Rate-Limiting Guanosine 5′-Triphosphate Hydrolysis during Nucleotide Turnover by FtsZ, a Prokaryotic Tubulin Homologue Involved in Bacterial Cell Division†

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ABSTRACT: FtsZ is a prokaryotic tubulin homologue that polymerizes into a dynamic ring during cell division. GTP binding and hydrolysis provide the energy for FtsZ dynamics. However, the precise role of hydrolysis in polymer assembly and turnover is not understood, limiting our understanding of how FtsZ functions in the cell. Here we investigate GTP hydrolysis during the FtsZ polymerization cycle using several complementary approaches that avoid technical caveats of previous studies. We find that at steady state ~80% of FtsZ polymer subunits are bound to GTP. In addition, we use pre-steady-state, single turnover assays to directly measure the rate of hydrolysis. Hydrolysis was found to occur at ~8/min and to be a rate-limiting step in GTP turnover; phosphate release rapidly followed. These results clarify previously conflicting results in the literature and suggest that pure FtsZ polymers, unlike microtubules, may not be able to undergo dynamic instability or to store energy in the polymer for force production.

FtsZ is a tubulin homologue essential to prokaryotic cell division (for recent reviews, see refs *1*−*4*). In vitro, FtsZ binds GTP to assemble into polymers; in vivo, it forms a ring that acts as the framework for the cell division machinery. The regulation of FtsZ assembly controls the timing and location of cell division. Such regulation occurs both during the normal cell cycle, when FtsZ's assembly properties change from net assembly to steady-state turnover to ring constriction and net disassembly, and also during responses to environmental stress when the pattern of cell division is altered.

GTP hydrolysis provides the energy for FtsZ polymer rearrangements, but the detailed mechanism by which this occurs is only beginning to be understood. Cycling between different nucleotide hydrolysis intermediates allows FtsZ to change its conformation and tendency to assemble. Like tubulin, GTP−FtsZ polymerizes into straight polymers (*5*, *6*). GTP hydrolysis is catalyzed by filament assembly (*7*, *8*), and polymers bound to GDP tend to curve (*9*, *10*) and are more labile (*11*). During steady-state GTP turnover, the properties of the polymer will be determined by the nucleotide species that predominates. In turn, the predominant nucleotide species will be determined by the rate-limiting step in FtsZ's hydrolysis cycle. This step has not yet been definitively determined.

The relative rates of different steps in the hydrolysis cycle are known to have important effects on eukaryotic cytoskeletal polymer turnover (for reviews, see refs *12*−*14*). For both actin and microtubules, hydrolysis and polymerization are tightly coupled. Nucleotide is only hydrolyzed after a subunit has polymerized, but it cannot be exchanged until the subunit has disassembled once again. In treadmilling actin filaments, hydrolysis and phosphate release occur slowly enough that filaments contain a mixture of ATP, ADP−P_i, and ADP-bound subunits. ATP subunits add to growing polymer ends, while ADP subunits release from the opposite, shrinking ends. In contrast, microtubules consist virtually entirely of GDP-bound subunits. Subunits within a microtubule hydrolyze GTP and release phosphate extremely rapidly (*15*, *16*). Depolymerization of the resulting GDP−tubulin is thermodynamically favorable but is kinetically blocked in growing microtubules. Thus in microtubules undergoing dynamic instability, the rate-limiting step in GTP turnover is an infrequent "catastrophe" that alters the structure of the microtubule end to allow rapid depolymerization of GDP-bound subunits (*15*).

For FtsZ, the details of the GTP hydrolysis cycle and its relationship to polymer turnover remain to be determined. Figure 1 shows a diagram of the FtsZ polymerization cycle (including rates determined in this study; see below). A key question is how to identify the rate-limiting step. Currently, it is unclear whether the cycle is rate limited by subunit dissociation from the polymer, as is the case for tubulin and actin, or by some other step. The data from several studies have implications for understanding the rate-limiting step. Two studies found that, in vitro, FtsZ polymers can exchange nucleotide much more rapidly than they hydrolyze it (*17*, *18*), suggesting that breaking protein interfaces is not the rate-limiting step for nucleotide turnover. Another study found that, in the Z ring in vivo, the rate of GTP turnover directly correlates with the rate of FtsZ subunit turnover (*19*), suggesting that hydrolysis itself may be rate limiting. The nucleotide intermediate to which FtsZ polymers are bound

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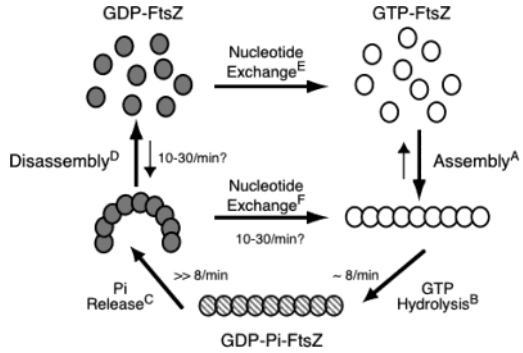

FIGURE 1: Model of FtsZ's hydrolysis cycle. The model includes rates determined in this study. Key: (open circle) GTP−FtsZ; (slashed circle) GDP−P_i−FtsZ; (solid circle) GDP−FtsZ. GTP−FtsZ assembles into straight polymers (A) and once assembled can hydrolyze nucleotide (B); $k_{hydrolysis} \approx 8$/min. Hydrolysis is a rate-limiting step, so that most subunits in the polymer are bound to GTP. Phosphate release (C) rapidly follows hydrolysis; $k_{P_i\ release} \gg 8$/min. A step following phosphate release is also partially rate limiting, leaving some fraction of FtsZ subunits bound to GDP. This step might be either the breaking or breathing of interactions between GDP subunits (D) or the rate of release of GDP directly from the polymer (F), as suggested in ref *17*. It must occur at ∼10−30/min in order to account for the total turnover rate in our experiments (4.5/min per FtsZ). Figure adapted from ref *3*.

at steady state remains unresolved. Polymers have been found to retain stoichiometric amounts of radioactivity from [γ-^{32}P]-GTP, but different studies have proposed that the nucleotide is in the form of GDP−P_i (*20*) or GTP (*17*). However, no assays have been developed that are capable of distinguishing between these two nucleotide species. The difficulty derives from the need to isolate polymer-bound nucleotide on a time scale in which little nucleotide exchange or hydrolysis occurs.

Our goal in this study was to understand the rate-limiting step in GTP turnover by single FtsZ protofilaments. This is a necessary starting point for future studies that probe the more complex assemblies that may predominate in vivo, for example, bundles cross-linked by other proteins. To avoid the technical caveats of previous studies, we used several complementary assays that do not require polymer isolation. We measured polymer-bound GDP in reactions that included a nucleotide regenerating system to convert all unbound nucleotide into GTP. In addition, single turnover assays were used to directly measure the rate of the hydrolysis step. In these latter experiments, the reaction was initiated by the addition of Mg^{2+} to polymers preassembled in EDTA.[1] The results from these assays indicate that hydrolysis is a rate-limiting step in GTP turnover and phosphate release rapidly follows.

EXPERIMENTAL PROCEDURES

Protein Preparation. FtsZ was expressed and purified as in ref *10*. On the day of each experiment, protein was cycled (assembled and then disassembled in calcium to select for active, nonaggregated protein) as in ref *10*, except that aliquots of FtsZ were diluted 3-fold prior to initial assembly, and for single turnover assays the final pellet was resuspended in 1× or 2× spin column buffer (50 mM NaMES, pH 6.5, 50 mM KCl, 1 mM EGTA).

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PNP, purine nucleoside phosphorylase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate.

For single turnover assays, excess nucleotide was removed by centrifugation through a 5 mL column of Sephadex G-25 fine resin (Sigma) prewashed with 30 mL of 1× or 2× spin column buffer. EDTA (1 mM) was included in the buffer when GTP was desired in the final FtsZ sample; EDTA was omitted when GDP was desired. The column was spun three times at 4 °C for 2 min at 670*g*. Between spins, a volume of buffer equivalent to the volume of protein to be desalted (150−400 μL) was added to the top of the column. The prepared column was kept at 4 °C to minimize subsequent FtsZ assembly in the column. When GTP was desired in the final protein sample, cycled FtsZ on ice was supplemented with 1 mM EDTA and 0.5 mM GTP. The protein was immediately loaded onto the column and spun at 4 °C for 2 min at 670*g*. Even at 4 °C, assembly of FtsZ−GTP caused solutions to be quite viscous, resulting in variable and frequently significant yield loss (∼25−75%).

Analysis of Protein and Nucleotide Concentrations. Protein concentrations were determined with a Bradford assay (Bio-Rad), using a BSA standard and adjusting the final concentration by a factor of 1.2 (*11*, *21*).

Nucleotide concentration in a protein sample was analyzed by one of two methods. (A) An FtsZ sample or GTP was diluted to 10−100 μM in polymerization buffer (50 mM NaMES, pH 6.5, 50 mM KCl, 2.5 mM $MgSO_4$, 1 mM EGTA). Protein was denatured and precipitated by addition of three volumes of 1 M perchloric acid and centrifugation at 4 °C for 10 min at 20800*g*. The 220−300 nm UV spectra of the supernatants confirmed that the protein had been removed. The OD_{253} was used to determine GNP concentration, using the GTP samples as standards. The GTP standards were handled identically to the experimental samples in order to account for any sample loss during precipitation, filtering, or column-loading steps; these standards had been previously calibrated using their extinction coefficient and OD_{253}. (B) Nucleotide was extracted from FtsZ and separated on a Smart System Mono-Q column (Pharmacia) (see GTP Hydrolysis in Single Turnover Assays). The area under the GTP and GDP peaks was calibrated relative to pure standards of known concentration. The nucleotide:FtsZ ratio in samples determined using method A was 1.36 ± 0.44 (nine measurements of six independent experiments); using method B, it was 0.69 ± 0.28 (ten measurements of eight independent experiments). Samples were prepared identically; the difference may be due to error in calibrating the two methods. Alternatively, the two techniques were used during different stages of the study; the gel filtration columns run in later experiments may have been more efficient at removing nucleotide.

Electron Microscopy. Negative stain electron microscopy was used to visualize FtsZ filaments. Carbon-coated 400 mesh copper grids were glow discharged for 2 min. Cycled protein was diluted in polymerization buffer. Reactions were initiated with the addition of GTP and, where indicated, a nucleotide regenerating system (see Detection of Polymer-Bound GDP). After incubation for 2 min at 30 °C, a drop was then applied to the carbon, and the reaction was incubated for 10 s before the excess was blotted. The grid was rinsed with 3−4 drops of 0.75% uranyl formate, blotted, and air-dried. Filaments were visualized using a Phillips 401 electron microscope at 50000× magnification.

Steady-State GTP Hydrolysis Measurements. The EnzChek phosphate assay kit (Molecular Probes) was used to detect steady-state GTP hydrolysis. Briefly, purine nucleotide phosphorylase (PNP) requires phosphate to cleave 2-amino-6-mercapto-7-methylpurine riboside (MESG), resulting in an absorbance change at 360 nm. Steady-state GTP hydrolysis assays were performed at 30 °C in polymerization buffer plus 1 mM GTP, 200 μM MESG, and 5 units/mL PNP. Sodium phosphate in polymerization buffer was used as a standard (0.009 $OD_{360}/\mu M$ P_i at 30 °C).

Detection of Polymer-Bound GDP. Protein was diluted in 25 μL of polymerization buffer containing 20 μM cold GTP, 0.8 μCi of [α-^{32}P]GTP, and a GTP regenerating system. The regenerating system consisted of 400−500 units/mL pyruvate kinase plus 2 mM phosphoenolpyruvate. For FtsZ, reactions were incubated for 3 min at 3 °C in order to reach steady state (*10*). For tubulin, the reactions were incubated on ice for 5 min to allow nucleotide exchange; Paclitaxel (Sigma) was then added at concentrations equimolar to tubulin, and the reaction was incubated for an additional 10 min at 37 °C. After incubation, all reactions were rapidly quenched with 3 volumes of 1 M cold perchloric acid/10 mM EDTA; mixing was completed within 1 s. The acid was neutralized with 2 volumes (relative to the original reaction) of 1 M K_2CO_3, and the resulting precipitate was removed by sedimentation at 4 °C at 20800*g* for 3 min.

To analyze the nucleotide in each sample, 5 μL of the supernatants was spotted onto poly(ethylenimine)−cellulose thin-layer chromatography (TLC) plates (J. T. Baker) and allowed to dry. The TLC plate was prerun in water, dried for 1−2 h, and run again in 1 M LiCl. Plates were exposed to a phosphor screen (Molecular Dynamics), read in a Bio-Rad molecular imager, and analyzed using QuantityOne software. The quantity of GDP was calculated by multiplying the total nucleotide in a reaction (nucleotide from the FtsZ sample plus 20 μM added GTP) by the fraction of the signal that was in the GDP spot. The fraction of polymer subunits bound to GDP was determined by plotting GDP versus total protein and calculating the slope.

Control experiments indicated that the regenerating system is sufficient to keep pace with FtsZ's steady-state hydrolysis rate but is rapidly inactivated during quenching. Varying the concentration of the regenerating system 2−3-fold did not affect results (data not shown). If the regenerating system was omitted from reactions, 88 ± 7% of the total nucleotide was hydrolyzed to GDP (up to 250% of the total FtsZ concentration). Similar results (90.5 ± 0.7%) were seen if the regenerating system was added after quenching with perchloric acid. These controls, along with the tubulin data presented below, indicate that little GDP released from the polymer during quenching is converted to GTP by the regenerating system.

GTP Hydrolysis in Single Turnover Assays. To detect hydrolysis of GTP in a single turnover assay, excess GTP was removed from FtsZ protein in the presence of EDTA, as described above. FtsZ was then diluted to the appropriate concentration in polymerization buffer (four separate experiments of 25, 25, 35, and 45 μM FtsZ). The protein was warmed to 30 °C and then rapidly added to 1.75 μL of $MgSO_4$ at the bottom of a tube at 30 °C, resulting in a final volume of 50 μL with 3.5 mM $MgSO_4$/1 mM EDTA. Reactions were incubated at 30 °C for the indicated times and rapidly quenched with 200 μL of cold 10 M urea/50 mM Tris, pH 7.5/10 mM EDTA. Samples were heated to 90 °C for 1 min, diluted with 100 μL of H_2O, and spun through a 30 kDa microcon filter (Millipore) for 12 min at 14000*g* at 4 °C. The 200 μL samples were loaded onto a 0.1 mL Smart System Mono-Q column (Pharmacia) and eluted with a 3 mL, 0.1−0.5 M ammonium bicarbonate gradient. Moles of GTP and GDP was determined by integrating under the peaks and comparing to known standards. For final rate determinations, GTP depletion rather than GDP appearance was monitored because the GDP peaks overlapped with a peak from the EDTA used to quench the reaction. The reaction rate was determined by plotting GTP versus time and fitting to a single exponential.

Phosphate Release in Single Turnover Assays. Phosphate release kinetics were determined using the EnzChek assay described above, except that 100 units/mL PNP was used. PNP at 100 units/mL could potentially convert phosphate to signal at a rate 3 orders of magnitude faster than FtsZ hydrolyzes GTP. However, in these assays, phosphate is significantly below the K_m for the PNP reaction (data not shown). Nonetheless, the effective rate of the system is >10 times that of the reactions being measured, as determined in control experiments using both a stopped-flow apparatus and manual mixing (data not shown).

For single turnover assays, excess GDP or GTP was removed from FtsZ as described above. Reactions were initiated with the addition of $MgSO_4$ (for GTP−FtsZ) or GTP (for GDP−FtsZ) as follows. FtsZ was combined with the EnzChek components in polymerization buffer at 30 °C (eight separate experiments of 7, 7, 20, 20, 37.5, 45, 46, and 50 μM final FtsZ). Using a prewarmed pipet tip, this mixture was rapidly added to 1−5 μL of magnesium sulfate or GTP at the bottom of a cuvette at 30 °C in a spectrophotometer. Final concentrations were 3.5 mM $MgSO_4$/1 mM EDTA (for GTP−FtsZ) or 1 equiv of GTP (for GDP−FtsZ). The first 2−4 s of each reaction was missed during mixing and closing of the spectrophotometer lid.

In control experiments in which GTP was added to the regenerating system in the absence of FtsZ, the OD_{360} readout was flat, indicating that within the dead time of the assay all inorganic phosphate preexisting in the GTP was converted to signal by the PNP. An instrument-derived slow baseline drift was occasionally visible in all traces from a single day; such drift was subtracted from all experiments for that day. Results were fit to a single exponential.

Calculation of the Rate of GDP Release. A step leading to GDP release was estimated to occur at between 10 and 30/s. These rates were calculated as follows. At the saturating GTP concentrations used here, the turnover rate, k_{cat} (=4.5/min per FtsZ), was assumed to be due to hydrolysis and one other rate-limiting step, X. In such situations, $1/k_{cat} = 1/k_{hydrolysis} + 1/k_X$. This equation was solved in two ways. In the first, the data from the single turnover assays were used to set $k_{hydrolysis} = 8.2$. Here, $k_X = 10$/min. In the alternative calculation, the data from the polymer-bound GDP assays were used to set $k_{hydrolysis} = x$ and $k_X = 4x$. In this case, $k_X = 30$/min.

RESULTS

Polymer Formation and Steady-State GTP Turnover. In this study, our goal was to investigate the nucleotide

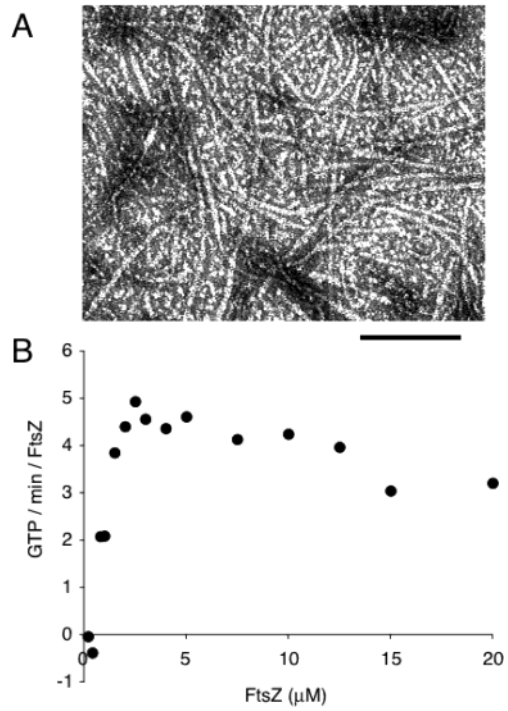

FIGURE 2: Concentration dependence of steady-state GTP turnover and polymer assembly. (A) Polymers visualized by negative stain electron microscopy; 10 μM FtsZ + 1 mM GTP. Polymers are largely single stranded although some lateral association of filaments also occurs. Bar indicates 100 nm. (B) Steady-state GTP hydrolysis (expressed as specific activity) versus FtsZ concentration. FtsZ was incubated with 1 mM GTP, and phosphate production was detected using a coupled enzyme system (Enzchek, Molecular Probes).

hydrolysis cycle of single FtsZ filaments as a baseline for future studies of more complex polymer forms. We therefore used negative stain electron microscopy (EM) to examine whether our FtsZ preparation polymerized into single protofilaments under the experimental conditions used here. In the presence of GTP, the minimum FtsZ concentration necessary to detect short single-stranded polymers was 0.5 μM (data not shown). Reactions with 10 μM FtsZ and 1 mM GTP consisted predominantly of single-stranded polymers (Figure 2A), although some lateral associations of protofilaments also existed and increased in prevalence at higher protein concentrations. Similar filaments were observed with only 20 μM added GTP if a nucleotide regenerating system was included in the reaction (data not shown).

The effect of FtsZ concentration on steady-state GTP turnover is shown in Figure 2B. Hydrolysis could be detected in reactions with $\geq$0.8 μM FtsZ. Reactions with between 2 and 10 μM FtsZ showed maximum specific activities (4.5 $\pm$ 0.4/min per FtsZ) that did not vary significantly with FtsZ concentration. Turnover rates began to decrease at higher FtsZ concentrations, presumably because polymer bundling led to decreased rates of polymer breakage or subunit dissociation.

The Majority of Subunits in FtsZ Polymers Are GTP Bound. It is difficult to determine the nucleotide intermediates bound to a polymer when the polymer is highly dynamic and nucleotide hydrolysis and product release are ongoing. Assays that detect such intermediates by separating bound from free nucleotide must be able to do so in much less than the half-time for hydrolysis and must also be able to prevent

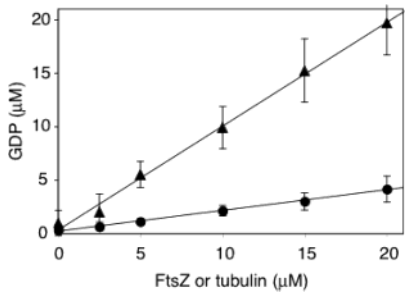

FIGURE 3: FtsZ polymers at steady-state contain only a small fraction of GDP-bound subunits. Polymers were assembled in the presence of a nucleotide regenerating system, so that the only GDP that exists in the reactions is polymer-bound. Key: (●) FtsZ + 20 μM GTP (19% of FtsZ subunits are bound to GDP); (▲) tubulin + equimolar Taxol + 20 μM GTP (97% of tubulin subunits are bound to GDP). Means $\pm$ standard deviations are shown.

further hydrolysis during sample handling. For FtsZ, the half-time for hydrolysis is less than 10 s, making such experiments technically challenging.

Here we develop an assay that does not require separation of bound from free nucleotide and in which samples were processed in <1 s with an acid quench that blocked further hydrolysis. FtsZ was incubated with low concentrations of [α-^{32}P]GTP in the presence of a nucleotide regenerating system. This regenerating system rapidly converted all nucleotide free in solution into GTP. As a result, the only GDP present in a reaction was that bound to the FtsZ and sequestered from the regenerating system (see Experimental Procedures for a discussion of control experiments). Reactions were allowed to reach steady state and then rapidly quenched with perchloric acid and EDTA. Extracted nucleotide was separated by thin-layer chromatography, and the GDP was quantitated. Figure 3 shows the results of reactions with 0−20 μM FtsZ polymers and control reactions with 0−20 μM microtubules.

Reactions with FtsZ polymers contained only 20% as much GDP as FtsZ, suggesting that four out of five FtsZ subunits were bound to GTP. The majority of these GTP-bound subunits must be in polymers, because the critical concentration for FtsZ assembly was 0.5 μM and our reactions contained between 2.5 and 20 μM FtsZ. The results suggest that, in FtsZ polymers, the main rate-limiting step in nucleotide turnover occurs at or before GTP hydrolysis. The linearity of the results with FtsZ concentration suggests either that the degree of polymer bundling does not change greatly over the FtsZ concentration range tested or that the loose lateral filament associations visible by EM do not significantly alter the rate-limiting steps in nucleotide turnover.

Control experiments with microtubules confirmed that the GDP/protein ratio in our assay is appropriately calibrated. Taxol-stabilized microtubules were found to consist of essentially 100% GDP-bound subunits, as expected. Taxol is a drug that allows assembly and hydrolysis but inhibits disassembly. The drug was included in these assays in order that tubulin's critical concentration be negligible [0.2 mg/mL (*22*)], so that the concentration of microtubules could be known with high accuracy.

Hydrolysis Is a Rate-Limiting Step in Nucleotide Turnover. The above results suggest that GTP hydrolysis is the major rate-limiting step in nucleotide turnover by FtsZ polymers.

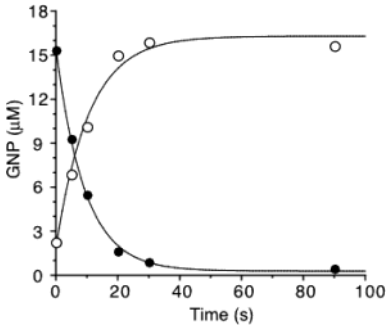

FIGURE 4: Kinetics of GTP hydrolysis in a single turnover assay. This representative experiment shows hydrolysis of GTP to GDP by 25 μM FtsZ. The FtsZ was purified with equimolar bound GTP and 1 mM EDTA; hydrolysis was initiated by the addition of 3.5 mM $MgSO_4$. Reactions were quenched at various times with perchloric acid, and the nucleotide was analyzed by anion-exchange chromatography. For $t = 0$, perchloric acid was added before the $MgSO_4$. Results were fit to a single exponential. Key: (○) GTP ($k_{GTP\ decrease} = 6.5 \pm 0.3$/min;) (●) GDP ($k_{GDP\ increase} = 5.6 \pm 0.9$/min).

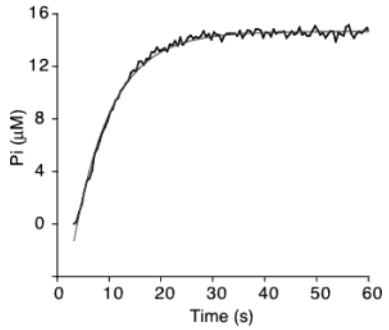

FIGURE 5: Kinetics of phosphate release in a single turnover assay. This representative experiment shows the release of phosphate from 20 μM FtsZ. The FtsZ was purified with equimolar bound GTP and 1 mM EDTA; hydrolysis was initiated by the addition of 3.5 mM $MgSO_4$ at $t = 0$. Phosphate release was detected using a spectroscopic coupled enzyme system (Enzchek, Molecular Probes). Results were fit to a single exponential. The apparent rate represents the rate of hydrolysis followed by phosphate release. $k_{hydrolysis + P_i\ release} = 8.3 \pm 0.1$/min.

To confirm this, we directly measured the rate of hydrolysis ($k_{hydrolysis}$, B in Figure 1) using single turnover assays. GTP-bound FtsZ polymers were assembled in the presence of EDTA (*24*) to prevent hydrolysis. Excess nucleotide was removed using a gel filtration column so that each subunit could undergo only a single round of hydrolysis. Reactions were initiated by the rapid addition of magnesium and quenched at various times with perchloric acid, and the nucleotide was analyzed by column chromatography.

The results of a typical reaction are shown in Figure 4. GTP decreased while GDP concurrently increased, both at rates of ~6/min. The rate of $k_{hydrolysis}$ measured in four separate reactions containing 25−45 μM FtsZ did not correlate with protein concentration (Figure 6, inset); the rates of GTP depletion are therefore plotted together in Figure 6. They ranged from 6.5 to 11/min and averaged 8.2 ± 2.4 min^{-1}.

Results again suggest that $k_{hydrolysis}$ is a rate-limiting step in nucleotide turnover, confirming the conclusions of the above experiments. The average rate of $k_{hydrolysis}$ (8.2 ± 2.4 min^{-1}) was on the same order as the steady-state hydrolysis rate (4.5 ± 0.4 min^{-1}). However, hydrolysis is somewhat faster than steady-state turnover ($p < 0.05$). If another step

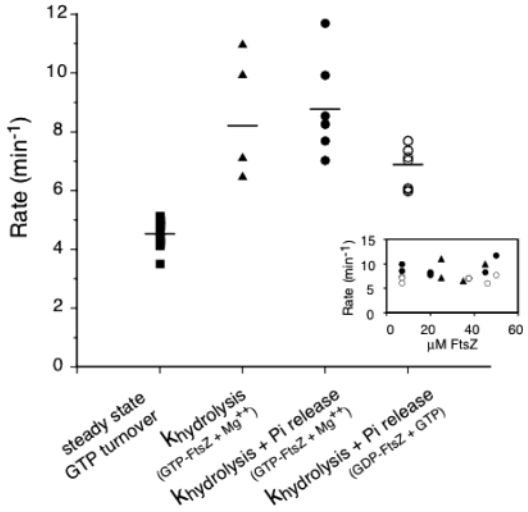

FIGURE 6: Hydrolysis is a rate-limiting step in GTP turnover, and P_i release rapidly follows. The steady-state turnover rate was compared to the rates of hydrolysis ($k_{hydrolysis}$) and of hydrolysis plus phosphate release ($k_{hydrolysis + P_i\ release}$) detected in single turnover assays. For the single turnover assays, two approaches were used: GTP−FtsZ was mixed with magnesium or GDP−FtsZ was mixed with equimolar GTP. Inset: Single turnover rates did not correlate with FtsZ concentration. Main graph: All FtsZ concentrations are plotted together. Key: (■) Steady-state hydrolysis rates, 2−10 μM FtsZ; (▲) $k_{hydrolysis}$, 25−45 μM GTP−FtsZ + $MgSO_4$; (●) $k_{hydrolysis + P_i\ release}$, 7−50 μM GTP−FtsZ + $MgSO_4$; (○) $k_{hydrolysis + P_i\ release}$, 7−50 μM GDP−FtsZ + equimolar GTP. Bars indicate the average of all experiments.

in nucleotide turnover besides hydrolysis were also partially rate limiting, it would account for the ~20% of FtsZ subunits bound to GDP.

Phosphate Release Rapidly Follows Hydrolysis. Two possibilities for an additional rate-limiting step included the release of phosphate from the polymer (C in Figure 1) and the release of GDP from the polymer (either D + E or F in Figure 1). To determine whether phosphate release was slow enough to contribute to the overall turnover time, we performed single turnover assays as above except that phosphate release rather than GTP hydrolysis was detected. In these experiments, the transit time measured is that of the two steps combined, hydrolysis followed by phosphate release ($k_{hydrolysis + P_i\ release}$, B plus C in Figure 1). If phosphate release were partially rate limiting, the rate detected here should be slower than that seen for hydrolysis alone and might possibly be equal to the steady-state turnover rate.

As above, FtsZ was preassembled with equimolar GTP in the presence of EDTA. Reactions were initiated with magnesium, and phosphate release was detected with a spectroscopically coupled enzyme system (see Experimental Porcedures for details). A sample result with 20 μM FtsZ is shown in Figure 5; here $k_{hydrolysis + P_i\ release} = 8.3$/min. Concentrations of FtsZ from 7 to 50 μM were assayed; again, results did not correlate with protein concentration (Figure 6, inset) and so are plotted together in Figure 6.

The results do not support the hypothesis that phosphate release is a slow step in nucleotide turnover. If phosphate release were a slow step, $k_{hydrolysis + P_i\ release}$ should have been significantly slower than $k_{hydrolysis}$ alone. Instead, the average rate measured was 8.8 ± 1.6/min, once again somewhat faster than the steady-state turnover rate ($p \ll 0.01$) and, within the accuracy of the data, not significantly different from the rate seen for hydrolysis alone ($p = 0.46$). It is

therefore unlikely that many GDP−P_i-bound subunits exist in FtsZ polymers.

In the above single turnover assays, high FtsZ concentrations were used in order that the majority of the protein be GTP bound and assembled at the start of a reaction. This was particularly important because EDTA can decrease nucleotide affinity and increase the critical concentration of polymerization to 3 μM (*23*, *24*). At these high protein concentrations, bundling of FtsZ polymers is enhanced, resulting in a decreased steady-state GTP turnover rate. However, the slower turnover is likely due to a slower rate of GDP release from the polymer (D+E or F in Figure 1) rather than to a change in the rate of $k_{hydrolysis}$ itself (B in Figure 1).

It was still possible that, at these high FtsZ concentrations, a slow diffusion of the magnesium into polymer bundles resulted in an apparent slow rate of $k_{hydrolysis}$. Arguing against this, although bundling increases with FtsZ concentration, the measured rates did not (Figure 6, inset). To further rule out any artifactual effects of preassembling the polymers at high protein concentrations, we performed an additional set of single turnover experiments in which the FtsZ began predominantly unassembled. FtsZ containing 1 equiv of GDP was rapidly mixed with an additional equivalent of GTP. In such experiments, the GDP−FtsZ is likely to start as small oligomers (*11*) that must release GDP, bind GTP, and assemble before hydrolysis can occur (E, A, and B in Figure 1). Such nucleotide exchange and assembly are likely to be quite rapid at these protein concentrations (>40/min; unpublished data) and thus contribute minimally to the measured rates. Using this alternative approach, $k_{hydrolysis + P_i\ release}$ occurred at a rate of 6.9 ± 0.7/min (Figure 6), similar to the previous results. The most parsimonious interpretation is that the step common to all reactions, hydrolysis itself, is rate limiting and occurs at ∼8/min.

DISCUSSION

Remodeling of cytoskeletal polymers is induced by transitions between nucleotide hydrolysis intermediates. The energy from hydrolysis can be used to destabilize a previously stable structure and to produce mechanical work. For FtsZ, the steps that limit nucleotide and polymer turnover are not fully understood. Conflicting reports have suggested different rate-limiting steps and polymer-bound nucleotide intermediates for FtsZ. Here we investigate GTP hydrolysis in FtsZ polymers using several complementary approaches that avoid the technical caveats of previous studies. The results indicate that GTP hydrolysis is a rate-limiting step in FtsZ's nucleotide turnover cycle and that, at steady state, single-stranded FtsZ polymers consist largely of GTP-bound subunits (see model, Figure 1). An additional step leading to GDP release was also found to be partially rate limiting. These results clarify the previously conflicting studies in the literature and have implications for the possible mechanisms of turnover of pure FtsZ polymers.

Rate-Limiting Hydrolysis Results in Predominantly GTP-Bound Polymer Subunits. $k_{hydrolysis}$ is a major rate-limiting step in GTP turnover, and as a result, FtsZ filaments predominantly contain GTP. The rate of $k_{hydrolysis}$ in FtsZ polymers (∼8/min, Figure 1) approximates some of the fastest turnover rates seen for *Escherichia coli* FtsZ [8.3/min per FtsZ, our unpublished observations; 6.5/min per FtsZ (*17*)]. These results are consistent with previous reports in which FtsZ polymers retained stoichiometric amounts of radioactivity from [γ-^{32}P]GTP (*17*, *20*) and suggest that, under certain conditions, $k_{hydrolysis}$ may be the only rate-limiting step for GTP turnover.

The hydrolysis kinetics of FtsZ filaments differ significantly from those of tubulin. The hydrolysis rate estimated to occur in rapidly growing microtubules is more than 2 orders of magnitude faster than the $k_{hydrolysis}$ seen here for FtsZ [hydrolysis occurs at >200/s for microtubules (*15*), corresponding to >900/min per protofilament]. Nucleotide hydrolysis in FtsZ polymers occurs at rates more similar to that seen for actin [18/min (*25*)].

One previously published study suggested that the nucleotide in FtsZ polymers was nearly entirely in the form of GDP−P_i rather than GTP (*20*). An explanation for the discrepancy between this study and ours may be in the different methods of sample handling. In the previous study, polymer was either bound to a nitrocellulose filter or precipitated using ammonium sulfate. Neither approach denatures FtsZ, so that hydrolysis may continue. Our results, coupled with studies that report rapid nucleotide exchange in FtsZ polymers (*17*, *18*), suggest that, in the previous study, the hydrolysis to GDP−P_i may have occurred during polymer isolation steps.

P_i Release Is Rapid, and a Step Leading to GDP Release Is Partially Rate Limiting. Although $k_{hydrolysis}$ is a major rate-limiting step in FtsZ's hydrolysis cycle, another step was also partially rate limiting in our assays. $k_{hydrolysis}$ was nearly 2-fold faster than the total turnover time, and ∼20% of the polymer subunits were bound to GDP rather than GTP. These results suggest that an additional rate-limiting step exists and occurs at ∼10−30/min (see Experimental Procedures for calculations).

Phosphate release was not found to be rate limiting (≫8/min; Figure 1), and thus in contrast to previous models (*20*), FtsZ polymers are unlikely to contain many GDP−P_i-bound subunits. The remaining subunits are therefore bound to GDP alone, and a step leading to release of this GDP is partially rate limiting. GDP release could occur either through subunit depolymerization followed by GDP release (D and E in Figure 1), as is necessary for tubulin, or by direct release of the nucleotide without alteration of the protein−protein interactions (F in Figure 1), as has been suggested for FtsZ (*17*). Intermediate between these extremes, subunit interactions might be able to breathe and release nucleotide without completely dissolving.

Implications for in Vitro Polymer Turnover Mechanisms. The rapid release of hydrolysis products from individual FtsZ filaments implies that the energy from GTP hydrolysis is quickly dissipated. This is in contrast to microtubules, in which virtually every subunit is bound to GDP and the energy from hydrolysis is stored as strain in the polymer (*26*). During microtubule catastrophes, this strain is released as tubulin protofilaments separate and rapidly peel away. The coordinated release of energy from multiple tubulin subunits during microtubule disassembly allows depolymerization to power movement of chromosomes and glass microspheres in vitro (*27*, *28*). Single-stranded FtsZ polymers containing GTP are unlikely to be able to similarly pool the energy of hydrolysis from multiple subunits to produce large forces. Instead, the energy from each hydrolysis event may be

released separately by the breaking of an individual polymer interface or the exchange of nucleotide on a single subunit.

Nonetheless, at any given time some fraction of the FtsZ subunits in our experiments had hydrolyzed nucleotide but not yet released GDP. These subunits may be dispersed throughout single-stranded polymers or may be located at the scattered sites of polymer bundling that occur occasionally at these high FtsZ concentrations. Alternatively, even in single-stranded filaments, GDP-bound subunits might cluster together. Electron microscopy pictures occasionally show individual filaments with sharply curled ends (*29*, *30*). If this is not a microscopy artifact, it may be a clue to the mechanism of FtsZ turnover. Clustering of GDP-bound subunits at the end of a polymer might indicate that single-stranded FtsZ polymers could turn over via treadmilling in a manner similar to that of actin filaments. It is also impossible to rule out that these small fractions of GDP-bound subunits could release from the end of a polymer in a concerted manner akin to microtubule disassembly. Distinguishing between these possibilities will be a challenging future goal.

Implications for in Vivo FtsZ Turnover. The rates measured here relate to the simplest, single-stranded FtsZ structures and provide a baseline for the comparison of more complicated systems, both in vitro and in vivo. Because $k_{hydrolysis}$ is only partially rate limiting, factors that cause relatively small changes in the rates of other steps in the hydrolysis cycle may result in polymers that exhibit quite different properties. Thus the hydrolysis cycle may be tuned to regulate FtsZ polymer dynamics.

In vivo, FtsZ filaments in the Z ring are likely to be a part of a higher order structure. The tight bundling of polymers in vitro has been found to inhibit nucleotide release (*17*) and to increase the fraction of GDP in the polymer (unpublished observations). Similar bundling of polymers in vivo may explain why turnover of subunits in the Z ring [$t_{1/2} = 12-30$ s (*19*, *31*)] occurs at a rate somewhat slower than that seen here for $k_{hydrolysis}$ ($t_{1/2} \approx 5$ s). Thus polymer bundles in vivo may couple hydrolysis to subunit turnover (*19*) more tightly than has been reported to occur in FtsZ polymers in vitro (*17*).

The coupling of hydrolysis and polymer turnover in FtsZ bundles could be used to regulate cell division in several ways. Inhibition of GDP release may allow polymers to use the energy from hydrolysis to curve and produce force during ring constriction. Alternatively, lateral interactions may stiffen FtsZ filaments, limiting both curvature and disassembly; the coordinated dissolution of such bundles might then allow concerted depolymerization during septation.

In the future, it will be important to study FtsZ's hydrolysis cycle under conditions in which higher order structures such as filament pairs and bundles predominate, both in pure FtsZ preparations (*18*, *32*, *33*) and in the presence of cell division proteins such as ZipA and ZapA (*34*−*36*). In addition, alternative mechanisms exist for altering the distribution of nucleotide intermediates in the polymers, for example, by directly enhancing $k_{hydrolysis}$, and these might also be used by FtsZ-interacting proteins to regulate assembly and disassembly in the cell.

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