GTP-Yeast Actin*

Received for publication, April 25, 2002, and in revised form, August 7, 2002
Published, JBC Papers in Press, August 20, 2002, DOI 10.1074/jbc.M204025200

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Because of the apparently greater conformational flexibility of yeast versus muscle actin and the ability of other members in the actin protein superfamily to efficiently use both ATP and GTP, we assessed the ability of yeast actin to function with GTP. Etheno-ATP exchange studies showed that the binding of GTP to yeast actin is about 1/9 as tight as that of ATP in contrast to the 1/1,240 ratio for muscle actin. Proteolysis of GTP-bound G-yeast actin suggests that the conformation of subdomain 2 is very much like that of ATP-bound actin, but CD studies show that GTP-bound actin is less thermostable than ATP-bound actin. GTP-actin polymerizes with an apparent critical concentration of 1.5 μM, higher than that of ATP-actin (0.3 μM) although filament structures observed by electron microscopy were similar. Yeast actin hydrolyzes GTP in a polymerization-dependent manner, and GTP-bound F-actin decorates with the myosin S1. Conversion of Phe306 in the nucleotide binding site to the Tyr hydroxyl will create a space for the C2 amino group of the GTP guanine.

Two cytoskeletal filament systems in the cell, actin microfilaments and tubulin-containing microtubules, depend on the ability to bind and hydrolyze nucleoside triphosphates for proper function. For microtubules, the nucleotide is GTP, and for the actin filaments, the nucleotide is assumed to be ATP (1). ATP F-actin is more stable than ADP F-actin, and the instability brought about by hydrolysis of the nucleotide is believed to be an important factor in the regulation of the dynamics of the actin cytoskeleton (2).

The ATP requirement for actin is based on two criteria: the isolation of actin from a number of sources with a bound ATP, and the demonstration that there is a 500-fold higher affinity of muscle actin for ATP than for GTP (3). This difference in nucleotide affinity was also observed in polymerization experiments. Martonosi and Gouvea (4) first reported that actin, in the presence of 0.1 M KCl, would not polymerize in the presence of a stoichiometric amount of GTP. Iyengar and Weber (3) also generated muscle G-actin free of nucleoside triphosphates and examined the ability of different nucleotides to affect polymerization when added back to the preparation in the presence of salt. Their results showed that whereas 20 μM G-actin would completely polymerize in the presence of a stoichiometric amount of ATP, 100 μM GTP was required to polymerize the actin. Apparently, the free GTP needed to be well above the nM-μM range required for ATP to saturate the high affinity nucleotide binding site of the actin.

We have previously used yeast actin as a model system for examining actin structure-function relationships in vitro, and our results have demonstrated significant quantitative differences in the behavior of yeast versus muscle actin. Yeast actin is more susceptible to controlled proteolysis and exchanges nucleotide faster (5). Kim et al. (6) showed that beginning with G-actin, the yeast protein reaches steady state polymer levels more rapidly than muscle actin. In another study, kinetic modeling demonstrated this acceleration was because of faster nucleation and filament fragmentation during polymerization leading to more ends for filament extension (7). These results suggest that yeast actin might assume a more open and flexible conformation than muscle actin, a hypothesis that is consistent with additional results from ours and other laboratories. Based on results from optical reconstruction of electron micrographs of yeast actin filaments, Belmont et al. (8) suggested that the area in the nucleotide cleft was more open in a yeast than in a muscle actin filament. Furthermore, we have demonstrated that His73 of yeast actin, unlike that of higher actins, is not methylated (9) and that this lack of a methyl group may allow the residues at the bottom of the nucleotide cleft to more readily assume a conformation that favors the open conformation (10) than is the case for other actins. If true, there might be a decrease in nucleotide specificity of yeast in comparison with other actins. Interestingly, Nyman et al. (11) have also shown with chicken β-nonmuscle actin produced in yeast (non-methylated) or isolated from tissue (methylated) that lack of methylation of this residue results in an uncoupling of ATP hydrolysis from filament formation.

Actin is a member of a superfamily of proteins consisting of hsc70, Escherichia coli DnaK protein, and sugar kinases, such as hexokinase (12). All share a similar three-dimensional structure around a nucleotide binding core, and all cleave the β-γ bond of ATP resulting in a significant conformational change in the protein. However, in a number of cases, the nucleotide specificity is rather low in comparison to actin. For example, DnaK hydrolyzes GTP with about one-half to one-fourth of the activity it has with ATP (13), and hexokinase from rainbow trout is only about twice as active with ATP as with GTP (14). Finally, it has recently been reported that MreB, thought to be a bacterial version of actin, will also polymerize in the presence of both ATP and GTP although relative efficiencies with each of these nucleotides was not determined (15).

We have recently investigated the interaction of Neisseria porins with yeast actin. These porins are nucleotide-gated channels that use either ATP or GTP (16), and we wanted to determine the effect of nucleotide on the interaction. As a control, we examined the effect of GTP on yeast actin alone because of the characteristics of yeast actin and the propensity of some actin superfamily members for GTP. The work reported

* This work was supported in part by National Institutes of Health Grants AI45728 and GM33689 (to P. A. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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here shows that yeast actin, as opposed to muscle actin, will work with GTP with relatively high efficiency.

**EXPERIMENTAL PROCEDURES**

**Materials**—Affi-Gel 10-activated resin and Bio-Spin® 30 Tris columns were purchased from Bio-Rad. DNase I affinity columns were made as described previously (17). ATP and GTP, purchased from Sigma, were dissolved in water to a final concentration of 50 mM. The pH was adjusted to 7.5 with NaOH prior to storing at −20 °C. The EnzChekTM phosphate assay kit and 1,2′-ethenoadenosine 5′-triphosphate (€ATP)1 as a 5 mM stock solution in 50 mM Tris-HCl, pH 7.5, were purchased from Molecular Probes (Eugene, OR) and stored at −20 °C. The Quick-Change site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA), and the DNA primers used for site-directed mutagenesis were obtained from Integrated DNA Technologies (Coralville, IA). Yeast cakes for preparation of wild-type actin were obtained from a local food store. The rabbit muscle actin powder and the bovine cardiac myosin S1 head preparation were generous gifts from Dr. E. Reisler and Dr. L. Tobacman, respectively. All other chemicals used were reagent grade quality.

**Actin Purification**—Yeast wild-type actin was purified in the Ca form by a DNase I affinity chromatography/DEAE ion exchange chromatography protocol as described previously (17), and it was stored in Ca G buffer (5 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM CaCl₂, and 0.1 mM dithiothreitol) at 4 °C and used within 3 days. Rabbit skeletal muscle F-actin was prepared from acetone powder according to Spudich and Watt (18). The Ca form of actin was converted to the Mg form by adding EGTA to a final concentration equal to the calcium concentration and MgCl₂ to a final concentration of 0.2 mM. The actin was then placed on ice for at least 10 min and used within hours.

**Oligodeoxynucleotide-directed Mutagenesis**—Using site-directed mutagenesis, we replaced the codon for Phe 306 in yeast actin with a Tyr

**Fluorescence Assays**—Fluorescence measurements were obtained using a Fluorolog-3 (Jobin Yvon Inc.) fluorescence spectrophotometer with a thermostatted bath attached to the cuvette chamber. Actin polymerization was monitored by a change in light scattering with excitation and emission wavelengths set at 360 nm. The €ATP fluorescence intensity change was monitored with excitation and emission wavelengths of 340 and 410 nm, respectively.

**Nucleotide-binding Titration**—Aliquots of 2.3 or 4.6 μM Ca €ATP-bound actin in the absence of free nucleotide were added to various concentrations of ATP or GTP at 25 °C. ATP or GTP to small aliquots from a 50 mM stock solution were added progressively with constant stirring at 25 °C to 2.3 or 4.6 μM Ca-€ATP-bound actin to achieve a final concentration of 700 μM. In both methods, the fluorescence decrease was monitored after the nucleotide was added until the fluorescence signal reached equilibrium. The results obtained from both methods were identical. The observed fluorescence intensity (€Fobs) obtained by the experiments was the sum of the fluorescence contributed by €ATP bound by actin and actin-free €ATP, and is shown in Equation 1,

\[
\text{€F}_{\text{obs}} = f_\varepsilon[\text{Actin}] + f_\varepsilon[\text{Actin}]
\]

where \( f_\varepsilon \) and \( f_\varepsilon \) are the specific fluorescence yields derived from experiments for the bound and free €ATP forms, respectively. [Actin] is the concentration for €ATP-bound actin, [Actin] is that for free €ATP, and the sum of \([\text{Actin}] + [\text{Actin}]\) is either 2.3 or 4.6 μM depending on the experiment. The fluorescence yield of €ATP from Equation 1 and were plotted as a function of total concentration of the competing ATP or GTP with the actin concentration constant. The solution to the quadratic expression modified from Ref. 20 was applied to the data with the dissociation constant ratio (Kp) as a fitting parameter using Microsoft Excel (Microsoft) and is shown in Equation 2,

\[
[\text{Actin}] = \frac{(K_p[N_T - E_R] + A_T + E_R)}{4K_p[1 - A_T]}
\]

where \( K_p \) is equal to \( K_{\varepsilon}/K_{\varepsilon} \). \( K_{\varepsilon} \) is the equilibrium dissociation constant for nucleotide (either ATP or GTP) binding to actin, \( K_{\varepsilon} \) is the equilibrium dissociation constant for €ATP, \([\text{Actin}] + [\text{Actin}]\) is the concentration of €ATP-bound actin, \( A_T \) is the concentration of the total ATP/GTP, and \( E_R \) is the concentration of the total concentration of actin.

**Thermal Denaturation**—The apparent melting temperature of G-actin was determined by circular dichroism according to Yao et al. (9). 1 μM G-actin in G buffer was heated at a constant rate of 1 °C/min from 20 to 80 °C with constant stirring. Changes in the actin ellipticity were monitored at 222 nm using an AVIV 62 DS spectropolarimeter. Data were normalized as the fraction of native protein based on the net change in ellipticity then fitted to a two-state model with a single transition between a native and a denatured form of the protein. The \( T_m \) value was designated as the temperature at which 50% G-actin was denatured.

**Limited Proteolysis of G-actin**—The digestion protocol was essentially the same as described by Kuang and Rubenstein (21) with modification. Briefly, 9.2 μM G-actin was incubated with trypsin (5 μg/ml), subtilisin (1 μg/ml), or €-chymotrypsin (22.5 μg/ml) at room temperature for the desired time. All the digestion reactions were stopped by adding SDS-sample loading buffer and then heating at 95 °C for 3 min. The samples were analyzed by SDS-PAGE on a 12.5% polyacrylamide gel, and the bands were visualized by staining with Coomassie Blue.

**Actin Polymerization**—Actin polymerization was triggered by the addition of MgCl₂ and KCl to final concentrations of 2 and 50 mM, respectively, and was monitored by the increase in light scattering as a function of time at 25 °C. The extent of polymerization of €ATP-actin was normalized against the extent of polymerization of an equivalent concentration of ATP-actin for comparison. To measure the critical concentration of ATP/€ATP-bound actin, increasing concentrations of actin were induced to polymerize until the steady state was reached, and the net change in light scattering in each experiment was recorded. The net changes were plotted against the concentration of actin, and the critical concentration of actin was determined by drawing a line through the points and determining its intersection on the x axis. To assess the ability of the actin filament to withstand cold temperature, 9.2 μM actin was polymerized at 25 °C until the steady state was reached. The temperature of the sample was then lowered from 25 to 5 °C using a slowly controlled thermostatted waterbath connected to the cuvette chamber.

**Phosphate Release Assay**—The inorganic phosphate released from F-actin during polymerization was measured in a coupled assay using the EnzChek™ assay developed by Webb (22). The assay is based on a F-dependent enzymatic conversion of 2-amino-6-mercapto-7-methylpurine-20-phosphate to 2-amino-6-mercapto-7-methylphosphonic acid by a purine nucleoside phosphorylase. The production of 2-amino-6-mercapto-7-methylpurine in F-bound actin was monitored at 295 nm (5 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 50 mM ribose, 0.2 mM dithiothreitol, and 0.2 mM nucleotide triphosphate) upon actin polymerization was monitored continuously by the change in absorbance at 360 nm. A standard curve allowed conversion of the absorbance change to Fₐ concentration.
Myosin Decoration and Electron Microscopy—To decorate actin filaments with myosin S1, GTP-bound actin (9.2 μM) was polymerized in G buffer with 100 μM GTP at 25 °C for 30 min and then mixed with 4.6 μM myosin S1 for another 30 min. To prepare the samples for electron microscopy, 4.6 μM F-actin or myosin-decorated actin filaments were diluted 4-fold with F-buffer (5 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 50 mM KCl, 0.1 mM GTP, and 0.1 mM dithiothreitol) and were applied to carbon-coated Formvar grids and visualized following negative staining with 1.5% (w/v) uranyl acetate using a Hitachi 7000 electron microscope (University of Iowa Electron Microscope Facility).

RESULTS

Relative Binding Affinity Assays—To determine the capacity of yeast actin to bind GTP, we measured the dissociation constant ratio of ATP and GTP versus eATP for the nucleotide tight binding site in yeast actin by adding free ATP or GTP to eATP-bound Ca-actin in nucleotide-free G buffer. Parallel ATP and GTP titrations were performed on the same preparation of eATP-actin to eliminate inconsistencies arising from differences between actin preparations.

Fig. 1, A and B demonstrates the relative ability of GTP or ATP to compete with eATP for binding to Ca yeast actin. These data were then fit to Equation 2. The results demonstrate that the dissociation constant ratio of eATP versus ATP, $K_{\text{ATP}}/K_{\text{eATP}}$, is 2.3 whereas the ratio of eATP versus GTP, $K_{\text{ATP}}/K_{\text{GTP}}$, is 0.25. Using these two numbers, we calculate a dissociation constant ratio of GTP versus ATP, $K_{\text{GTP}}/K_{\text{ATP}}$, of 9.2. This value is ~50-fold less than the ratio, 500, previously reported by Iyengar and Weber (3) for muscle actin.

To ensure that the difference observed between yeast and muscle actins was not simply due to differences in experimental methodologies employed in the two studies, we repeated the identical experiments with Ca muscle actin. From the results shown in Fig. 1, C and D, we calculated the ratios for Ca muscle actin to be 6.2 for $K_{\text{ATP}}/K_{\text{eATP}}$ and 0.005 for $K_{\text{ATP}}/K_{\text{GTP}}$. Thus, the dissociation constant ratio of GTP versus ATP, $K_{\text{GTP}}/K_{\text{eATP}}$, is $1.24 \times 10^3$ for our Ca muscle actin experiments. This number is within about a factor of 2 of what was reported previously (3), indicating that yeast actin is not as highly selective as muscle actin in its ability to interact with GTP at the tight binding pocket. The 6.2-fold stronger affinity of muscle actin for ATP compared with eATP reported here is slightly higher than the 4-fold difference reported by others (23, 24), and the small difference in results may reflect different methods used in the different studies.

Effect of Divalent Cation on Nucleotide Binding—When Mg²⁺ is bound in the high affinity divalent cation binding site of actin, it changes the flexibility of the actin molecule (25). We, thus, examined the dissociation constant ratio of GTP versus ATP for yeast Mg-actin. Fig. 2 shows that $K_{\text{ATP}}/K_{\text{GTP}}$ is 9.44, and $K_{\text{ATP}}/K_{\text{eATP}}$ is 0.8 for Mg yeast actin, demonstrating an ~11-fold stronger interaction for Mg yeast actin with ATP than GTP. The slight difference in the values of $K_{\text{GTP}}/K_{\text{ATP}}$ for Ca-actin, 9.2, and Mg actin, 11, indicates that the divalent cation bound in the cation tight binding site does not significantly alter the ability of actin to interact with GTP.

The Conformation of Subdomain 2 of GTP-bound Yeast Ca-actin—Limited proteolysis has been used widely to probe the conformation of actin subdomain 2. Strzelecka-Golaszewska et al. (26) showed that ADP-bound muscle actin is more susceptible to trypsin digestion and less susceptible to subtilisin digestion than ATP-bound muscle actin, indicating distinct conformations of subdomain 2 when muscle actin is bound to ATP or ADP. We thus used limited proteolysis to assess the effect of GTP binding on the structure of subdomain 2. We subjected G-actin to limited proteolysis with trypsin, subtilisin, or chymotrypsin, which specifically cleave G-actin at Arg⁶²-Gly⁶³, Lys⁶⁶-Tyr⁶⁷, Met⁴⁴-Gly⁴⁵, and Met⁴⁴-Val⁴⁵/Leu⁴⁷-Lys⁶⁸, respectively.

SDS-PAGE analysis (Fig. 3) shows no significant difference in the digestion pattern between the GTP-bound and ATP-bound yeast Ca-actins suggesting that the exposure of the subdomain 2 sites is not significantly different for the two actins. Actin crystallographic data (10, 27, 28) all show that the

![Fig. 1. Ability of ATP versus GTP to compete with eATP for binding to Ca yeast and skeletal muscle actins. Different amounts of ATP or GTP were added to 4.6 μM yeast actin or 2.3 μM muscle actin containing eATP, and the decrease in fluorescence was observed until equilibrium had been reached. eATP-actin concentrations were calculated from the final fluorescence values as described under “Experimental Procedures.” Open circles represent the experimental data, and the lines through the points represent those predicted with Equation 2 under “Experimental Procedures.” Panel A, yeast actin with ATP; panel B, yeast actin with GTP; panel C, muscle actin with ATP; and panel D, muscle actin with GTP.](https://example.com/fig1.png)
nucleotide locates at the interface of actin’s two major domains near the base of the interdomain cleft; the polyphosphate part of the molecule interacts with both domains, and the base portion interacts with subdomains 3 and 4. Thus, the bound nucleotide functions as a connecting bridge between the two domains. The amino acids rearrange when they interact with either the tri- or diphosphate and cause a subdomain 2 conformational change reported by Strzelecka-Golaszewska et al. (26). Therefore, our results imply that the triphosphate of GTP locates near the base of the interdomain cleft; the polyphosphate part of the nucleotide locates at or near the same position as the triphosphate of ATP. (26). Therefore, our results imply that the triphosphate of GTP might not be detected by limited proteolysis. Thus, the bound nucleotide functions as a connecting bridge between the two domains. The amino acids rearrange when they interact with either the tri- or diphosphate and cause a subdomain 2 conformational change reported by Strzelecka-Golaszewska et al. (26). Therefore, our results imply that the triphosphate of GTP locates at or near the same position as the triphosphate of ATP. 

Thermal Stability of GTP-bound G-actin—A structural change in yeast actin subdomains 3 and 4 needed to accommodate GTP might not be detected by limited proteolysis. Thus, we examined the thermal stability of GTP-bound Ca yeast actin by circular dichroism. The molar ellipticity at 222 nm, which reflects the content of α-helical structure in G-actin, was monitored as a function of temperature. The thermal stability of a protein is determined by the apparent melting temperature \( T_m \), which is defined as the midpoint of the thermal transition curve. Although the transition is not reversible for actin, a comparison of the curves generated by two different actins gives a relative indication of their thermal stabilities. This approach was first used for actin by Strzelecka-Golaszewska et al. (29) and Bertazon et al. (30) for muscle actin and validated for yeast actin by us previously (19). The results in Fig. 4 show that the \( T_m \) is 49.5°C for GTP-bound Ca yeast G-actin, whereas the \( T_m \) is 59°C for ATP-bound G-actin. This substantial decrease in the “melting temperature” of the actin in the presence of GTP may result either from a weaker binding of GTP versus ATP to actin giving rise to a less stable actin conformation at higher temperature or from a GTP-induced conformational change in the protein itself.

Polymerizibility of GTP-bound Yeast Ca-actin—To determine the effect of the replacement of ATP with GTP on actin polymerization, we examined the polymerizability of GTP-bound yeast Ca-actin at different GTP concentrations at 25°C. The starting material was a solution of a 1:1 complex of actin and GTP from which unbound nucleotide had been removed. Fig. 5 shows that GTP-bound yeast Ca-actin, contrary to the case for muscle actin, polymerizes in the absence of exogenous nucleotide. Samples at the end of each polymerization assay were observed by electron microscopy, and the structure of GTP-bound yeast Ca-actin filaments appears similar to ATP-bound yeast Ca-actin filaments (Fig. 6).

The purine binding region of actin lies near the intersection of subdomains 3 and 4. Because thermal denaturation results suggested that the binding of GTP altered the conformation of actin, it is possible that the integrity of the 3/4 boundary was affected. Previous results from our laboratory demonstrated that mutations in this region might produce a cold-sensitive polymerization defect (19, 21). To determine whether the binding of GTP to actin produced such an effect, we polymerized 9.2 μM GTP-bound yeast Ca-actin at 25°C until a steady state was reached. We then lowered the temperature to 5°C and monitored light scattering as a function of temperature. We observed no cold sensitivity of the preformed filaments (data not shown).

Critical Concentration of GTP-bound Actin—At a concentration of 4.6 μM, the extent of polymerization of GTP-bound actin is ~35% less than that of ATP-bound actin as shown in Fig. 5 suggesting a significant difference in the critical concentration of the GTP- and ATP-actin forms. We thus determined the critical concentrations of the two actin preparations by measuring the increase in light scattering when solutions of different actin concentrations were allowed to polymerize and graphing the magnitude of these changes against the actin concentration. Fig. 7 shows that although the critical concentration of ATP-actin is 0.3 μM, that of the GTP-actin is 1.5 μM. To determine whether the GTP- and ATP-actin conformations were interconvertible, we added ATP to a sample of GTP F-
Fig. 5. Polymerization of GTP Ca yeast actin. Ca yeast ATP- or GTP G-actin, 4.6 μM, in the presence of either ATP or different concentrations of GTP, respectively, was polymerized by the addition of MgCl₂ and KCl to 2 and 50 mM, respectively, at 25 °C, and the change in light scattering observed was as described under "Experimental Procedures." 100 μM ATP (X); GTP concentrations were as follows: 0 μM, ○; 10 μM, Δ; and 100 μM, ●.

Fig. 6. Electron micrographs of GTP and ATP yeast Ca F-actin. At the conclusion of the experiments in Fig. 5, actin was removed, stained with uranyl acetate, and viewed under the electron microscope as described under “Experimental Procedures.” Panel A, ATP-actin; panel B, GTP-actin. Bar = 50 nm.

Fig. 7. Critical concentration determined for ATP versus GTP Ca yeast actin. As described under “Experimental Procedures,” different concentrations of the actins were allowed to polymerize in the presence of 100 μM of their respective nucleotides, and the final light scattering values were plotted against the total actin concentration. The critical concentration was calculated at the point of intersection of each line with the x axis. ATP-actin, ○; GTP-actin, Δ.

Fig. 8. Stimulation of additional actin polymerization by the addition of ATP to Ca GTP F-actin. Both 4.6 μM Ca ATP- (○) and GTP- (Δ) Ca yeast actin in the presence of 100 μM of their respective nucleotide were polymerized until the steady state was approached. At 1600 s, equivalent amounts of 100 μM ATP was then added to each sample, and the resulting change in light scattering followed. Both samples reached the same final light scattering value. The sharp deflection in the middle of the plot is because of the opening and closing of the sample changer.

Higher critical concentration of GTP-bound actin described previously suggest that polymerization-induced hydrolysis of bound nucleotide might be affected. Therefore, we assessed the ability of actin to hydrolyze bound GTP. Yao and Rubenstein (31) reported that ATPase activity and Pᵢ release are concurrent during yeast actin polymerization; there is no appreciable retention of Pᵢ following nucleotide hydrolysis contrary to what is observed with muscle actin. Therefore, as an indication of actin’s ability to hydrolyze GTP, we monitored Pᵢ release from GTP-bound actin following the induction of polymerization. Fig. 9 shows that until polymerization reaches a steady state, Pᵢ release and actin polymerization curves with either GTP-bound actin or ATP-bound actin are superimposable. The continued release of Pᵢ following attainment of the polymerization

ACTIN. Fig. 8 demonstrates that the extent of polymerization increased to that of a parallel sample of ATP F-actin showing the GTP did not cause irreversible changes in actin conformation. Pi Release Assay—ATP hydrolysis and Pᵢ release have been proposed to be important determinants of the rate of actin filament turnover (2). The slow polymerization rate and the
steady state results from continued addition of nucleotide triphosphate-actin monomers to and release of nucleotide diphosphate monomers from the ends of the filaments in the absence of net filament growth. The greater divergence of the two curves in the ATP versus GTP plots may reflect the differences in the relative on and off rates for GTP versus ATP monomers. These results indicate that the mode of binding of GTP to yeast actin places the γ-phosphate in a position that allows normal hydrolysis to occur.

Interaction of GTP-bound Yeast Actin with Myosin S1—GTP-actin appears to form filaments similar to ATP-actin filaments. If their structure is normal, they should be able to interact with myosin S1 to generate the appearance of arrowheads on the filament surface. Fig. 10 shows an electron micrograph of GTP-actin filaments decorated with myosin S1 head in a typical arrowhead pattern, further indicating the normal structure of these filaments. Significantly, this experiment was carried out in the presence of exogenous GTP to help stabilize the actin, and this decoration was prevented by the presence of exogenous ATP instead of GTP because of the ability of ATP to displace S1 from actin.

Molecular Basis for Determination of Nucleotide Binding Specificity—The crystal structure of muscle actin shows that three residues in the protein provide the principal points of contact with the adenine ring of the bound ATP or ADP: Ghu, Lys, and Tyr (32). In yeast actin, Tyr is replaced with a Phe. We used the Swiss-PDB Viewer 3.7 program (www.expasy.org/spdbv/mainpage.html) to fit the residues around the adenine binding pocket in yeast (PDB number 1YAG) and muscle actin (PDB number 1ATN) and found no significant differences in the nature of the contacts with the adenine. We then used the program to calculate cavities of 1.4 Å or greater diameter in the adenine binding pockets of these two proteins. Fig. 11 shows that a cavity with a surface area of 35 Å² and a volume of 16 Å³ is adjacent to the adenine in yeast actin (panel A). This cavity is not present in either muscle actin (panel B) or in β-nonmuscle actin in either the tight or open conformation (data not shown). This cavity appears to be at the position usually occupied by the Tyr hydroxyl group in a position that might allow the C2 amino group of guanine to insert with a minimum amount of distortion of the protein structure. To further substantiate our hypothesis, we substituted a Tyr for Phe in the yeast actin structure in the same rotamer conformation and recomputed the presence of a possible cavity. The cavity was no longer present, strongly suggesting that this difference between the yeast and muscle actins may play a significant role in the relatively greater ability of yeast actin to function with GTP instead of ATP.

To test this hypothesis, we used site-directed mutagenesis to substitute Tyr for Phe at position 306 of yeast actin. Cells expressing this mutant as the sole actin were easily obtained, and they exhibited no obvious defect in growth on complex medium, hypertonic medium, or on medium containing glycerol as the carbon source. Actin was purified from these cells and tested for its relative ability to bind GTP versus ATP using the eATP displacement assay employed earlier. The results in Fig. 12 show that the ratio of GTP to ATP dissociation constants goes from 9.2:1 observed with the Phe actin to about 125:1 with the Tyr actin. Thus, with our assay system, this single substitution regains one order of magnitude of the two-order of magnitude difference between the yeast and muscle actins.

DISCUSSION

Actin had been thought to be highly selective toward adenine versus guanine nucleotides in terms of the nucleotide it could use for structural stabilization and possibly as a determinant of filament turnover within the cell. Our results with yeast actin in terms of affinity, polymerizability, and nucleotide hydrolysis demonstrate that among actins, this selectivity is certainly not universal. There is only about a 9-fold difference in the ability of yeast actin to use GTP versus ATP instead of the 1,000-fold
difference observed with muscle actin. However, with GTP the behavior of yeast actin is somewhat affected. Overall polymerization from initiation of the process to establishment of the steady state is slower. We did not dissect the process further to rigorously differentiate between the effects of the nucleotide on nucleation versus elongation. The critical concentration is higher, and thermal stability of the monomer is lower for GTP-compared with ATP-actin. These differences suggest that whereas the yeast actin structure can more easily accommodate the guanine than can that of muscle actin, the guanine nucleotide must still cause a distortion in the normal structure of the protein.

Based on an analysis of the difference in the structures of yeast and muscle actin, we hypothesized that the relaxed specificity of yeast actin might be due, in part, to the presence of Phe at residue 306 instead of the Tyr found in muscle actin. The absence of the Tyr -OH group appeared to create a cavity in which the side chain amino group of the guanine might insert. Conversion of this Phe to Tyr in yeast actin resulted in a recovery of one of the two-orders of magnitude difference in nucleotide selectivity that normally differentiate yeast from muscle actin, in agreement with our hypothesis.

Another factor that may play a major contribution in accommodating the GTP in yeast actin is a propensity for the yeast actin monomer to assume a more open conformation than muscle actin. Actin has been crystallized as a monomer in two conformations, a more closed conformation which is most often seen (33–35) and a more open conformation relative to the size of the interdomain nucleotide binding cleft (10). A number of studies, both biochemical and structural, have suggested that yeast actin may be more prone to the open conformation compared with muscle actin. Optical reconstructions of electron micrographs of yeast actin filaments (8) reveal a decreased density in the nucleotide pocket of monomers within the filament that can best be modeled by the open monomer confor-
mation described by Chik et al. (10) whereas muscle actin filaments are best modeled using the closed conformation. Yeast actin polymerizes more rapidly than does muscle actin, a phenomenon that may result from more rapid filament nucleation (7, 17), and it exchanges nucleotide 10 times faster than does muscle actin (5, 31). Whereas muscle actin retains the P\textsubscript{i} generated by polymerization-dependent hydrolysis of ATP for a substantial time before releasing it into the solution to generate ADP F-actin (36), the release of P\textsubscript{i} from yeast actin is almost immediate following its hydrolysis of the bound nucleotide (31). Finally, in a series of mutagenesis experiments with yeast actin involving the conserved His\textsuperscript{73} near the bottom of the nucleotide cleft, Yao et al. (31) demonstrated that the H73E mutation caused several polymerization and monomer structural abnormalities in yeast actin. Two acidic residues, Asp\textsuperscript{179} and Asp\textsuperscript{184}, on the opposite side of a cleft are potentially in a position to interact electrostatically with the cationic His\textsuperscript{73}. In an H73E mutant, we asked which of the two residues when converted to Arg, 184 or 179, would be most effective in restoring the H73E mutant to a wild-type phenotype. A D179R mutation, as opposed to a D184R mutation, was much more effective in restoring the wild-type properties to the H73E actin, and the H73E/D179R interaction appeared, on the basis of molecular dynamics modeling, to be more highly favored in the open conformation (37).

The biological significance of the ability of yeast actin to function efficiently with GTP is unclear. The concentration of GTP in the cell is \(-0.2 \text{ mM}\), and that of ATP is about 1 mM (38). Coupled with the roughly 10-fold difference in yeast actin for GTP versus ATP, on the average no more than about 2% of the actin would be expected to be in the GTP form. Unless a particular actin-binding protein had a particular affinity for GTP versus ATP-actin, or unless there were relatively large local concentrations of GTP in the cell, the impact of the GTP-actin that did form should be minor. However, the ability of yeast to utilize GTP, the small but significant effects it exerts on some parameters of actin function, and the availability of different fluorescently labeled yeast actins that have been generated may lead to a unique insight into the way in which the bound nucleotide controls domain/domain interactions in yeast actin and the processes that depend on them. Realization of the potential importance of a Phe versus Tyr at residue 306 in controlling conformational flexibility of subdomains 3 and 4 will also provide an avenue for obtaining new insight into the importance of this interacting network in regulating polymerization and filament dynamics.

Although the significance of the ability of yeast actin to more easily utilize GTP is not clear, the evolutionary segregation of the Phe and Tyr residues at position 306 strongly suggests that the identity of the amino acid here is important to the organism. Based on actin sequences deposited in the NIH protein database (www.ncbi.nlm.nih.gov/entrez/), metazoans including insects, frogs, worms, sea urchins, birds, fish, and mammals have a Tyr at residue 306. On the other hand, actins from plants including chlamydomonas, slime molds including physisarum, dictyostelium, and acanthamoeba, and the protist Naegleria gruberi all carry a Phe at position 306. Fungal actins display both alleles. Filamentous fungi including neurospora, aspergillus, and penicillium all have Tyr. The budding yeasts Saccharomyces cerevisiae, Candida albicans, Pichia pastoris, and Kluyveromyces lactis all have Phe. Interestingly, the actin from Schizosaccharomyces pombe, a fission yeast, carries a Tyr and not the Phe found in the budding yeast. The line ending in S. pombe, believed to have diverged from that of S. cerevisiae, a budding yeast, around 400 million years ago, is thought to have undergone rapid evolutionary change leading to the organism we know today (39). Whether other fission yeasts share the same sequence at this position could not be determined. The pervasiveness of Tyr\textsuperscript{306} among the metazoans and Phe\textsuperscript{306} among the plants suggests that in these evolutionarily advanced groups of organisms, the particular allele has been fixed, because it offers some selective advantage to the cells that reflect the particular development of the cytoskeletal machinery in the cells in which these actins are expressed.

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