The Bundling of Actin with Polyethylene Glycol 8000 in the Presence and Absence of Gelsolin

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ABSTRACT Actin filament and bundle formation occur in the cytosol under conditions of very high total macromolecular concentration. In this study we have utilized the inert molecule polyethylene glycol 8000 (PEG) as a means of simulating crowded conditions in vitro. Column-purified Ca-actin was polymerized in the absence and presence of gelsolin (to regulate mean filament lengths between 50 and 5000 mers) and PEG (2–8%) using various concentrations of KCl and/or 2 mM divalent cations. Bundling was characterized by the scattered light intensity and mean diffusion coefficients obtained from dynamic light scattering, as well as by fluorescence and phase-contrast microscopy. The minimum concentration of KCl required for bundling decreases both with increasing concentration of PEG at a fixed mean filament length, and with decreasing filament length at a fixed concentration of PEG. In the absence of divalent cation, bundling is reversible on dilution, as determined by intensity levels, diffusion coefficients, and microscopy. However, with either 2 mM Mg^{2+} or Ca^{2+} added, bundling is irreversible under conditions of higher PEG concentrations or longer filaments, indicating that osmotic pressure effects cannot fully explain actin bundling with PEG. Weaker divalent cation-binding sites on actin as well as disulfide bonds appear to be involved in the irreversible bundling.

INTRODUCTION

The role of actin in cell motility and in the maintenance of cellular morphology relies, to a great extent, on the formation and dissociation of ordered structures composed of bundled actin filaments. Within the peripheral cytoplasm, actin is dynamically regulated between monomeric, polymeric, and bundled structures in association with a multitude of specific actin-binding proteins. In vitro experiments with purified actin have shown the formation of aligned domains and long-range order at modest concentrations of actin (Newman et al., 1989a; Kerst et al., 1990; Suzuki et al., 1989; Coppin and Leavis, 1992; Furukawa et al., 1993; Kas et al., 1996). It is important, therefore, to have a detailed understanding of the self-association interactions between purified actin filaments in solution as a mechanism for generating both structure and force in vivo, even in the absence of actin-binding proteins.

A number of investigations have shown that by increasing the concentration of inert (noninteracting) macromolecules in solution (macromolecular crowding or increased osmotic pressure) it is possible to initiate the formation of parallel bundles of actin filaments (Tellam et al., 1983; Suzuki et al., 1989; Cuneo et al., 1992; Grazi et al., 1992). High concentrations of inert macromolecules simulate macromolecular crowding found in cells and serve to increase the local osmotic pressure. The concentration of protein present in cells is sufficiently high that F-actin should normally be in the form of bundles while in the absence of actin-binding proteins (Suzuki et al., 1989). Unfortunately, the mechanisms by which cells can regulate the conversions between F-actin and bundled actin are still not completely understood. It has been demonstrated that increasing the concentration of macromolecules in solution favors bundle formation (Suzuki et al., 1989) and that this transition is regulated by accessory cytoskeletal proteins (Cuneo et al., 1992). It was suggested that local gradients of such ancillary proteins are responsible for the bundling or debundling of F-actin in certain regions of the cell (Cuneo et al., 1992).

Other osmotic pressure effects on actin filaments have also been identified. An early study showed that the association rate constant of actin monomers to an actin filament increased in the presence of inert macromolecular crowders (Drenckhahn and Pollard, 1986). Studies on the effects of osmotic stress on volume flow rates through actin solutions in the absence and presence of actin-associated proteins were interpreted in terms of anisotropic and heterogeneous structures formed (Ito et al., 1987, 1992). By studying the packing of actin filaments in bundles, it was shown that the actin filament diameter decreases in response to increased osmotic pressure (Schwienbacher et al., 1995). These studies point to profound effects on actin filaments that can occur from macromolecular crowding, the natural state of existence in a cell.

A number of theoretical studies have discussed the effects of macromolecular crowding on various aspects of the structure and function of proteins (Minton, 1992; Minton et al., 1992; Zimmerman and Minton, 1993; Han and Herzfeld, 1993). Herzfeld has published a series of three papers concerned with crowding-induced organization of cytoskeletal elements (Madden and Herzfeld, 1993, 1994; Kulp and Herzfeld, 1995). The theme of these papers is that actin bundling under crowded conditions occurs spontaneously, is entropically driven, and is aided, but not caused, by actin-binding cross-linkers. This work suggests that native

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bundling of actin filaments in a cell does not fundamentally require actin-binding proteins; these may serve to modulate the bundling by stabilizing or destabilizing bundles or by preventing their formation.

In this study we confirm previously published work that has shown that the addition of PEG acts as a macromolecular crowder, forcing filamentous actin into bundled structures. Dynamic and static light scattering as well as fluorescence and phase contrast microscopy are used to characterize the bundling transition. These studies are extended to include the bundling characteristics of length-regulated filaments using gelsolin, a barbed-end capping and severing protein. In the absence of divalent cation, we find that reversible bundling is produced by a threshold level of KCl that is lowered by increasing PEG concentrations or by decreasing the mean filament length. With the addition of 2 mM Ca^{2+} or Mg^{2+} the bundling becomes irreversible under conditions of higher concentrations of PEG or longer filaments. This implies that osmotic pressure effects cannot fully explain PEG-induced actin bundling. We have examined a number of variables in an attempt to understand the irreversibility observed with divalent cations, including the tightly bound divalent cation, possible interactin disulfide bridges, and filament flexibility. Our results lead us to suggest the involvement of the weaker divalent binding sites on actin.

MATERIALS AND METHODS

Materials

Actin was extracted from rabbit muscle acetone powder and column purified as previously described (Newman et al., 1985, 1989a,b), resulting in G-actin with Ca^{2+} bound to the high-affinity site (Ca–G-actin). Mg–G-actin was prepared by adding 50 μM Mg^{2+}/EGTA to a solution of Ca–G-actin in G-buffer and incubating for 5 min at room temperature. G-buffer was 0.2 mM ATP, 0.02 mM $CaCl_2$, 0.1% NaN_3, and 5 mM HEPES, pH 7.0, as previously described (Newman et al., 1989b). F-buffer was freshly prepared and contained G-buffer as well as the same concentrations of KCl and divalent cations as in the sample to be diluted. Gelsolin was purified from human plasma as previously described (Kurakowa et al., 1990). Polyethylene glycol (PEG) with molecular weight 8000 was purchased from Sigma Chemical Company and used without further purification. A stock solution of PEG-8000 (200 g/liter) was prepared in G-buffer.

Actin polymerization as measured by dynamic light scattering

Samples of G-actin were added to stock PEG so that the final concentrations of PEG were 0, 2, 4, 6, 7, or 8% (w/w) PEG. Length-regulated actin solutions were prepared by the addition of 200 μM $CaCl_2$ and gelsolin to monomeric actin before dilution to 10 μM with G-buffer. All samples were filtered through 80-nm-pore ultrafilters directly into previously cleaned optical cuvettes. Polymerization was initiated by the introduction of concentrations of KCl (75–150 mM) determined to be below the threshold for bundling for the PEG concentration used. The cuvette was then inverted gently to ensure complete mixing.

Dynamic light scattering experiments were performed at room temperature using a previously described apparatus (Newman et al., 1985, 1989a,b) at a scattering angle of 90° and light of 514.5-nm wavelength. Data containing the average scattered light intensity (normalized to incident laser intensity) as well as the mean diffusion coefficient were obtained contemporaneously. Typical experimental durations of 15–30 s resulted in intensities and diffusion coefficient values with uncertainties of 1–2%, smaller than the symbols in all figures. The time courses of polymerization and/or bundling were followed with repeated experiments until a steady state was reached, typically between 30 and 120 min, depending on the conditions. We measured the viscosities of solvents at room temperature with various PEG concentrations in three different ways. Direct measurements with a Cannon viscometer or a Rheometrics RFS II rheometer agreed with indirect determinations from dynamic light scattering within a few percent. These latter measurements were made by measuring the diffusion coefficient of monodisperse polystyrene latex spheres in the various solvents and using the Stokes-Einstein relation

$$D = \frac{kT}{6\pi\eta R}$$

Viscosity corrections were made to the measured actin diffusion coefficients in different PEG solutions.

PEG-induced bundle formation as measured by dynamic light scattering

Bundle formation was initiated in several ways: 1) through the addition of KCl, with or without divalent cation (2 mM Ca^{2+} or Mg^{2+}), exceeding the bundling threshold to a G-actin/PEG solution; 2) through the addition of a small increment of KCl and/or divalent cation to an F-actin/PEG solution polymerized with a subbundling KCL concentration; or 3) through the addition of PEG to a cuvette containing F-actin. In all cases the cuvettes were gently inverted several times after additions to ensure complete mixing. All samples contained a final actin concentration of 10 μM. Dynamic light scattering was performed as described above, although the cuvette was placed in a piezoelectrically driven mount, allowing us to slowly scan the entire height of the sample. Because of the inhomogeneous nature of samples containing bundles, all such data were taken while the cuvette was scanned, to obtain sample averages (San Biagio et al., 1991).

Fluorescence and phase contrast microscopy

Freshly prepared samples or samples from previous light scattering measurements were stored at 4°C and observed not more than 24 h after polymerization. For the fluorescence microscopy, F-actin samples were visualized using rhodamine-labeled phalloidin. Observation was performed with a Nikon Diaphot inverted fluorescent microscope.

RESULTS

PEG-induced bundle formation as measured by dynamic light scattering

For most of these measurements, actin polymerization was initiated by the addition of subbundling concentrations of KCl, and changes in the light scattering intensity and mean diffusion coefficient were monitored at a 90° scattering angle until a steady state was reached. At steady state, a solution of 10 μM actin consists of a network of entangled polydisperse filaments. Mean diffusion coefficients do not correspond to independently diffusing F-actin. Upon further addition of KCl, the transition from actin filaments to bundles was accompanied by a 10–30-fold increase in the average scattered light intensity as well as a 10–30-fold decrease in the mean diffusion coefficient. A typical time

course of scattered intensity and mean diffusion coefficient data is shown in Fig. 1.

To study the threshold for bundling in some detail, titrations with KCl (25 mM increments) were performed with solutions of F-actin in 2, 4, 6, 7, or 8% (w/w) PEG and varying molar ratios of actin to gelsolin (from 50:1 to 5000:1 and in the absence of gelsolin). In Fig. 2 the KCl threshold for bundling, determined by gross changes in scattered light intensity and diffusion coefficients, is plotted as a function of PEG concentration for different amounts of added gelsolin. Bundle formation occurs at lower KCl concentrations when the concentration of PEG is increased, reaching a plateau at 7% PEG. At 2% (w/w) PEG it was not possible to form bundles even at 400 mM KCl. Fig. 2 also shows that bundle formation is induced at lower salt concentrations for actin filaments with a shorter mean length. The shortest filaments required the least amount of salt to induce filament bundling, whereas filament bundling in the absence of gelsolin required the highest concentrations. We were unable to observe a time course for bundling under most conditions, as bundling was complete within the 30 s or so required for the addition and gentle inversion of the cuvette several times to ensure mixing of the added salt. For shorter filaments (50 mers and 500 mers) we were sometimes able to observe a time course for bundling, as shown in Fig. 3. In some experiments (not shown) PEG and sufficient salt to produce bundling were added directly to G-actin, immediately mixed by cuvette inversion, and left undisturbed. In those experiments we generally observed a short phase (<30 min) of slow growth in scattered intensity consistent with polymerization, followed by a more rapid intensity increase due to filament bundling. The two phases of intensity increase were not separable.

Bundles formed in the absence of divalent cation were completely reversible on a 1:1 dilution in F-buffer (with the KCl concentration kept constant). This 1:1 dilution decreases the PEG concentration (as well as the actin concentration) to a point where bundles do not form from polymer. A typical example of such data for both scattered intensity and mean diffusion coefficients is shown in Fig. 4. The reversibility of bundling was determined by 1) a decrease in the average scattered light intensity to a stable value slightly below that of 10 μM F-actin, 2) an increase in mean diffusion coefficients to a stable value slightly above that of 10 μM F-actin, and 3) phase-contrast microscopy, to confirm the absence of bundles. The differences in final values from the initial steady-state values are due to the dilution of the actin filaments themselves on 1:1 dilution in F-buffer.

Irreversible bundle formation

Irreversible bundling was induced in the presence of divalent cations (2 mM Mg^{2+} or Ca^{2+}) under conditions of higher PEG concentrations (7–8%) at all mean filament lengths or in the absence of gelsolin at PEG concentrations of 4–8%. Reversible bundling was observed for length-regulated samples at PEG concentrations below 7% in the presence of divalent

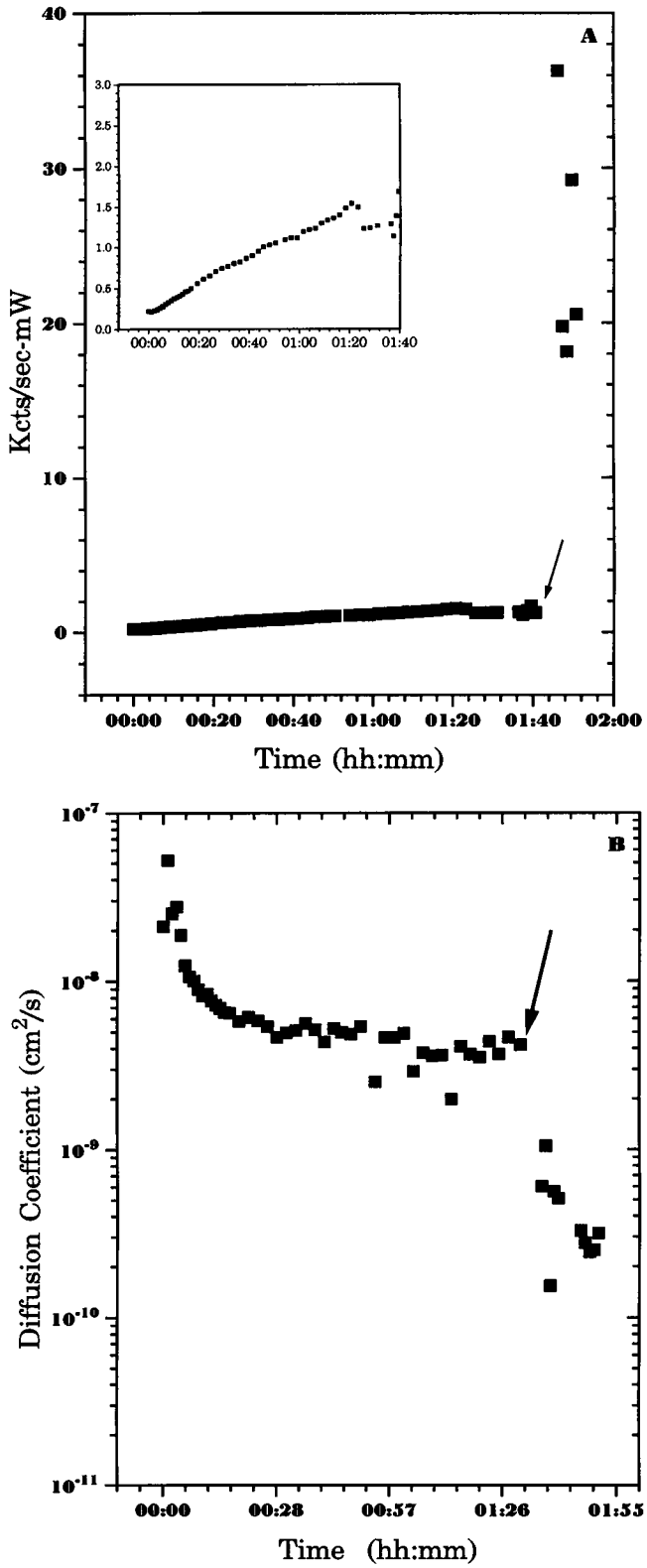


FIGURE 1 Monomeric Ca-actin (10 μM) was polymerized with 100 mM KCl in the presence of 8% PEG. (*A*) Normalized scattered intensity as a function of time after the addition of salt. The inset shows the initial polymerization in more detail. (*B*) Mean diffusion coefficients as a function of time, with these data taken contemporaneously. Note the log scale for diffusion coefficients. The arrow in both figures indicates the time at which the [KCl] was increased to 150 mM.

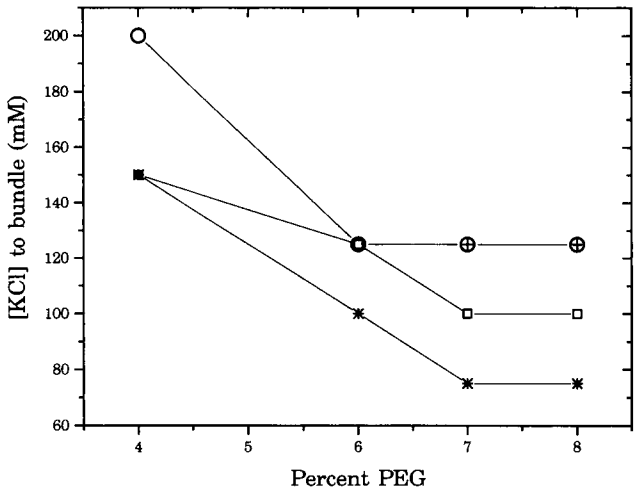


FIGURE 2 Monomeric Ca-actin (10 μM) was polymerized with KCl in the presence of varying molar ratios of gelsolin. The amount of salt needed to bundle the actin filaments is shown as a function of percentage PEG (w/w) for different actin:gelsolin ratios (O, no gelsolin; + 5000:1; □, 500:1; *, 50:1). At a [PEG] of 2% no bundling was observed at ionic conditions of up to 400 mM KCl. The lines are drawn to guide the eye.

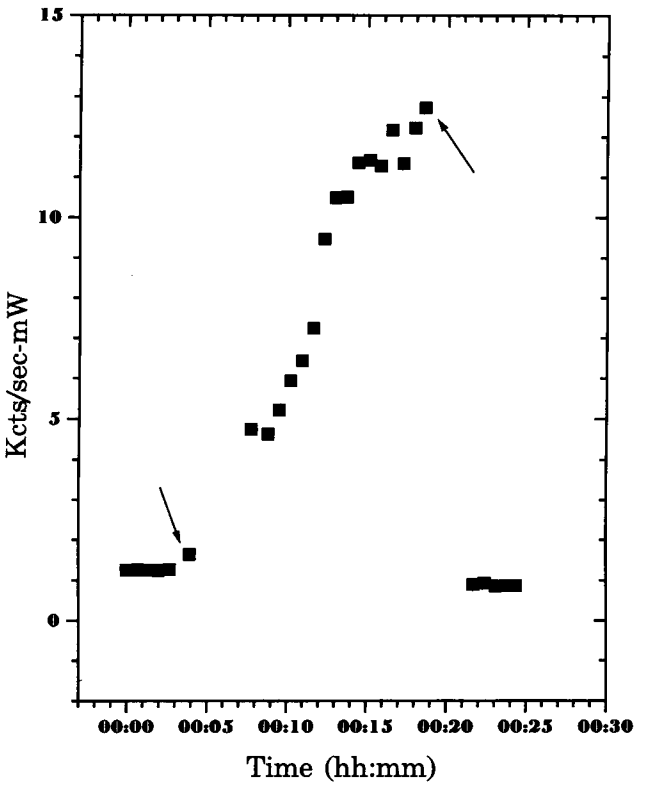


FIGURE 3 Normalized scattered light intensity from a 10 μM Ca-actin sample in 8% PEG with gelsolin added to an actin:gelsolin ratio of 50:1, as a function of time. At time 0 polymerization is initiated with 75 mM KCl, below the bundling threshold at these conditions. The first arrow indicates the addition of 25 mM KCl, and the bundling time course is then monitored. At the second arrow the sample is diluted 1:1 in F-buffer containing 100 mM KCl, diluting the PEG to nonbundling conditions.

cations. We formed bundles with divalent cations using either of two protocols: 1) the addition of 2 mM Mg^{2+} or Ca^{2+} along with KCl directly to a solution containing G-actin, or 2) the addition of 2 mM Mg^{2+} or Ca^{2+} to a cuvette containing F-actin at steady state. A typical experiment using protocol 2 is shown in Fig. 5. Titrations with KCl showed that, using either protocol, bundles formed at lower concentrations of KCl (typically by about 50 mM) than in the absence of divalent cation. Irreversibility was characterized by a scattered light intensity substantially higher than at 10 μM F-actin as well as a mean diffusion coefficient substantially lower than 10 μM F-actin after a 1:1, or in many cases even a 2:1, dilution in F-buffer (in which the KCl and divalent cation concentrations were kept constant). It is important to note that the 1:1 dilutions resulted in PEG concentrations that would not have induced bundle formation of the original actin solution, even at the original actin concentration. Routinely, solutions of actin bundles were monitored continuously for 1 h to check for reversibility and, in certain cases, were observed the next day.

A majority of the divalent cation data were recorded using Mg^{2+} to induce bundling. For a variety of conditions, we checked that similar results were also obtained when we used Ca^{2+}. Fig. 6 illustrates average intensity data as well as mean diffusion coefficients for Ca^{2+}-induced bundles, which were shown to be partially reversible upon 1:1 dilution in F-buffer. Although the extent of irreversibility varied, we found no significant differences between Mg^{2+}- or Ca^{2+}-induced bundles.

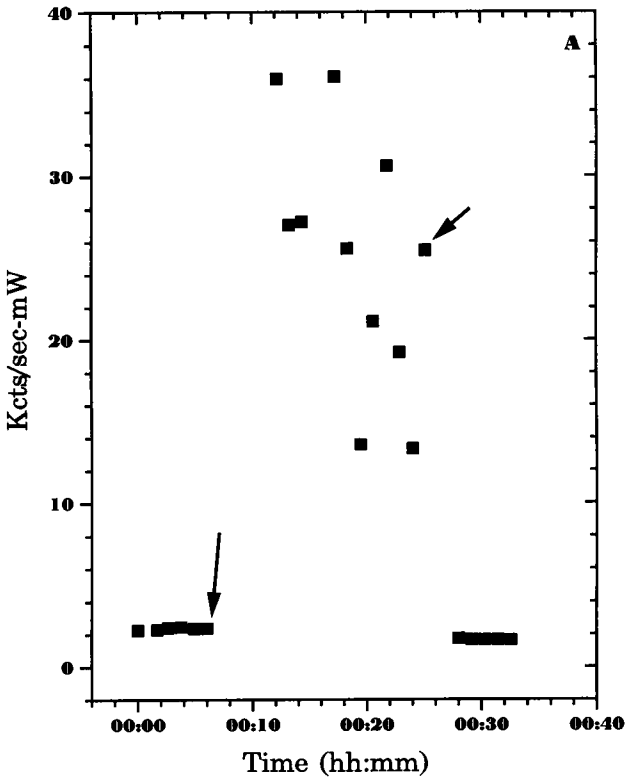
Phase-contrast microscopy

Bundled actin filaments were detectable by phase-contrast microscopy, which we used to confirm the presence or absence of bundles under various conditions. The lengths of the bundles were found to decrease with decreasing mean filament length, although it should be noted that the lengths of the bundles were much longer than the mean filament length. Fig. 7 shows an example of a phase-contrast view of actin bundles formed using a relatively high concentration of gelsolin.

DISCUSSION

A knowledge of the mechanism whereby microfilaments are converted into, and dissociate from, bundles is essential to our understanding of cell motility. It has previously been demonstrated that F-actin can be induced to form ordered bundled structures in the presence of elevated concentrations of small macromolecules in solution, a condition that simulates crowded conditions in the cytoplasm (Suzuki et al., 1989). Crowded conditions may be induced through the use of the protein ovalbumin or the inert molecule PEG (Tellam et al., 1983; Suzuki et al., 1989).

Our PEG studies support these findings. Mapping out the "phase space" for bundling of gelsolin length-regulated filaments, we have shown that bundles appear to form more



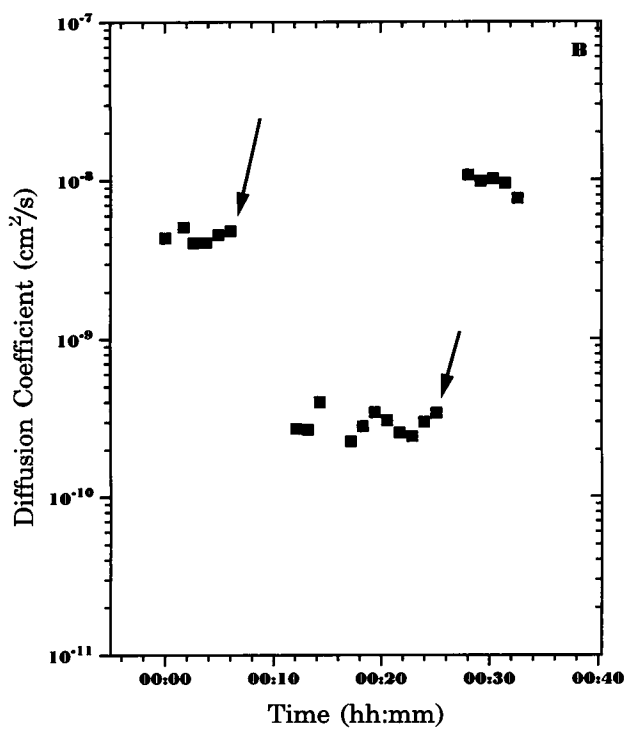
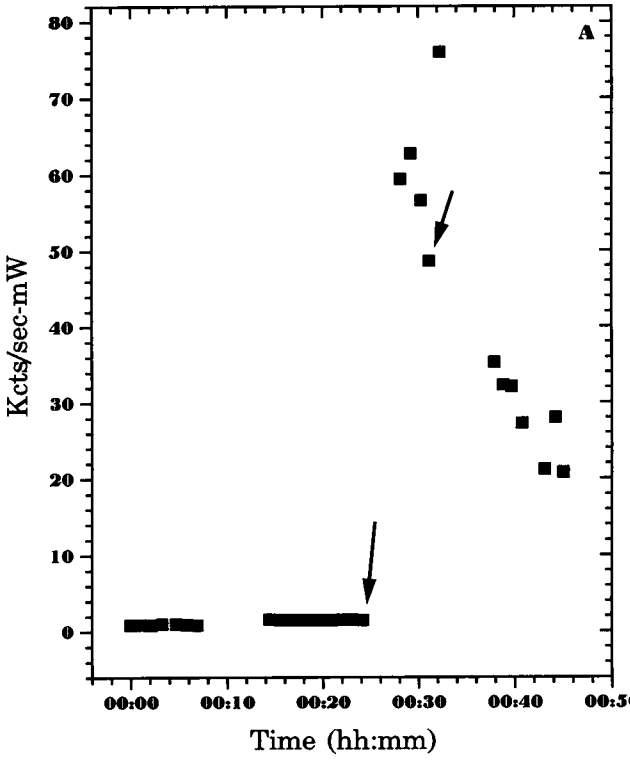


FIGURE 4 Data are similar to those in Fig. 1, except that 100 mM KCl was added at time 0 and the actin was in 6% PEG. The first arrow in each figure indicates the time at which the KCl concentration was increased to 125 mM, inducing rapid bundling. The second arrow in each figure indicates the time when the solution was diluted 1:1 with F-buffer containing 125 mM KCl, decreasing the PEG to 3%, conditions under which bundles do not normally form from polymer. The final intensities and diffusion coefficients differ from initial polymer values because of the dilution to 5 μM actin.



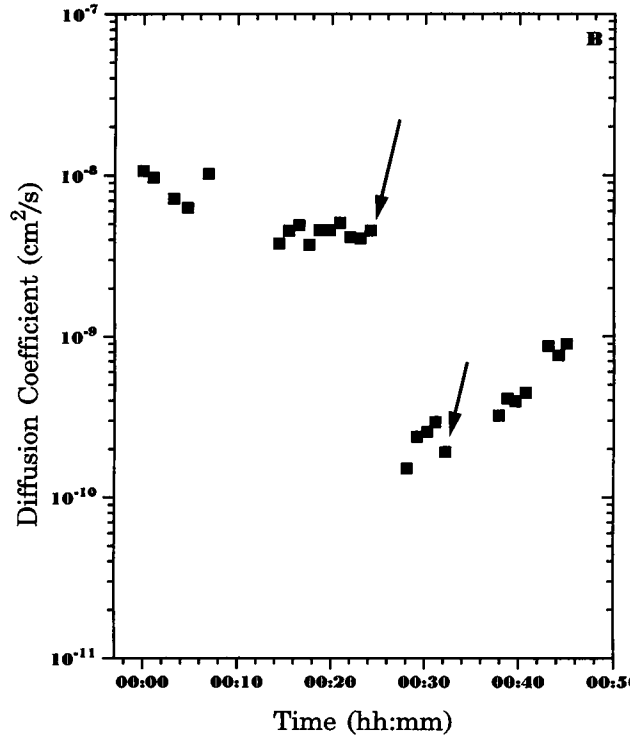


FIGURE 5 Data similar to those in Fig. 4, except that the PEG concentration was 7%. After reaching a polymerization steady state as in Fig. 4, the first arrow in each figure indicates the time when the [KCl] was increased to 150 mM and 2 mM $MgCl_2$ was added. The second arrow indicates the time at which the solution was diluted 1:1 with F-buffer containing 150 mM KCl and 2 mM $MgCl_2$. It is clear that neither the scattered intensity nor the mean diffusion coefficient has returned to the same level as the polymer steady-state values, indicating that most of the actin filaments remain bundled.

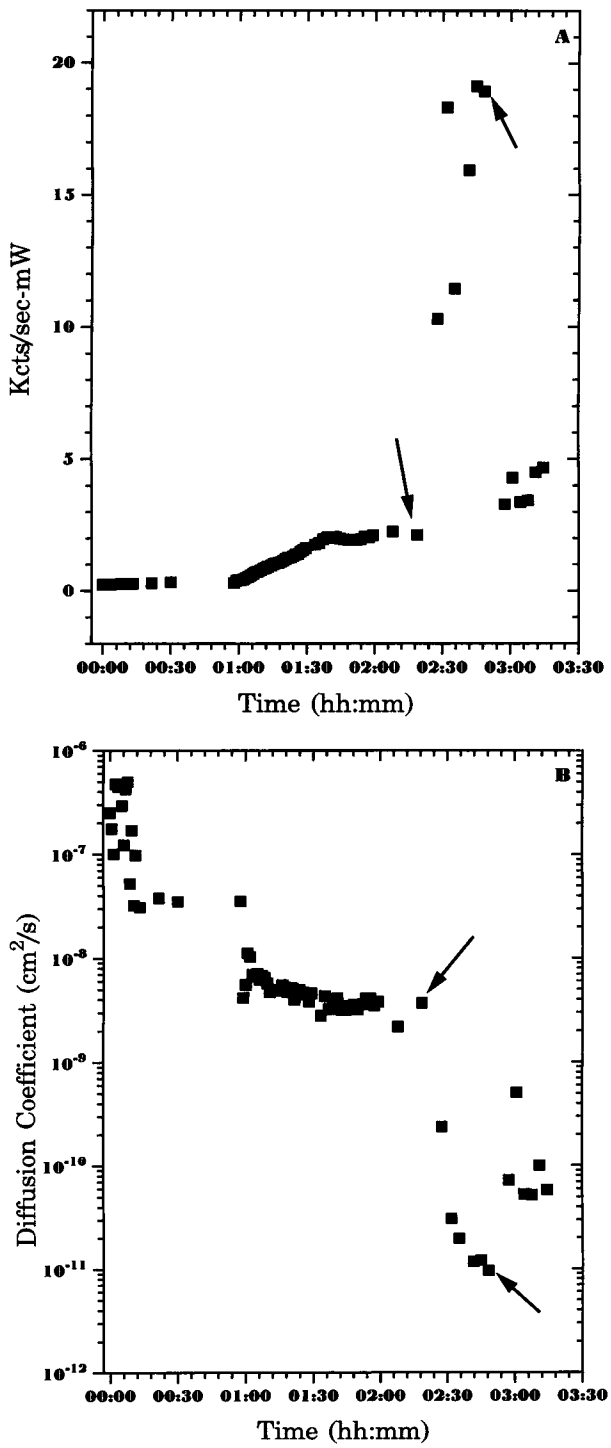


FIGURE 6 Data similar to those in Fig. 4, but in 8% PEG. In this case the first arrow indicates the addition of 2 mM $CaCl_2$, inducing bundling. The second arrow indicates 1:1 dilution with F-buffer containing 100 mM KCl and 2 mM $CaCl_2$. It is clear that the bundling is not fully reversible in this case.

readily (that is, at lower KCl concentrations) for actin filaments when in the presence of higher PEG concentrations. As Suzuki et al. (1989) have suggested, the effects of PEG on actin bundling are not caused by a direct interaction of

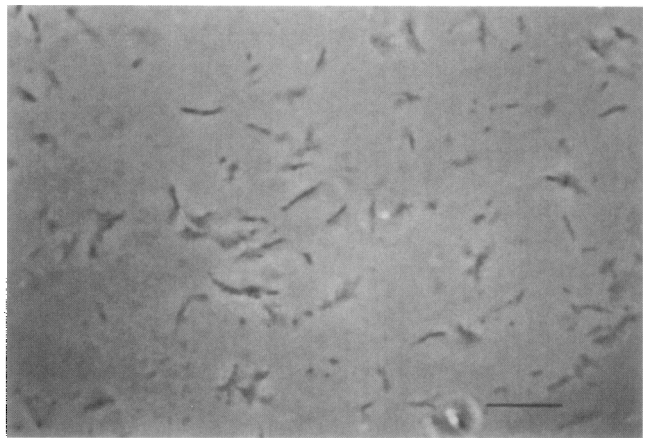


FIGURE 7 Phase-contrast micrograph of actin bundles formed at 10 μM actin in 8% PEG, 75 mM KCl, 2 mM $MgCl_2$, and with an actin:gelsolin ratio of 50:1. The sample was diluted 1:1 with F-buffer for microscopy. The bar indicates a length of 10 μm.

the PEG with the actin filaments. Rather, PEG acts (indirectly as an inert crowder molecule) to simulate the high protein concentrations inside cells. The increased KCl concentration needed to bundle actin filaments at lower PEG concentrations is in agreement with similar data of Suzuki et al. (1989) and suggests that a balance between osmotic stress and electrostatic repulsion is important to actin filament bundling.

Our experimental results differ from those of Suzuki et al. (1989) for experiments in which the salts and PEG necessary for bundling were added to G-actin and the sample was left undisturbed. In our experiments bundling always occurred within about 30–60 min and not over periods of 24 h, as was reported. When small volumes of highly concentrated additional salts are added to polymerized actin filament solutions, bundle formation is extremely rapid after the addition of appropriate salts and several gentle cuvette inversions. As suggested by Suzuki et al. (1989), this more rapid bundling is probably due to the alignment of filaments induced by flow when the cuvette is inverted to ensure complete mixing of salts. Bundling was observed to occur in the sample region in which salt was added, even without cuvette inversion, although these inhomogeneous solutions with locally indeterminate salt concentration were not studied. We routinely inverted the cuvettes to ensure uniform and known salt concentrations. A time course for bundle formation using this protocol is observed only for the shortest mean filament length samples. These samples are probably less well oriented by flow on cuvette inversion, so that a time course of bundling is observable. At 2% (w/w) PEG bundles did not form, even at 400 mM KCl, when we used our standard protocols of cuvette inversion and monitored for up to several hours. It is well known that the addition of 20–100 mM $MgCl_2$ to solutions of G- or F-actin in the absence of PEG results in the formation of actin paracrystals (Hanson, 1973; Yamamoto et al., 1975; Hartwell et al., 1980; Fowler and Aebi, 1982; Tang et al., 1996).

We have shown that at a given PEG concentration, bundles form at lower KCl concentrations for actin filaments with a shorter mean length. This finding is somewhat surprising and is not the expected behavior based on entropic arguments (Madden and Herzfeld, 1994). One possible explanation is that gelsolin itself may play a significant role in the bundling process. The S2–3 (F-actin) binding site on gelsolin may serve as a cross-link between the capped actin filament and another F-actin. Under shorter mean filament conditions, the ratio of gelsolin to actin is high and the shorter filaments might also be expected to be more stabilized in bundles than longer filaments would be by a cross-linker.

Actin filament bundling with PEG in the absence of divalent cations is completely reversible. When the PEG and actin levels are diluted to nonbundling conditions, the bundles rapidly and completely dissociate. In the presence of divalent cations, under certain conditions, PEG-induced bundles do not completely dissociate on 1:1 dilution with F-buffer. The irreversibility of bundling under these conditions suggests that osmotic pressure effects cannot fully explain actin bundling with PEG. Divalent cations are required for the observed irreversibility, which occurs particularly under conditions of longer filament lengths and higher PEG concentrations.

In attempts to understand the origin of the irreversibility observed with divalent cations present, we conducted a series of experiments. Because there is a single, specific, tight binding site on actin for such divalent cations (with a 2 nM binding constant), we performed experiments with Ca- and Mg-actin separately bound at the tight binding site. These experiments showed that in the absence of free divalent cation the bundles formed from both types of actin were fully reversible. Furthermore, Ca- and Mg-actin demonstrated similar irreversibility when bundled in the presence of 2 mM Mg^{2+} or Ca^{2+}. These findings implicate the importance of weak divalent binding sites on actin (with binding constants of 20–200 μM) to the irreversibility of bundling (Selden et al., 1989).

In further experiments we attempted to reverse bundling by adding 2 mM EDTA to chelate divalent cations. No noticeable effect on actin bundling was observed, implying that either the EDTA was excluded from the bundles or that the presence of the divalent cation is only required for irreversibility during bundle formation.

We also studied the effect on reversibility when the reducing agent dithiothreitol (DTT) was added to these samples. There was no effect on bundle reversibility when 5 mM DTT was added to an irreversibly bundled actin sample. However, when 5 mM DTT was added before the addition of the final salts that otherwise cause irreversible bundling, the bundles that form completely reversed on a 1:1 dilution to nonbundling conditions. Western blots of irreversibly bundled actin using an actin antibody under reducing conditions showed no evidence for any covalent cross-linking of actin monomers. These results suggest that irreversibly bundled actin may be due to disulfide bonds that form between actin filaments. We can only speculate that the binding of divalent cations at weak binding sites along an actin filament allows disulfide bonds to form between adjacent filaments. Supporting this possibility is the fact that divalent cation binding affects the fluorescence of fluorophores bound at cysteines. Our finding that DTT does not lead to the complete dissolution of the bundles when subsequently added suggests that these disulfide bonds are probably not accessible within the actin bundles.

Because irreversibility in the presence of divalent cations is particularly prevalent for filaments that are not length-regulated, we surmised that the irreversible bundling may be affected by the flexibility of the actin filaments. In an attempt to investigate this, phalloidin was used to stiffen actin filaments, changing the persistence length by about a factor of 2 to a value near that of actin with tropomyosin and troponin bound in the off state (Isambert et al., 1995). These experiments produced no significant differences in the bundling of actin, indicating either that filament flexibility is not an important factor in the formation and reversal of irreversible bundles, or that stiffening the filaments by only a factor of 2 is not sufficient to disrupt the bundles formed. Previous experiments on the bundling of tropomysoin-actin filaments have shown a requirement of millmolar levels of free Mg^{2+} at about 7% PEG for bundling under the solvent conditions of the study (Grazi et al., 1992).

A recent study of counterion-induced bundling of actin reports on the reversible bundling of actin in the presence of various positively charged multi-ion complexes, including divalent cations (Tang et al., 1996). This work finds that lateral associations of rodlike polyelectrolytes form because of counterion condensation. Monovalent salts or polyvalent coions (negatively charged) tend to dissolve these bundles, either by an ionic strength shielding effect or by a reduction in free counterion concentration, respectively. Tang et al. found that under actin and salt conditions comparable to those of this study, but in the absence of PEG, more than 10–20 mM Ca^{2+} or Mg^{2+} was required to induce reversible bundling. The length dependence of bundling induced by polylysine (with a mean of 18 lysines) was examined in this case as well and was shown to be roughly independent of length, except for the shortest filaments (50 mers), which required higher concentrations of polylysine to induce bundling.

Although our study uses uncharged PEG, the report by Tang et al. (1996) may have some implications for our results with divalent cations. In the presence of sufficient PEG and monovalent KCl, actin filaments will reversibly bundle without divalent cations. In the presence of divalent cations, bundles formed from all mean length samples studied at higher PEG concentrations, as well as all those formed from longer filaments at all PEG bundling concentrations, were not dissolved on dilution to nonbundling conditions. It is possible that the actin bundles formed under crowded conditions are further stabilized by a counterion condensation due to divalent cations at the weak binding sites on actin, although at lower, physiologically relevant, divalent cation concentrations. This stabilization may also promote disulfide bond formation and explain why the

bundles are not reversible on dilution. Such a combination of osmotic pressure and electrostatic effects may play a significant role in the functioning of actin in the peripheral cytosol.

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