The Interactions of Cell Division Protein FtsZ with Guanine Nucleotides

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Prokaryotic cell division protein FtsZ, an assembling GTPase, directs the formation of the septosome between daughter cells. FtsZ is an attractive target for the development of new antibiotics. Assembly dynamics of FtsZ is regulated by the binding, hydrolysis, and exchange of GTP. We have determined the energetics of nucleotide binding to model apoFtsZ from Methanococcus jannaschii and studied the kinetics of 2′/3′-O-(N-methylanthraniloyl) (mant)-nucleotide binding and dissociation from FtsZ polymers, employing colorimetric, fluorescence, and stopped-flow methods. FtsZ binds GTP and GDP with $K_D$ values ranging from 20 to 300 μM under various conditions. GTP-Mg$^{2+}$ and GDP-Mg$^{2+}$ bind with slightly reduced affinity. Bound GTP and the coordinated Mg$^{2+}$ ion play a minor structural role in FtsZ monomers, but Mg$^{2+}$-assisted GTP hydrolysis triggers polymer disassembly. Mant-GTP binds and dissociates quickly from FtsZ monomers, with ~10-fold lower affinity than GDP. Mant-GTP displacement measured by fluorescence anisotropy provides a method to test the binding of any competing molecules to the FtsZ nucleotide site. Mant-GTP is very slowly hydrolyzed and remains exchangeable in FtsZ polymers, but it becomes kinetically stabilized, with a ~30-fold slower hydrolysis turnover and with the reported subunit turnover in FtsZ polymers. Although FtsZ polymers can disassemble upon either GTP consumption or GDP addition (18, 19), GDP binding destabilizes FtsZ polymers the main rate-limiting step (20). In E. coli FtsZ polymers, the main rate-limiting step in nucleotide turnover was found to be nucleotide hydrolysis, rapidly followed by phosphate release, whereas a second rate-limiting step could be nucleotide dissociation. However, whether nucleotide dissociation took place directly from the polymer or through depolymerization into subunits, followed by GDP release, was not determined (21).

An important problem yet to be solved for FtsZ assembly dynamics is whether, following GTP hydrolysis (i) GDP dissociates from subunits in the FtsZ polymer which directly reload with GTP, (ii) polymer subunits exchange with GTP-bound subunits in solution, or (iii) the FtsZ-GDP polymer fully disas-

FtsZ is a cytoskeletal protein essential to bacterial cytokinesis and a member of the tubulin family of GTPases, which also includes αβ-tubulin (1), γ-tubulin (2), bacterial tubulin BtubA/B (3, 4), and TubZ (5). FtsZ assembles by forming filaments that constitute the Z-ring at the cell division site in bacteria. The Z-ring, a dynamic structure maintained by assembly and disassembly of FtsZ, recruits the other elements of the division machinery following chromosome segregation (6–10). bacterial cell growth and division are regulated by nutrient availability; a metabolic sensor has been recently identified in Bacillus subtilis, including an effector, the glucosyltransferase UgtP, which modulates FtsZ assembly (11). GTP binding, hydrolysis, and exchange constitute the regulatory mechanism responsible for dynamics of FtsZ and tubulin polymers. The nucleotide switches of these assembling GTPases appear to involve polymerization-driven structural changes (12), although FtsZ and tubulin form different end polymers. The GTPase activity of FtsZ is modified by the polymerization inhibitory protein MipZ (13) and, weakly, by EzrA (14).

The hydrolyzable nucleotide bound to tubulin becomes occluded in microtubule protofilaments (15). Microtubules hydrolyze all bound GTP to GDP except at their very ends and become metastable, giving rise to microtubule dynamic instability (16). In contrast, polymers of FtsZ from E. coli were reported to contain mostly GTP, and, under certain conditions, nucleotide exchange proceeds faster than hydrolysis (17). This suggested that the nucleotide binding site remains exchangeable in FtsZ polymers, which would therefore be devoid of dynamic instability. Polymers of Methanococcus jannaschii FtsZ were found to contain different proportions of GTP and GDP (depending on the hydrolysis rate) and to rapidly depolymerize upon either GTP consumption or GDP addition (18, 19). GDP binding destabilizes M. jannaschii FtsZ polymers compared with polymers with GTP or without a bound nucleotide (20). In E. coli FtsZ polymers the main rate-limiting step in nucleotide turnover was found to be nucleotide hydrolysis, rapidly followed by phosphate release, whereas a second rate-limiting step could be nucleotide dissociation. However, whether nucleotide dissociation took place directly from the polymer or through depolymerization into subunits, followed by GDP release, was not determined (21).

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Assembles and reassembles again from GTP-bound subunits. Consistent with an exchangeable nucleotide in FtsZ polymers, the nucleotide was observed to be largely accessible in the crystal structure of a protofilament-like dimer of *M. jannaschii* FtsZ (22). On the other hand, exchange of GFP-FtsZ fusions in bacterial Z-rings was found to proceed with a half-time of 8–9 s in vivo, by means of fluorescence recovery after photobleaching (23, 24). As observed in an in vitro fluorescence resonance energy transfer assay, subunit turnover in filaments of *E. coli* FtsZ took place with a half-time of 7 s with GTP, which was slowed down under conditions reducing the nucleotide hydrolysis rate (25). This rate of subunit turnover is comparable with the turnover rate of GTP hydrolysis (21) and with the rate of depolymerization in GDP excess, suggesting that GDP does not exchange into intact filaments (23). This favors the interpretation that the rapid assembly dynamics of FtsZ filaments may operate by a mechanism related to microtubule dynamic instability (25). In addition, subunit turnover and GTPase in FtsZ from *Mycobacterium tuberculosis* are both about 10 times slower than in *E. coli* FtsZ (26).

FtsZ and its nucleotide binding site are attractive targets for cell division inhibitors, which may lead to new classes of antibacterial compounds (27) to fight the continuous emergence of antibiotic resistance. Small molecules reported to modulate FtsZ assembly include 8-bromo-GTP (28) and other nucleotide analogues (29), 3-methoxybenzamide (30), viriditoxin (31), ruthenium red (32), zantrins (33), SRI-3072 (34), polyphenols (35), mant-GTP, and mant-GDP to soluble apoFtsZ was measured spectrophotometrically employing an extinction coefficient $\varepsilon_{280} = 6990$ M$^{-1}$ cm$^{-1}$ (calculated for 1 Trp, 1 Tyr). ApoFtsZ was frozen and stored in liquid nitrogen and was melted on ice before use.

**Experimental Procedures**

**Nucleotides**—GDP was obtained from Sigma, and GTP (lithium salt) was from Roche Applied Science or Sigma. mant-GTP and mant-GDP were from Jena Bioscience. [8-3H]GTP (6 Ci/mmol) and [α-32P]GTP ($\approx$400 Ci/mmol) were from American Biosciences. Nucleotides were analyzed (after extraction with perchloric acid in the case of protein samples) (18) by HPLC with a Grace Vydac 3021c4.6 anion exchange column (0.46 x 25 cm) eluted with a linear gradient of 25 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 2.8, to 125 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 2.9. All other chemicals (analytical grade) used were from Merck or Sigma.

**Preparation of Nucleotide-free FtsZ**—Nucleotide-free FtsZ (apoFtsZ) was prepared as described (20) with minor modifications. FtsZ was incubated in 2.5 mM guanidinium chloride (GdmCl) for 30 min at room temperature, followed by gel filtration in a 0.9 x 25 cm Sephadex G-25 column in 25 mM Pipes-KOH and 2.5 mM GdmCl, pH 7.5, to separate the released nucleotide from protein (monitored spectrophotometrically at 254 and 280 nm). A second G-25 column in 25 mM Pipes-KOH, 50 mM KCl, and 1 mM EDTA, pH 7.5 (Pipes-KCl buffer) was used to eliminate GdmCl and equilibrate the protein in this experimental buffer. ApoFtsZ concentration was measured spectrophotometrically employing an extinction coefficient $\varepsilon_{280} = 6990$ M$^{-1}$ cm$^{-1}$ (calculated for 1 Trp, 1 Tyr).

**Differential Scanning Calorimetry (DSC)**—Measurements were performed using a VP-DSC microcalorimeter (Microcal, Inc.). Samples were degassed at room temperature prior to calorimetric experiments. Calorimetric cells (operative volume ~0.5 ml) were kept under an extra constant pressure of 2 atm to prevent degassing during the scan. Standard VP-Viewer and Origin-DSC software (MicroCal) were used for data acquisition and analysis. Excess heat capacity ($C_p$) was obtained after subtraction of the buffer-buffer base line, and the denaturation enthalpy ($\Delta H_d$) was determined from the area under the absorption peak. Measurements were performed at a scan rate of 30 °C/h in Pipes-KCl buffer using 12 μM FtsZ. GXP and Mg$^{2+}$ concentrations were 100 μM and 10 mM, respectively.

**Isothermal Titration Calorimetry (ITC)**—Calorimetric titrations of FtsZ with GXP, GXP-Mg, and Mg$^{2+}$ were performed at 25 °C using a MCS titration calorimeter (MicroCal). Measurements were carried out in Pipes-KCl buffer, supplemented with 10 mM Mg$^{2+}$ in both protein and nucleotide solutions for titration experiments with GXP-Mg$^{2+}$ (EDTA was omitted for titration with Mg$^{2+}$). Samples were dialyzed against buffer before measurements. Ligand solutions (~150 μM GXP or 50 mM Mg$^{2+}$) were prepared in the dialysis buffer. FtsZ (10–25 μM) solution was loaded into the calorimeter cell and titrated, typically, by adding 1 × 1 μl, plus 16 ~22 injections (10 ~12 μl), of a concentrated solution of the ligand. Heats of titration were determined in separate runs and subtracted, when required, to obtain the heat of binding. Binding isotherms were analyzed by nonlinear regression analysis to a single set of sites model, using software supplied by the manufacturer, to calculate the number of binding sites (n), the binding constant ($K_b$), and the enthalpy of binding ($\Delta H$).

**Stoichiometry of Binding of Nucleotides and ApoFtsZ Polymerization**—The stoichiometry of binding of GTP, GDP, mann-GTP, and mann-GDP to soluble apoFtsZ was measured using a centrifugation assay. ApoFtsZ (6 or 8 μM) was incubated at 25 °C for 30 min with nucleotides at different known concentrations (3~15 μM) in a final volume of 0.6 ml of Pipes-KCl buffer. Samples were then centrifuged for 2.5 h at 100,000 rpm and 25 °C in a TLA-120.2 rotor employing a Beckman Optima
Affinity of Binding of [3H]GTP to ApoFtsZ—Binding of [8-3H]GTP to apoFtsZ was measured by protein depletion (44) as follows. Varying [8-3H]GTP concentrations were added to aliquots of apoFtsZ (500 nM) in Pipes-KCl buffer (0.2 ml). Mixtures were centrifuged for 1 h at 100,000 rpm and 25 °C in a Beckman TLA-100 rotor. The total [8-3H]GTP concentration was determined in the bottom half, and the free concentration was determined in the protein-depleted top half of tubes, after dilution in 1.5 ml of Beckman ReadySafe solution, employing a Wallac Trilux 1450 Microbeta liquid scintillation counter (PerkinElmer Life Sciences). In each assay, controls with [8-3H]GTP alone were included, and concentrations were corrected for the small amount of nucleotide sedimented in the absence of protein. When we measured the binding of [8-3H]GTP to an excess of apoFtsZ, 1.8% of inactive ligand was found in the stock solution. This percentage was subtracted from the concentration of free [8-3H]GTP calculated in each assay.

Affinity of Binding of Mant-nucleotides to ApoFtsZ—Binding of mant-nucleotides to FtsZ was measured by the increase in fluorescence intensity and anisotropy of the probe. It was first confirmed that more than 95% of mant-GXP co-sedimented with an excess of apoFtsZ upon high speed centrifugation. Fluorescence of free and FtsZ-bound mant-GXP was measured with a Fluorolog 3-221 instrument (Jobin Yvon-Speks, Longi-umeau, France) employing an excitation wavelength of 357 nm and an emission wavelength of 445 nm, with 3- and 5-nm bandwidths, respectively, and 2 × 10-mm cells. Anisotropy was measured in T-format with 5-nm excitation and emission slits) using standards of FtsZ-bound mant-GTP.

Affinity of Ligands Competing with Mant-GTP—Competition assays were performed by measuring, through the decrease in fluorescence anisotropy, the displacement of mant-GTP from FtsZ. Different concentrations of competing ligand were mixed with apoFtsZ (500 nM) and mant-GTP (500 nM) in Pipes-KCl, 10 mM MgCl₂ buffer (final volume of 0.4 ml), and the anisotropy was measured at 25 °C. The fraction of the reference ligand mant-GTP bound was plotted against the competing ligand concentration, and data were fitted assuming that the two ligands bind to the same site. The resulting system of equations (45) was numerically solved with the program Equigra version 5.0 (46) or with a MATLAB script (available upon request), which provided the best fitted value of the equilibrium binding constant of the competing ligand.

The relative affinity of FtsZ for GDP and GTP was directly determined by incubating apoFtsZ with solutions of different ratios of GTP/GDP for 1 h at 25 °C in 50 mM Tris-HCL, 50 mM KCl, 1 mM EDTA, pH 7.5 (Tris-KCl buffer). Excess nucleotide was removed by a chromatography in a fast desalting column HR 10/10 (Amersham Biosciences) equilibrated in the same buffer with 10 µM nucleotide at the same GDP/GTP ratio. Eluted protein was precipitated with perchloric acid, and nucleotide content was measured by HPLC.

Kinetics of Binding and Dissociation of Mant-nucleotides to ApoFtsZ—Kinetic measurements were made with a Bio-Logic SFM-400 T-format stopped-flow device equipped with a fluorescence detection system. A wavelength of 368 nm in the excitation pathway and a filter with a cut-off of 450 nm in the emission pathway was employed. When measuring light scattering at the same time, a 350-nm band pass filter was included in the second emission pathway. 5–10 separate curves were averaged for each condition, and the curves so obtained were fitted to a single-, double- or triple-exponential equation of the form

\[ y(t) = a_1 + b + \Sigma A_i e^{-\lambda_i t} \]  

where the slope (\( a \)) and offset (b) correspond to the linear drift after the reaction. The best fitting rate constants (\( k \)) and amplitudes (\( A \)) were determined with the Bio-Kine software (Bio-Logic) or with a nonlinear least squares fitting program based on the Marquardt algorithm (47).
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### RESULTS

**Effects of Nucleotide on FtsZ Secondary Structure and Thermal Stability**—Prior to studying the interactions of FtsZ with nucleotides, effects of the bound nucleotide on FtsZ stability were evaluated. The circular dichroism spectrum of stable nucleotide-free FtsZ from *M. jannaschii* (20) was not significantly different from that of FtsZ. The reversible unfolding profiles with GdmCl were also very similar in the absence and presence of 50 μM GTP plus 1 mM MgCl₂, with a [GdmCl]½ value of 3.1 M (supplemental Fig. 1); this is compatible with the release of the nucleotide at lower GdmCl concentration (48).

Nucleotide binding would be expected to stabilize the protein against denaturation. This was examined by differential scanning calorimetry, which was done with GDP, in order to avoid FtsZ polymerization and GTP hydrolysis at high temperatures. Irreversible thermograms (Fig. 1A) showed that this thermophilic apoFtsZ (T<sub>m</sub> = 90.16 ± 0.03 °C, ΔH<sub>D</sub> = 190 ± 20 kcal/mol) is further stabilized by GDP (100 μM), which increased the temperature of the transition by 10 °C (T<sub>m</sub> = 100.72 ± 0.09 °C, ΔH<sub>D</sub> = 220 ± 10 kcal/mol). Magnesium (10 mM MgCl₂) does not significantly stabilize apoFtsZ (T<sub>m</sub> = 90.25 ± 0.02 °C, ΔH<sub>D</sub> = 230 ± 10 kcal/mol) but apparently induces a destabilization of FtsZ-GDP (FtsZ-GDP-Mg<sup>2+</sup>) (T<sub>m</sub> = 96.2 ± 0.7 °C, ΔH<sub>D</sub> = 190 ± 10 kcal/mol). The contribution of GDP dissociation to the denaturation enthalpy, ΔH<sub>ΔG</sub>, could not be estimated from these experiments, due to errors of the large denaturation enthalpy values.

**Binding Equilibrium of Guanine Nucleotides to FtsZ**—The stoichiometry of nucleotide binding to apoFtsZ was checked first. Different known concentrations of GTP, GDP, mant-GTP, or mant-GDP were added to FtsZ, and the solutions were centrifuged at high speed. The free nucleotide in the protein-depleted top half of tubes was measured, and the bound nucleotide was calculated by difference from the total. The stoichiometry values were as follows: 0.94 ± 0.03 GDP or GTP, 0.94 ± 0.06 mant-GTP, 0.83 ± 0.08 mant-GDP (i.e. essentially one nucleotide per FtsZ).

The energetics of the interaction of apoFtsZ (10–25 μM) with GDP and GTP were systematically examined by ITC. Nucleotide binding is moderately exothermic (Fig. 1, B and C, and Table 1) and the average stoichiometry of GXP binding from

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**FIGURE 1.** A, DSC traces of ApoFtsZ (12.5 μM) with MgCl₂ (10 mM), with GDP (100 μM), and with GDP (100 μM) plus MgCl₂ (10 mM) in Pipes-KCl buffer. B and C, calorimetric titration (ITC) of FtsZ binding to GDP and GTP-Mg<sup>2+</sup>, respectively, at 25 °C. Each peak (upper panels) represents the heat (integrated area) resulting from ligand injection into FtsZ solution (see “Experimental Procedures”). Each point in the bottom panels is the heat evolved per mol of injected ligand in the corresponding peak in the upper panel, after subtraction of ligand dilution heat. Solid lines are the best fits to experimental data (see Table 1 for binding parameters).

Histidine-tagged FtsZ Polymers—FtsZ-His<sub>6</sub> and mutant FtsZ-W319Y-His<sub>6</sub> were overproduced in *E. coli* and affinity-purified as described (19). ApoFtsZ-W319Y-His<sub>6</sub> was prepared as apoFtsZ above, and its concentration was measured with an extinction coefficient ε<sub>280</sub> = 2980 M<sup>−1</sup> cm<sup>−1</sup> (2 Tyr). It was diluted at 55 °C into Pipes-KCl buffer, pH 6.5, supplemented with 6 mM MgCl₂ and nucleotides (the His-tagged protein has a tendency to precipitate at pH <7 at room temperature). FtsZ polymers were negatively stained and observed under a Jeol 1230 electron microscope.

Copolymers of FtsZ-W319Y-His<sub>6</sub> and FtsZ-His<sub>6</sub>, formed in 50 mM Mes, 50 mM KCl, 1 mM EDTA, pH 6.5 (Mes-KCl buffer) with 6 mM MgCl₂ and 0.1 mM GTP at 55 °C, were pelleted by centrifugation at 60,000 rpm for 6 min at 55 °C in a prewarmed TLA-100 rotor. They were resuspended in 1% SDS, and the concentration of the FtsZ-His<sub>6</sub> single Trp was measured fluorometrically by excitation at 295 nm, employing FtsZ-His<sub>6</sub> standards. Concentration of FtsZ-W319Y-His<sub>6</sub> polymers was measured with the Bio-Rad assay (43) with FtsZ-W319Y-His<sub>6</sub> standards. Exchange of [α-<sup>32</sup>P]GTP into FtsZ-W319Y-His<sub>6</sub> or FtsZ-His<sub>6</sub> in Mes-KCl buffer with 6 mM MgCl₂ and 1 mM GTP at 55 °C, was measured employing a nitrocellulose filtration assay (17).
**TABLE 1**

| Ligand   | $K_b$ (μM) | $\Delta G_b$ (kcal mol$^{-1}$) | $\Delta H_b$ (kcal mol$^{-1}$) | $\Delta S_b$ (cal K$^{-1}$ mol$^{-1}$) |
|----------|------------|-------------------------------|-------------------------------|--------------------------------------|
| GDP      | 50 ± 10    | -10.5 ± 0.1                   | -6.80 ± 0.20                  | 12.4 ± 0.3                           |
| GDP-Mg$^{2+}$ | 20 ± 8    | -10.0 ± 0.2                   | -3.78 ± 0.09                  | 20.9 ± 0.4                           |
| GTP      | 300 ± 100  | -11.6 ± 0.2                   | -5.88 ± 0.08                  | 19.1 ± 0.4                           |
| GTP-Mg$^{2+}$ | 30 ± 10   | -10.2 ± 0.2                   | -3.31 ± 0.03                  | 23.1 ± 0.6                           |
| Mg$^{2+}$ | 0.0001 ± 0.0003 | -2.7 ± 0.2                   | 4.2 ± 0.7                     | 23 ± 2                               |

ITC experiments were 0.81 ± 0.06. Binding affinity increased in the presence of the nucleotide γ-phosphate (6-fold without Mg$^{2+}$, 1.5-fold with Mg$^{2+}$) but decreased (2.5–10-fold) when an excess of Mg$^{2+}$ is added to provide nucleotide-Mg$^{2+}$ complexes. Mg$^{2+}$ alone binds with very low affinity (Table 1).

The ITC binding measurements were made under conditions in which FtsZ does not polymerize but self-associates into dimers and trimers, in a magnesium- and nucleotide-insensitive manner; however, FtsZ is predictably monomeric at submicromolar concentrations (18). Therefore, the binding affinity of [8-3H]GTP to 500 nM apoFtsZ was also measured by protein depletion, counting the top and bottom half of each solution. The best fitting equilibrium association constant was $K_b = 700 ± 100$ μM$^{-1}$, which decreased in excess Mg$^{2+}$ to $K_b = 90 ± 20$ μM$^{-1}$ (Fig. 2A). These values are 2–3-fold larger than association constants estimated by ITC (Table 1). This may be due to a slightly weaker binding of the nucleotide to FtsZ oligomers and the disparity of the methods. The average of the best fitting stoichiometries was 0.9 ± 0.1 [8-3H]GTP per FtsZ.

In order to conveniently measure the binding of nucleotides to FtsZ monomers with fluorescent methods, we employed the analogs mant-GTP and mant-GDP that contain a methyl-anthraniloyl group attached to the ribose moiety and have been widely employed to study nucleotide binding by proteins (49–51) and were found to bind specifically to FtsZ. The addition of apoFtsZ produced both a 3.5-fold increase of the mant-nucleotide fluorescence intensity and a shift of the emission maximum from 449 to 440 nm. Magnesium in the millimolar concentration range quenched the fluorescence of FtsZ-bound mant-GTP, but not that of free mant-GXP. This impeded intensity measurement of the equilibrium binding of the fluorescent nucleotides to FtsZ in Mg$^{2+}$ containing buffers. However, a protein concentration-dependent increment of anisotropy, $\Delta r$, over that of the free fluorophore (0.04) was also observed, with a maximum value, $\Delta r_{\text{max}}$, practically insensitive to Mg$^{2+}$. Titration of mant-GTP with apoFtsZ in 10 mM MgCl$_2$ allowed determination of best fitted values of $\Delta r_{\text{max}} = 0.24 ± 0.01$ and $K_b = 4.2 ± 0.4$ μM$^{-1}$ (Fig. 2B); titration of apoFtsZ with mant-GTP, employing the $\Delta r_{\text{max}}$ value, gave a coincident $K_b$ value (4 ± 1 μM$^{-1}$) and a stoichiometry of 1.12 ± 0.06 mant-GTP bound per FtsZ (Fig. 2C). Affinities of binding of mant-GDP and mant-GTP to FtsZ were systematically measured under several conditions (Table 2 and supplemental Fig. 2). Apparent affinities of mant derivatives are 3–16-fold lower than those of natural nucleotides. Triphosphate/diphosphate affinity ratios are small, and a similar weakening effect of Mg$^{2+}$ is observed. Smaller Mg$^{2+}$ concentrations (50 nM to 100 μM in buffer without EDTA) did not increase the apparent affinity of mant-GTP. On the other hand, the minor differences found between values...
are equilibrium constants measured by fluorescence intensity.

Nucleotide | [MgCl₂] | Kₛ (25 °C) | Kₛ (55 °C)
--- | --- | --- | ---
Mant-GDP | 0.0 | 17 ± 2 (19 ± 2) | 10 ± 1 (18 ± 3)
 | 2.0 | 10 ± 3 | 11 ± 1
 | 10.0 | 1.6 ± 0.2 | 1.4 ± 0.2
Mant-GTP | 0.0 | 18 ± 5 (32 ± 4) | 35 ± 5 (21 ± 2)
 | 2.0 | 5 ± 2 | 5.3 ± 0.9
 | 10.0 | 4.2 ± 0.4 | 3 ± 1

FIGURE 3. Relative affinity of FtsZ for guanine nucleotides. A, displacement curves of mant-GTP (500 nM total) from FtsZ (500 nM apoFtsZ total) by GTP (solid circles), GDP (open circles), and GMP (squares) in Pipes-KCl buffer, 10 mM MgCl₂, at 25 °C. Data were determined from the change in mant-GTP anisotropy with the competing ligands (see “Experimental Procedures”). Lines (solid, dashed, and dashed and dotted) correspond to the best fitted Kₛ to each data set (see “Experimental Procedures” and “Results”). B, relative affinity of FtsZ for GTP and GDP. apoFtsZ (32 μM) was incubated with GTP/GDP mixtures (total GXP 200 μM) in 50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, pH 7.5 (0.5 ml) at 25 °C for 60 min, excess nucleotide was removed, and protein-bound nucleotides were analyzed. For two ligands binding to the same site, the slope of this plot is the ratio of their equilibrium binding constants. Empty triangles, no MgCl₂; solid triangles, 10 mM MgCl₂.

GMP were 330 ± 80, 110 ± 40, and 0.022 ± 0.007 μM⁻¹, respectively. The Kₛ value of GTP determined by competition is 3.6-fold larger than the one determined directly by [8-3H]GTP co-sedimentation with FtsZ. The relative affinity of FtsZ for GTP and GDP was measured directly by incubating apoFtsZ with nucleotide mixtures of varying GDP/GTP ratios, separating and quantifying the protein-bound nucleotides. The affinity of GTP binding is slightly larger than GDP binding, 11 ± 1- and 3.2 ± 0.3-fold with 0 and 10 mM Mg2+, respectively (Fig. 3B).

**Kinetics of Nucleotide Interactions with Unassembled FtsZ**—The kinetics of mant-GXP binding and dissociation from FtsZ were studied by employing stopped-flow methods at 25 °C (in the presence and absence of magnesium) and at 55 °C (without magnesium to avoid polymer formation). To measure the association under pseudo-first-order conditions, mant-nucleotide was mixed with a large excess of apoFtsZ, and the increments in fluorescence intensity (Fig. 4A) and anisotropy (Fig. 4B) of mant were recorded. The reaction time courses were fitted by single exponentials. The rate constant values determined by intensity and anisotropy were identical within experimental error, although the noise was smaller for the intensity measurements (and it could be further reduced by removing the polarizers). The small increase in fluorescence intensity with 10 mM MgCl₂ could also be monitored with the stopped-flow instrument. The observed rate constant values, kₘₐₓ, depend linearly on the concentration of binding sites (apoFtsZ) (Fig. 4C), which is compatible with a one-step binding mechanism, for which the following relationship holds.

\[
k_{\text{app}} = kₐ[\text{protein}] + kₜ.
\]

We could determine the association rate constant, kₐ, from the slope of the regression line, but not, with sufficient precision, the dissociation rate constant kₜ. The association rate constant is reduced by Mg²⁺ and increases weakly with temperature (Table 3).

The dissociation rate was determined in displacement experiments in which an excess of unlabeled GTP was used to displace mant-GXP from its complex with FtsZ. Time courses of mant-GXP dissociation monitored by the decrease in fluorescence intensity could be fitted to single exponentials (Fig. 5), giving the first-order dissociation rate constant values (Table 3). The dissociation rate was increased by Mg²⁺ and temperature (Table 3).

**Kinetics of Nucleotide Binding and Dissociation from FtsZ Polymers**—The interaction kinetics of mant-nucleotides with FtsZ polymers were compared with the interaction kinetics of unassembled protein. The experiments were facilitated by polymerization of nucleotide-free FtsZ (20), although a com-
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A complete kinetic analysis is hampered by the system heterogeneity, consisting of unassembled FtsZ (monomers and oligomers) and FtsZ polymers.

FtsZ polymer stability was examined first. Polymerization measurements with 10 mM MgCl₂ at 55 °C showed that apoFtsZ formed pelletable polymers above a critical protein concentration.

The rate constant of GDP-induced depolymerization of apoFtsZ, mant-GTP-FtsZ, and GTP-FtsZ polymers were 2.6 ± 0.1, 0.055, and 0.42 ± 0.05 s⁻¹, respectively. Measurements of depolymerization rates by dilution in buffer without GDP were carried out in parallel (Fig. 6B) and gave the observed values of 0.16 ± 0.02 s⁻¹ (apoFtsZ), 0.03 ± 0.01 s⁻¹ (mant-GTP-FtsZ), and 1.2 ± 0.1 s⁻¹ (GTP-FtsZ). FtsZ-mant-GTP polymers were observed under the electron microscope, and their morphology was found to be similar to that of GTP polymers. However, no polymers were detected with mant-GDP and up to 20 mM FtsZ by pelleting or electron microscopy. These observations resemble previous ones with GTP and GDP (18, 20).

ApoFtsZ polymers bind mant-GTP in a molar ratio of 1.0 ± 0.1, determined by sedimentation of polymers at different times after adding mant-GTP and measuring protein and mant-GTP concentrations in the pellet and in the supernatant. These polymers were stable for at least 30 min after adding equimolar mant-GTP, suggesting that FtsZ very slowly hydrolyzes mant-GTP, in contrast with GTP, which is required in a larger excess to maintain the polymers.
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**TABLE 3**

Kinetics of binding and dissociation of mant-nucleotides from FtsZ

| Rate constant | Mant-GTP | Mant-GDP |
|---------------|----------|----------|
|               | 25 °C    | 55 °C    | 25 °C    | 55 °C    |
| \( k_1 \) (\( \mu M^{-1} \) s\(^{-1} \), no MgCl\(_2\)) | 28 ± 2 (26 ± 5) | 37 ± 7 | 25 ± 1 (23 ± 5) | 35 ± 4 |
| \( k_2 \) (\( \mu M^{-1} \) s\(^{-1} \), 10 mM MgCl\(_2\)) | 12 ± 1 (11 ± 2) | ~16 predicted | 6 ± 1 (7 ± 2) | ~7 predicted |
| \( k_3 \) (s\(^{-1} \), no MgCl\(_2\)) | 0.82 ± 0.01 | 2.25 ± 0.01 | 2.00 ± 0.01 | 11.0 ± 0.1 |
| \( k_4 \) (s\(^{-1} \), 10 mM MgCl\(_2\)) | 6.30 ± 0.02 | ~17 predicted | 7.17 ± 0.03 | ~40 predicted |

The hydrolysis rate of mant-GTP (20 \( \mu M \)) to mant-GDP by polymers of FtsZ (12 \( \mu M \)) was \( 4 \times 10^{-3} \) s\(^{-1} \), 10 mM MgCl\(_2\) at 55 °C (determined by HPLC), whereas the hydrolysis rate of GTP is 0.1 s\(^{-1} \) under related conditions (19).

Once the FtsZ polymer stability was determined, we proceeded to measure the mant-nucleotide association. Binding of mant-GTP to unassembled and polymerized apoFtsZ was first compared during the same experiment (with 10 mM MgCl\(_2\) at 55 °C) by loading, in the thermostated syringe of the stopped-flow instrument, either unassembled apoFtsZ (3 \( \mu M \), under the 7.0 \( \mu M \) Cr of polymer formation) or partially polymerized apoFtsZ (15 \( \mu M \)) and then mixing it with the ligand to the same final concentration (1.5 \( \mu M \)). Nucleotide binding to the FtsZ polymer-containing solution was markedly slower than to unassembled FtsZ (note that the fast initial rise was smaller). Interestingly, both were complete within a few seconds and were clearly faster than the dilution-induced depolymerization measured in the same experiment (Fig. 7A); this is a model-free observation. The time courses in these experiments were best fitted by a sum of three exponentials. The slowest of them was independent of protein concentration and had a constant rate value of 0.42 ± 0.05 s\(^{-1} \). This phase was attributed to an uncharacterized rearrangement of the system, and its value was constrained in further analysis. The apparent rate constants of the fastest and second fastest phases in this experiment (Fig. 7A) were as follows: unassembled, ~70 and 4.6 s\(^{-1} \); polymerized, ~40 and 4.3 s\(^{-1} \). Their relative amplitudes were 10:1 in the unassembled sample and 0:1 in the polymerized sample. This suggests parallel reactions with two types of binding sites, fast ( unassembled) and slow (polymerized FtsZ), present in different proportions in each sample. To estimate the bimolecular rate constants of binding of mant-nucleotides to unassembled and polymerized FtsZ, apoFtsZ solutions were mixed at different final concentrations in excess over the nucleotide, time courses were fitted as above, and observed rates were plotted against total protein concentration (Fig. 7, B and C). The results (Table 4) indicate that unassembled apoFtsZ binds mant-GTP with a rate constant \( k_1 \approx 30 \mu M^{-1} s^{-1} \). This fast rate is twice the rough value predicted from measurements under related conditions (Table 3). The equivalent fast component, which is observed with decreased amplitude in polymerized apoFtsZ solutions (Table 4), can be attributed to the fraction of unassembled protein. It may then be proposed that the second rate constant, \( k_2 \approx 1 \mu M^{-1} s^{-1} \), whose amplitude increases upon FtsZ polymerization (Table 4), reflects the binding of mant-GTP to apoFtsZ polymers. This process is more than 1 order of magnitude slower than the binding to unassembled apoFtsZ (the fact that this slow component can also be detected in a small proportion at FtsZ concentrations below the Cr measured by sedimentation might be explained by formation of polymer nucleation species which fail to pellet). Apparent rates of binding of mant-GDP and mant-GTP to polymerized apoFtsZ were similar (Fig. 7C). The inherent limitations of this phase analysis should be kept in mind, including the possibility that we are approximating a continuum of reaction rates from diverse...
unassembled and polymeric FtsZ species with a simple sum of a few exponentials. The dissociation rate of mant-GTP from unassembled FtsZ under polymerization conditions, $\sim 40$ s$^{-1}$, estimated from the y axis intercepts in Fig. 7, B and C, is compatible with rough predicted values (Table 3); the dissociation rate from polymers could not be determined by this method.

Following the analysis of binding kinetics, the dissociation of mant-nucleotide from unassembled FtsZ, oligomers, and FtsZ polymers were compared. To do this, mant-GXP-FtsZ was mixed with a large excess of GDP or GTP in the stopped flow, with a minimal (10%) dilution of protein to avoid depolymerization. Dissociation of mant-GTP and mant-GDP from unassembled FtsZ (0.55 $\mu M$ initial concentration, well below the 2.2 $\mu M$ Cr for polymerization) took place with rate constants of $\sim 20$ and $\sim 50$ s$^{-1}$, respectively (Table 5 and supplemental Fig. 3), which are compatible with predicted values (Table 3). Dissociation of mant-GDP from oligomeric FtsZ (10 $\mu M$ FtsZ-mant-GDP initial concentration) included a principal component with a rate constant of 0.20 s$^{-1}$ (Table 5 and supplemental Fig. 3) possibly due to the FtsZ oligomers. Dissociation of mant-GTP was found to be markedly slower in polymerized FtsZ solutions (10 $\mu M$ FtsZ-mant-GTP initial concentration, well above the 2.2 $\mu M$ polymerization Cr). In an excess of GTP, dissociation consisted of two phases (0.018 ± 0.001 s$^{-1}$, 85% amplitude; 0.20 ± 0.01 s$^{-1}$, 15% amplitude). It was followed by partial depolymerization at a rate of 0.012 ± 0.001 s$^{-1}$ (Fig. 8, trace 1, and Table 5) and by new GTP-induced polymerization at longer times (not shown). These results indicate that mant-GTP-FtsZ polymers depolymerize upon substitution of the fluorescent derivative by GTP, suggesting that GTP-FtsZ and mant-GTP-FtsZ do not freely co-polymerize into exactly the same polymer, possibly due to some structural perturbation induced by the fluorescent group, which also inhibits the nucleotide hydrolysis. With an excess of GDP, mant-GTP dissociation proceeded at a rate of $\sim 0.057$ s$^{-1}$ (which could not be fitted by a sum of exponentials) and was closely followed by depolymerization at $\sim 0.055$ s$^{-1}$ monitored by light scattering (Fig. 8, trace 2, and Table 5). Since dilution-induced depolymerization takes place in a similar time scale (see above), an experimental comparison of dissociation from unassembled and polymerized FtsZ at the same final low protein concentration (as in the case of the association) could not be made.

The 0.02–0.06 s$^{-1}$ mant-GTP dissociation rate, which may be attributed to FtsZ polymers, is several hundred-fold slower than dissociation from unassembled FtsZ under the same solution conditions. These results could be interpreted as either a lower intrinsic dissociation rate of mant-GTP from FtsZ polymers or as the result of a rate-limiting exchange of FtsZ monomers in these polymers, followed by fast nucleotide dissociation from the unassembled subunits. It should be kept in mind that this, necessarily simplified, analysis only partially resolves the dissociation rates of the nucleotide from the multiple FtsZ species present.

**Accessibility of the Nucleotide Binding Site in Stable Sheet of FtsZ-W319Y-His$_6$**—In order to probe the accessibility of the nucleotide binding site in FtsZ polymers without the complications due to subunit exchange, it was desirable to use stabilized FtsZ polymers. Under standard conditions, histidine-tagged

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**FIGURE 7.** A, kinetics of binding of mant-GTP to unassembled (trace 1) and to polymerized (trace 2) apoFtsZ. Polymers were formed from 15 $\mu M$ apoFtsZ with 10 mM MgCl$_2$ at 55 °C (8 $\mu M$ apoFtsZ in polymers) and mixed in the stopped flow at final concentrations of 1.5 $\mu M$ mant-GTP (0.8 $\mu M$ polymers) and 400 nM mant-GTP (trace 2). Light scattering was recorded during the experiment with apoFtsZ polymers to monitor their stability (trace 3). The light scattering signal changed from 2.22 to 2.18 during the ligand binding measurement and to 2.10 later when depolymerization was complete. Unassembled apoFtsZ (3 $\mu M$, under the same conditions) was mixed to 1.5 $\mu M$ final concentration (trace 1). Data were fitted by a sum of three exponentials, $k_{1app}$, $k_{2app}$, and $k_{3app}$: 72 s$^{-1}$ (69%), 4.6 s$^{-1}$ (7%), and 0.42 s$^{-1}$ (24%) (trace 1); 43 s$^{-1}$ (16%), 4.3 s$^{-1}$ (32%), and 0.42 s$^{-1}$ (52%) (trace 2). Residuals of the fit are shown in the small panel above. B, dependence of the observed rate constants $k_{1app}$ and $k_{2app}$ on the concentration of unassembled apoFtsZ (solid circles, 400 nM mant-GTP; empty circles, mant-GDP shown for comparison). C, dependence of the observed rate constants $k_{1app}$ and $k_{2app}$ on the final total concentration of apoFtsZ under polymerization conditions (400 nM mant-GXP); a very slow third rate constant, $k_{3app} = 0.42$ s$^{-1}$, was independent of protein concentration and is not plotted.
TABLE 4
Association kinetics of mant-GTP to apo-FtsZ under polymerization solution conditions

| Initial [apoFtsZ] | $k_1$ (unassembled) | $k_0$ (unassembled) | $k_1$ (polymer) | $k_0$ (unknown) |
|-------------------|--------------------|---------------------|----------------|----------------|
| Below Cr for polymerization | $30 \pm 6$ (49 ± 8%) | $38 \pm 7$ | $1 \pm 2$ (7 ± 3%) | $0.42 \pm 0.05$ (44 ± 10%) |
| Above Cr for polymerization | $35 \pm 15$ (24 ± 10%) | $44 \pm 20$ | $1.1 \pm 0.3$ (20 ± 6%) | $0.42 \pm 0.05$ (56 ± 8%) |

TABLE 5
Observed dissociation rate constants (s$^{-1}$) of mant-GXP from FtsZ under polymerization solution conditions

ApoftsZ with added mant-GTP or mant-GDP (1.1 nucleotide/1 FtsZ) was allowed to polymerize at 55 °C with 10 mM MgCl$_2$ and mixed in the stopped flow with an excess (1 mM) of GTP or GDP as indicated. The reaction time courses were recorded during 400 s. NO, a second phase was not observed.

| Initial conditions | $k_1$ (GDP excess) | $k_0$ (GDP excess) | $k_1$ (GTP excess) | $k_0$ (GTP excess) |
|--------------------|--------------------|--------------------|-------------------|-------------------|
| Unassembled mant-GDP-FtsZ (0.5 mM FtsZ) | NO | 52 ± 9 | NO | |
| Unassembled mant-GTP-FtsZ (0.5 mM FtsZ) | 22 ± 2 | NO | 18 ± 3 | NO |
| Mant-GDP-FtsZ oligomers (9 mM FtsZ) | 40 ± 10 (10%) | 0.20 ± 0.05 (90%) | 33 ± 10 (10%) | 0.21 ± 0.05 (90%) |
| Mant-GTP-FtsZ polymers (9 mM FtsZ) | NO | 0.057 ± 0.005 | 0.20 ± 0.01 (15%) | 0.018 ± 0.001 (85%) |

FtsZ-His$_6$ polymerizes, hydrolyzes GTP, and depolymerizes similarly to FtsZ, but large stable sheets are formed by the non-hydropolizing point mutant FtsZ-W319Y-His$_6$ (19), which were employed as stable model FtsZ polymers. X-ray structures of FtsZ-W319Y and FtsZ-His$_6$ are superimposable (22). The FtsZ-W319Y-His$_6$ sheets are made up of double protofilaments with the same 4-nm tubulin-like subunit spacing as in wild-type FtsZ filaments. They hardly disassemble with an excess of GDP or in the cold (19). Polymerized apoftsZ-W319Y-His$_6$ readily binds mant-GTP, with a marked increase in fluorescence intensity of the ligand; the addition of an excess of GTP reduced fluorescence to the level of free mant-GTP (Fig. 9A). Both mant-GTP association and dissociation were essentially complete (>90%) within the dead time of measurement (~20 s, therefore proceeding at an apparent rate of ~0.1 s$^{-1}$). This implies, for the reactant concentrations employed (12.5 μM mant-GTP and ~6 μM polymerized FtsZ-W319Y-His$_6$ determined by sedimentation) a bimolecular association constant of ~0.05 μM$^{-1}$ s$^{-1}$ for the slowest FtsZ species (52) and a dissociation constant of ~0.1 s$^{-1}$. These rate constants are compatible with the corresponding values for wild-type FtsZ polymers (Table 5). The association time course of 0.4 μM mant-GTP to 9 μM apoftsZ-W319Y-His$_6$ (~4 μM polymers) (Fig. 9B) was biphasic, with apparent rate constants of 8.7 ± 0.2 s$^{-1}$ (48%) and 0.29 ± 0.003 s$^{-1}$ (52%), which may be assigned to FtsZ-W319Y-His$_6$ monomer and polymer, respectively. The dissociation time course could not be measured due to destruction of the FtsZ-W319Y-His$_6$-mant-GTP polymers in the stopped flow.

In order to estimate the rate of subunit exchange in the polymers of FtsZ-W319Y-His$_6$, a small proportion (5%) of tracer FtsZ-His$_6$ was added to the solution of preformed polymers. FtsZ-His$_6$ (in a 1–10% proportion) had been observed to freely copolymerize with FtsZ-W319Y-His$_6$ (from which it can be distinguished by fluorescence of the single tryptophan Trp$^{319}$) without significantly perturbing polymer formation. The exchange process was very slow (Fig. 9C), with an observed rate constant of 0.0004 s$^{-1}$, which clearly cannot account for the much faster observed binding of the mant-nucleotide (apparent rate of >0.1 s$^{-1}$). Finally, mant-GTP binding results were complemented by independent measurements of the exchange of [α-32P]GTP into un assembled and polymerized FtsZ-W319Y-His$_6$ and FtsZ-His$_6$, which exchanged one GTP in less than 20 s in all cases (Fig. 9D). It can therefore be concluded that the nucleotide binding site is accessible in FtsZ-W319Y-His$_6$ stable model polymers.

**DISCUSSION**

**Energetics of Nucleotide Binding to FtsZ and Functional Consequences**—Guanine nucleotide binding and dissociation are central to the dynamics of FtsZ and tubulin polymers, which are in turn essential for their respective cellular functions. Thermophilic apoftsZ from *M. jannaschii* was employed in this work as a conveniently stable model protein for the study of the interactions of FtsZ with nucleotides. Similar experiments with nucleotide-free mesophilic FtsZ from *E. coli* were precluded by its instability. GDP stabilizes FtsZ against thermal denaturation. The destabilizing effect of Mg$^{2+}$ on FtsZ-GDP

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may be explained by a reduction in the binding affinity of GDP (see below); alternately, the cation may be increasing the rate of irreversible thermal denaturation of the protein and therefore decreasing the apparent \( T_m \). GTP binding imperceptibly modifies the average secondary structure of the protein, in agreement with the similar polymerization properties (20) and crystal structures of the nucleotide-free and GTP-liganded forms of this FtsZ (22). These results support the notion that the bound nucleotide has little structural role in \( M. jannaschii \) FtsZ monomers and polymers, but it is employed to trigger disassembly upon hydrolysis (20).

ApoFtsZ binds guanine nucleotides with relatively high affinity. \( K_b \) values ranging from 20 to 300 \( \mu M \) weakly increased with the presence of the nucleotide \( \gamma \)-phosphate and decreased with a chelating \( Mg^{2+} \) ion (Table 1). An equivalent effect is observed with mant-GDP and mant-GTP (Table 2). MgCl\(_2\) moderately reduces the association rates and enhances the dissociation rates of mant-GDP and mant-GTP (Table 3). These results indicate that the \( Mg^{2+} \) ion bound to the nucleotide \( \beta \) - and \( \gamma \)-phosphates and Gln\(_{75}^{75}\) observed in the crystal structure of FtsZ (22), suggested to assist the hydrolysis of the \( \gamma \)-phosphate by FtsZ polymers, provides little additional stability to the FtsZ monomer-nucleotide complex. This may be explained by (i) \( Mg^{2+} \) binding to another low affinity site that has to be displaced by the nucleotide binding or that allosterically weakens the observed nucleotide binding affinity or (ii) the existence of a slightly unfavorable process that makes the binding of the nucleotide-\( Mg^{2+} \) complex less favorable than the binding of the nucleotide alone. The first explanation is consistent with the quenching of the fluorescence of FtsZ-bound mant-GXP (see "Results") induced by \( Mg^{2+} \) and with the low affinity binding of \( Mg^{2+} \) to tubulin (53). The second explana-

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**FIGURE 9. Interactions of nonhydrolyzing mutant FtsZ-W319Y-His\(_8\) with nucleotides.**

A, mant-GTP binding to polymers of FtsZ-W319Y-His\(_8\). Solid line, fluorescence emission spectrum of 12.5 \( \mu M \) mant-GTP in a solution of 12.5 \( \mu M \) polymerized apoFtsZ-W319Y-His\(_8\); dashed line, mant-GTP alone; dotted line, mant-GTP + polymerized FtsZ-W319Y-His\(_8\) + 1 \( mM \) GTP; dashed and dotted line, polymerized FtsZ-W319Y-His\(_8\) alone. The spectrum of mant-GTP + GTP was identical to that of mant-GTP alone. Excitation was at 357 nm (1-nm bandwidth); emission maxima of bound mant-GTP was at 439 nm, free at 446 nm (2-nm bandwidth). Inset, an electron micrograph of the FtsZ-W319Y-His\(_8\)-mant-GTP polymers; the bar indicates 100 nm. B, association time course of 0.4 \( \mu M \) mant-GTP to 9 \( \mu M \) apoFtsZ-W319Y-His\(_8\) monitored with the stopped flow. Gray trace, data; dark line, biexponential best fit. C, time course of incorporation of 0.63 \( \mu M \) FtsZ-His\(_6\) into \( \sim 6 \mu M \) preformed polymers of the FtsZ-W319Y-His\(_8\) Trp-less mutant (12.5 \( \mu M \) total concentration), measured by polymer sedimentation. The solid line is a single exponential fit to data, giving a pseudo-first order rate constant of \( 4 \pm 1 \times 10^{-5} \) s\(^{-1}\) and an amplitude of 0.06 \pm 0.01 (marked fit); maximum incorporation was 0.053 \pm 0.003 (marked exp), determined by copolymerizing the FtsZ-wt-His\(_8\) together with FtsZ-W319Y-His\(_8\). D, time courses of exchange of \([\alpha-\text{32P}]\text{GTP}\) into 12.5 \( \mu M \) FtsZ-W319Y-His\(_8\), (solid circles) or FtsZ-His\(_8\), (empty circles) under no assembly (left, no \( MgCl_2\)) or polymerization conditions (right, 6 \( mM \) \( MgCl_2\), measured with a filtration assay. Note that FtsZ-His\(_8\) polymers hydrolyze GTP, whereas FtsZ-W319Y-His\(_8\) ones do not.
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FtsZ and tubulin form a distinct family of GTPases (1), but there are structural (22) and important functional differences between the FtsZ and tubulin nucleotide binding sites. Unlike FtsZ, nucleotide γ-phosphate and Mg2+ binding are linked in αβ-tubulin (54). The nucleotide γ-phosphate and the coordinated Mg2+ ion bound at the functional GTP/GDP binding site of β-tubulin control microtubule stability, whereas the Mg2+ bound to the nonfunctional GTP site of α-tubulin controls the stability of the αβ-dimer (55). In classical GTPases, GTP is bound in complex with Mg2+, which is coordinated to oxygens from the β- and γ-phosphates. However, the functional roles of the γ-phosphate and Mg2+ vary among different G-proteins. Thus, Ras and EF-Tu form tight GDP-Mg2+ complexes, Mg2+ binding reduces the GDP off rate by 4 orders of magnitude, and GDP binds more tightly than GTP (56). As another example, Mg2+ is not required for GDP binding to eRF3 but strengthens GTP binding; no structural changes were observed for GTP-Mg2+ and GDP-Mg2+ binding to eRF3 (57). In Rho proteins, the Mg2+ cofactor does not affect the nucleotide binding affinity per se but rather acts as a kinetic stabilizer for bound nucleotides by slowing down both the off and on rates (58). The different properties of the FtsZ nucleotide binding site in comparison with tubulin and other GTPases suggest the possibility of fine tuning specific inhibitors for the FtsZ-GTP interaction.

Interactions of FtsZ Monomers with Fluorescent Mant-nucleotides, Kinetics of Binding, and Competitive Assay for Ligands of the FtsZ Nucleotide Site—Interactions of FtsZ monomers with GTP and GDP were probed by employing the fluorescence anisotropy change of their mant derivatives in dilute solutions. The kinetics of association of mant-nucleotides to unassembled FtsZ is compatible with a one-step reaction, with fast association rate constant values (10 < kₐ < 40 μM⁻¹ s⁻¹) and dissociation rates (1 < kₐ < 10 s⁻¹), depending on solution conditions (Table 3). Rate constant values are weakly dependent on temperature, suggesting small activation energies for nucleotide association and dissociation from an easily accessible site.

The bound mant-GTP is specifically displaced by nonfluorescent nucleotides. Except for the possible offset in absolute Kₐ values determined by competition and ITC methods, the ratio Kₐ(GTP-Mg₂⁺)/Kₐ(GDP-Mg₂⁺) determined with the competition method is 3 ± 2, which is comparable with the 3.2 ± 0.3 ratio directly determined with GTP and GDP (Fig. 3B), with the ~1.5 ratio from ITC (Table 1), and with the 2.6 ± 0.6 ratio of the respective mant-derivatives (Table 2).

The mant-nucleotide displacement method outlined here is a homogeneous fluorescence assay that may, in principle, be conveniently employed to characterize the binding of any nucleotides or other substances, such as small molecule modulators of FtsZ assembly (see Introduction), to its nucleotide site, as well as to measure the effects of ligand modifications on binding affinity. This method may be eventually scaled up to screen for inhibitors binding to the FtsZ nucleotide site.

Exchangeable Nucleotide Is Kinetically Stabilized in FtsZ Polymers—How FtsZ polymers exchange the hydrolyzed nucleotide is a major unresolved issue, which impacts on the mechanism of their dynamics being possibly different or similar to microtubules (see Introduction). The results of this study indicate that the nucleotide remains exchangeable in polymers of FtsZ from M. jannaschii. The accessibility of the nucleotide binding site in FtsZ polymers was probed by measuring the kinetics of interactions of FtsZ with mant-GTP under polymerization conditions. The scheme in Fig. 10A summarizes relevant rate constants determined with unassembled FtsZ (at protein concentrations below Cr) and estimated for FtsZ polymers (above Cr). The apoFtsZ polymers are obviously devoid of any dynamics related to nucleotide hydrolysis, and FtsZ-mant-GTP polymers do not hydrolyze the nucleotide on the time scale of these experiments. Under polymerization conditions, binding kinetics becomes multiphasic due to the different FtsZ aggregation species found in solution. A nucleotide association phase with a rate constant 1 μM⁻¹ s⁻¹, whose amplitude...
increases upon polymerization (see “Results”), was ascribed to the binding of mant-GTP to FtsZ polymers, 30-fold slower than to unassembled FtsZ. This binding rate constant cannot come from the dissociation of apoFtsZ subunits or from nucleotide binding to the unassembled protein, since the apoFtsZ polymer dissociation rate is much lower than the observed mant-GTP binding rates (Fig. 7).

Dissociation of mant-GTP from FtsZ polymers proceeds at an observed rate of 0.06 s\(^{-1}\) in GDP excess (0.02 s\(^{-1}\) in GTP excess), which is 3 orders of magnitude slower than dissociation from unassembled FtsZ. The value of 0.21 s\(^{-1}\) estimated for mant-GTP dissociation from FtsZ polymers using the rate and equilibrium constants depicted in the reaction box of Fig. 10A is only 3.5-fold higher (not too bad, considering the difficulty of several of the kinetic measurements). The fact that the mant-GTP dissociation time course shortly precedes polymer disassembly (Fig. 8) would be compatible with direct dissociation of mant-GTP from the polymer, closely followed by disassembly of the GDP-bound polymer at the rates indicated (Fig. 10A). However, we do not think that monitoring the polymer concentration by scattering is accurate enough to warrant this interpretation. Given the similarity of the apparent ligand dissociation rate and the polymer disassembly rate, this result may also be interpreted as due to FtsZ depolymerization followed by fast mant-GTP dissociation from FtsZ monomers. According to this interpretation, the 0.06 s\(^{-1}\) value would be only an upper limit to the true rate constant of mant-GTP dissociation from the polymers. The slower dissociation rate in excess of GTP indicates the participation of polymer disassembly in this process. In either case, our results indicate that the nucleotide is kinetically stabilized in FtsZ polymers with respect to monomers. This agrees with an accessible nucleotide binding site located between two consecutive monomers along the FtsZ protofilament (22). Mant-GTP binding and FtsZ polymer elongation moderately favor each other, with a linkage free energy of only \(-1.1 \pm 0.4\) kcal mol\(^{-1}\), calculated from data in Fig. 10A.

In order to unequivocally prove whether FtsZ polymers can bind and dissociate nucleotide without subunit exchange, stabilized FtsZ polymers were needed. These have been provided by the mutant FtsZ-W319Y-His\(_6\) which forms an inactive GTPase sheet (further stabilized by the His tag (19)) and co-polymerizes with wild-type FtsZ-His\(_6\). Wild-type subunits slowly exchange into mutant polymers at a rate of 0.0004 s\(^{-1}\), whereas polymers bind and dissociate mant-GTP nucleotide at a much faster rate, >0.1 s\(^{-1}\), under the same conditions. This shows that exchange of the bound nucleotide without subunit exchange is possible in these model FtsZ polymers.

*Implications for FtsZ Polymer Dynamics*—The observation that mant-nucleotide exchange can take place without hydrolysis in polymers of *M. jannaschii* FtsZ gives insight into FtsZ polymer dynamics. These results might superficially seem to favor models in which FtsZ is devoid of any microtubule-like dynamics. However, the problem is quantitative; the kinetic pathway actually operative will depend on the effective reaction rates under given conditions. Once FtsZ polymers eventually hydrolyze mant-GTP and release P\(_i\), mant-GDP would be expected to induce disassembly, but, since mant-GTP hydrolysis is much slower than the mant-nucleotide exchange, it does not influence polymer dynamics. This is not the case with the natural nucleotide GTP. Models for FtsZ assembly with GTP are schematized in Fig. 10B.

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The observation that subunit exchange is very slow in polymers of the GTPase-deficient mutant FtsZ-W319Y-His\(_6\) (19) compared with the fast disassembly of FtsZ-His\(_6\) active GTPase and the important findings that (i) the turnover of FtsZ-GFP subunits in the Z-rings of *E. coli* cells is reduced in mutant *ftsZ84* (24), (ii) FtsZ subunit exchange in a fluorescence resonance energy transfer assay is strongly reduced by the slowly hydrolyzable nucleotide GMPCMP (25), and (iii) the correlation very recently found, between the slower subunit turnover, GTPase, and GDP-induced disassembly in *Mycobacterium tuberculosis* FtsZ (26) favors a polymer recycling model of FtsZ assembly (Fig. 10B). In conclusion, FtsZ polymers can be observed to exchange nucleotide, unlike microtubules, but GDP dissociation may be slow enough for FtsZ polymer disassembly to take place first, as in microtubules, resulting in FtsZ polymers cycling with GTP hydrolysis. Since FtsZ polymers are typically single flexible protofilaments a few hundred nanometers long (60), it is possible that their relatively rapid assembly and disassembly but proceeds between membrane attachment points and provides continuously updated positional information for the assembly and operation of the septosome.

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