

# Direct Demonstration of Actin Filament Annealing In Vitro

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**Abstract.** Direct electron microscopic examination confirms that short actin filaments rapidly anneal end-to-end in vitro, leading over time to an increase in filament length at steady state. During annealing of mixtures of native unlabeled filaments and glutaraldehyde-fixed filaments labeled with myosin subfragment-1, the structural polarity within heteropolymers is conserved absolutely. Annealing does not appear to require either ATP hydrolysis or the presence of exogenous actin monomers, suggesting that joining occurs through the direct association of filament ends. During recovery from sonication the initial rate of annealing is consistent with a second-order reaction involving the

collision of two filament ends with an apparent annealing rate constant of  $10^7 \text{ M}^{-1}\text{s}^{-1}$ . This rapid phase lasts  $<10 \text{ s}$  and is followed by a slow phase lasting minutes to hours. Annealing is calculated to contribute minimally to filament elongation during the initial stages of self-assembly. However, the rapid rate of annealing of sonicated fixed filaments observed in vitro suggests that it may be an efficient mechanism for repairing breaks in filaments and that annealing together with polymer-severing mechanisms may contribute significantly to the dynamics and function of actin filaments in vivo.

**A**T steady state in vitro, preparations of actin filaments (Nakaoka and Kasai, 1969; Kawamura and Maruyama, 1970) and microtubules (Mitchison and Kirschner, 1984) grow longer and become progressively fewer in number. While monomer exchange at polymer ends is clearly an important factor in determining the growth and stability of polymers of actin and tubulin, it is likely that polymer fragmentation and annealing are also involved in determining the lengths and numbers of polymers at steady state. In recent studies of microtubule assembly, we demonstrated that microtubule polymers can rapidly join end-to-end (anneal) (Rothwell et al., 1986) and that, under certain conditions, mechanisms of polymer annealing and monomer exchange both play important roles in determining microtubule dynamics in vitro (Rothwell et al., 1987). In the present paper, we examine the role of annealing in the assembly of actin filaments.

There are divided opinions regarding the existence of both fragmentation and annealing during actin filament assembly. There is irrefutable evidence that actin filaments can break into smaller pieces when subjected to strong mechanical forces (Asakura, 1961), although it is less certain that thermal energy alone is strong enough to break filaments in samples that have not been stirred. Mathematical models for the spontaneous polymerization of actin fit the time course of assembly better when a term for spontaneous fragmentation is included (Wegner, 1982; Wegner and Savko, 1982; Cooper et al., 1983). However, the contribution of fragmentation and annealing to filament assembly are thought to be complex (Frieden and Goddette, 1983). In these papers, a fragmentation term was not required for all polymerization con-

ditions and there were contradictions between the papers as to which conditions required a correction for fragmentation. In addition, Pantaloni et al. (1984, 1985) and Carlier and co-workers (1984, 1985) argued that there are other equally plausible models other than fragmentation that can also explain the data.

Similarly, there are arguments both for and against end-to-end annealing of actin filaments during recovery from sonication. It is agreed that actin filaments rapidly increase in length and decrease in number after sonication, as judged by electron microscopy, viscosity, flow birefringence, and nucleation assays. Nakaoka and Kasai (1969) and Kawamura and Maruyama (1970) concluded that endwise annealing is the main mechanism responsible. Since Oosawa and Maruyama (1987) found that ADP-actin filaments stabilized with phalloidin do not depolymerize during sonication and yet rapidly increase in length after sonication, they argue that end-to-end annealing is the predominant mechanism. On the other hand, Carlier et al. (1984) showed that a model with random loss of monomers from the ends of short filaments and their reincorporation at the ends of longer growing filaments during the recovery of ADP-actin filaments from sonication fit their data better than an annealing mechanism. In a completely different type of experiment, Kondo and Ishiwata (1976) examined the growth of ADP-actin from the ends of actin filaments decorated with heavy meromyosin by EM. They observed a small population of filaments with alternating decorated and undecorated segments. Annealing seemed like the only plausible explanation for the formation of these heteropolymers, but we were still left with the question of whether annealing is important quantitatively in the

redistribution of filament lengths during recovery from sonication or spontaneous fragmentation.

To document actin filament annealing directly by EM and examine the mechanism of this process, we mixed together samples of native unlabeled actin filaments with filaments that were labeled with myosin subfragment-1 (S-1)<sup>1</sup> and fixed with glutaraldehyde. As originally observed by Kondo and Ishiwata (1976) in their nucleated growth experiments, long filaments with alternating labeled and unlabeled segments with uniform polarity form in the mixture. We also determined that annealing doubled the average filament length in <2 s in dilute preparations of actin filaments (0.5–5  $\mu\text{M}$ ) containing short polymers (0.15  $\mu\text{m}$  mean length). The dependence of the initial annealing rate on the concentration of filament ends suggests that annealing is a second-order process with an apparent annealing rate constant on the order of  $10^7 \text{ M}^{-1}\text{s}^{-1}$ . In contrast, more concentrated samples with longer filaments ( $\sim 20 \mu\text{M}$ , 1–3  $\mu\text{m}$  mean length) exhibited a second, slower annealing rate lasting several minutes or hours that was independent of filament concentration, indicating that annealing may be influenced by certain physical constraints such as the length, concentration, and alignment of polymers in solution.

## Materials and Methods

### Preparation of Actin

Actin was prepared from an acetone powder of rabbit skeletal muscle by the method of Spudich and Watt (1971) and was purified by chromatography on Sephadex G-150 as described by MacLean-Fletcher and Pollard (1980). Actin concentration was determined by measuring absorbance at 290 nm using an extinction coefficient of  $2.66 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  (Houk and Ue, 1974). Alternatively, the Bradford (1976) protein assay was performed using an actin standard whose concentration was initially determined from its absorbance at 290 nm. Actin monomer (2 mg/ml) was stored for up to 3 wk at 4°C in buffer G: 2 mM imidazole, pH 7.5, 0.2 mM ATP, 0.2 mM  $\text{CaCl}_2$ , 0.5 mM dithiothreitol (DTT), and 1.0 mM  $\text{NaN}_3$ . Actin polymerization was initiated by adding 0.1 vol of a  $10 \times$  stock solution of actin assembly buffer to give final concentrations of 10 mM imidazole, pH 7.5, 50 mM KCl, 1 mM  $\text{MgCl}_2$ , and 1 mM EGTA. As determined by sedimentation and determination of the equilibrium monomer concentration, the critical concentration for self-assembly was determined to be 0.4  $\mu\text{M}$  and the half-life of actin assembly activity 10 d.

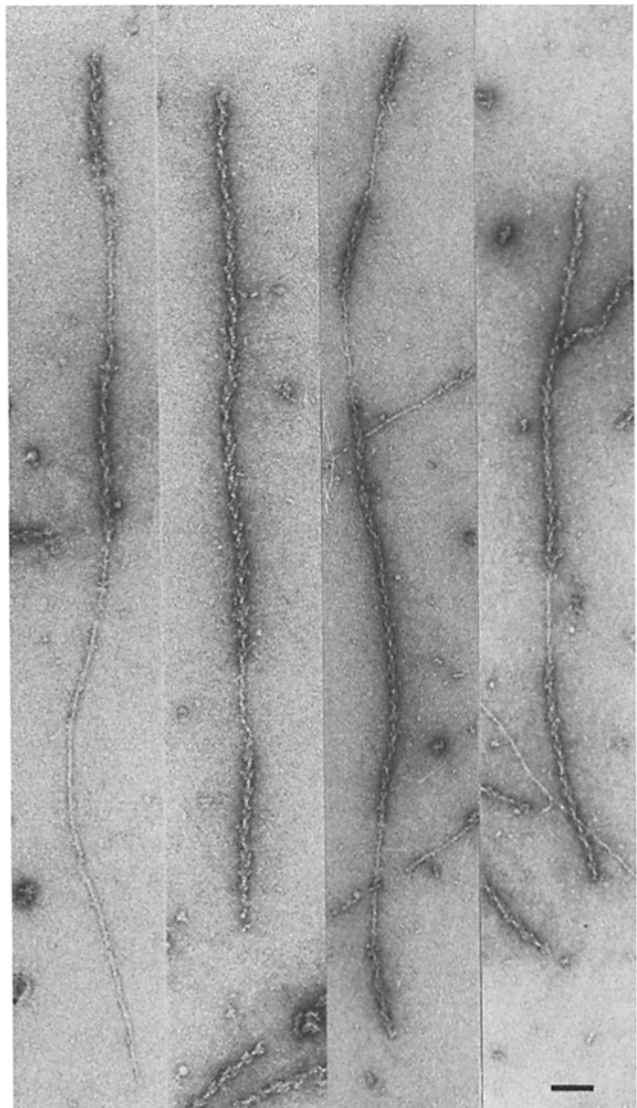
### Preparation of Myosin S-1 and S-1-decorated Actin Filaments

Myosin S-1 was prepared from rabbit skeletal myosin by minor modifications of the method of Weeds and Taylor (1975). Preparations of myosin S-1 retained actin-binding activity for up to 3 mo but were generally used within 1 mo of preparation. To study annealing using filaments decorated with myosin S-1 in assembly buffer containing ATP, it was necessary to couple myosin S-1 covalently to the actin filaments (S-1/actin weight ratio, 3:1) with 0.1% glutaraldehyde (Cooper and Pollard, 1982). Residual glutaraldehyde was reacted with a fivefold molar excess of ethanolamine, and the fixed filaments were passed over a  $0.8 \times 10\text{-cm}$  column containing Sephadex G-25 preequilibrated with assembly buffer. Labeled filaments retained their ability to elongate bidirectionally in the presence of actin monomers and anneal with unlabeled actin filaments.

### Electron Microscopy

EM grids with Parlodion and carbon films were glow discharged and coated with 0.2 mg/ml cytochrome c in 0.1% isoamyl alcohol before use. Protein samples were usually diluted 3–10-fold in assembly buffer containing 0.1% glutaraldehyde and incubated for 2–5 min before negative staining. Control

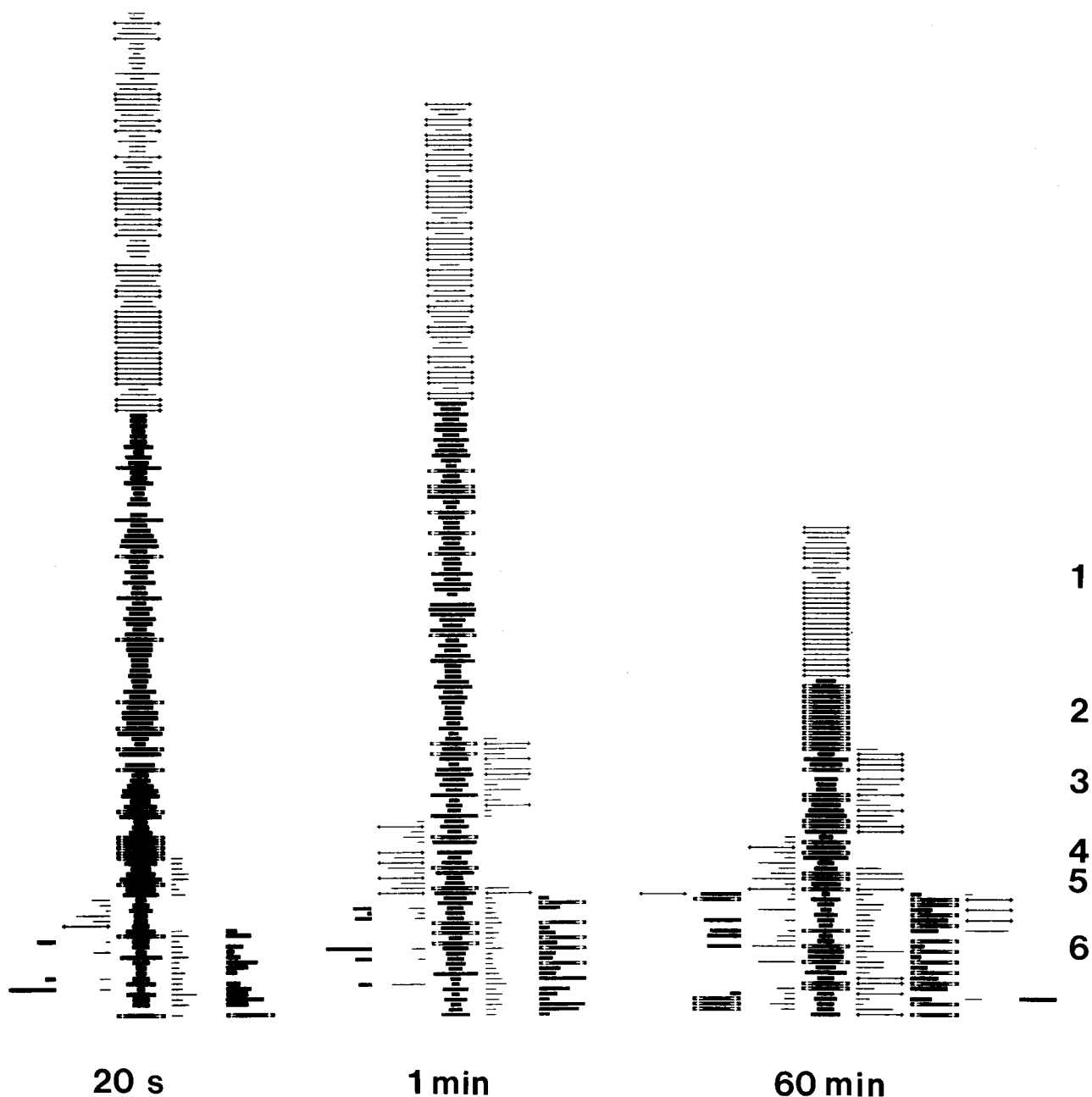
1. Abbreviation used in this paper: S-1, subfragment-1.



**Figure 1.** Actin heteropolymers formed by polymer annealing. Filaments labeled with myosin S-1 were fixed and mixed with unlabeled native actin filaments. Samples taken after mixing were diluted in 0.1% glutaraldehyde in assembly buffer and negatively stained with 1% uranyl acetate. Heteropolymers such as these composed  $\sim 15\text{--}20\%$  of the filaments in the sample. The polarity of S-1-labeled domains within individual heteropolymers is conserved. Bar, 0.1  $\mu\text{m}$ .

experiments showed that diluted filaments (4.8  $\mu\text{M}$ ) in 0.1% glutaraldehyde maintained a constant length for times  $\leq 1$  h. It is not known if the arrest in annealing observed under these conditions was due to glutaraldehyde, dilution, or the combination of these factors. Grids were floated successively on drops containing fixed actin filaments (10 s), water (1 s), and 2 drops of 1% aqueous uranyl acetate (drop 1 for 1 s, drop 2 for 10 s). Excess stain was removed with a filter paper, and grids were examined and photographed with an electron microscope (model EM 10A, Carl Zeiss, Inc., Thornwood, NY).

For each sample and time point, we measured 150  $\mu\text{m}$  of filaments, representing  $\sim 250$  individual domains (labeled and unlabeled segments). This was done by examining individual filaments on the microscope viewing screen at a magnification of  $25,000\times$  and counting the number of units comprising each domain up to a cumulative total length of 5,000 U for each sample. 1 U (0.03  $\mu\text{m}$ ) was defined as the length of a 0.75-mm reference marker on the viewing screen. Since increasing the sample size to 400  $\mu\text{m}$  did not significantly change the mean and standard deviation of the filament length, we felt confident that 150–200  $\mu\text{m}$  total length reliably defined the sample.



**Figure 2.** Dynamics of actin filament annealing. Fixed labeled filaments and native unlabeled filaments were combined and sampled at various times after mixing. Each interrupted horizontal line represents a single actin filament with alternating unlabeled (—) and labeled (▬) segments. For clarity, the segments are shown with a maximum length of 1.0  $\mu\text{m}$ ; segments longer than this are marked at their ends with dots. Filaments are oriented according to polarity, with the pointed ends of labeled segments facing right, and are grouped according to the following patterns of morphology: (1) unlabeled filaments, (2) labeled filaments, (3) unlabeled segment attached to the pointed end of a labeled filament, (4) unlabeled segment attached to the barbed end of a labeled filament, (5) unlabeled segments attached to both the pointed and barbed ends of labeled segments, and (6) “annealed” filaments containing multiple labeled and unlabeled domains.

## Results

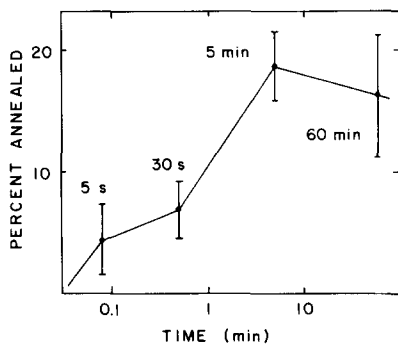
To study actin filament annealing by EM, we used two different methods. (a) We used two morphologically distinct filaments to document annealing by EM. (b) We used glutaraldehyde-fixed filaments to study the time course of annealing and estimate an annealing rate constant.

### Direct Observation of Actin Filament Annealing

In the following two sections we used labeled and unlabeled

polymers (total actin concentration, 12–24  $\mu\text{M}$ ) that were sheared to a mean length of 0.6  $\mu\text{m}$  by passing them four times through a 26-gauge syringe needle. The preparations were mixed 1:1 either immediately after shearing or at various times up to 5 min after disruption. Dilution into glutaraldehyde stabilized the filaments and stopped further annealing.

As originally observed by Kondo and Ishiwata (1976) in nucleated growth experiments, heteropolymers rapidly formed in these mixtures (Fig. 1). Annealed filaments with alternat-



**Figure 3.** Time course of actin filament annealing. The data from the experiments included in Table I were pooled to show the mean and SD of the percent of annealed filaments observed at 5 s, 30 s, 5 min, and 60 min after mixing labeled and unlabeled filaments. General conditions are described in the text and in the legend to Fig. 1, although specific details regarding nucleotide content and shearing varied between experiments. Filaments annealed slowly and continuously over several minutes.

ing labeled and unlabeled domains formed within 5 s and continued to increase in length and complexity with time. The polarity of S-1-decorated domains in heteropolymers was always the same, indicating that each annealing event involved the endwise joining of barbed and pointed ends.

We conclude that heteropolymers arise through annealing, since (a) the myosin heads on the labeled segments are covalently cross-linked so that they do not dissociate or redistribute even in ATP and (b) the concentration of free myosin S-1 in the fixed gel-filtered fraction of labeled filaments is too low to promote additional labeling of unlabeled filaments. The endwise joining of filaments is probably specific, since filaments were never observed to make side-to-side or end-to-side attachments and because the structural polarity of la-

beled domains within annealed filaments was always the same.

Fig. 2 shows graphically how the morphology of a mixture of labeled and unlabeled polymers changes with time. Six patterns of filament morphology were observed. We scored as annealed only those filaments with multiple labeled and unlabeled domains or with a single unlabeled domain at the pointed end of a labeled domain (3 and 6 in Fig. 2) but excluded those heteropolymers that could have arisen from either annealing or monomer addition such as those labeled domains showing bidirectional or barbed end growth (4 and 5 in Fig. 2). Therefore, these criteria give a very conservative estimate of the extent of annealing.

The initial rate of annealing in the heteropolymer assay was both rapid and variable (Figs. 2 and 3; and Table I). Approximately 5 and 15% or more of the filaments annealed within the first 5 s and 5 min, respectively, but the reaction slowed down thereafter (Fig. 3). In some cases, 20% or more of the final extent of annealing was observed after 5 s. This variability in the apparent rate of annealing was typical of these preparations and may be due to the scoring method, a rapid initial phase of annealing which could have been missed by taking samples over several minutes of time, or differences in the concentrations, lengths, and orientations of filaments in solution.

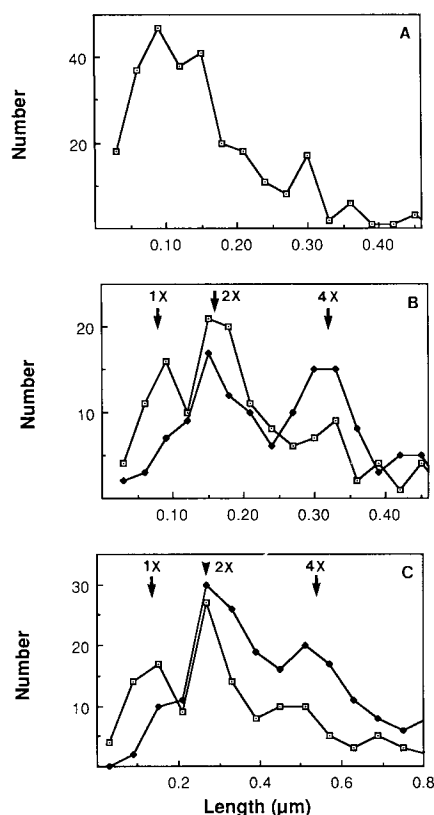
#### **Annealing Does Not Require ATP Hydrolysis or the Incorporation of Free Monomers**

The heteropolymer system was used to compare the initial rates of annealing in assembly buffer containing 0.2 mM ATP or ADP. In addition, filaments were sheared to expose ADP-actin monomers contained within the polymer cores, and initial annealing rates were compared for preparations mixed 10 s vs. 5 min after shearing. None of these treatments significantly affected the initial rates of annealing (Table I),

**Table I. Effects of Nucleotides and Shearing on Actin Filament Annealing**

Experimental conditions	Filament length		Concentration of ends <i>nM</i>	Molar ratio (Unlabeled/labeled)	Annealed filaments at 5 s %
	Labeled $\mu\text{m}$	Unlabeled $\mu\text{m}$			
<b>ATP-actin</b>					
Mix at 10 s	0.5	0.7	118	2.7	2.0
	0.2	1.0	93	2.0	2.7
	0.3	0.7	110	5.7	21.4
Mix at 5 min	0.5	0.8	117	2.1	10.5
<b>ADP-actin</b>					
Mix at 10 s	0.3	0.4	198	2.9	3.0
	0.6	0.9	96	1.8	2.7
Mix at 5 min	0.7	1.4	192	3.2	0.6
	0.5	0.6	122	2.6	4.0
<b>ATP/ADP-actin</b>					
Mix at 10 s	0.7	1.0	88	2.5	7.8
	0.5	1.1	83	2.4	7.1
Mix at 5 min	0.5	0.4	202	6.7	5.9

Initial rates of polymer annealing are shown for actin filaments in 0.2 mM ATP or ADP or an equimolar mixture of both nucleotides. Samples with ADP were prepared from ATP-containing samples by adding 25 U/ml hexokinase and 10 mM glucose. Chromatographic analysis of samples containing gamma-<sup>32</sup>P]ATP showed that the residual ATP concentration was reduced to <1  $\mu\text{M}$ , or 25 times less than the total actin molarity (25  $\mu\text{M}$ ). Samples containing 25  $\mu\text{M}$  actin and the appropriate nucleotide were sheared four times with a 26-gauge needle to a mean length of 0.6  $\mu\text{m}$  and were mixed 10 s or 5 min after shearing. The concentration of ends was calculated from the actin concentration and the mean filament length. The percentage of annealed filaments at 5 s indicates the extent of annealing that occurred during the first 5 s after mixing labeled and unlabeled filaments together.



**Figure 4.** Length-frequency distributions of sonicated actin filaments. (A) The distribution of actin filaments fixed during sonication. Fixed unlabeled filaments (3.1  $\mu\text{M}$ ) were diluted with fixative 5 s after the initiation of sonication. The mean filament length was 0.16  $\mu\text{m}$ ; the population contained a principal peak at 0.08  $\mu\text{m}$ . (B) The length distributions of fixed filaments during recovery from sonication. Fixed unlabeled filaments were sonicated for 5 s and aliquots were removed and fixed after 13 s ( $\square$ ) and 2 min ( $\blacklozenge$ ) recovery. (C) Length distributions of myosin S-1-labeled domains during recovery from sonication. Preparations containing myosin-labeled fixed filaments and native unlabeled filaments were sonicated for 5 s and aliquots were removed and fixed after 2 ( $\square$ ) and 22 ( $\blacklozenge$ ) s recovery. Each sample depicted above includes 120–150 filaments.

the differences being within the wide range of variability observed for annealing in assembly buffer containing ATP. The near equivalence of annealing rates in ATP- and ADP-containing buffers suggests that nucleotide hydrolysis is not required for polymer annealing.

We also examined the rate of polymer annealing at various actin monomer concentrations in preparations where both the labeled and unlabeled filaments were fixed and the ratio and concentrations of labeled and unlabeled filaments remained the same. Since microtubule annealing requires substantial rearrangements of tubulin monomers at the site of endwise polymer contacts, it was possible that actin annealing would require the incorporation of exogenous monomers. At the same time, monomers might inhibit annealing if they competed with other polymers for addition onto the ends of filaments. To examine the effect of monomers on the rate of actin filament annealing, we added actin monomers to preparations where both labeled and unlabeled filaments were fixed with glutaraldehyde and the ratio and concentrations of the two filament types remained the same. Control

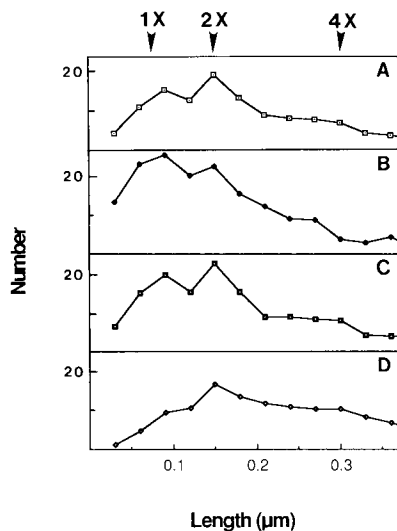
experiments showed that fixed chromatographed filaments annealed together, although at a somewhat reduced rate, in the absence of native monomers (11% of the filaments annealed after 60 min). Over a wide range of actin monomer concentrations (0.4–3.6  $\mu\text{M}$ , corresponding to a molar ratio of monomers to filament ends of 4–36), no major differences in annealing rates were observed, although some slight inhibition may have occurred at higher monomer concentrations (data not shown). These observations suggest that over this range of concentrations, monomers do not significantly affect the rate of polymer annealing.

#### **Examination of Fixed Actin Filaments during Recovery from Sonication**

To examine the possibility that annealing occurs rapidly on a time scale of just a few seconds, we sonicated fixed filaments and measured their lengths at intervals of 2–5 s during recovery. Fixation with 0.1% glutaraldehyde for 5 min at 22°C stabilizes the filaments and electrophoresis of fixed actin on SDS-polyacrylamide gels reveals that the great majority of protein does not enter the gel. While the exact physical state of the fixed polymers is not known, it is likely that many monomers are not cross-linked to neighbor monomers within polymers and that during sonication filaments break at sites where the number of cross-links between monomers is reduced or absent. The experiments described above established that fixed filaments annealed after gel filtration chromatography and that dilution into glutaraldehyde stopped annealing for at least 1 h. When diluted with glutaraldehyde during sonication, the distribution of filament lengths was observed to contain a principal peak corresponding to a length of 0.08  $\mu\text{m}$  (Fig. 4 A). The drop-off in filament length below values of 0.08  $\mu\text{m}$  is real. Filaments half this length were occasionally observed, and in areas of good negative staining we are confident that we could have seen even shorter oligomers.

During recovery from sonication, the length-frequency distributions evolved into a series of peaks that were multiples of the primary peak observed during sonication (Fig. 4 B). With time, the proportion of longer filaments increased at the expense of the smaller species. Both unlabeled (Fig. 4 B) and labeled (Fig. 4 C) filaments behaved the same way. The positions and relative magnitudes of the peaks were the same with samples of 120–400 filaments. Although the positions of these peaks remained close to the integral multiples of the initial polymer length, there was a subtle shift to slightly longer than integral lengths with time, which may reflect the fact that the lengths of filaments within any one size class are distributed over a range of values. The slight shifts upwards in mean length may be due to the fact that shorter filaments are the first to anneal owing to their larger magnitude diffusion coefficients (Hill, 1983, 1987).

The unique length distributions observed in individual samples containing 120–150 filaments were also seen in a summary plot containing the lengths of  $\sim 6,000$  fixed sonicated actin filaments (6 experiments, 44 different samples; Fig. 5). A, which shows the length distribution of all 6,000 filaments, reveals the same peaks observed in individual samples consisting of only 120 filaments (Fig. 4 B). The effects of annealing on the patterns of length distribution are shown in the bottom panels of the figure. Since concentrated

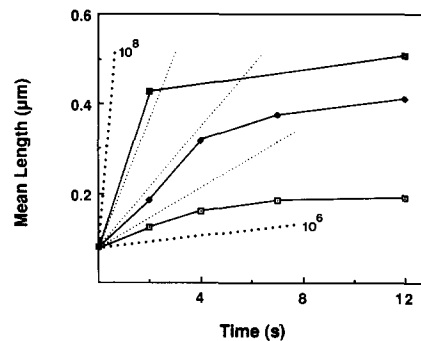


**Figure 5.** Length–frequency distributions of fixed actin filaments during recovery from sonication grouped according to the product of polymer concentration and time after sonication. Fixed unlabeled actin filaments at 0.6, 1.2, 2.4, and 4.8  $\mu\text{M}$  were sonicated for 5 s, and aliquots were removed at various times during recovery  $\leq 30$  s. *A* shows the cumulative length–frequency distribution of  $\sim 6,000$  filaments from 44 different samples in 15 experiments. The remaining panels show length distributions for the same filaments after subdividing them according to the product of the time of incubation (s) and actin concentration (mg/ml) as follows: (*B*) product  $< 0.1$ ,  $n = 680$  filaments from five samples; (*C*) product = 0.1 to 1.0,  $n = 3,100$  filaments from 23 samples; (*D*) product  $> 1.0$ ,  $n = 2,160$  filaments from six samples. The data in all four panels are normalized to show the distributions expected for samples containing 150 filaments.

samples and longer time points would be expected to exhibit more annealing, we divided the total population of filaments into subgroups according to the magnitude of the product of actin concentration and the time of sampling (Fig. 5, *B–D*). The presence of multimodal peaks and changes in the relative magnitudes of the peaks with time are clearly visible. Since these polymers were stabilized by fixation, we conclude that the rapid increase in length is due to polymer annealing. This conclusion is strongly supported by incremental growth in multiples of the average length of sonicated polymer.

The rate of annealing is very high and strongly dependent on the concentration of filaments (Fig. 6). The time course of these reactions is consistent with a simple bimolecular reaction involving the joining of two filament ends. Annealing rate =  $k^+ [\text{ends}]^2$ , with a rate constant of  $10^7 \text{ M}^{-1}\text{s}^{-1}$  (estimated from the initial rates of change in mean length and the calculated molarities of filament ends from nine sonication experiments). This is obviously only an order of magnitude estimate, since the EM method does not provide the time resolution needed for an accurate determination of the rate constant.

Using  $10^7 \text{ M}^{-1}\text{s}^{-1}$  as the association rate constant, a simple bimolecular reaction mechanism fits the experimental data reasonably well for up to two cycles of annealing (Fig. 6), but thereafter the reaction slows or stops. Clearly the initial burst of rapid annealing is terminated soon after it starts. Annealing continues (Figs. 1–3), but at a much slower rate.



**Figure 6.** Dependence of the rate of change in mean filament length on the concentration of filament ends. Preparations of fixed unlabeled filaments containing 1.2 ( $\square$ ), 2.4 ( $\diamond$ ), and 4.8 ( $\blacksquare$ )  $\mu\text{M}$  total actin were sonicated and allowed to recover for various times as described in Materials and Methods, and the mean lengths of 120–150 filaments contained in each sample were determined. Since the time required to manually transfer an aliquot to fixative was  $\sim 1$ –2 s, 2 s was added to the time of sampling for each sample and a length of  $0.08 \mu\text{m}$  was used as an estimate of the length at 0 s (Fig. 4 *A*). For each of the three actin concentrations there are corresponding theoretical plots with  $k^+ = 10^7 \text{ M}^{-1}\text{s}^{-1}$  (fine dotted lines). The theoretical plots were calculated for an irreversible bimolecular reaction mechanism starting with a uniform population of 30-mers ( $0.08 \mu\text{m}$ ). Theoretical plots are also shown for 2.4  $\mu\text{M}$  actin with  $k^+ = 10^6$  and  $10^8 \text{ M}^{-1}\text{s}^{-1}$  (coarse dotted lines). Note that the theoretical plots of mean length are linear with time even though the annealing rate declines rapidly. This is due to the fact that the species binding together grow progressively longer as the reaction proceeds.

## Discussion

We have used two different EM methods to study the annealing of actin filaments. Formation of heteropolymers from two populations of filaments confirmed the observations of Kondo and Ishiwata (1976) and established the polarity of the reactions. Analysis of length distributions during recovery from sonication showed that the annealing process consists of a very rapid transient phase followed by a much slower phase. As first suggested by Nakaoka and Kasai (1969), the rapid phase appears to be a second-order reaction with a rate proportional to the square of the concentration of filament ends. Our EM data are consistent with an association rate constant of  $\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$ , as expected for a diffusion-limited process.

It is interesting to note that glutaraldehyde-fixed filaments resemble native actin filaments in many respects: (*a*) annealing is specific for joining between the ends of filaments; (*b*) filament structural polarity is conserved during annealing; (*c*) fixed filaments elongate in the presence of native actin monomers; and (*d*) when annealing among fixed actin filaments was compared in the presence and absence of added monomers, the rate of annealing was observed to remain the same. Thus, the ends of fixed filaments behave like native ends with respect to their ability to anneal. We cannot rule out the possibility that fixation biases the results in some way (e.g., fixed filaments would not be able to exhibit monomer-polymer exchange), but at the present time the method appears promising for future studies of actin filament dynamics.

Although annealing of actin filaments did not exhibit an obvious requirement for ATP hydrolysis or exogenous monomers under the conditions we used, the details of the process still remain unclear and, in some cases, even appear to contradict expectations. In the case of microtubules, for example, we observed that imperfections in the microtubule wall at the site of joining quickly become filled in or repaired, suggesting a possible requirement for the rearrangement of monomers in the polymer wall or the incorporation of exogenous tubulin monomers (Rothwell et al., 1986). However, our experiments showed that filament annealing was not affected in an obvious way by the addition of actin monomers, even at relatively high molar ratios of monomers to filament ends. Further, for both actin filaments and microtubules, and contrary to intuition, the order of the annealing process was not always dependent on polymer concentration as predicted for a second-order reaction. Rather, for both actin filaments and microtubules, annealing appeared to be a zero-order process when samples were taken over several minutes of time. This is an important observation and may eventually provide insights regarding the conditions that permit annealing to occur. In future studies it will also be valuable to examine annealing on a millisecond time scale to obtain better quantitative measurements during the rapid phase to understand the mechanism in more detail.

Why does the rapid early phase of annealing slow down after a few cycles? The most likely explanation is that after two or three cycles of annealing, certain physical constraints such as the length, concentration, and orientation of filaments preclude the free diffusion of the filaments. At the concentrations of actin typically used in our experiments (2.5–25  $\mu\text{M}$ ), long polymers may align spontaneously into bundles (Oosawa and Asakura, 1975; Nordh et al., 1986; Buxbaum et al., 1987) or form gels (Tait and Frieden, 1982; Sato et al., 1985; Buxbaum et al., 1987), either of which could reduce filament mobility and annealing rates.

The available evidence strongly supports the existence of annealing during recovery from shearing or sonication, but the relative contributions of polymer annealing and monomer-polymer exchange to the steady-state dynamics of actin filaments remains an interesting question. In the studies reported here using fixed filaments, annealing contributed in an obvious way to increases in filament length and decreases in filament number. From inspection of experiments in which we mixed equal portions of fixed and native filaments, the contribution of annealing to the increase in filament length for annealed heteropolymers and the overall filament population is estimated to be 25 and 50%, respectively. In the second series of studies, where we used only glutaraldehyde-fixed filaments, nearly all of the redistribution of length would be expected to be due to annealing. However, as pointed out above, chemical fixation may introduce a bias to the results, since monomer-polymer exchange is not possible for fixed filaments.

The steady-state dynamics of actin filaments have been interpreted up until now solely in terms of the exchange of actin monomers at filament ends. Carlier et al. (1984) argued that monomer exchange alone accounts for the change in polymer number during recovery of ADP-actin from sonication and that annealing is not important during polymerization at steady state. For such a mechanism to occur, monomer exchange would have to be much faster than annealing, a situa-

tion favored by the particular conditions used by Carlier et al. (a) Their observations were made during a period of time corresponding to our slow phase of annealing. (b) They used ADP-actin that dissociates from filaments much faster than ATP-actin (Pollard, 1986). This dissociation rate determines the rate of monomer exchange. This may account for why their pure monomer exchange model did not fit their data for ATP-actin.

We argue that under *in vitro* conditions both annealing and monomer exchange contribute to steady-state dynamics of ATP-actin filaments and that annealing probably also occurs during spontaneous polymerization. At steady state with 20  $\mu\text{M}$  ATP-actin in a KCl-Mg buffer, the rate of annealing will be low because the filaments are very long,  $>5 \mu\text{m}$  (Pollard, 1983). As a consequence, the number concentration of ends is in the nanomolar range. This alone would limit the rate of annealing, but the length also restricts the motion of the ends that is required for annealing. Similarly, the rate of monomer dissociation is low at both ends in ATP (Pollard, 1986) so that neither process will produce rapid changes in polymer length.

During spontaneous polymerization, annealing contributes to a small extent to the elongation of the filaments. Consider, for example, the situation at the 100-s time point with ATP-actin shown in Fig. 4 by Cooper et al. (1983). The filament number concentration was  $10^{-11} \text{ M}$ , the polymerized actin concentration was  $3 \times 10^{-8} \text{ M}$ , and the actin monomer concentration was  $6 \times 10^{-6} \text{ M}$ . Given that the rate constants for elongation and annealing are both  $\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$ , the rate of growth due to monomer addition was  $6 \times 10^{-10} \text{ M}\cdot\text{s}^{-1}$  or 60 molecules/filament per s, and the rate of annealing was  $10^{-15} \text{ M}\cdot\text{s}^{-1}$ . This is a low rate, but since the average filament had 3,000 monomers, each annealing event would have a dramatic effect on the length of the involved filaments. During this initial phase of self-assembly and filament elongation, polymer growth due to annealing is estimated to be only a hundredth or a thousandth that due to monomer addition. However, at monomer concentrations close to the critical concentration, annealing may significantly affect filament growth, involving by our own very conservative estimate 15–20% of the filament ends.

The possible role of polymer annealing *in vivo* is not yet known, but it may constitute an important repair mechanism. For example, it has now been observed in several different *in vitro* models (e.g., Toyoshima et al., 1987) that myosin (even single myosin heads) can exert enough force to break actin filaments, making it conceivable that filaments break during muscle contraction or cell movements. At the concentrations of actin found in cells, short actin filaments would anneal on a millisecond time scale, especially if the broken ends remained proximal due to other constraints. Even more importantly, annealing after severing by specific proteins may contribute to rapid rearrangements of actin filaments associated with cell motility and morphogenesis. Further work is required to explore these interesting possibilities.

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