dissolved in dimethyformamide (~24 mg/mL) was added while vortexing to aid in dispersal, and the solution was incubated overnight in the dark. The F-actin was collected by centrifugation, and the pellet was taken up to the original volume and dialyzed overnight against 2 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.01% NaN_3. The solution was clarified by centrifugation at 170000*g* for 3 h. The resulting pyrenyl-actin was 95−100% labeled with pyrene as determined from $\epsilon^{422} = 0.022$ μM^{-1} cm^{-1} (Kouyama & Mihashi, 1981).

Severing Experiments. Mg-Actin (5−20 μM) was polymerized with 2 mM $MgCl_2$, 0.1 M KCl, and either equimolar phalloidin (pyrenyl-Mg-actin) or equimolar TRITC-phalloidin (unlabeled Mg-actin) and diluted into F-buffer to the desired concentrations. Gelsolin severing experiments were carried out by rapidly mixing phalloidin-stabilized pyrenyl-actin or TRITC-phalloidin-stabilized native actin with gelsolin either in a 1 × 1 cm cuvette with constant stirring or in a Hi-Tech Model SFA-11 stopped-flow apparatus with a dead time of approximately 20 ms. All experiments were conducted at 20 °C. The reaction buffer consisted of 0.1 M KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2 mM ATP, and 5 mM HEPES, pH 7.0. Fluorescence intensity was monitored with an SLM SPF 500 spectrofluorometer. Each TRITC-phalloidin experiment is the average of 4−6 consecutive time courses in the stopped flow. The fluorescence data were fit with a single exponential term or the sum of two exponential terms as appropriate, using Sigmaplot (Jandel Scientific).

Monomer Add-On Assay for Filament Ends. The amount of severing by gelsolin was assayed by monitoring the rate of monomeric pyrenyl-actin addition to the pointed ends of severed F-actin. At various times after addition of 50 nM gelsolin to 1 μM unlabeled F-actin (in the presence of $CaCl_2$), excess EGTA and 2 μM pyrenyl-G-actin were added, and the resultant fluorescence intensity increase was recorded. EGTA was added to inactivate gelsolin and stop the severing reaction. The rate of the resultant nucleated polymerization is proportional to the number concentration of actin filaments present and was analyzed with the equation:

$$F(t) = F_0 + \Delta F\{1 - \exp(-k_{obs}t)\}$$

where F_0 is the initial fluorescence value, ΔF is the change in fluorescence, and k_{obs} is the observed first order rate constant for polymerization. The observed rate constant, $k_{obs} = mk_p$, where m is the number concentration of actin filaments and k_p is the second-order rate constant for monomer addition onto the pointed ends of filaments (the barbed ends are gelsolin-capped); k_p was estimated as described below (Results).

KINETIC MODEL AND DATA ANALYSIS

We assumed a simple model for gelsolin severing of F-actin in which gelsolin initially binds reversibly to F-actin and then irreversibly severs the actin filament:

$$G + A \underset{k_-}{\overset{k_+}{\leftrightarrow}} GA \xrightarrow{k_{sev}} GA^{sev}$$

where A is an F-actin monomeric subunit, G is gelsolin, GA is a gelsolin−F-actin complex, and GA^{sev} is a gelsolin-capped actin filament barbed end formed by severing. The rate equations for this scheme are

$$d[A]/dt = -k_+[A][G] + k_-[GA]$$

$$d[G]/dt = -k_+[A][G] + k_-[GA]$$

$$d[GA]/dt = k_+[A][G] - (k_- + k_{sev})[GA]$$

$$d[GA^{sev}]/dt = k_{sev}[GA]$$

The overall reaction is not easily solved in the general case, but solutions exist for the pseudo-first-order approximation when either reactant is in large excess, and these solutions may be further approximated as the sum of two exponential terms when the concentration of the reactants is large enough so that $k_+[G]$ or $k_+[A] \gg k_{sev}$. Under these conditions, there would be a rapid buildup of the intermediate GA complex which decays approximately exponentially, to form the severed filaments. The fluorescence time course due to the association reaction would be

$$F(t) = F_0 - \Delta F\{1 - \exp(-k_1 t)\} \quad (1)$$

where $k_1 = k_+[G] + k_-$ and the fluorescence time course due to both the association reaction and the subsequent severing reaction would be

$$F(t) = F_0 - \Delta F_1\{1 - \exp(-k_1 t)\} - \Delta F_2\{1 - \exp(-k_2 t)\} \quad (2)$$

where F_0 is the initial fluorescence value at $t = 0$, ΔF_1 and ΔF_2 are the magnitudes of the change in fluorescence intensity for each phase, and $k_2 = k_+[G]k_{sev}/(k_+[G] + k_-)$. Under conditions where $k_+[G] \gg k_-$, $k_2 \cong k_{sev}$.

Our model implies a 1:1 stoichiometry for binding of gelsolin to F-actin which is consistent with the observation that gelsolin has one F-actin binding site located in the S2 domain (Yin et al., 1988). The stoichiometry of the gelsolin−actin complex after the initial binding by the gelsolin S2 segment is not addressed by our model; however, our analysis of the severing step is concerned with the generation of new actin filaments by severing events which occur one per each gelsolin.

It has been shown that one gelsolin binds two actin monomers (Bryan & Kurth, 1984), so that at a ratio of 1:2, this equilibrium binding stoichiometry should be obtained. Titration of F-actin with gelsolin suggests that as this 1:2 gelsolin:actin ratio is approached, the apparent rate of gelsolin interaction with F-actin slows, perhaps because of steric hindrances to gelsolin binding at adjacent F-actin monomer subunits (data not shown). The pseudo-first-order construction of our model is experimentally symmetrical so that the concentration of either one of the reactants, G or A, may be in large molar excess and thus be considered approximately constant and rate-determining. Thus, for time courses greater than about 1−2 s, we have found it simpler to interpret the TRITC-phalloidin fluorescence intensity kinetics from experiments in which the gelsolin does not saturate the actin (see Figure 4, below).

RESULTS

Phalloidin Stops Depolymerization of F-Actin Severed by Gelsolin. When gelsolin is added to 0.2 μM pyrenyl-F-actin, there is a multiphasic fluorescence intensity decrease as the F-actin is severed and subsequently depolymerizes from the pointed ends, as shown in the lower curve of Figure 1. The rate of depolymerization is proportional to the number of actin filaments and has been used as an assay for the gelsolin severing activity (Harris & Weeds, 1983). The addition of phalloidin to F-actin reduces the rate of depolymerization so much (Estes et al., 1981) that depolymerization can be neglected. The upper curve in Figure 1 shows a rapid decrease in pyrenyl-F-actin fluorescence intensity in the presence of phalloidin, while the subsequent slower phase is no longer apparent. We were able to study this initial pyrenyl-F-actin fluorescence intensity decrease using stopped-flow. The very low actin critical concentration in the presence of phalloidin allows experiments to be performed in a concentration range which results in second-order kinetics which are slow enough to be easily measured.

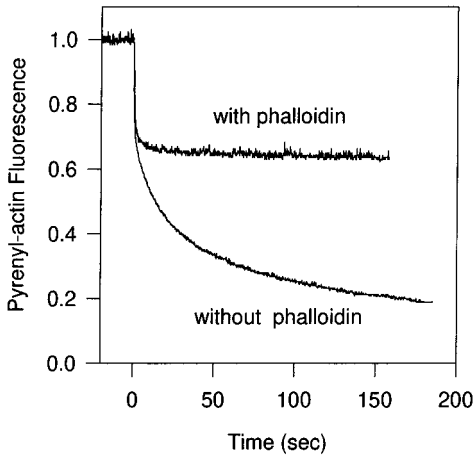

FIGURE 1: Stabilization of F-actin with phalloidin upon severing by gelsolin. 20 nM gelsolin was added to 200 nM pyrenyl-F-actin in F-buffer with 0.5 mM $CaCl_2$, in the presence or absence of 2 μM phalloidin. The curve without phalloidin shows the typical fluorescence intensity decrease which results from depolymerization of F-actin subsequent to the fluorescence intensity decrease which occurs upon binding of gelsolin.

Rapid Kinetics of Gelsolin Interaction with F-Actin Determined in Stopped-Flow Experiments. The initial time courses of the pyrenyl-actin and TRITC-phalloidin data are well fit by a single exponential. Figure 2A shows representative stopped-flow fluorescence data in which 20 nM, 100 nM, or 500 nM gelsolin was added to phalloidin-stabilized pyrenyl-F-actin. Figure 2B shows similar data using F-actin with bound TRITC-phalloidin to which 20 nM, 100 nM, or 400 nM gelsolin was added. Each TRITC-phalloidin data curve is an average of 4−6 consecutive acquisitions using the stopped-flow cell.

The Observed Rate of Interaction between Gelsolin and F-Actin Increases Linearly with Increasing Gelsolin Concentration. Figure 3 shows values for the observed rate constants determined from data such as shown in Figure 2, plotted as a function of gelsolin concentration. The upper panel shows values for pyrenyl-actin. The slope of the plot yields a value for the second-order association rate constant, $k_+ = 1.8 \times 10^7$ M^{-1} s^{-1} (SE = $\pm 0.1 \times 10^7$ M^{-1} s^{-1}), and the intercept yields a value for $k_- = 0.41$ s^{-1} (SE = ± 0.38 s^{-1}). The lower panel shows values for TRITC-phalloidin data with $k_+ = 2.1 \times 10^7$ M^{-1} s^{-1} (SE = $\pm 0.2 \times 10^7$ M^{-1} s^{-1}) and $k_- = 1.2$ s^{-1} (SE = ± 0.42 s^{-1}). The value for the equilibrium constant for gelsolin binding to F-actin can be calculated from these kinetic constants; $K_d = 23$ and 60 nM as determined from pyrenyl-actin and TRITC-phalloidin-labeled actin, respectively.

Separation of the TRITC-Phalloidin Fluorescence Intensity Decreases Due to Binding and Subsequent Severing. Our model for gelsolin−actin interaction uses the simplifying condition that one of the reactants is in large molar excess over the other so that the reaction is pseudo-first-order. The

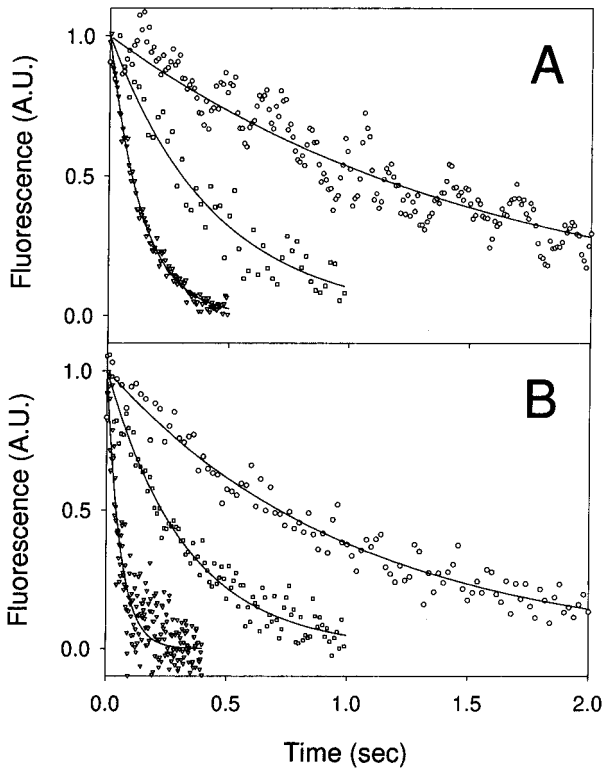

FIGURE 2: Rapid interaction of gelsolin and F-actin. Various concentrations of gelsolin were added to F-actin in F-buffer with 0.5 mM $CaCl_2$, using a stopped-flow apparatus as described under Materials and Methods. Panel A: Pyrenyl-F-actin with 2 μM phalloidin. Concentrations are 10 nM F-actin, 20 nM gelsolin, ○; 20 nM F-actin, 100 nM gelsolin, □; and 100 nM F-actin, 500 nM gelsolin, ▽. Panel B: 10 nM F-actin with equimolar bound TRITC-phalloidin. Concentrations of gelsolin are 20 nM, ○; 100 nM, □; and 400 nM, ▽. The data are normalized.

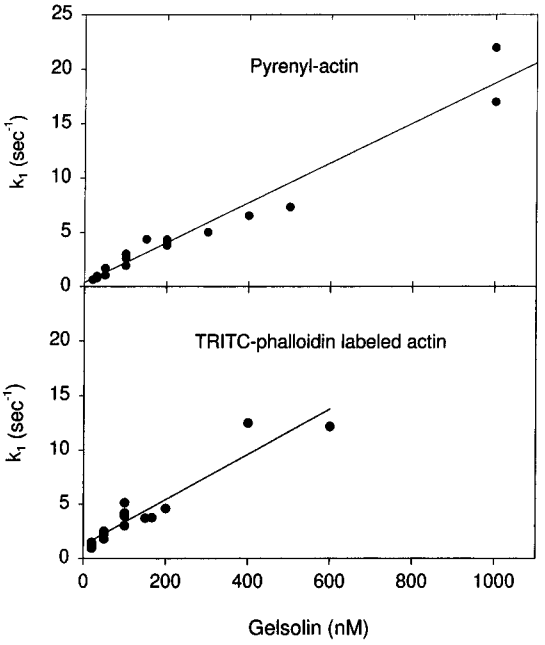

FIGURE 3: Observed rate constant for the initial interaction of gelsolin with F-actin. Gelsolin, in molar excess of the F-actin concentration, was mixed with F-actin in F-buffer plus 0.5 mM $CaCl_2$. Data were acquired as in Figure 2, and values for k_1 (from eq 1) were determined from fits to the time courses. The upper panel shows data from experiments using pyrenyl-F-actin; the slope of the line gives a value for $k_+ = 1.8 \times 10^7$ M^{-1} s^{-1} (SE = $\pm 0.1 \times 10^7$ M^{-1} s^{-1}), and the intercept yields a value for $k_- = 0.41$ s^{-1} (SE = ± 0.38 s^{-1}). The lower panel shows data from experiments using actin F-actin with bound TRITC-phalloidin with $k_+ = 2.1 \times 10^7$ M^{-1} s^{-1} (SE = $\pm 0.20 \times 10^7$ M^{-1} s^{-1}) and $k_- = 1.2$ s^{-1} (SE = ± 0.42 s^{-1}).

experiments described above have used an excess of gelsolin over the F-actin concentration; however, our model is symmetrical, and we find that the k_+ can be determined using F-actin in molar excess. Figure 4 illustrates the biphasic nature of the fluorescence signal which results from gelsolin interaction with TRITC-phalloidin-labeled F-actin. In these experiments, the gelsolin:F-actin monomer unit ratio is 1:10 for each F-actin concentration, and the fluorescence values are normalized. Under these conditions, the observed rate constant for the first kinetic phase is $k_1 = k_+[A] + k_-$, and that for the second phase is $k_2 = k_+[A]k_{sev}/(k_+[A] + k_-)$. In each panel, the solid lines are fits to the data using eq 2. The dashed lines are calculated using the differential equations and values for the rate constants $k_+ = 2 \times 10^{-7}$ M^{-1} s^{-1}, $k_{sev} = 0.27$ s^{-1}, and either $k_- = 0$ (dashed−dotted line), $k_- = 0.5$ s^{-1} (dashed line), or $k_- = 1$ s^{-1} (dashed−double-dotted line).

Figure 5 shows the values for k_1 and k_2 from eq 2 determined from fits to the time courses of TRITC-phalloidin fluorescence intensity decrease for a number of experiments using the conditions of Figure 4. The values determined for k_1 are plotted in the upper panel as a function of F-actin concentration. From the slope and intercept of the line, respectively, $k_+ = 1.7 \times 10^7$ M^{-1} s^{-1} (SE = 0.25×10^7 M^{-1} s^{-1}) and $k_- = 1.0$ s^{-1} (SE = 0.67 s^{-1}). This k_+ value is in good agreement with that determined from the gelsolin concentration dependence of the gelsolin−actin interaction rate (Figure 3). The values determined for k_2 from the fit to eq 2 are plotted in the lower panel. The line is a fit to $k_2 = k_+[A]k_{sev}/(k_+[A] + k_-)$ using the above values for k_+ and k_- and a value determined for $k_{sev} = 0.27$ s^{-1} (SE = 0.076 s^{-1}).

The Severing Rate Determined by Fluorescence Is Supported by an Independent Assay of the Increase in the Number of Actin Filaments with Time. Gelsolin was incubated with unlabeled, phalloidin-stabilized F-actin for various times; then monomeric pyrenyl-actin was added, and the resultant pyrenyl-actin fluorescence intensity increase was recorded. The inset of Figure 6 shows sample polymerization time courses. The observed polymerization rate constant, k_{obs}, is plotted in Figure 6 (circles) as a function of incubation time, and the line represents a fit to the data by a single exponential with a rate constant $k_{sev} = 0.23$ s^{-1} (SE = ± 0.05 s^{-1}). This value is in good agreement with $k_{sev} = 0.27$ s^{-1} determined from TRITC-phalloidin fluorescence experiments (Figure 5). At the end point for the severing reaction, the number of actin filaments (m) is equal to the concentration of gelsolin, 50 nM, and the value for $k_{obs} = 0.26$ s^{-1}. The value for the rate constant for monomer addition to the pointed end of the actin filaments (k_p) can be calculated: $k_p = k_{obs}/m = 5 \times 10^6$ M^{-1} s^{-1}. This is in reasonable agreement with the value 2.2×10^6 M^{-1} s^{-1} reported by Pollard and Mooseker (1981) under similar ionic conditions.

DISCUSSION

While values for a rate constant for gelsolin association with F-actin have been previously reported (Allen & Janmey, 1994; Ditsch & Wegner, 1994), neither a dissociation rate

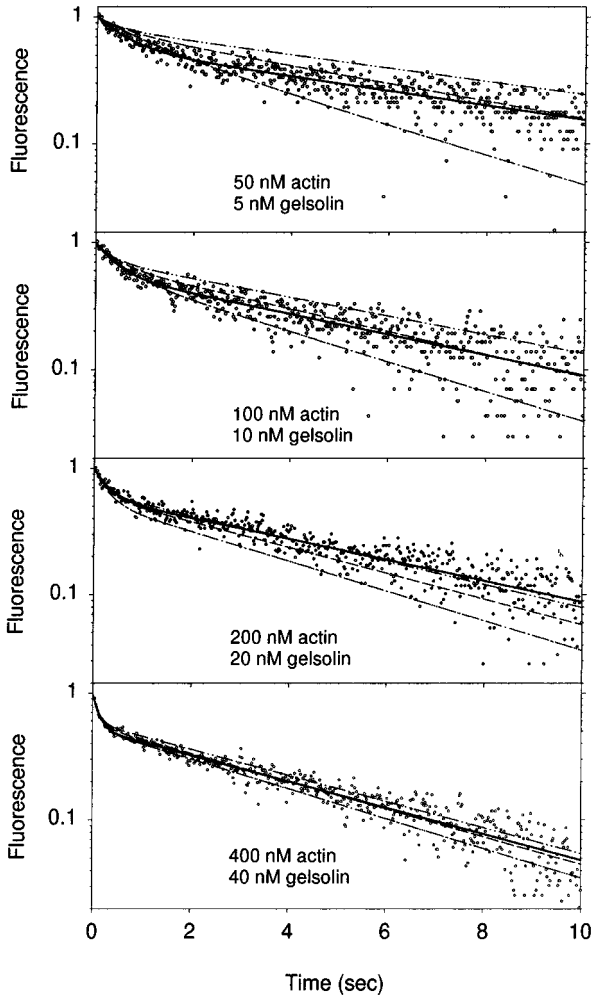

FIGURE 4: Time course of TRITC-phalloidin fluorescence intensity decrease due to gelsolin binding and severing activity. Gelsolin was added to F-actin in F-buffer plus 0.5 mM $CaCl_2$. The gelsolin and F-actin concentrations are listed in each panel. The data were fit using eq 2 (solid lines), normalized to the extent of fluorescence change and plotted here in a semi-log format. The dashed lines are calculated using the differential equations and the following values for the rate constants: $k_+ = 2 \times 10^7$ M^{-1} s^{-1}, $k_{sev} = 0.27$ s^{-1}, and either $k_- = 0$ (dashed−dotted line), $k_- = 0.5$ s^{-1} (dashed line), or $k_- = 1$ s^{-1} (dashed−double-dotted line).

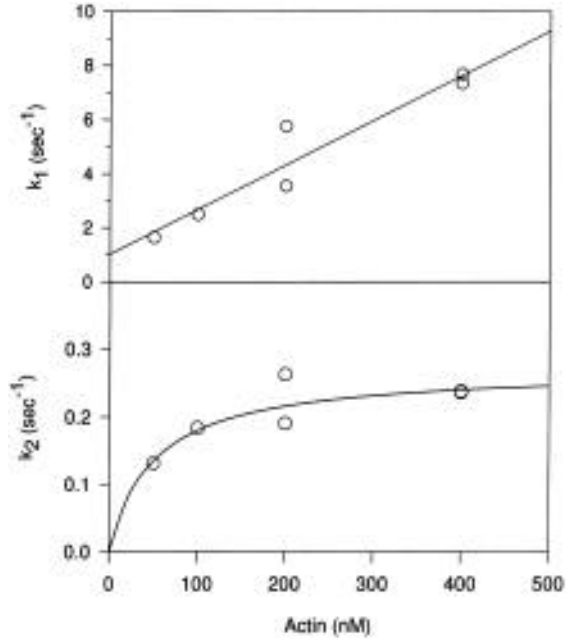

FIGURE 5: Observed rate constants for gelsolin binding and severing activity. The values determined from data fits using eq 2 are plotted as a function of F-actin concentration. The values for k_1 are shown in the upper panel. The slope and intercept of the line give values for $k_+ = 1.7 \times 10^7$ M^{-1} s^{-1} (SE $= 0.25 \times 10^7$ M^{-1} s^{-1}) and $k_- =$ 1.0 s^{-1} (SE $=$ 0.67 s^{-1}). The lower panel shows the values determined for k_2. The line is a fit to $k_2 = k_+[A]k_{sev}/(k_+[A] + k_-)$ and yields a value for $k_{sev} = 0.27$ s^{-1} (SE $= 0.076$ s^{-1}).

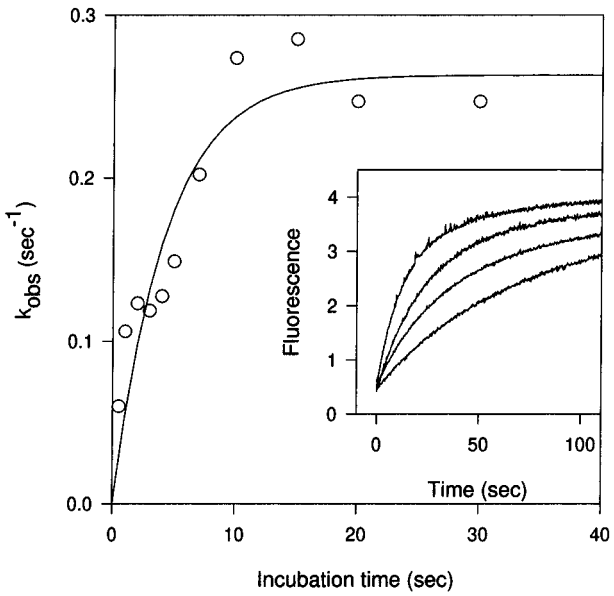

FIGURE 6: Rate of actin monomer addition to actin filaments severed by gelsolin as a function of incubation time. As described under Material and Methods, the observed rate constant for actin polymerization, k_{obs}, was plotted as a function of the time of F-actin incubation with gelsolin. The values for k_{obs} (circles) are fit with a single exponential with a rate constant for severing $k_{sev} = 0.23$ s^{-1} (SE $= \pm 0.05$ s^{-1}). The inset shows typical data curves of the pyrenyl-actin fluorescence intensity increase resulting from nucleated polymerization onto F-actin preincubated with gelsolin for 0.5, 1, 5, and 10 s (from bottom to top, respectively).

constant nor a rate constant for F-actin severing by gelsolin has been previously published. It has been proposed that the mechanism by which gelsolin severs F-actin includes multiple steps. Initially, the gelsolin S2 domain binds to F-actin, reducing its translational entropy followed by binding of its S1 and S4 domains at other sites on the actin filament to then enable the severing reaction (Way et al., 1989). The severing step described by the rate constant for severing, k_{sev}, considered in our kinetic model may include molecular steps such as the positioning of the gelsolin S1 and S4 domains, destabilization of actin−actin bonds within the filament, and conformational shifts which ultimately result in the breaking of the actin−actin bonds and high-affinity binding of gelsolin to the nascent filament end.

This study has determined a value for the association rate constant for gelsolin with F-actin, $k_+ = 2 \times 10^7$ M^{-1} s^{-1}, which is about an order of magnitude higher than previously reported values. The higher value for k_+ reported here is probably due to our use of the stopped-flow technique which allows rapid measurements of the reaction rate of gelsolin binding to F-actin with bound TRITC-phalloidin in a protein concentration range where the association reaction is faster than the severing reaction. Our use of phalloidin-stabilized pyrenyl-actin allows measurements of the time course of gelsolin binding with minimal interference from fluorescence intensity changes due to actin depolymerization or severing. It may be that the k_+ values determined previously by others

(Allen & Janmey, 1994; Ditsch & Wegner, 1994) resulted from observed rates which were partially due to binding and partially due to severing by gelsolin. Ditsch and Wegner (1994) indirectly determined a value for k_+ from modeling the actin polymerization time course in the presence of gelsolin. Their model assumed that severing of actin by gelsolin was instantaneous; thus, the rate of severing was only limited by the rate of the association reaction. Allen and Janmey (1994) reported a [Ca^{2+}] dependence of k_+ and suggested that if the reaction could be driven fast enough, the binding and severing reactions could be separated, although they were not able to achieve this in their experimental system. Our value for k_+ was determined in the presence of 0.5 mM $CaCl_2$ (free [Ca^{2+}] > 300 μM), which, in agreement with Allen and Janmey (1994), we found to give a maximal rate of gelsolin binding to F-actin (data not shown).

The observation that upon reaction with gelsolin, the fluorescence intensity decrease of F-actin-bound TRITC-phalloidin contains two kinetic phases (Figure 4) suggested that the reaction of gelsolin with F-actin was not as simple as had been previously described. This led to the finding that when the concentration of either one of the reactants is greater than approximately 0.1 μM, the binding and severing events could be separated kinetically. The results of the monomer add-on experiment in Figure 6 correlate well with the determinations of k_{sev} using TRITC-phalloidin, and both indicate that severing of phalloidin-stabilized actin is a first-order process with $k_{sev} \cong 0.25$ s^{-1}. This independent assay for gelsolin severing activity justifies the interpretation of the TRITC-phalloidin fluorescence intensity change as being due to severing as well as binding. The value we report for k_{sev} has been determined for F-actin which has phalloidin or TRITC-phalloidin bound; however, it has been reported that phalloidin binding to F-actin does not significantly reduce the rate of severing by gelsolin (Allen & Janmey, 1994).

The values we determined for k_- (Figures 3 and 5) vary in the range 0.4−1.2 s^{-1}. The k_- value determined from pyrenyl-actin appears smaller than those determined from TRITC-phalloidin data. This could possibly result from the characteristics of the probes; pyrene is covalently bound to the actin whereas the TRITC-phalloidin bound through noncovalent interactions and dissociates from the actin after gelsolin binding. In order to illustrate the effect of various values for k_- on the calculated time courses of gelsolin binding and severing, Figure 4 also shows the time courses using values for $k_- = 0$, $k_- = 0.5$ s^{-1}, and $k_- = 1$ s^{-1}. This effect appears most pronounced at protein concentrations near the range of the equilibrium dissociation constant for gelsolin binding to the side of the actin filament, $K_d \approx 25-60$ nM calculated from the above data. We have attempted, without success, to measure k_- directly by using EGTA to rapidly lower the free calcium concentration in a sample of gelsolin bound to pyrenyl-F-actin; a rebound increase in fluorescence would suggest dissociation of bound gelsolin before it had a chance to sever. We observed no such fluorescence increase, suggesting that gelsolin may remain bound to the actin filament after chelation of Ca^{2+}. This is similar to the situation which occurs when EGTA is added to the GA_2 complex formed from gelsolin and two actin monomers; one actin monomer remains bound to gelsolin after chelation of Ca^{2+} (Bryan & Kurth, 1984). It may be that a rapid conformation change upon binding of gelsolin S2 to F-actin leads to a high-affinity complex which precedes the severing event. Further work will be needed to clarify this aspect of gelsolin binding and severing kinetics.

The equilibrium constant for fragmentation of an actin filament has been reported to be 0.4 pM (Kinosian et al., 1993). The binding of gelsolin to the barbed end of the filament may be stronger than this, since the free energy of the final state of severing in which gelsolin caps the barbed end of the actin filament must be lower than the free energy of the actin−actin bonds which were disrupted. This implies an effectively irreversible reaction relative to the time frame of severing. The equilibrium constant for capping of the barbed end of the actin filament by the gelsolin−actin complex was reported to be less than 10 pM (Selve & Wegner, 1986).

In conclusion, we have used the fluorescence intensity of both pyrenyl-actin and TRITC-phalloidin to monitor the interaction between gelsolin and phalloidin-stabilized actin and have determined the number of actin filaments by a polymerization assay (Figure 6). The data support a model of gelsolin severing activity with an association rate constant $k_+ = 2 \times 10^7$ M^{-1} s^{-1}, a dissociation rate constant $k_- = 0.4-1.2$ s^{-1}, and severing rate constant $k_{sev} = 0.25$ s^{-1}. The degree of reversibility of the initial rapid binding of gelsolin to actin is still somewhat unclear, and it is possible that a rapid conformational change stabilizes gelsolin binding and leads irrevocably to severing. Establishment of these basic parameters for our simple model of F-actin severing by gelsolin will provide a basis for further studies of gelsolin's severing activity, and, in particular, for analysis of the regulation of the binding and severing phases by calcium and by phosphatidylinositol phosphates [reviewed in Stossel (1994)].

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