Actin Filament Annealing in the Presence of ATP and Phalloidin†

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ABSTRACT: The re-formation of actin filaments after fragmentation by sonication in the presence of phalloidin and ATP has been found to follow second-order kinetics. The data are described by a model in which the rate of actin filament annealing is proportional to the square of the number concentration of actin filaments and the rate of fragmentation is proportional to the actin polymer concentration. In the presence of 100 mM KCl, 1 mM $MgCl_2$, and equimolar phalloidin, the second-order rate constant for annealing of actin filaments is 2.2×10^6 M^{-1} s^{-1} and the first-order rate constant for fragmentation is 7×10^{-7} s^{-1}. In addition, the observed pseudo-first-order rate constant for annealing was found to increase with increasing ionic strength. Thus, annealing may play a major part in the length redistribution phase of actin polymerization and may be important for actin filament rearrangement in the cell.

Monomeric actin can polymerize to form a two-stranded helical polymer, approaching several micrometers in length. The rate constants for polymerization and depolymerization of actin filaments by addition and removal of monomers at filament ends have been well-characterized under a variety of conditions [for review, see Pollard and Cooper (1986)]. However, while there is evidence to indicate that actin polymers can anneal, except for one report by Murphy *et al.* (1988), there is little quantitative data about the rate constants for this process. Moreover, it has been reported that annealing does not always follow the expected kinetics of a second-order process (Kawamura & Maruyama, 1970; Murphy *et al.*, 1988), and it has also been reported that annealing of actin filaments may be quantitatively unimportant under certain conditions (Carlier *et al.*, 1984).

Nakaoka and Kasai (1969) determined that the initial rate of increase of viscosity following sonication of actin polymer was proportional to the square of the actin polymer concentration, and they concluded that annealing was the predominant mechanism during the recovery from sonication. Using electron microscopy, Kawamura and Maruyama (1970) measured the length redistribution of actin filaments after sonication. They found that filaments annealed with second-order kinetics for the initial stage of recovery, but after 2 min, the rate became very slow. More recently, Murphy *et al.* (1988) reported very similar results, in which annealing appeared to be a two-phase process: for the first few seconds, annealing was a second-order process with a rate constant of approximately 10^7 M^{-1} s^{-1}, but this fast phase was followed by a much slower phase which appeared to be a zero-order process. Oosawa and Maruyama (1987) reported that phalloidin-treated actin filaments anneal after sonication, based on measurements of filament number concentration by the method of Pantaloni *et al.* (1984). Although several laboratories (Murphy *et al.*, 1988; Oosawa & Maruyama, 1987; Rickard & Sheterline, 1988; Kawamura & Maruyama, 1970) have interpreted the mechanism of length redistribution which follows fragmentation as being due primarily to annealing, another group (Carlier *et al.*, 1984) has described the recovery of actin filaments after fragmentation, in the absence of ATP, as a diffusion-like random walk mechanism in which monomeric actin dissociation and association events are responsible for the observed kinetics. The relative contribution of either of the mechanisms mentioned above to the recovery of actin filaments after fragmentation is apparently dependent on the solution conditions, especially the presence of ATP.

In the present report, we have used phalloidin to minimize the contribution of monomeric events to the rearrangement of actin filaments. Phalloidin stabilizes actin filaments and reduces the actin critical concentration (c_c) by decreasing the dissociation rate constant (k_-) of actin monomers from filaments (Estes *et al.*, 1981). This allows an assessment of the process of end-to-end filament annealing with a minimum of actin monomer reactions, which might otherwise alter the length distribution of the actin filaments. While it may be argued that phalloidin might artificially alter the annealing mechanism, Burlacu *et al.* (1992) showed that phalloidin does not affect the flexibility or length distribution of actin filaments. Thus, data obtained in the presence of phalloidin offer an opportunity for quantitative determinations of the annealing process which are difficult to obtain otherwise.

Our laboratory has analyzed the decrease in the number concentration of actin polymers subsequent to fragmentation by sonication. So that annealing rates would be in a range amenable to our measurement technique, and to minimize complications due to filament entanglements, we chose to work at low actin concentrations with initial filament lengths less than 0.1 μm. This allowed us to test a reversible second-order model for annealing over a wide range of concentrations, something which has not been previously reported. In the presence of phalloidin, 100 mM KCl, and 1 mM $MgCl_2$, we have determined values for a second-order constant for annealing, k_a, of 2.2×10^6 M^{-1} s^{-1} and a first-order constant for fragmentation, k_f, of 7×10^{-7} s^{-1}. We also determined that the annealing rate constant is significantly dependent on ionic strength in a manner analogous to the situation for monomer addition to a filament end.

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MATERIALS AND METHODS

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

Actin Preparation. Actin was prepared from rabbit skeletal muscle acetone powder (Szent-Gyorgyi, 1951) which was purified as described by Selden *et al.* (1986) except that the eluant for the final gel-filtration chromatography step contained 5 mM HEPES, 0.2 mM ATP, 0.02 mM $CaCl_2$, and 1.5 mM NaN_3, pH 7.0 (G buffer).

Fluorescence. Actin polymerization was followed by measuring the fluorescence intensity increase of actin labeled with *N*-(1-pyrenyl)iodoacetamide (pyrene–actin), approximately 10% labeled, prepared as previously described (Selden *et al.*, 1986; Gershman *et al.*, 1989). Measurements were made with an SLM SPF 500-C spectrofluorometer using excitation at 365 nm and emission at 386 nm.

Experimental Procedures. Actin prepared as described above contains 1 tightly bound Ca^{2+} per actin monomer (Ca-actin). Ca-actin was converted to Mg-actin by a 10-min incubation in G buffer plus 50 μM $MgCl_2$ and 50 μM EGTA at pH 7.5 (ME buffer).

Actin filaments were prepared from Mg-actin (10–20 μM) which was polymerized in the presence of equimolar phalloidin in ME buffer plus 1 mM $MgCl_2$ and 100 mM KCl or 1 mM $MgCl_2$ alone. This Mg-actin was diluted to the desired concentrations with ME buffer plus the desired salt concentrations prior to sonication.

Aliquots of polymeric actin were sonicated with a Heat Systems Ultrasonics Inc. sonifier cell disrupter manufactured by Branson Sonic Power Co. The polymer was sonicated for approximately 5 s/mL of solution, which was long enough to reach a fragmentation equilibrium as evidenced by a consistent polymer length obtained for varying volumes and concentrations of polymer.

The number concentration of filaments was determined by adding a small aliquot of the actin polymer solution to an aliquot of monomeric Mg-actin (approximately 10% pyrene–actin) along with 1 mM $MgCl_2$ and 100 mM KCl (final concentrations) and evaluating the apparent rate constant of polymerization (mk_+) from the curve fit to the fluorescence intensity increase. A single-exponential equation was used to fit the fluorescence intensity time course data, where the apparent rate constant is equal to mk_+, the product of the number concentration of actin filaments, m, and the rate constant for elongation, k_+ (Gershman et *al.*, 1989). The end point steady-state fluorescence value was measured and used as a fixed parameter in the curve fit. The value for m was then calculated from mk_+ (Kinosian *et al.*, 1991) by assuming that the rate constant of polymerization (k_+) is 10^7 M^{-1} s^{-1} for Mg-ATP-actin under these conditions [see Pollard (1986) for a summary of reported rate constants].

ANALYSIS

Our analysis is derived from a model of end-to-end annealing of actin filaments in which the rate of fragmentation is proportional to the total polymer concentration and the rate of annealing is proportional to the square of the number concentration of filaments. The rate equation for this process is

$$\frac{dm}{dt} = Ak_f - m^2k_a \qquad (1)$$

where m is the number concentration of polymers, A is the actin polymer concentration (of monomer subunits), k_a is the rate constant for annealing, and k_f is the rate constant for spontaneous fragmentation in the absence of (*i.e., after*) sonication. According to Hill (1983), there are theoretical reasons for k_a and k_f to vary with polymer length. However, this would make the model unmanageably complicated. Thus, in accord with previous investigators [e.g., Wegner and Savko (1982)] we assume here that k_a and k_f are independent of polymer length. Integration of eq 1 yields

$$m(t) = m_\infty + \frac{2m_\infty}{\left(\frac{m_0 + m_\infty}{m_0 - m_\infty}\right)e^{2kt} - 1} \qquad (2)$$

where $k = (Ak_fk_a)^{1/2}$, the observed pseudo-first-order rate constant for annealing; m_∞ is the number concentration of actin filaments at equilibrium, $m_\infty = (Ak_f/k_a)^{1/2}$; m_0 is the number concentration of actin filaments at $t = 0$, which can be expressed as $m_0 = A/L_0$, where L_0 is the initial filament length expressed as the average number of monomers per filament at $t = 0$. We have used eq 2 to determine values for k, m_0, and m_∞ from the time course of actin filament annealing at various actin concentrations. Values for k_f and k_a were then determined from the actin polymer concentration dependence of k and m_∞. Alternatively, k_f, k_a, and L_0 were used directly as fit parameters in eq 2 by substituting appropriate expressions for m_∞ and k; values for k_f, k_a, and L_0 were determined from fits to annealing time courses and averaged to determine a single set of parameters which describe the annealing time courses for a wide range of actin concentrations.

The experimentally determined variable $m(t)$ which is fit in the analysis is calculated as described under Materials and Methods using an assumed value for the polymerization rate constant, k_+. Thus, $m(t)$ is inversely proportional to the value of k_+. A change in the assumed value for k_+ would result in a proportional change in the fit parameter k_a and an inversely proportional change in k_f. Note that since the observed rate constant for annealing, k, depends on the product, k_ak_f, it is independent of k_+.

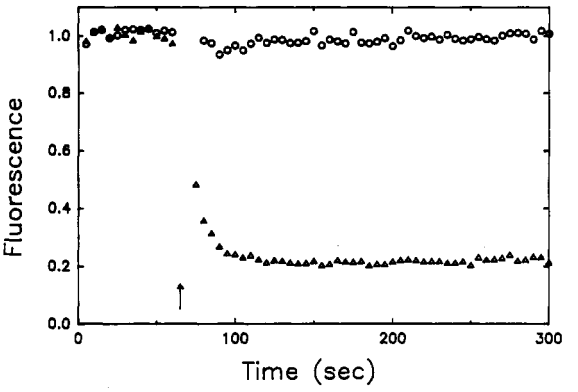

FIGURE 1: Actin polymer stabilization by phalloidin. Polymeric actin with (circles) or without (triangles) phalloidin was diluted to 0.1 μM in 1 mM $MgCl_2$ and 100 mM KCl. Actin was sonicated for 15 s at the time indicated by the arrow. The control sample shows a rapid loss of fluorescence intensity indicative of depolymerization while the phalloidin-stabilized sample remains polymerized.

RESULTS

Figure 1 shows that phalloidin-stabilized actin filaments do not depolymerize when subjected to sonication. The symbols represent the relative fluorescence intensity of 10% pyrene–actin which has been diluted to 0.1 μM in 100 mM KCl and 1 mM $MgCl_2$. The circles represent actin stabilized with equimolar phalloidin, and the triangles represent actin

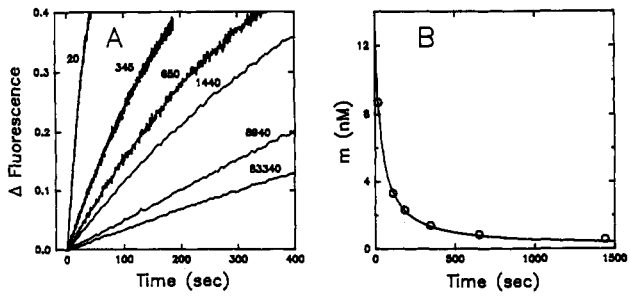

FIGURE 2: Time course of phalloidin-stabilized actin filament recovery after fragmentation by sonication. Panel A illustrates the assay for the number concentration of actin filaments, which was determined by the rate of addition of monomeric pyrene–actin onto the phalloidin-stabilized filaments. The rate of fluorescence intensity increase of the pyrene–actin is proportional to the number concentration of filaments present. The fluorescence curves are labeled with the time, in seconds, after sonication of the filaments was stopped. Panel B shows the number concentration of filaments determined from such an assay versus the time after sonication of the filaments was stopped (circles) and the fit by eq 2 (line).

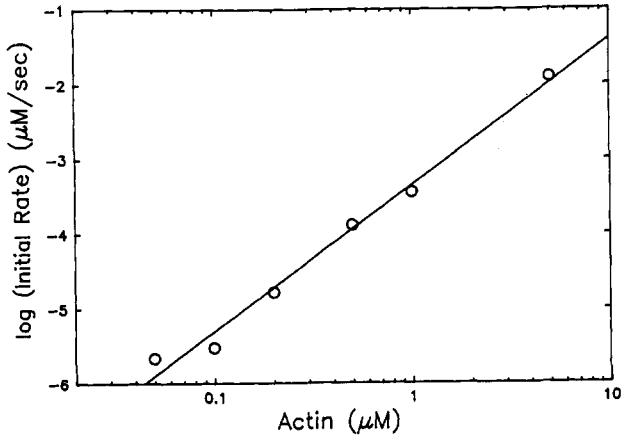

FIGURE 3: Initial rate of actin filament annealing as a function of polymer concentration. The initial rates were determined from curve fits to individual annealing time courses using eq 2. The slope of the log–log plot is 1.95, which indicates that the initial rate varies with the square of the actin concentration.

without phalloidin. Sonication was applied to the samples for 15 s, beginning at the time indicated by the arrow. The phalloidin-stabilized actin showed no decrease in fluorescence intensity, indicating no loss of polymer, but the control shows a rapid loss of polymer after application of sonication.

Changes in the relative number of filaments with time were measured using a polymerization assay as shown in Figure 2. In this experiment, the polymer solution was sonicated to equilibrium as described under Materials and Methods. At measured times after cessation of sonication, aliquots of the sonicated polymer solution were added to a 2 μM Mg-ATP-actin solution along with 100 mM KCl and 1 mM $MgCl_2$. Figure 2A shows an example of the data from such an experiment; the time after sonication was stopped (in seconds) is indicated for each assay curve. The rate of nucleated actin polymerization is proportional to the number concentration of the actin filaments present. The values for the rates of actin polymerization, mk_+, were determined from computer fits to the fluorescence intensity data and were used to calculate the number concentration of filaments, m, assuming $k_+ = 10^7$ M^{-1} s^{-1} (see Materials and Methods). The values determined for m were plotted as a function of time (after cessation of sonication) in Figure 2B (symbols), and the data were fit by eq 2 (solid line).

Figure 3 illustrates the initial rate of actin filament annealing subsequent to sonication for a series of actin concentrations.

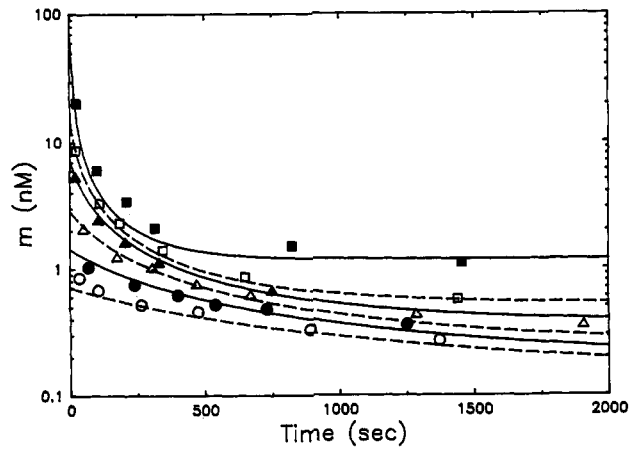

FIGURE 4: Time course of actin filament annealing subsequent to sonication at several F-actin concentrations. The number concentration of actin filaments, m, was assayed at various times subsequent to fragmentation by sonication (see Materials and Methods) using various concentrations of F-actin in the presence of equimolar phalloidin, 100 mM KCl, and 1 mM $MgCl_2$. The actin concentrations used are, from bottom to top, 0.05, 0.1, 0.2, 0.5, 1.0, and 5.0 μM. Symbols represent data points and lines represent values calculated using eq 2 and the following values: $k_f = 7.0 \times 10^{-7}$ s^{-1}, $k_a = 2.2 \times 10^6$ M^{-1} s^{-1}, and $L_0 = 65$ subunits/filament.

Because the actin filament assay employed in these experiments does not allow for direct evaluation of the initial phase of annealing, the initial rate of annealing for each actin concentration was determined from the fit of eq 2 to the annealing time course. The log–log plot of the initial rate of annealing versus actin concentration has a slope of 1.95, which indicates a second-order dependence on actin polymer concentration, as is expected for annealing.

Further evaluation of time courses of annealing allowed us to fit the annealing data using one set of parameters for a range of actin concentrations. Figure 4 shows the time courses of filament annealing after fragmentation by sonication for a 100-fold concentration range of F-actin (0.05–5 μM). The fit to the data set was calculated using eq 2 and the values for $L_0 = 65$, $k_f = 7.0 \times 10^{-7}$ s^{-1}, and $k_a = 2.2 \times 10^6$ M^{-1} s^{-1}. It can be seen that these constants provide a description of the annealing process for the range of actin concentrations shown.

Values for the initial number concentration of filaments, m_0, were determined from curve fits to annealing time courses in the presence of 100 mM KCl and 1 mM $MgCl_2$ (circles) or 1 mM $MgCl_2$ alone (triangles) and are plotted in Figure 5. The number concentration of actin filaments at $t = 0$ (when sonication was stopped) should be proportional to the concentration of polymer if the sample has reached an equilibrium during sonication. This was shown to be the case for DNA (Freifelder & Davison, 1962), and was confirmed to be the case for actin by Carlier et al. (1984, 1985). Figure 5 shows that the value of m_0 varies linearly with F-actin concentration. In Figure 5, the slope of the line fit to the data is equal to $1/L_0$, with $L_0 = 65$ monomers/filament.

Figure 6 shows m_∞ values determined from curve fits to annealing time courses (circles) and also m_∞ assayed from samples allowed to incubate overnight (triangles). At equilibrium, $m_\infty = (Ak_f/k_a)^{1/2}$; thus, a plot of m_∞ versus $A^{1/2}$ is linear with the slope equal to $(k_f/k_a)^{1/2}$. The value for $k_f/k_a = 3 \times 10^{-13}$ M; this represents an equilibrium dissociation constant for filament fragmentation and is in good agreement with the theoretical value of 10^{-13} M calculated by Erickson (1989). The data depicted in Figure 6 suggest that annealing is the predominant mechanism for length redistribution of actin filaments under these conditions in which monomeric

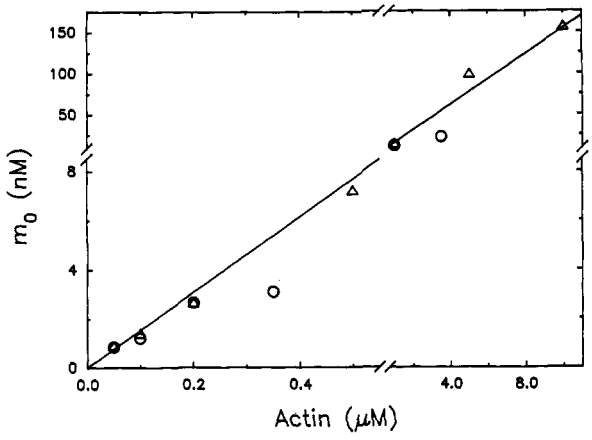

FIGURE 5: Initial number concentrations of actin filaments as a function of actin concentration. Actin with equimolar phalloidin, in 5 mM HEPES, pH 7.0, 0.2 mM ATP, and either 1 mM $MgCl_2$ (triangles) or 100 mM KCl and 1 mM $MgCl_2$ (circles), was sonicated and the filament number concentration was assayed. The values for m_0 were determined from extrapolation of the time course data to $t = 0$ using eq 2. The data are fit by a linear function which indicates that the average size of the actin filaments is 65 monomeric subunits.

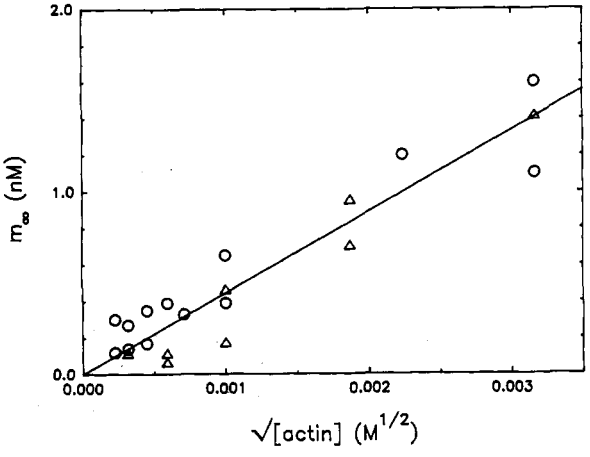

FIGURE 6: Equilibrium number concentration of filaments as a function of the square root of the F-actin concentration. Actin with equimolar phalloidin in 5 mM HEPES, pH 7.0, 0.2 mM ATP, 100 mM KCl, and 1 mM $MgCl_2$ was sonicated and the filament number concentration was assayed. The values for m_∞ were determined from extrapolation of the time course data to $t = \infty$ using eq 2 (circles), or the samples were allowed to incubate overnight and then assayed (triangles). The linear slope is equal to $(k_f/k_a)^{1/2}$; here the value of k_f/k_a is 3×10^{-13} M, the equilibrium dissociation constant for filament fragmentation.

events at filament ends are minimized by the action of phalloidin.

The rate of annealing was found to be dependent upon ionic strength. Figure 7 depicts the values determined for k_a in 1 mM $MgCl_2$ and varying KCl concentrations. Experiments were conducted with the same procedures as those shown in Figure 2. The time courses of filament annealing were fit using eq 2. The initial filament number concentration, m_0, was found to be independent of ionic strength (see Figure 5), and thus the increase in annealing rate with ionic strength must reflect an increase in the rate constant for annealing, k_a. Figure 7 shows that k_a is roughly proportional to the log of the ionic strength; this is somewhat similar to the salt dependence of the actin polymerization rate constant (Selden *et al.*, 1986). With somewhat lower confidence, it appeared that k_f increased in a similar manner with ionic strength so that m_∞ was relatively independent of salt concentration.

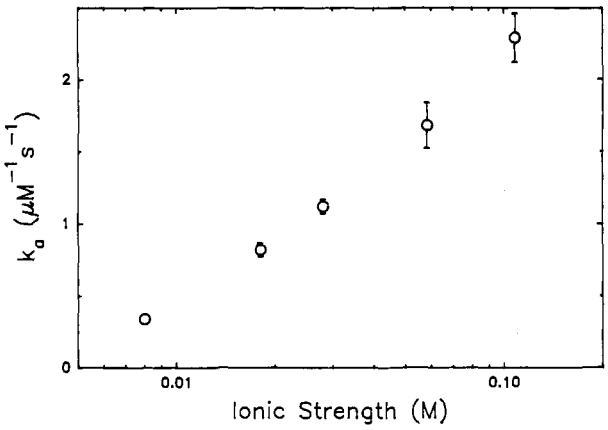

FIGURE 7: Effect of ionic strength on the rate of actin filament annealing. The values determined for k_a for 0.1 μM actin in the presence of equimolar phalloidin, 5 mM HEPES, pH 7.0, 0.2 mM ATP, 1 mM $MgCl_2$, and various KCl concentrations is shown as a function of ionic strength. The concentrations of KCl added to the experiments shown in the figure are 0, 10, 20, 50, and 100 mM. The ionic strengths were calculated using a computer program and published dissociation constants and included the HEPES, ATP, and $MgCl_2$ as well as the added KCl. The symbols represent the values determined from fits to time course data by eq 2, and error bars represent 1 standard deviation.

DISCUSSION

This study shows that in the presence of phalloidin, 0.1 M KCl, and 1 mM $MgCl_2$, actin filaments rearrange subsequent to fragmentation by sonication with a second-order rate constant for annealing, $k_a = 2 \times 10^6$ M^{-1} s^{-1}, and a first-order rate constant for fragmentation, $k_f = 7 \times 10^{-7}$ s^{-1}. This k_a value is in reasonable agreement with the second-order rate constant estimated by Murphy *et al.* (1988), $k_a = 10^7$ M^{-1} s^{-1}. In 1 mM $MgCl_2$ (no KCl), $k_a = 3 \times 10^5$ M^{-1} s^{-1}. This value for k_a is orders of magnitude larger than the maximum value for k_a estimated by Carlier *et al.* (1988), $k_a = 3$ M^{-1} s^{-1}. The equilibrium dissociation constant for filament fragmentation reported here, $K_F = k_f/k_a = 0.4$ pM, is in reasonable agreement with values calculated from average filament length data measured by optical microscopy ($K_F = 0.5$–2 pM; Burlacu *et al.*, 1992) and the theoretical value calculated by Erickson (1989) of 0.1 pM. These studies of the fragmentation and annealing of phalloidin-stabilized actin filaments clearly demonstrate that filament annealing is an important process in establishing the equilibrium length distribution for actin filaments.

Phalloidin blocks depolymerization events, markedly decreasing the rate constants for dissociation of actin monomers from filaments (Estes *et al.*, 1981). The actin critical concentration, c_c ($= k_-/k_+$), is thus very low in the presence of phalloidin even at low ionic strength or in the absence of ATP. It has been shown that the c_c of ATP-actin increases during sonication and approaches that for ADP-actin (Pantaloni *et al.*, 1984), but the c_c of ATP-actin in the presence of phalloidin is shown here to be unchanged by sonication (Figure 1). Thus, with phalloidin present, the quantity of polymer in solution can be considered to be approximately equal to the total actin concentration, even during prolonged sonication. Our results show that phalloidin-stabilized actin polymer at a concentration as low as 0.05 μM is stable under sonication, even at low ionic strength. A recent report (Burlacu *et al.*, 1992) has shown that the use of phalloidin in solutions of F-actin does not alter the length distribution or average length of actin filaments. This suggests that phalloidin does not interfere with the annealing/fragmentation equilibrium

and that monomer events may be of lesser importance in actin filament length redistribution.

Our model assumes that k_a and k_f are independent of polymer length. While there are theoretical arguments for length dependence of k_a and k_f (Hill, 1983), the good fit of the model to the data set suggests that these effects of polymer length on k_a and k_f may not be large over the range covered in our experiments. Entanglements at long polymer lengths would be expected to cause major changes in k_a and k_f (as we discuss in the following paragraph). To assess effects of filament length on k_a and k_f in the semidilute regions (i.e., in the absence of entanglements) would probably require rapid assay techniques applied to samples with shorter L_0, or perhaps use of different physical techniques, such as quasi-elastic light scattering.

As actin polymers anneal, their length increases with each annealing event and, given an adequate total concentration, can reach lengths at which they are no longer free to diffuse in solution without interacting with each other by overlap or entanglement (Sawyer *et al.*, 1988; Newman *et al.*, 1989a,b). Measurements from fluorescence photobleaching recovery experiments (Pan & Ware, 1988; Wang *et al.*, 1989) indicate that D for actin polymer (at high concentrations) is 100–1000-fold lower than for actin monomer. This suggests that at these concentrations there is an interaction among actin filaments that would substantially decrease the rate of annealing as filaments become long. These diffusion limitations discussed above have been pointed out by others (Murphy *et al.*, 1988; Sawyer *et al.*, 1988). We have generally designed our experiments to use low concentrations of actin polymer—concentrations in the semidilute region where polymer entanglement is not likely. An exception to this may be observed in the 5 μM actin sample of Figure 4, where the data suggest a deviation from second-order kinetics; perhaps in this sample the polymers become long enough to entangle and thus slow the annealing reaction. The lower concentration samples of Figure 4 did not show significant deviation from second-order kinetics throughout the time course, suggesting that overlap and entanglements did not occur. The experiments reported by Murphy *et al.* (1988) were performed at 0.6–4.8 μM actin concentration, at the border of the semidilute region, and this may explain the mixture of first- and second-order kinetics they observed. The experiments of Carlier *et al.* (1984) extended to actin polymer concentrations further above the semidilute region, and this may well have affected the kinetics they observed.

It is difficult to reconcile the results reported here with the analysis of ADP-actin filament length redistribution reported by Carlier *et al.* (1984). The experiments they described were on Mg-actin polymer in the presence of 1 mM $MgCl_2$ and 0.2 mM ADP, with a monomer concentration (c_c) of 8 μM; these conditions are clearly much different than for the experiments reported here, and the high monomer concentration suggests that monomer-cycling events may have predominated, as their analysis concluded. The time course data in their study (Carlier *et al.*, 1984, Figure 3) is very similar to our data (our Figure 4). However, we found that the initial rate of decrease in polymer number concentration is clearly second order (our Figure 3), whereas the equivalent data from their study (Carlier *et al.*, 1984, Figure 4) appears first order. An important difference between the studies is that their data covered an actin polymer concentration range of only 1–8 μM, whereas our experiments covered an actin polymer concentration range of 0.05–5 μM. Perhaps if the data of Carlier *et al.* (1984) had covered a similar wide range of actin polymer concentration, the second-order dependence would have been observed. The "diffusion-like random walk mechanism of (filament) length redistribution" proposed by Carlier *et al.* (1984) will not fit our data. Furthermore, the random walk mechanism does not correctly describe the approach to equilibrium, whereas the model proposed here does [$m_\infty = (Ak_f/k_a)^{1/2}$], and its predictions have been experimentally confirmed (Figure 6). The results presented here clearly show that annealing is a major mechanism of actin polymer rearrangement, particularly when there is a high concentration of short polymers present. This may be relevant to the *in vivo* situation in cells actively undergoing cytoskeletal rearrangements, where filament annealing probably plays a significant role.

The data reported here suggest that actin filament annealing is energetically very favorable under ionic conditions similar to those of the intracellular milieu. It is possible that annealing of actin filaments may be important *in vivo* during cell motile events that require the rearrangement of filament networks. In a system in which addition of monomers to actin filament ends might be unlikely (due perhaps to a very low free actin concentration or other regulatory mechanisms), rapid annealing of small actin polymers may well be a plausible mechanism for filament network rearrangement.

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