

# Polymerization of ADP-Actin

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**ABSTRACT** Using hexokinase, glucose, and ATP to vary reversibly the concentrations of ADP and ATP in solution and bound to *Acanthamoeba* actin, I measured the relative critical concentrations and elongation rate constants for ATP-actin and ADP-actin in 50 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1 mM nucleotide, 0.1 mM CaCl<sub>2</sub>, 10 mM imidazole, pH 7. By both steady-state and elongation rate methods, the critical concentrations are 0.1 μM for ATP-actin and 5 μM for ADP-actin. Consequently, a 5 μM solution of actin can be polymerized, depolymerized, and repolymerized by simply cycling from ATP to ADP and back to ATP. The critical concentrations differ, because the association rate constant is 10 times higher and the dissociation rate constant is five times lower for ATP-actin than ADP-actin. These results show that ATP-actin occupies both ends of actin filaments growing in ATP. The bound ATP must be split on internal subunits and the number of terminal subunits with bound ATP probably depends on the rate of growth.

Although it has been more than 30 years since Straub and Feurer (1) discovered that ATP bound to actin monomers is dephosphorylated to ADP during polymerization, the role of the bound nucleotide continues to be enigmatic. There are a number of elegant studies of the affinity of actin for ATP and ADP (2–10) including estimates of the association and dissociation rate constants (8, 9), but almost nothing is known about the influence of ATP and ADP on the quantitative aspects of actin polymerization or exactly when dephosphorylation of the bound nucleotide occurs during polymerization (11). These issues are important for understanding the function of actin in cells, because ATP hydrolysis is responsible for the potential ability of actin monomers to treadmill or flux through a filament at steady state (12–19) and may also be involved with the regulation of actin assembly (11).

A great deal has been learned about the mechanism of ATP-actin polymerization (11, 20). Self-assembly begins with the slow formation of trimers that act as nuclei for further growth of the filament (21–23). Nucleation is much faster in MgCl<sub>2</sub> than CaCl<sub>2</sub>, even though the mechanism in MgCl<sub>2</sub> involves a first-order activation reaction (presumably exchange of bound Ca<sup>++</sup> for Mg<sup>++</sup>) not present in CaCl<sub>2</sub> (21, 23). The filaments grow bidirectionally (24), about 10 times faster at the barbed end than the pointed end (13). The dependence of the elongation rate on the actin concentration allows one to measure the rate constants for elongation (13, 18). These rate constants are defined in Fig. 1. At the barbed end, the association rate constant ( $k_+^B$ ) is  $\sim 10^7$  M<sup>-1</sup>s<sup>-1</sup> and the dissociation rate constant ( $k_-^B$ ) is  $\sim 2$  s<sup>-1</sup> in MgCl<sub>2</sub>. At the

pointed end, these constants ( $k_+^P$ ,  $k_-^P$ ) have values of  $\sim 2 \times 10^6$  M<sup>-1</sup> s<sup>-1</sup> and 1 s<sup>-1</sup>. Consequently, the critical concentrations ( $k_-/k_+$ ) at the two ends differ:  $\sim 0.15$  μM at the barbed end and 0.5 μM at the pointed end (18). Under other conditions the critical concentrations may be the same at the 2 ends (13, 18).

ADP-actin can also polymerize (2, 3, 6, 7, 25) but the rate and extent of polymerization are lower (6, 7) than for ATP-actin, especially in the cold (25). In 100 mM KCl, 0.1 mM MgCl<sub>2</sub>, the critical concentration for ADP-actin polymerization is about three times higher than for ATP-actin (7). Because no nucleotide hydrolysis takes place, this should be the critical concentration for both ends (11, 14). ADP-actin adds almost exclusively to the barbed end of the filament (26, 27), but the elongation rates have not been quantitated, nor has the ADP-actin concentration been varied to assess the rate constants at the two ends of the filament. In principle, the analysis of the mechanism should be much simpler than for ATP-actin, because ADP-actin is the only species involved (Fig. 1E).

During the polymerization of ATP-actin, the bound nucleotide is hydrolyzed, because virtually all of the nucleotide bound to the polymer is ADP, but the site and mechanism of hydrolysis are not yet clear (11). Fig. 1A shows the monomeric species and reactions that might participate in elongation but does not specify the site of ATP hydrolysis. Both ATP-actin and ADP-actin can, in principle, associate and dissociate from both ends of the filament. The association rates are equal to the products of second-order rate constants and the concen-

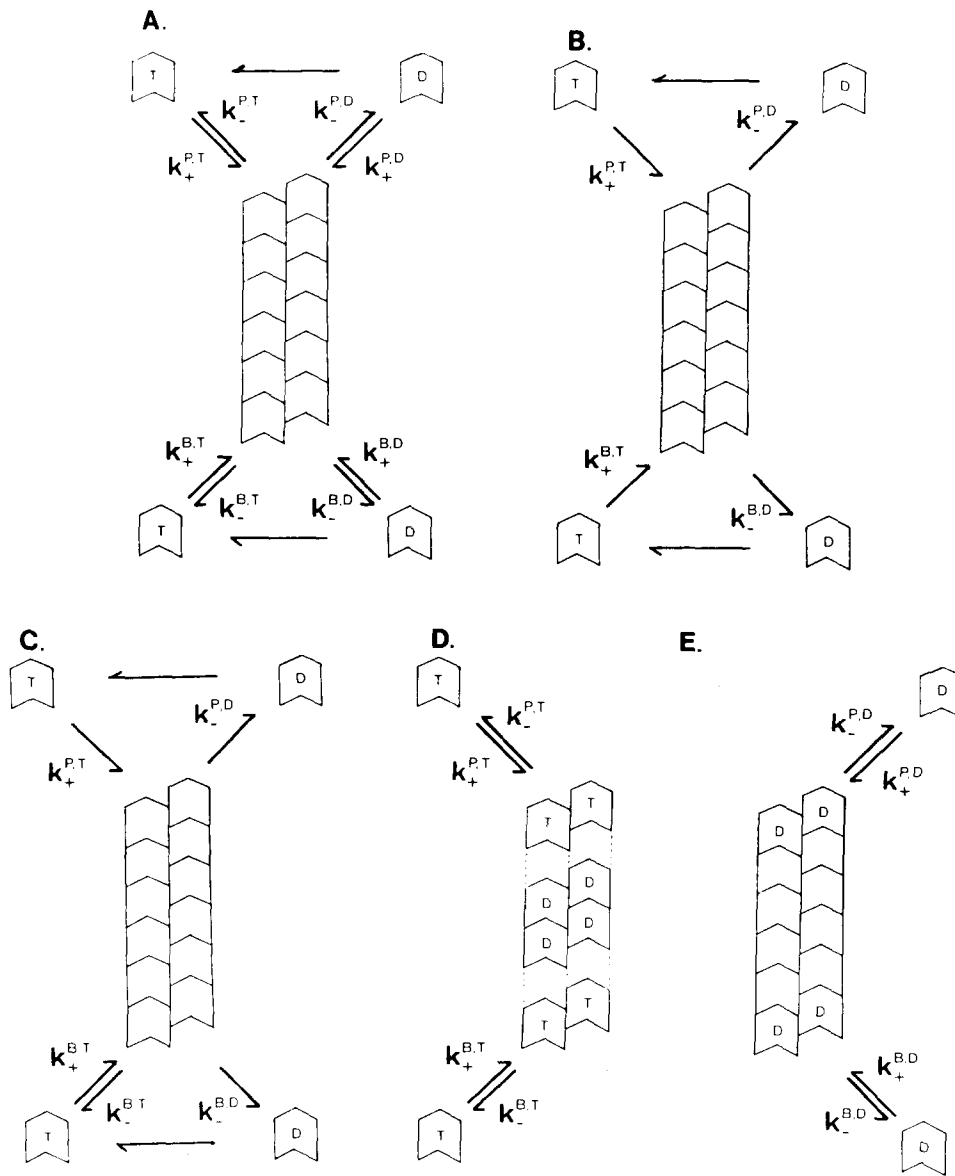


FIGURE 1 Models for the elongation of actin filaments. The subunits are drawn with barbed and pointed ends that represent the two ends of the filament that can be identified by decoration with myosin heads. The letters *T* and *D* represent bound ATP and ADP. The rate constants (*k*) are specified with subscripts to represent association (+) or dissociation (−) and two superscripts to represent barbed (*B*) or pointed (*P*) ends and bound ATP (*T*) or bound ADP (*D*). Thus,  $k_{+}^{B,T}$  is the association rate constant for ATP-actin at the barbed end of the filament. For one end of a single filament, the rate of growth in ATP is  $R = (A_1)k_{+}^{B,T}$  for an actin concentration of  $A_1$ . The exchange of ADP for ATP is considered to be rapid and to strongly favor bound ATP when ATP is present in large excess of ADP in the medium. (A) Complete model after Pollard and Moosker (13) and Hill and Kirschner (14). (B) Model of Wegner (12) with nucleotide hydrolysis coupled to association at both ends. (C) Model of Neuhaus et al. (19) with nucleotide hydrolysis coupled to association at the pointed end and occurring on internal subunits at the barbed end. (D) Model with ATP hydrolysis occurring exclusively on internal subunits at both ends. (E) Model with ADP-actin alone.

trations of ATP-actin and ADP-actin. In a medium with free ATP, the concentration of ADP-actin monomers will be low due to rapid exchange of the ADP with ATP. Consequently, the ADP-actin association rate will be negligible, and ATP-actin must be the major species that binds to the ends of the filaments. Therefore the observed association rate constants are  $k_{+}^{P,T}$  and  $k_{+}^{B,T}$ . It has sometimes been assumed that binding is coupled tightly with ATP hydrolysis at one (Fig. 1 C; reference 19) or both (Fig. 1 B; reference 12) ends, so that ADP-actin is the only species that dissociates from that end of the filament. An implication of such a mechanism is that hydrolysis of ATP on the terminal subunit is required for the addition of the next actin molecule with the consequence that hydrolysis might become rate limiting under some conditions. However, hydrolysis would have to be very fast ( $>50 \text{ s}^{-1}$ ) at the barbed end, because the elongation rate depends directly on the actin monomer concentration up to rates of at least  $50 \text{ s}^{-1}$  (13, 28).

While ADP-actin is the only dissociating species during the polymerization of ADP-actin (Fig. 1 E), there is no reason to exclude the possibility that ATP-actin can both bind to and dissociate from one (Fig. 1 C) or both (Fig. 1, A and D) ends

of a filament during elongation or at steady state. In fact, the steady-state rate of actin subunit exchange appears to exceed the rate of hydrolysis of bound ATP (29), suggesting that ATP-actin occupies the end(s) of the filament and can dissociate under some conditions. Similarly, ATP hydrolysis can lag behind polymerization under some (30), but not all (29), conditions, suggesting that ATP-actin can occupy the end(s) of the polymer and that ATP hydrolysis can be separated from binding in both time and space. Even those experiments where polymerization and ATP hydrolysis appear to follow exactly the same time course (29) are consistent with a mechanism where ATP is hydrolyzed on internal subunits, because the binding of individual actin molecules is so fast ( $>100 \text{ s}^{-1}$  at  $10 \mu\text{M}$  actin) that hydrolysis on the terminal subunit cannot be distinguished from hydrolysis 10 or more subunits from the end.

The most straightforward way to answer the questions about the mechanism of actin polymerization and the hydrolysis of the bound ATP is to identify all of the possible reactions and to measure their rate constants. In this study, I have compared the polymerization properties of ATP-actin and ADP-actin using hexokinase, glucose, and ATP to vary

reversibly the species of nucleotide in the solution and bound to the actin. The use of hexokinase for these experiments was suggested by Dr. Michael Caplow of the University of North Carolina. In a buffer containing KCl, MgCl<sub>2</sub>, and EGTA, the critical concentration for *Acanthamoeba* actin polymerization is 50 times higher for ADP-actin than ATP-actin. This is due to major differences in the elongation rate constants: the association rate constant is 10 times higher and the dissociation rate constant is five times lower for ATP-actin than ADP-actin. Since the dissociation rate constant for ATP-actin is lower than for ADP-actin, ATP-actin is the major species occupying and dissociating from the ends of an actin filament during elongation in ATP. Consequently, ATP hydrolysis must occur on internal actin subunits rather than at the ends. These conclusions have been supported by a subsequent study (31), where we found that hydrolysis of ATP bound to actin lags behind elongation under the same conditions used here. The hydrolysis of bound ATP appears to be a first-order process, because the rate depends on the concentration of polymerized ATP-actin, not the elongation rate. The first-order rate constant is about 0.07 s<sup>-1</sup>. Knowledge of these rate constants provides a number of insights regarding the mechanism of polymerization.

## MATERIALS AND METHODS

**Materials:** ATP (grade I), yeast hexokinase (Type C-302), Sephadex G150, imidazole (grade III), and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO. Pyrene-iodoacetamide was from Molecular Probes, Junction City, OR. [ $\alpha$ -<sup>32</sup>P]ATP prepared by the method of Johnson and Walseth (32) was a generous gift of Dr. Peter Devreotes of the Johns Hopkins Medical School. Polyethyleneimine-cellulose thin-layer plates were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ.

**Actin Purification:** Actin was purified from *Acanthamoeba castellanii* by a modification of the method of Gordon et al. (33). A 0.34 M sucrose extract was chromatographed on DEAE-cellulose (34) and the actin-containing fractions eluting between 0.22 and 0.30 M KCl were pooled. The actin was polymerized in 2 mM MgCl<sub>2</sub> at 25°C and pelleted by centrifugation for 2 h at 38,000 rpm in a Ti45 rotor at 20°C. The pellets were homogenized in buffer G (2 mM imidazole, pH 7.0, 0.5 mM dithiothreitol, 0.2 mM CaCl<sub>2</sub>, 0.2 mM ATP) and dialyzed against several changes of buffer G over 2–3 d. After clarification by centrifugation at 20,000 g for 10 min at 4°C, most of the actin was repolymerized in 50 mM KCl, 2 mM MgCl<sub>2</sub>, 10 mM imidazole, pH 7.0, at 25°C for 30 min. After pelleting and depolymerization, purification was completed by gel permeation chromatography on a 4 × 51-cm column of Sephadex G-150 equilibrated with buffer G. Peak fractions typically had concentrations of 40–42 μM. The remainder of the clarified, once-polymerized actin was polymerized and labeled with pyrene-iodoacetamide as described by Cooper et al. (35) using a 7:1 ratio of dye to actin and mixing slowly with a magnetic stirring bar for 5–18 h. Depending on the time of the reaction, 0.7–1.0 pyrene molecules were coupled to the actin. After pelleting and depolymerization, the pyrene-actin was purified by chromatography on G-150. Pyrene actin was mixed with unlabeled actin to give 0.02–0.05 pyrenes per actin.

**Polymerization Assays:** Actin polymer concentration and the time course of polymerization were measured by fluorometry exactly as described by Cooper et al. (35). The critical concentrations were measured by the method of Tobacman et al. (36) where actin is polymerized at high concentration (e.g., 24 μM) and then diluted in the same buffer to a series of lower concentrations. The diluted samples were sonicated for 5 s twice in a bath sonicator (Laboratory Supplies Company, Inc., Hicksville, NY) and then incubated for 10 h at 25°C to reach a new steady state. Elongation rates were measured using ADP-actin filaments as nuclei (28).

**Analysis of Nucleotides:** Actin monomers (10 μM) in buffer G were mixed with a trace of [ $\alpha$ -<sup>32</sup>P]ATP to give ~3 × 10<sup>6</sup> cpm/ml and equilibrated for 24 h at 4°C. Samples for the analysis of total nucleotide composition were prepared by mixing 25-μl aliquots of actin solution with 50 μl of acetone and heating to 100°C in a water bath for 2–3 min. The acetone was removed under vacuum and the nucleotides were separated using internal standards by thin-layer chromatography on polyethyleneimine-cellulose plates with 1.0 M formic acid, 0.5 M LiCl for the solvent (37). Protein-bound nucleotides were prepared for thin-layer chromatography by mixing 30 μl of actin with a 12-μl

pellet of Dowex-1 (Dow Chemical Co., Midland, MI) in 10 mM imidazole, pH 7 for 2 min to bind free nucleotides. The Dowex-1 was pelleted by centrifugation for 30 s in an Eppendorf microcentrifuge. A 25-μl sample of the supernatant containing the actin and bound nucleotides was treated as above for total nucleotides. Radioactive spots on the thin-layer plates were identified by autoradiography, cut out, and counted in a liquid scintillation counter. The background counts on the plates were determined by cutting out and counting blank regions of the plate between ATP and ADP, having an area equal to the nucleotide spots.

## RESULTS

Actin polymerizes with a normal time course in the presence of hexokinase (Fig. 2A), but when glucose is added to the fully polymerized actin there is a rapid decrease in the polymer concentration (Fig. 2B). Glucose in the absence of hexokinase has no effect. The decline in the polymer concentration is more rapid and complete when the sample is briefly sonicated. Sonication of fully polymerized actin has no effect on the steady-state fluorescence. Without sonication (Fig. 2D) hexokinase plus glucose causes the polymer concentration to fall, with a half-time in the range of 15–40 min, to an intermediate level, followed by a slower decline over a period of many hours. Plots of log fluorescence vs. time after the addition of glucose are highly nonlinear, as expected from a population of filaments that vary in length.

The hexokinase and glucose convert very efficiently all of the ATP in the samples of polymerized actin to ADP. Within 2 min all of the detectable nucleotide is converted from ATP

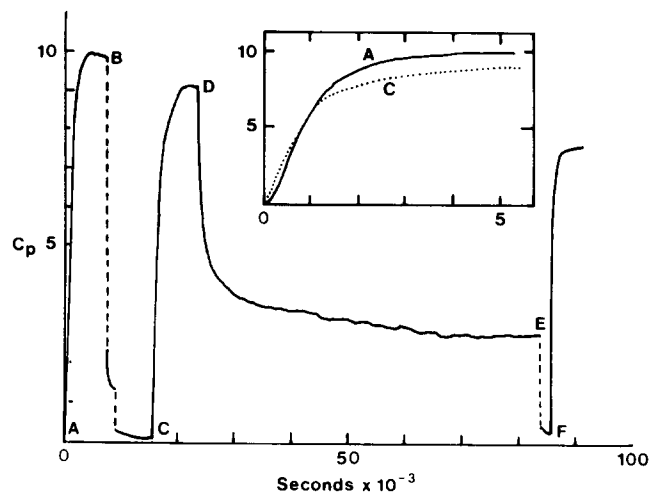


FIGURE 2 Reversible polymerization of 5 μM *Acanthamoeba* actin by change in the concentrations of ATP and ADP. Polymer concentration in arbitrary units was measured by the fluorescence of pyrene-labeled actin. Conditions: 50 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1 mM CaCl<sub>2</sub>, 10 mM imidazole (pH 7.0), 25 U/ml yeast hexokinase, 25°C. (A) Initial ATP concentration was 100 μM. The solid line in the inset shows this time course of polymerization in more detail. (B) When the steady state was reached, D-glucose was added to a concentration of 200 μM and the sample was sonicated twice for 5 s directly in the cuvette. (C) When a new steady state was reached, ATP was added to a concentration of 200 μM. At this point [ATP] = [total ATP] - [glucose] = 100 μM and the [ADP] = [total glucose] = 200 μM. The detailed time course of repolymerization is given by the dotted line in the inset. (D) When a new steady state was reached, D-glucose was added to a concentration of 200 μM to observe the time course of depolymerization without sonication. (E) After prolonged incubation, the sample was sonicated to hasten depolymerization. (F) ATP was added to a concentration of 200 μM to repolymerize the actin. At this point [ATP] = 100 μM and [ADP] = [glucose] = 400 μM.

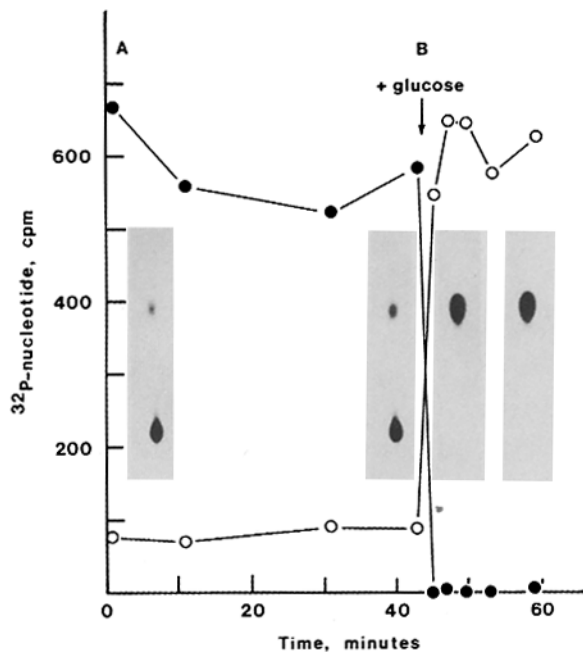


FIGURE 3 The concentrations of ATP (●) and ADP (○) in an experiment similar to that in Fig. 2. The nucleotides were labeled in the  $\alpha$  position, separated by thin-layer chromatography, and quantitated by scintillation counting. A and B correspond to A and B in Fig. 2. At A, polymerization is initiated. At B, glucose was added to the solution of polymerized actin and hexokinase. The insets illustrate the separation of ATP and ADP on the thin-layer plates.

to ADP (Fig. 3). Assays for nucleotide bound to actin revealed ADP and no ATP after treatment with hexokinase and glucose, providing that the actin was polymerized. In contrast, if actin monomers were treated with hexokinase and glucose, ATP bound to actin was detectable for  $>6$  h.

The effect of hexokinase plus glucose on actin polymerization is almost completely reversible; addition of ATP in excess of the glucose results in rapid repolymerization of the actin to a steady-state level within 10% of the first cycle of polymerization (Fig. 2C). The time course of repolymerization after addition of excess ATP differs slightly from the initial cycle of polymerization (Fig. 2, inset). The repolymerizing sample has little or no lag, presumably due to a few remaining actin filaments that act as nuclei. In some cases the half-time is also slightly longer during repolymerization, perhaps due to the time required for nucleotide exchange. Samples of actin have been taken through four cycles of polymerization and depolymerization in this way, but the extent of the repolymerization in excess ATP declines with each cycle (Fig. 2). This lack of complete recovery is related to the duration of the experiment, because recovery is best when each cycle is carried out quickly using sonication to hasten depolymerization.

The extent to which hexokinase plus glucose can reverse polymerization depends on the actin concentration. A concentration of 4–5  $\mu\text{M}$  is the maximum to obtain nearly complete reversal of polymerization. At higher concentrations of actin, for example 8  $\mu\text{M}$ , hexokinase plus glucose cause the polymer concentration to fall to a lower value (about 3  $\mu\text{M}$  in this example) that remains constant even after multiple treatments with the sonicator.

The critical concentration for polymerization in KCl,  $\text{MgCl}_2$ , and EGTA is 0.10–0.15  $\mu\text{M}$  for ATP-actin and  $\sim 5.5$

$\mu\text{M}$  for ADP-actin in hexokinase and glucose (Fig. 4). Furthermore, the slopes of the linear plots of fluorescence vs. total actin are the same for ADP-actin and ATP-actin above the critical concentration. Most of the actin in these samples must remain native during the assay, because the effect of hexokinase and glucose is reversed almost completely simply by adding excess ATP to the glucose sample and glucose to the ATP sample (Fig. 4). The critical concentrations can be reversed exactly, but the slopes of the linear plots are 10–15% lower after the conditions are reversed. The fact that the critical concentration is completely reversible shows that the actin is not denatured by these treatments with hexokinase and glucose. The 10% loss of fluorescence is the same as the loss observed in continuously monitored samples subjected to similar nucleotide-induced changes in the critical concentration (Fig. 2). The large difference in the critical concentrations for ADP-actin and ATP-actin explain the ability of hexokinase and glucose to depolymerize actin completely at low concentrations and partially at concentrations above the critical concentration.

The reason for the difference in the critical concentrations is clear from an analysis of elongation rates (Fig. 5). Plots of elongation rates vs. monomer concentration (Fig. 5E) give critical concentrations similar to those measured from the steady-state polymer concentration: 0.1  $\mu\text{M}$  for ATP-actin and 4.5  $\mu\text{M}$  for ADP-actin. In addition, these plots provide some information about the relative values of the elongation rate constants, providing that the number concentration of

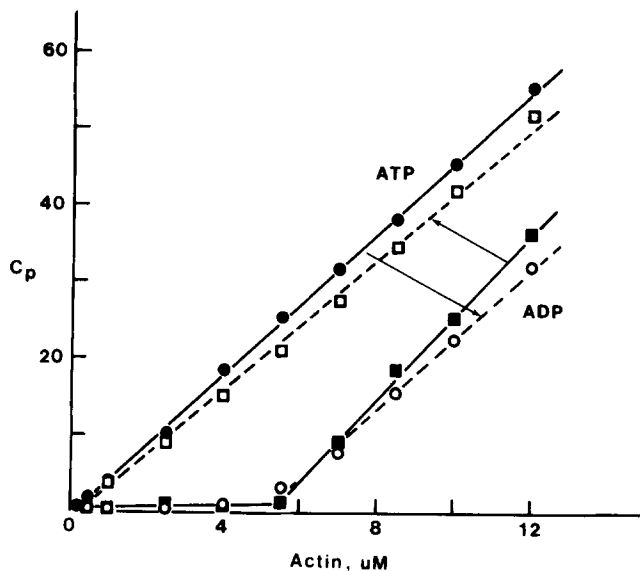


FIGURE 4 Critical concentrations for ADP-actin and ATP-actin, measured by the dependence of the steady-state polymer concentration on the total actin concentration. Conditions: 50 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, 0.1 mM  $\text{CaCl}_2$ , 10 mM imidazole (pH 7.0), 10 U/ml hexokinase. The ATP and ADP concentrations were varied. Set No. 1. Initial conditions (●) were 100  $\mu\text{M}$  ATP, no glucose for 10 h. After reading the fluorescence, glucose was added to give 0  $\mu\text{M}$  ATP and 100  $\mu\text{M}$  ADP, the samples were sonicated and then incubated for 13 h at 25°C, and the fluorescence was recorded (○). Set No. 2. Initial conditions (■): 200  $\mu\text{M}$  glucose was added to give 0  $\mu\text{M}$  ATP and 100  $\mu\text{M}$  ADP for 10 h at 25°C. After reading the fluorescence, ATP was added to 250  $\mu\text{M}$  to give 150  $\mu\text{M}$  ATP and 200  $\mu\text{M}$  ADP, the samples were sonicated and then incubated for 13 h at 25°C, and the fluorescence was recorded (□). The points were fit by linear regression.

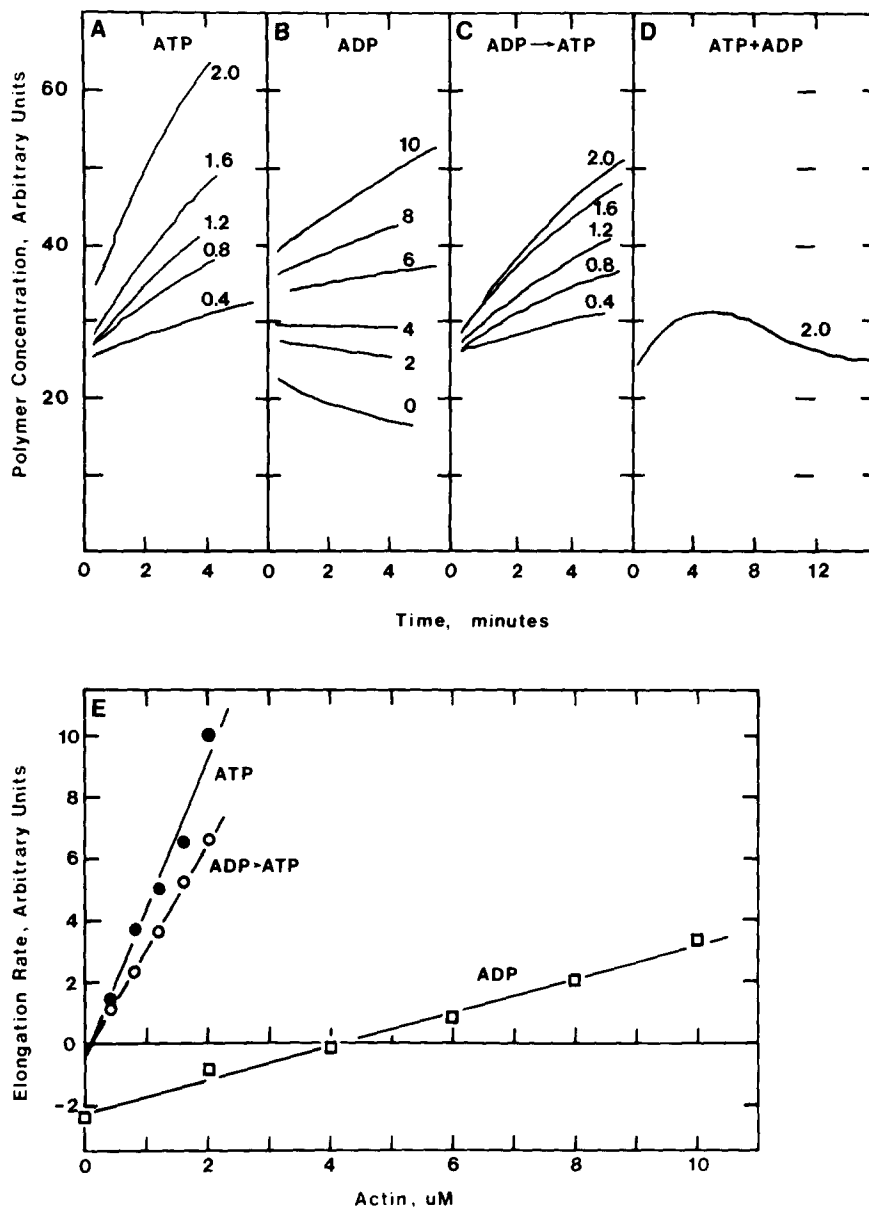


FIGURE 5 Nucleated rate of polymerization of ATP- and ADP-actin by the method of Pollard (28). Conditions: 50 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1 mM CaCl<sub>2</sub>, 10 mM imidazole (pH 7.0). The ADP-actin filament nuclei were prepared by treating a 20-μM stock of polymerized actin with 20 U/ml hexokinase and 400 μM glucose overnight at 25°C in polymerization buffer. The final concentration of nuclei was 1 μM. (A) Time course of polymerization of ATP-actin. Subunit concentrations in micromolar are indicated next to each curve. (B) Time course of polymerization of ADP-actin. The ADP-actin monomers were prepared by incubating 20 μM ATP actin monomers in buffer G with 50 μM MgCl<sub>2</sub>, 400 μM glucose, and 25 U/ml hexokinase for 70 h at 4°C. (C) Time course of polymerization of ATP-actin prepared from the ADP-actin in B by incubating for 10 h at 4°C with 200 μM ATP in excess of the glucose. (D) Time course of polymerization of 2 μM actin incubated in hexokinase and glucose for 4 h. (E) Dependence of the nucleated rate of polymerization on the actin concentration. All plots were fit by linear regression. (●) ATP-actin as in A; (○) ADP-actin treated with ATP as in C; (□) ADP-actin.

filaments is the same in the samples being compared (28). Assuming that this is true here, the experiment in Fig. 5E shows that the critical concentration for ADP-actin is about 45 times higher than for ATP-actin, because the association rate constant (slope) is about 10 times lower and the dissociation rate constant ( $\gamma$ -intercept) is five times higher for ADP-actin. As in the steady-state critical concentration experiment (Fig. 4), the fluorescence of ATP-actin is slightly lower after it has been treated with hexokinase and glucose followed by excess ATP to displace the ADP.

The slow exchange of ATP bound to actin monomers for ADP in the solution after hexokinase and glucose are added to the monomer sample complicates this experiment. It is necessary to incubate the monomers with hexokinase and glucose for 2–3 d before all traces of ATP-actin are gone. The presence of ATP-actin in such a sample is detected most sensitively from the anomalous time course of polymerization in the presence of ADP-actin filament nuclei (Fig. 5D). ATP-actin polymerizes with a hyperbolic time course (28). ADP-actin does the same though at much lower rates. In contrast, mixtures of ATP-actin and ADP-actin can produce complex

time courses (Fig. 5D). For example, if elongation of a 2 μM mixture of ATP-actin and ADP-actin is measured, the initial rate is moderately fast as the ATP-actin adds to the ends of the nuclei. However, after a few minutes the polymer concentration plateaus and then declines. Presumably the plateau occurs when the concentration of ATP-actin monomer falls to its critical concentration, whereas the subsequent depolymerization is necessary to reset the monomer concentration to a level appropriate for the ADP-actin critical concentration.

## DISCUSSION

### *Evaluation of the Data and Comparison with Other Work*

The conclusions to be drawn from this work depend on the reliability of the methods that I have used for measuring the critical concentration and rate constants. The fluorescence assay for polymer concentration has been tested and verified in several laboratories (e.g., references 21–23, 28, 29, 35, 36). Further, the specific fluorescence of polymer is the same in

ATP and ADP (Fig. 4). The critical concentrations for ADP-actin and ATP-actin should be reliable, because nearly the same values are obtained by both steady-state (Fig. 4) and elongation-rate (Fig. 5) methods. The difference in the critical concentrations in ADP and ATP is not due to denaturation of the actin in ADP, because actin can be incubated in ADP for at least 20 h at 25°C and then returned to ATP where it has the same critical concentration as ATP-actin that was never exposed to ADP (Fig. 4).

The most important assay is the dependence of the nucleated polymerization rate on actin concentration (Fig. 5), because it provides the elongation rate constants that are required to interpret the experiments. This assay appears to measure the elongation exclusively according to several criteria: (a) at the concentrations used, actin monomer alone does not polymerize during the time required to record the initial rate (28); (b) the time course of nucleated polymerization is first order over the whole time course of the reaction (data not illustrated) showing that the number of filaments is constant throughout the reaction; and (c) plots of initial rate vs. actin monomer concentration are linear above the critical concentration (Fig. 5E), as expected from the accepted mechanism (11, 13, 20) for elongation. On the other hand, it must be recognized that there are several potential problems with this assay. The most important is the assumption that the filament number concentration is the same in the two buffers used here, one with ATP and the other with ADP. Every effort was made to handle the samples identically, but it is conceivable that the ADP-actin filaments differ in their mechanical stability when mixed with ATP or ADP. I consider this to be improbable, because free nucleotides do not exchange rapidly with nucleotides bound to the subunits in filaments (38), and the vast majority of subunits in the filament have bound ADP whether in an ATP or ADP solution. However, any difference in mechanical stability could give different filament number concentrations under the two different conditions. This would not affect the measurement of the critical concentration, but it would change the slope of the plot of rate vs. actin concentration and the apparent rate constants. For example, if the filaments fragmented more readily in ADP, the number concentration would be higher than in ATP, and the observed slopes in Fig. 4 would give an overestimate of  $k_+$  and underestimate of  $k_-$  compared with the rate constants in ATP. A second problem is the difficulty in obtaining reliable initial rates below the critical concentration. The filaments used as nuclei shorten and the fluorescence decreases, but the reaction is not first order, presumably because the number concentration of filaments changes rapidly as the short filaments disappear. A third problem is the unexplained difference in the slope of the ATP-actin plots before and after exchange of the bound nucleotide for ADP. This may be due to a small amount of denaturation, the presence of ADP, and/or a subpopulation of monomers that retain bound ADP, but it probably does not alter the main conclusions. Finally, the fluorometric assay does not distinguish between the ends of the filament. Some of these problems will be addressed in the future using electron microscopic assays for elongation.

Turning now to other work on the polymerization of ADP-actin, the data reported by Cooke (7) for muscle actin is similar to my result at least qualitatively. He found that the critical concentration for polymerization is higher for ADP-actin than ATP-actin, but did not study the mechanism in

any detail. However, the data in Fig. 3 of his paper are particularly interesting and can be reinterpreted in light of the data presented here. In that experiment Cooke measured the dependence of the initial rate of polymerization on the actin concentration. No nuclei were added to the actin, but given the way that the actin was prepared, and the absence of a lag at the outset of polymerization, the actin samples must have contained nuclei. Therefore, he was looking largely at elongation. If one calculates a least squares fit of the points for ATP-actin and ADP-actin, including the values for the critical concentrations that he measured at steady state, the fit is better than the lines drawn in the paper. The corrected plots of rate vs. actin concentration show that the critical concentration ( $x$ -intercept) is higher for ADP actin, because the slope ( $k_+$ ) is lower, although the  $y$ -intercept ( $k_-$ ) for ADP-actin and ATP-actin are about the same. Strictly speaking, this data should not be interpreted this way because the experiment was really not designed to measure elongation rates, but the trends illustrated in this experiment are strikingly similar to the results obtained here with *Acanthamoeba* actin under different conditions (Fig. 5).

Lal and colleagues (39, 40) have also presented a preliminary report on the polymerization of ADP-actin from muscle. They found that the critical concentration for ADP-actin is higher than for ATP-actin by a factor ranging from 2 to 10, depending on the solution conditions. They used a different method with trimers as the nuclei to estimate minimum values for the elongation rate constants and reported  $k_+^T = 2-6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_+^D = 3-4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_-^T = 0.4-2 \text{ s}^{-1}$ , and  $k_-^D = 2-7 \text{ s}^{-1}$ . As in my experiments there was a substantial difference in the dissociation constants, but they found little difference in the association rate constants. The apparent disagreement in our results is probably attributable to the assay methods, but there may be a real difference between muscle actin and *Acanthamoeba* actin.

### Interpretation

The simple experiments reported here provide a number of insights into the fate of the bound nucleotide during the polymerization of actin.

First, the bound nucleotide affects the elongation rate constants at both ends of the filament. The situation at the barbed end is clear. The large differences in the slopes of plots of elongation rate vs. monomer concentration for ATP-actin and ADP-actin (Fig. 5) can only be explained if the bound nucleotide affects the association rate constant at the barbed end where most of the growth takes place (13, 18, 26). The conclusion that ATP-actin and ADP-actin have different rate constants at the pointed end is based on the following reasoning. For ADP-actin the critical concentrations should be the same at the two ends (11, 12, 14). This is,  $A_1^\infty = k_-^{P,D}/k_+^{P,D} = k_-^{B,D}/k_+^{B,D} = 5 \mu\text{M}$  as measured by both steady-state and elongation-rate methods. This critical concentration is not only higher than the critical concentration for ATP-actin at the barbed end (estimated to be 0.10–0.15  $\mu\text{M}$  under these conditions), but also higher than the critical concentration for ATP-actin at the pointed end (estimated to be  $\sim 0.6 \mu\text{M}$  by electron microscopy (18) and by experiments with a barbed end capping protein from *Acanthamoeba* [41]). Thus the critical concentrations at both ends are higher for ADP-actin than ATP-actin and at least one rate constant at each end must be different for ADP-actin and ATP-actin. The differ-

ence could be in either the association constants or the dissociation constants, or both. Given the overall association rate constant  $k_+^T = k_+^{B,T} + k_+^{P,T} = (10 \times 10^6) + (2 \times 10^6) = 12 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (13, 18) and a critical concentration of 0.10  $\mu\text{M}$  in ATP, the overall dissociation rate constant,  $k_-^T = k_-^{B,T} + k_-^{P,T}$ , is  $1.2 \text{ s}^{-1}$ . From electron microscopic measurements (13, 18),  $k_-^{B,T}$  is slightly larger than  $k_-^{P,T}$ , but both are  $\sim 0.6 \text{ s}^{-1}$  under these conditions. Considering now ADP-actin, the overall association rate constant,  $k_+^D = k_+^{B,D} + k_+^{P,D}$ , can be estimated by comparing the slopes of the plots in Fig. 5E. This gives a value of  $\sim 1.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  for  $k_+^D$ . From the critical concentration in ADP and this value for  $k_+^D$ , the overall dissociation rate constant for ADP-actin,  $k_-^D = k_-^{B,D} + k_-^{P,D}$ , is calculated to be  $\sim 6 \text{ s}^{-1}$ . It is more difficult to learn how the two ends contribute to these overall rate constants without measuring all of the rate constants directly. However, because neither Hayashi and Ip (26) nor Kondo and Ishiwata (27) observed any addition of ADP-actin to the pointed end of decorated actin filaments by electron microscopy, and because the overall dissociation constant is not extremely large,  $k_+^{B,D}$  is approximately equal to  $k_+^D$  and  $k_+^{P,D}$  is likely to be smaller than  $k_+^{P,T}$ . Thus the association rate constants for ADP-actin are smaller than for ATP-actin at both ends. A similar argument cannot be made about the dissociation rate constants, so direct measurements by electron microscopy will be required to establish how each of the two ends contributes to the overall dissociation constant for ADP-actin.

Second, ATP-actin is the major species that dissociates from the filament during the polymerization of ATP-actin (Fig. 1D). This conclusion is based on the  $y$ -intercepts for ATP-actin and ADP-actin in Fig. 5E. The observed dissociation rate of ADP-actin at zero monomer concentration is five times larger than the dissociation rate for ATP-actin estimated by linear extrapolation from positive growth rates. If this extrapolation gives an accurate value for the dissociation rate in ATP and if ADP-actin were the species that dissociates from the ends of the filament during ATP-actin polymerization (as predicted by the mechanisms in Fig. 1, B and C), then the  $y$ -intercepts would be the same in ATP and ADP. Instead, ADP-actin dissociates faster than the apparent dissociation rate in ATP, so that the more slowly dissociating ATP-actin or some other species must occupy the end of the filament. The only other species that has been considered is ADP- $P_i$ -actin (11, 42). It is unlikely to be a major dissociating species (unless ATP is resynthesized when ADP- $P_i$ -actin dissociates), because the amount of ATP hydrolyzed during polymerization is equal to the final amount of actin incorporated into polymer, not the amount polymerized plus the number of actin molecules that dissociate during assembly (11). The data are adequate to conclude that ATP-actin is the major dissociating species during elongation in ATP and that the dissociation of ADP-actin from the two ends together is more rapid than the dissociation of ATP-actin. However, measurement of the two ADP-dissociation constants will be required to know whether the dissociation rates in ADP are approximately equal, as shown for ATP-actin, or whether there is a stronger bias toward one end—a result that would have important consequences for events at steady state where it is possible for both ATP-actin and ADP-actin to occupy the ends of the polymer.

Third, because the barbed end (and perhaps the pointed end) of growing actin filaments is occupied by ATP-actin, the ATP bound to the subunits must be hydrolyzed predomi-

nantly at some internal position rather than on the terminal subunit. The data presented here do not provide direct information about the site of hydrolysis, but they provided the impetus for subsequent experiments where we found that the rate of hydrolysis of ATP by actin during polymerization depends on the concentration of polymerized ATP-actin, not the growth rate (31). This is consistent with a first-order reaction mechanism as predicted theoretically by Hill and Kirschner (14). Thus ATP hydrolysis probably occurs randomly on internal positions, and the number of ATP-actin subunits near the end of a filament depends on the rate of elongation and its immediate past history.

### Mechanism of Polymerization

In ATP with a concentration of actin monomers well above the critical concentrations for the two ends, the polymer will grow by the mechanism illustrated in Fig. 1D. ATP-actin will bind to both ends at rates determined by the concentration of monomer and the association rate constants. ADP-actin will not participate because its concentration will be low. ATP-actin will also dissociate from the polymer ends at low rates ( $\sim 1 \text{ s}^{-1}$ ) determined by the dissociation constants, but there will be net growth because the association rates will be higher. The bound ATP is hydrolyzed relatively slowly ( $k_{ATPase} = 0.07 \text{ s}^{-1}$ ) in a first-order reaction (31). When the growth rate is high, the probability of this occurring at the end of the filament is negligible, so virtually all of the ATP is hydrolyzed at internal positions. Consequently ATP-actin will occupy the ends of the growing polymer. Since ATP-actin has a lower dissociation constant than ADP-actin, the separation of binding from hydrolysis will stabilize the growing polymer.

When the actin monomer concentration is less than or equal to the critical concentration, there will be a higher probability that ADP-actin will occupy the end of the polymer and ADP-actin may also represent a substantial fraction of the monomer pool reacting with the filament, so that the system will be described by the more complex set of reactions illustrated in Fig. 1A. First consider the two ends individually at steady state. If the ATP concentration is high, ATP-actin will be the major species binding to the end of the filament at a rate that balances the dissociation rate ( $\sim 1 \text{ s}^{-1}$ ). If the ATPase rate constant for bulk polymerization applies to events at the end of the filament, the ends undergoing subunit exchange at the critical concentration will be occupied mainly by ATP-actin because the rate of hydrolysis ( $0.07 \text{ s}^{-1}$ ) is lower than the exchange rate for ATP-actin. This conclusion is consistent with measurements made at steady state (29) and the rate constants calculated from presteady-state experiments (13, 18, 31, present study). Note, however, that although  $< 1$  in 10 of the ATP-actins that bind to the end will hydrolyze their ATP while bound to the end, this event will transiently destabilize the end of the polymer owing to the larger dissociation rate of ADP-actin. Thus the presence of ATP-actins at the end of a filament composed of ADP-actin subunits will tend to block the dissociation of internal ADP-actin subunits. It is expected that substantial fluctuations in length may result. A filament with internal ADP-subunits will shorten rapidly when an ADP-actin is exposed on the end by dissociation of terminal ATP-actin(s) or by hydrolysis of ATP bound to the terminal subunit(s), until another ATP-actin binds to the end. Then the polymer will be more stable and more likely to lengthen by adding another ATP-actin.

When the ATP-actin monomer concentration is below the

critical concentration, subunit dissociation will exceed association and ADP-actin will occupy the end a greater fraction of the time. ATP-actin binding from the monomer pool will still occupy the end part of the time and will temporarily stabilize the end. This will lead to some variation in the rate of depolymerization.

These complications must be taken into account during the quantitative analysis of steady-state subunit exchange reactions (12, 15, 29) or the time course of depolymerization. At steady state the association rate will be  $A_1^T \cdot k_+^T$ , but the off rate will be neither  $k_-^T$  nor  $k_-^D$ , but rather  $F^T \cdot k_-^T + F^D \cdot k_-^D$ , (where  $F^T$  is the fraction of terminal subunits with bound ATP and  $F^D$  is the fraction of terminal subunits with ATP). This should give a steady state monomer concentration ( $A_1^\infty$ ) that is slightly higher than the critical concentration measured from the dependence of the elongation rate on the ATP-actin concentration. That is,  $A_1^\infty = (F^T k_-^T + F^D k_-^D) / k_+^T > k_-^T / k_+^T =$  critical concentration for elongation. This effect can be observed as an overshoot during the polymerization of actin under some conditions, such as illustrated dramatically during the nucleated polymerization of a mixture of ATP-actin and ADP-actin (Fig. 5D).

The actual conditions at steady state in  $MgCl_2$  and ATP will be more complex than described above, because the two ends of the filament have different critical concentrations (13, 18). The reactions illustrated in Fig. 1A will still apply, but the steady-state concentration of monomers will be above the critical concentration at the barbed end and below the critical concentration at the pointed end. Consequently, ADP-actin will probably occupy the pointed end a larger fraction of the time than at the barbed end, but without knowing the values of the ADP-actin dissociation constants at the two ends, the details of the reactions cannot be predicted.

These conclusions highlight the importance of the bound nucleotide in determining the properties of the elongation reaction. Because it now appears that hydrolysis does not usually take place on the terminal subunit either during elongation in ATP (30, 31) or at steady state (29), one must consider mechanisms by which bound nucleotides on subunits other than the end of the filament can have an indirect effect on the association and dissociation of monomers and give rise to different critical concentrations at the two ends under some conditions.

There are many interesting parallels in the mechanisms of polymerization of actin and tubulin. In both cases the association of NTP subunits is very fast (perhaps diffusion limited) at one end and somewhat slower at the other end (13, 43). In both cases NTP hydrolysis seems to be a slow first-order reaction that occurs after the incorporation of subunits into the polymer (31, 44), but the NTPase rate constant is 10 times larger for actin than tubulin. In both cases NDP subunits dissociate faster than NTP subunits. For tubulin the difference in the dissociation rate constants may be >100-fold (45). This leads to a dramatic difference in the stability of microtubules when GTP-tubulin or GDP-tubulin occupy the ends, with the result that plots of elongation rate vs. the concentration of GTP-tubulin are nonlinear below the critical concentration. Above the critical concentration, GTP-tubulin with a low dissociation constant occupies the end(s) of the polymer, but below the critical concentration GDP-tubulin occupies the ends a larger fraction of the time and its large dissociation constant comes to dominate the assembly process. For actin there is only a fivefold difference in the dissociation constants

for ADP-actin and ATP-actin, so the species of actin occupying the ends has a more subtle effect on the stability of the polymer as shown in Fig. 5E. In both cases, some important parameters, especially the association and dissociation rate constants for NDP-subunits still need to be measured directly. Then a fuller comparison will be possible.

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