An Intermediate Form of ADP-F-actin*

Keith E. Bryan and Peter A. Rubenstein‡
From the Department of Biochemistry, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242

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With yeast actin, contrary to other actins, filament formation, ATP hydrolysis, and P_i release are concurrent at low actin concentrations, the condition usually employed to assess actin polymerization. This observation leads to a question concerning the conformation of the filament barbed end that might be recognized by specific actin-binding proteins. To try to detect possible new actin polymer conformations that might be intermediate in the pathway leading to mature F-actin, we monitored the change in intrinsic tryptophan fluorescence of yeast and muscle actins polymerized at pH 6 to accelerate the rate of filament formation. This allowed temporal resolution of the P_i release process from the slower process of polymerization. With both actins, we detected a biphasic instead of the usual monophasic fluorescence change, a rapid decrease that tracks with filament formation followed by a slower rebound (the second phase). This second phase postpolymerization conformational change requires P_i release and occurs nearly coincident with its release. The addition of P_i causes this second phase response to disappear, and the inclusion of P_i during polymerization prevents its appearance. At pH 7.5, with higher yeast actin concentrations to accelerate polymerization, a two-phase fluorescence change is also observed. In this case, the second phase change lags substantially behind P_i release. P_i release could also be resolved from polymer formation. V159N yeast actin, hypothesized previously as remaining in a postpolymerization ATP-like state, exhibits the same two-phase intrinsic tryptophan fluorescence behavior as wild-type yeast actin. Together, these observations demonstrate the presence of an intermediate filament state between ADP-P_i and mature ADP-F-actin.

Actin, a highly conserved and abundant protein in virtually every eukaryotic cell, plays an important role in a number of cellular processes, including cell structure determination, cytokinesis, locomotion, and the production of contractile force (1). Participation of actin in the majority of these biological processes requires that it polymerize from its globular form (G-actin) into its filamentous (F-actin) form. In vivo, actin filaments are dynamic structures that constantly undergo cycles of polymerization and depolymerization in response to cellular needs.

Actin binds a molecule of adenine nucleotide with high affinity in the central cleft separating the two domains of the protein (2). G-actin exhibits a slow ATPase activity that is enhanced by more than 3 orders of magnitude during polymerization (3). With most actins and especially with muscle actin, subsequent to polymerization and ATP hydrolysis, the P_i is retained, creating an intermediate ADP-P_i state prior to its release to yield the ADP-bound form (4, 5). Hence, inside most eukaryotic cells, newly polymerized actin will be composed of either ATP- or ADP-P_i-actin subunits, and as the filament ages, this filament composition shifts to a mixture composed primarily of ADP-bound subunits with some ADP-P_i, and only a few ATP subunits at the filament ends (6, 7).

An extensive body of evidence indicates that the regulation of cytoskeletal dynamics depends on two major factors: 1) the hydrolysis of actin-bound ATP and subsequent P_i release and 2) a wide array of actin-binding proteins. Progression from one nucleotide-bound form to another has several functional implications for filament dynamics. First, ATP and ADP-P_i filaments have essentially equivalent stabilities, whereas ADP filaments are less stable (8). The addition of millimolar P_i or a phosphate analogue such as aluminum or beryllium fluoride to ADP-F-actin in vitro will force it back to an ADP-P_i state (9, 10). Second, the state of the bound nucleotide influences filament polarity. The barbed or plus end of the filament, enriched in ATP or ADP-P_i, actin, has an approximate 10-fold lower critical concentration, the minimum concentration below which filament growth will not occur, than that of the pointed or minus end (11). Therefore, under conditions in which the concentration of free actin monomers in solution is between the critical concentration of both ends of the filament, the rapid addition of ATP-actin to the barbed end will compensate for the release of the ADP-actin from the pointed end, resulting in the treadmilling of actin monomers through the filament (12). Because ATP hydrolysis is faster than P_i release for most actins, the rate of P_i release functions as an intrinsic timer for destabilizing the older end of the filament (6, 7).

This nucleotide-dependent filament stability must be reflected in nucleotide-dependent changes in actin conformation. Crystallographic studies of G-actin modified at the residue penultimate to the C terminus by the attachment of tetramethylrhodamine revealed structural differences between ATP- and ADP-actin, particularly in the loops bordering the bound nucleotide and in the DNase I loop in subdomain 2 of the protein (13, 14). In the case of the DNase I loop, there is an alteration of a 12-residue segment from an unstructured loop in the ATP-bound form to an α-helix in the ADP-bound form. Structural changes in subdomain 2 have also been observed by optical reconstruction of electron micrographs of muscle actin filaments formed under various conditions (15, 16). Egelman and co-workers (15, 16) have data suggesting that in newly formed actin filaments, the monomers are incorporated loosely in the filament in a more “tilted” and “open” conformation, creating filaments that look “kinked” or “ragged,” which then collapse into the more closed conformation observed generally with mature actin filaments. They also present results indicat-
ing that the conformation of the monomers that comprise the barbed and pointed ends of the filaments are structurally different (17). This result provides supporting evidence that the conformational changes linked to P, release occur following F-actin formation.

The dynamic equilibrium that exists between the monomeric and filamentous forms of actin inside cells is modulated by an extensive array of actin-binding proteins. These include capping proteins that control filament length and access to the two filament ends, nucleating proteins that initiate polymer growth, filament-severing proteins, and monomer-sequestering proteins that regulate the concentration of free G-actin. Several studies have demonstrated that many of these proteins are sensitive to the state of the actin bound nucleotide (11). Profilin, a G-actin-binding protein that enhances nucleotide exchange, displays a higher binding affinity for ATP-actin than ADP-actin (18, 19), whereas thymosin β-4, a G-actin-sequestering protein, preferentially binds ATP-actin with higher affinity (20). ADF/cofilin, an actin filament-severing protein, which also binds monomers, prefers ADP-actin to ATP-actin (21–23). The Arp2/3 complex functions by binding to existing actin filaments and nucleating new filament formation (branches) at an angle of ~70° to the parent filament. Direct observation of the polymerization process reveals that there may be a preference for Arp2/3-dependent filament branching at or near the barbed end of an existing filament, suggesting that Arp2/3 may have a higher affinity for either ATP- or ADP-P,F-actin subunits versus ADP-F-actin (24–26).

The recent work of Yao and Rubenstein (27) indicates that with yeast actin neither the ATP- nor the ADP-P, state exists for any appreciable amount of time in actively growing filaments when polymerization is carried out at low actin concentrations. This observation is the opposite of the result observed with muscle actin under similar conditions in which an appreciable phosphate lag is observed between filament formation and P, release (4, 5). This initial observation suggests that Arp2/3-dependent barbed end branching, at least in yeast actin, does not result from a higher affinity of the complex for ATP- or ADP-P,F-actin but from an immature ADP-actin conformation (ADP*) at or near the barbed end of the filament.

Post-P, release intermediate conformations prior to mature ADP-F-actin formation have yet to be observed in solution studies. The absence of this documentation may be the result, in part, of conditions in which actin polymerization is usually studied, consisting of dilute actin concentrations in which an appreciable phosphate lag is observed between filament formation and P, release (4, 5). This initial observation suggests that Arp2/3-dependent barbed end branching, at least in yeast actin, does not result from a higher affinity of the complex for ATP- or ADP-P,F-actin but from an immature ADP-actin conformation (ADP*) at or near the barbed end of the filament.

**EXPERIMENTAL PROCEDURES**

Materials—DNase I (grade D) was purchased from Worthington. DE52 DEAE-cellulose was purchased from Whatman. Micro Bio-Spin P-30 Tris gel filtration chromatography columns and Affi-Gel 10-activated resin were purchased from Bio-Rad. N-(1-pyrenyl)maleimide, ATP, and AMPPNP4 were purchased from Sigma. The EnzChek® phosphate assay and eATP were purchased from Molecular Probes. All other compounds used were reagent grade quality. The A197C mutant yeast actins were purified in the Ca2+ form using a combination of a DNase I affinity column and DEAE ion exchange chromatography followed by subsequent polymerization-depolymerization cycling according to Cook et al. (29) and stored in Ca2+ G-Buffer (2.9 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM MgCl2, and 0.1 mM dithiothreitol) at 4 °C. Wild-type actin was purified from commercially available yeast cakes. For the preparation of mutant yeast actin, yeast cells expressing the V159N mutant actin as their sole actin were grown in yeast extract (1%), bactopeptone (2%), and dextrose (2%) liquid media at 30 °C for 36–48 h. The cells were harvested by centrifugation and frozen prior to purification of the actin. Rabbit skeletal muscle F-actin was purified from rabbit skeletal muscle acetone powder according to Spudich and Watt (30) and was stored at 4 °C in F-Buffer (5 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 2 mM MgCl2, 1 mM dithiothreitol, and 0.01% NaN3). To prior use, the muscle F-actin was depolymerized by dialysis against at least three changes of G-Buffer followed by ultracentrifugation at 4 °C in a TLA 100.2 Beckman rotor at 75,000 rpm for 1 h using a Beckman TL100 instrument to remove any polymerized or denatured protein. The concentration of G-actin was determined from the ultraviolet absorbance at 290 nm using an extinction coefficient of 0.63 μg · cm−1 · nm−1. All actins were used within 4 days after the completion of purification.

Actin Polymerization—Polymerization experiments were monitored using a Fluoromax-3 (Jobin Yvon, Inc.) fluorescence spectrometer containing a computer-controlled, thermostatted cuvette chamber set at a constant temperature of 25 ± 0.1 °C. Actin polymerization was triggered by the addition of F-salts consisting of a 1:9 volume of 120 mM MgCl2 and 1 mM KCl to yield final concentrations of 2 and 50 mM, respectively. The total reaction volume was 120 μl. To assess polymerization by the change in light scattering, both the excitation and emission wavelengths were set to 360 nm, and slit widths were set at 1 and 2 nm, respectively. Actin was labeled at Cys-374 with N-(1-pyrenyl)maleimide according to Feng et al. (31) and was combined with unlabeled actin at a ratio of 1 to 9. The polymerization-dependent increase in intrinsic tryptophan fluorescence was monitored by exciting the pyrene probe at 365 nm and following the change in pyrene fluorescence at 386 nm as a function of time. Slit widths were 1 and 2 nm for excitation and emission, respectively. Assessment of the polymerization-dependent change in intrinsic tryptophan fluorescence as a function of time was performed by using an excitation wavelength of 300 nm and monitoring the change in the emission of tryptophan fluorescence at 335 nm with slit widths of 1 and 3 nm, respectively. The fluorescent signal was recorded every 10 s, and the integration time for each point reading was 1 s. All polymerization experiments were performed in quartz microcuvettes at least three times with different actin preparations.

**pH Adjustment**— Acidification of actin solutions to pH 6 was carried out by adding equal parts of a 2.9 mM Tris-HCl G-Buffer/actin solution, pH 7.5, and a 6 mM MES buffer, prepared pH 7.5, to yield final concentrations of 2 and 50 mM, respectively. Adjustment of the pH in the PBS assay at pH 6.0 was achieved by using the same scheme described above; however, the addition of 100 mM MES buffer, pH 5.6, was added in place of an equal amount of 4 mM MES buffer, pH 5.6. The final pH before and after the addition of F-salts was verified using a pH meter equipped with an NMR pH electrode.

Nucleotide Depletion—Unbound nucleotide and P, were removed from the samples using Micro Bio-Spin P-30 Tris gel filtration columns that had been equilibrated with 2.9 mM Tris-HCl buffer, pH 7.5, and 0.2 mM CaCl2 at 4 °C according to the manufacturer’s specifications. Following depletion, the protein was kept on ice, and all nucleotide-depleted actins were used within 4 h.

**Release (from Actin)**—Release of P, at pH 7.5 from poly- merizing actin samples following ATP hydrolysis was assessed using the commercially available EnzChek® phosphate assay at 25 °C (purine nucleoside phosphorylase). Briefly, this spectrophotometric assay utilizes the phosphorylase of 6-mercaptop-7-methylurine riboside to ribose 1-phosphate and 2-amino-6-mercaptop-7-methylurine, the latter of which has a characteristic absorbance at 360 nm that is not shared by the phosphate substrate at pH values greater than 6.5 (15, 32). Following induction of polymerization of 11.9 μM actin, the absorbance was monitored as a function of time, with readings taken automatically at 10-s intervals at 360 nm using a thermostatted cuvette holder set to 25 °C ± 0.1 °C.

1 The abbreviations used are: AMPPNP, adenosine 5’-O-(β,γ-imido)triphosphate; PBP, phosphate-binding protein; MES, 2-morpholine-ethanesulfonic acid; WT, wild-type.

2 X. Yao and P. A. Rubenstein, unpublished data.
Because of the pH dependence of the purine nucleoside phosphorylase assay, we also monitored Pi release using the E. coli mutant PBP, constructed originally and characterized by Brune et al. (33) (see “Experimental Procedures”). The excitation and emission wavelengths were 425 and 464 nm, respectively, and both excitation and emission slit widths were 1 nm. The increase in fluorescence of the PBP on binding to free Pi was recorded every 10 s, and the signal was averaged for 1 s. To decrease the initial background Pi levels, excess nucleotide and Pi were depleted as stated above prior to the initiation of actin polymerization. The final ratio of actin/PBP concentration was 11.9–110 μM, respectively. Under our conditions, the assay was linear up to 30 μM Pi, based on standard curves, with all experimental conditions producing phosphate concentrations within this range. Again, these experiments were performed in a Fluoromax-3 fluorometer at 25 ± 0.1 °C using quartz microcuvettes. All experiments were performed at least three times with different actin preparations.

**Actin-dependent ATP Hydrolysis**—Both the purine nucleoside phosphorylase and PBP assays can detect only free (unbound) Pi, not bound Pi. Therefore, the determination of the rate of ATP hydrolysis was accomplished by removing an aliquot from an F-actin mixture and rapidly denaturing the protein by chelating both Mg²⁺ and Ca²⁺ with a solution of 3 mM EDTA, 0.3 mM EGTA prior to exposing the protein to 100 °C for 1 min in preheated glass tubes. Immediately following thermal denaturation, the samples were cooled to 4 °C. The samples were transferred to new Eppendorf tubes and vortexed, and the remaining denatured protein was removed by centrifugation at 14,000 rpm for 5 min using an Eppendorf microcentrifuge. A fixed volume of the supernatant was removed from each tube, and the amount of total Pi was measured using either the EiinzChek™ phosphate assay following adjustment of the pH to 7.5 or via PBP as described previously. Controls showed that free ATP was not substantially hydrolyzed under the conditions of the assay.

**Effect of Pi on Polymerization-dependent Change in Tryptophan Fluorescence**—Polymerization of 11.9 μM actin was induced and monitored via the change in intrinsic tryptophan fluorescence as described above. Potassium monobasic phosphate, adjusted to the pH of the reaction, was added to an actin solution at a final concentration of 15 mM, either initially or following completion of polymerization.

**Data Fitting**—The observed first-order rate constants for the second phases of intrinsic tryptophan fluorescence curves were determined independently by fitting only the second phase of the unmanipulated polymerization curve with Bio-Kine (version 3.26, Bio-Logic SA) using the following equation.

\[
y = at + b + \sum_{i=1}^{N} c_i e^{-kt_i} \quad (Eq. 1)
\]

Parameters of the fit were minimized using the Simplex algorithm. After the first-order rate constant (kt) was obtained, the amplitude of the second phase (Δ2) was determined by extrapolating that portion of the polymerization curve back to t = 0. Both the average first-order rate constants and the average Δ2 values for the second phase were obtained from the sum of the individually calculated rate constants and the total change in magnitude of the second phase based on the values determined from the extrapolation along with the standard deviations. A two-sample Student’s t test analysis was used to determine whether the rate constants were statistically similar or different.

**RESULTS**

**Intrinsic Tryptophan Fluorescence Behavior of Polymerizing Yeast Actin**—To accelerate actin polymerization relative to Pi release to determine whether we could temporally resolve the two processes, we carried out polymerization of yeast actin at pH 6.0 based on the previous work of Zimmerle and Frieden (34). We examined polymerization using the change in intrinsic tryptophan fluorescence, which is characterized typically by a time-dependent decrease following induction of the process (35–37). Yeast and muscle actins contain four tryptophan residues, all of which are located in subdomain I. Doyle et al. (38), using site-directed mutagenesis of yeast actin, demonstrated that the polymerization-dependent fluorescence decrease is the combined contribution of three of the four tryptophan residues, Trp-79, Trp-86, and Trp-340, all of which are near the nucleotide binding site in subdomain I.

![Fig. 1. Change in intrinsic tryptophan fluorescence of polymerizing yeast actin at pH 7.5. A, polymerization-dependent change is shown in intrinsic tryptophan fluorescence of 11.9 μM WT yeast actin at pH 7.5. A.U., arbitrary units. B, a comparison is shown between the polymerization-dependent change in intrinsic tryptophan fluorescence (—) and pyrene fluorescence (▲) of 10% Cys-374 pyrene-labeled WT yeast actin. The intrinsic tryptophan fluorescence curve was inverted, and the curves were normalized so that the total change in signal was equal to 1. All curves were corrected for the dilution caused by addition of the F-salts. ■, the starting G-actin fluorescence at t = 0.](image-url)

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![Fig. 1A shows that the intrinsic tryptophan fluorescence behavior on induction of polymerization of 11.9 μM WT yeast actin at pH 7.5 is characterized generally by a monophasic decrease in fluorescence as shown previously for muscle actin (35) and yeast actin (36, 37). The small rapid initial increase in fluorescence we observed probably resulted from the conversion of Ca²⁺-actin to Mg²⁺-actin following the introduction of MgCl₂ and KCl to initiate polymerization (35). A method generally employed to assess filament formation is the change in fluorescence of a pyrene moiety attached to Cys-374. When we compared the polymerization-dependent changes in fluorescence of 10% Cys-374 pyrene-labeled actin and intrinsic tryptophan fluorescence, we observed that the rate of change in intrinsic tryptophan fluorescence was somewhat faster than the change in pyrene fluorescence (Fig. 1B). This result suggested that the change in orientation of the pyrene probe on the flexible C-terminal arm occurred more slowly than the change in environment sampled by the tryptophan residues.

Surprisingly, Fig. 2A shows that the polymerization-dependent change in intrinsic tryptophan fluorescence is very different at pH 6.0 compared with 7.5. At pH 6, there is a rapid decrease in the intrinsic tryptophan fluorescence (first phase) followed by a slower rebound (second phase). To determine the relationship of each of these phases to filament formation, we compared the normalized polymerization-dependent tryptophan fluorescence change with that obtained using Cys-374-pyrene maleimide-labeled actin as described under “Experimental Procedures” (Fig. 2B). For ease in comparison, we inverted the change in the intrinsic tryptophan fluorescence
an appreciable lag between polymerization and Pᵢ release was noted previously by Yao and Rubenstein (27).

At pH 6, however, Pᵢ release was reproducibly slower than the polymerization-dependent first phase change in intrinsic tryptophan fluorescence (Fig. 3B). The second phase fluorescence change and Pᵢ release appeared to occur over a similar time frame. Fig. 3C indicates that Pᵢ release is characterized by a slightly but reproducibly faster rate constant than the second phase fluorescence change. The average $k_{obs}$ for the second phase (0.0059 s⁻¹) was slower than then the average rate constant for Pᵢ release (0.0115 s⁻¹ ± 0.0008, n = 6). These rates were statistically different ($p < 0.0001$). Furthermore, in all iterations of this experiment, Pᵢ release appeared to occur slightly before the second phase fluorescence change. This temporal displacement may actually be an underestimate because the PBP assay requires release of the Pᵢ followed by its reaction with the phosphate-binding protein, resulting in an unavoidable, although small, delay in generation of the signal. These results suggested that the postpolymerization conformational change might actually depend on the prior release of Pᵢ.

A number of possibilities might explain the pH 6-dependent lag in Pᵢ release compared with polymerization. First, acidification could accelerate polymerization with no change in retardation of the rate of Pᵢ release. Second, acidification could accelerate both but affect polymerization to a greater extent than Pᵢ release, resulting in a temporal dissociation of the two curves. Finally, acidification could actually retard ATP hydrolysis relative to polymerization. To eliminate the last possibility, we monitored the rate of ATP hydrolysis by measuring the amount of total Pᵢ generated, both free and bound (see “Experimental Procedures”), as a function of time following the induction of polymerization. Our results from these experiments demonstrated that ATP hydrolysis and polymerization remained coincidental (Fig. 3D). The Pᵢ reading at $t = 0$ was corrected for the small amount of Pᵢ present in the nucleotide-depleted G-actin sample just prior to induction of polymerization. Moreover, because of that nature of the assay, it was not possible to collect time points earlier than 10 s. A direct comparison of the Pᵢ release curves at pH 6 and 7.5 demonstrated clearly that lowering the pH to 6.0 decelerated the rate of Pᵢ release relative to pH 7.5 while actually accelerating the rate of actin polymerization (Fig. 3E).

As the pH is increased to 7.5, the rate of polymerization may slow down to such an extent that the second phase increase becomes submerged under the first phase polymerization curve. If this is the case, increasing the actin concentration might increase the rate of polymerization relative to the $k_{obs}$, resulting in an observable second phase rebound. Increasing the yeast actin concentration to 40 µM at pH 7.5 resulted in two resolvable fluorescence phases (Fig. 4) similar to that observed at pH 6.0 using 11.9 µM actin. The average $k_{obs}$ under these conditions was approximately half that (0.0035 ± 0.0004, n = 4) of the rate obtained at pH 6 ($p = 0.02$).

**Effect of Exogenous Pᵢ on the Second Phase Fluorescence Change**—If the second phase change required Pᵢ release from actin, the inclusion of exogenous Pᵢ in the assay might depress or eliminate the second phase. Carlier and Pantaloni (10), using muscle actin, demonstrated that 15 mM Pᵢ was sufficient to drive ADP-actin back into an ADP-Pᵢ-F-actin state. Fig. 5 shows the change in response of the second phase fluorescence of yeast actin on addition of 15 mM Pᵢ, either after the polymerization “steady state” is reached (Fig. 5, A and B) or simultaneously with salts during the induction of polymerization (Fig. 5C). Postpolymerization addition of Pᵢ resulted in a decrease in the magnitude of the second phase fluorescence at pH 6.0 and decreases in the final steady-state fluorescence as well
at pH 7.5. Elimination of the second phase was observed when Pi was added at the same time as salts to induce polymerization. Control experiments performed with 10% pyrene-labeled actin revealed little if any effect of adding 15 mM Pi on the extent of polymerization (data not shown). These results are consistent with the hypothesis that the second phase fluorescence rebound of yeast actin is the result of the release of Pi.

A similar kinetic comparison was performed at pH 6.0 with muscle actin. Fig. 6B indicates that the rate of the second phase rebound (0.0045 ± 0.001, n = 4) had essentially the same rate constants as the Pi release (0.0031 ± 0.0002, n = 4). To determine whether the second phase in muscle actin was caused by the release of Pi, we conducted similar Pi addition experiments (Fig. 6A). These results indicated that the second phase observed with muscle actin was also caused by the release of Pi.

Temporal Relationship of Polymerization, Pi Release, and the Second Phase Change of 40 μM Yeast Actin at pH 7.5—Our ability to observe a second phase fluorescence change during yeast actin polymerization at physiological pH at elevated actin concentrations led to the question of whether the second phase represented a transition to the mature ADP-F-actin conformation following Pi release. Fig. 7 shows the results we obtained by examining all of these processes with the same yeast actin preparation at a concentration of 40 μM. Pi release begins shortly after the onset of polymerization and is roughly 65% complete before the initiation of the second phase fluorescence change. Furthermore, there is a delay of roughly 200 s between the times needed for Pi release and the second phase fluorescence to reach 50% of their maximum values. The partial temporal displacement of Pi release from polymerization we observed here, in contrast to the concurrence of these processes at lower actin concentrations, probably resulted from the actin-concentration dependent acceleration of polymerization relative to the first-order rate constant that governs Pi release from the filament.

One possible explanation for the appearance of the second phase conformation change is a postpolymerization filament bundling that could affect the environment of the subdomain 1 tryptophan. However, two pieces of evidence argue against this hypothesis. First, in Fig. 7, the onset of the second phase change occurs well after the change in light scattering reached a plateau. The occurrence of bundling would have been detected by a continual increase in light scattering during the time that fluorescence was increasing, which we did not observe. Second, centrifugation of the actin samples polymerized at either pH 6.0 or 7.5, which is sufficient to pellet actin...
bundles (39), failed to produce a detectable pellet. Thus, this second phase fluorescence change resulted from a change in conformation of the filament that requires Pi release. Furthermore, with yeast actin at physiological pH, the results indicated that a substantially long-lived ADP*-F-actin intermediate exists following Pi release prior to its conversion to the mature ADP-F-actin state.

"ATP-like" Conformation of the Yeast Actin Mutant V159N—V159N mutant yeast actin forms hyperstable filaments in vitro even though it hydrolyzes ATP and releases Pi normally (15, 28). Belmont and Drubin (28) proposed that this hyperstability results from the maintenance of the actin in an ATP-like conformation despite the occurrence of ATP hydrolysis and Pi release. If their hypothesis is correct, based on our fluorescence results, we might expect to observe an absence of a second phase fluorescence change with this actin following the induction of polymerization at pH 6. However Fig. 8 demonstrates the appearance of a biphasic fluorescence curve for this mutant actin similar to that observed with WT yeast actin under the same conditions. This result suggests that, at least as far as the environment sampled by the tryptophans in subdomain 1 is concerned, this part of the actin does not remain in an ATP-like state.

**FIG. 5.** Effect of the addition of 15 mM Pi on the intrinsic tryptophan fluorescence behavior of yeast actin. Actin polymerization was triggered by the addition of F-salts, and the polymerization-dependent change in intrinsic tryptophan fluorescence behavior was monitored as a function of time. Shown is the dilution-corrected effect that the postpolymerization addition of 15 mM Pi has on the tryptophan fluorescence of 11.9 µM WT actin at pH 7.5 (A) and pH 6.0 (B). A.U., arbitrary units. Once steady state was reached, 15 mM Pi was added at the time denoted by the arrows (A and B). C, effect is shown of the prepolymerization addition of 15 mM Pi, on the polymerization-dependent intrinsic tryptophan fluorescence response 11.9 µM WT actin at pH 7.5 (—) and pH 6.0 (□). ■, the starting G-actin fluorescence at t = 0.

**FIG. 6.** Correlation of the second phase fluorescence change and Pi release in polymerizing muscle actin. A, shown is the dilution-corrected effect that the postpolymerization addition of 15 mM Pi has on the intrinsic tryptophan fluorescence of 11.9 µM muscle actin at pH 6. The arrow denotes the time of the addition of the Pi to the sample. B, comparison is shown between the second phase fluorescence change of muscle actin (—) and the rate of Pi release (●) under the same conditions. The two curves were normalized so that the total change in fluorescence was equal to 1. Pi release was measured with the PBP assay (see “Experimental Procedures”). ■, denotes the starting G-actin fluorescence at t = 0. A.U., arbitrary units.

**FIG. 7.** Correlation of filament formation, second phase, and Pi release in polymerizing 40 µM yeast actin at pH 7.5. Shown is a comparison of polymerization (□), Pi release (●), and the second phase (—) of polymerizing 40 µM yeast actin at pH 7.5. Note the large temporal displacement of the second phase relative to Pi release. All curves were normalized so that the total change in fluorescence equaled 1. Filament formation was monitored by light scattering, and the rate of Pi release was monitored via the p-nitrophenyl phosphate assay (see “Experimental Procedures”). Polymerization was triggered by the addition of F-salt. ΔF, change in fluorescence; ΔS, change in light scattering.

**DISCUSSION**

Conformational changes in G- or F-actin induced either as a result of hydrolysis of its bound nucleotide (13–16, 40–48) or its interaction with various actin-binding proteins (17, 49–53) have been described in a number of structural studies. However, the elucidation of these changes in real time has been more problematic. The attachment of probes to actin to detect such changes can alter the structure and behavior of the protein. Another problem is that sequential conformational
changes may not be easily resolvable because of the relative rates at which they occur. The work presented here provides new insight into postpolymerization remodeling of the actin filament and the role played by nucleotide hydrolysis and P_i release from the actin in these changes.

Previous work demonstrates that actin polymerization is accompanied by a single phase decrease in intrinsic tryptophan fluorescence (35–37) as a result of alterations in the environment of three tryptophan residues in subdomain 1 (38). We reasoned that if we accelerated polymerization by lowering the pH of the solution, we might resolve postpolymerization changes that might otherwise be overlooked. Our results with yeast actin showed clearly two changes in tryptophan fluorescence when polymerization occurs at pH 6.0, an initial rapid decrease that seems to correlate with monomer assembly into a filament and hydrolysis of bound nucleotide followed by a second, slower first-order increase in fluorescence that appears to depend on the release of the P_i from the actin. Control experiments demonstrated that these changes did not arise as a result of pH or salt effects on the inherent fluorescence properties of the tryptophan indole ring. We demonstrated that this conformational change occurred with muscle actin as well. Thus, this two-phase fluorescence behavior is not confined to yeast actin. Because the tryptophans that give rise to this polymerization-dependent response are near the nucleotide binding pocket of the actin, the changes in the state of the nucleotide during polymerization may be a major factor in this fluorescence change.

This newly observed second phase is pH-dependent in that it decreased in magnitude and in its apparent rate constant by ~2-fold as the pH increased from 6.0 to 7.5. Concomitantly, the magnitude of the first phase fluorescence decrease became larger. At pH 6, the addition of P_i to the solution decreased the magnitude of the second phase, in agreement with our hypothesis that this change arises from the release of P_i from the nucleotide cleft of actin. We also observed a similar effect at pH 7.5, a depression of the final fluorescence value following the addition of P_i.

Our studies with higher yeast actin concentrations at pH 7.5 led to two significant observations. The first observation was the detection of a detectable lag in P_i release following polymerization. Contrary to the case with other actins, this had not been present when polymerization studies were carried out previously at lower actin concentrations. This result suggests that even with yeast actin at concentrations that are likely to occur locally within the cell the presence of an ADP-P_i cap at the barbed end may play a significant role in the regulation of cytoskeletal dynamics. Second, our results provide spectral evidence for a previously undetermined intermediate ADP-F-actin state (ADP*) prior to the formation of mature ADP-F-actin. Because new monomer addition to the filament occurs primarily at the barbed end, the existence of this intermediate state should be localized primarily there as well. Recently, a number of studies have demonstrated that some actin-binding proteins may display barbed-end side- or end-on binding specificity. One such protein is the Arp2/3 complex, the biological function of which is to provide a platform on which daughter filaments can branch from the side of the mother filament at an angle of ~70°. Recently, evidence has been presented (24, 26) suggesting that the Arp2/3 complex displays a mild preference for ATP- or ADP-P_i-F-actin over ADP-F-actin and also that there may be a preference of the complex for binding at or near the barbed end of the filament. It is possible that in areas of the cell undergoing active actin polymerization, where the actin concentration is very high, part of the barbed-end selectivity exhibited by proteins such as the Arp2/3 complex may originate from a preferred interaction with the ATP, ADP-P_i, or ADP* forms found near the barbed end. Our result may also have implications in terms of the mechanism by which other actin side-binding proteins, such as formin (54), may discriminate between the barbed and pointed ends. Whether the ADP* intermediate state is equivalent to the open tilted state of the filament and the role played by nucleotide hydrolysis and P_i release from the actin in these changes.

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