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-methylanthranilyl) (mant)-nucleotide binding and dissociation from FtsZ polymers, employing calorimetric, fluorescence, and stopped-flow methods. FtsZ binds GTP and GDP with $K_b$ values ranging from 20 to 300 μM$^{-1}$ 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 GTP. 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 $k_+$ and ~500-fold slower $k_-$ than in monomers. The mant-GTP dissociation rate from FtsZ polymers is comparable with the GTP hydrolysis turnover and with the reported subunit turnover in Escherichia coli FtsZ polymers. Although FtsZ polymers can exchange nucleotide, unlike its eukaryotic structural homologue tubulin, GDP dissociation may be slow enough for polymer disassembly to take place first, resulting in FtsZ polymers cycling with GTP hydrolysis similarly to microtubules.

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 assemblies 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 glycosyltransferase 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).

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-

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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), and taxanes (32), azasulphorhodamine (33), taxol (34), polyphenols (35), PC58538 and PC170942 (36), sanguinarine (37), certain taxanes (38), A189 (39), amikacin (40), totarol (41), and cinna-maldehyde (42).

This study focused on fundamental processes of FtsZ-nucleotide interactions. We have determined the energetics of GTP and GDP binding to FtsZ and the kinetics of binding and dissociation in FtsZ monomers and polymers using fluorescent (mant)-nucleotides. The results reveal functional differences with nucleotide binding to tubulin that will facilitate screening for compounds binding to the nucleotide site of FtsZ. They also indicate a slowed down nucleotide exchange in FtsZ polymers, which provides insight to their dynamics.

**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-35P]GTP (400 Ci/mmol) were from Amer sham 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 × 25 cm) eluted with a linear gradient of 25 mM NaH2PO4/Na2HPO4, pH 2.8, to 125 mM NaH2PO4/Na2HPO4, pH 2.9. All other chemicals (analytical grade) used were from Merck or Sigma.

**Preparation of Nucleotide-free FtsZ from M. jannaschii—**FtsZ (without histidine tag) was overproduced in *E. coli* BL21 (DE3)pLys and was purified as described (18, 19). Nucleotide-free FtsZ (apoFtsZ) was prepared as described (20) with minor modifications. FtsZ was incubated in 2.5 M guanidinium chloride (GdmCl) for 30 min at room temperature, followed by gel filtration in a 0.9 × 25-cm Sephadex G-25 column in 25 mM Pipes-KOH and 2.5 M 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 ε280 = 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.

**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 (Cp) was obtained after subtraction of the buffer-buffer base line, and the denaturation enthalpy (ΔHds) 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 mM FtsZ. GXP and Mg²⁺ concentrations were 100 μM and 10 mM, respectively.

**Isothermal Titration Calorimetry (ITC)—**Calorimetric titrations of FtsZ with GXP, GXP-Mg, and Mg²⁺ were performed at 25 °C using a MCS titration calorimeter (MicroCal). Measurements were carried out in Pipes-KCl buffer, supplemented with 10 mM MgCl₂ in both protein and nucleotide solutions for titration experiments with GXP-Mg²⁺ (EDTA was omitted for titration with Mg²⁺). Samples were dialyzed against buffer before measurements. Ligand solutions (~150 μM GXP or 50 mM Mg²⁺) 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 titrant dilution 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 (Kd), and the enthalpy of binding (ΔH).

** Stoichiometry of Binding of Nucleotides and ApoFtsZ Polymerization—**The stoichiometry of binding of GTP, GDP, mant-GTP, and mant-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
action was determined as reported (45), with modifications. Fixed concentrations of mant-GXP (10–500 nM) were first titrated with different apoFtsZ concentrations (0–6 μM) in Pipes-KCl buffer, with or without 10 mM Mg²⁺, to obtain the anisotropy increment, Δrₚₐₓₙ, corresponding to all of the mant-GTP bound. To do this, the increase in anisotropy was plotted against apoFtsZ concentration and iteratively least-squares fitted with an isotherm of binding to one site. The estimated values of Δrₚₐₓₙ were used to approximate the free apoFtsZ concentrations, and these new values were employed again, until an unchanging Δrₚₐₓₙ value was obtained. The convergent data were used to calculate the binding constant of apoFtsZ to mant-GXP. Titration of apoFtsZ (500 nM) with different mant-GTP concentrations was also measured, and the data were model-fitted (employing the Δrₚₐₓₙ value) to yield the number of binding sites and the equilibrium binding constant of mant-GTP to apoFtsZ.

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 Equtira 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

\[ y(t) = a + b + \sum A_k e^{-k/t} \]

(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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Histidine-tagged FtsZ Polymers—FtsZ-His$_6$ and mutant FtsZ-W319Y-His$_6$ were overproduced in *E. coli* and affinity-purified as described (19). ApoFtsZ-W319Y-His$_6$ was prepared as apoFtsZ above, and its concentration was measured with an extinction coefficient $\varepsilon_{280} = 2980$ M$^{-1}$ cm$^{-1}$ (2 Tyr). It was diluted at 55 °C into Pipes-KCl buffer, pH 6.5, supplemented with 6 mM MgCl$_2$ 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$_6$ and FtsZ-His$_6$ were formed in 50 mM Mes, 50 mM KCl, 1 mM EDTA, pH 6.5 (Mes-KCl buffer) with 6 mM MgCl$_2$ 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$_6$ single Trp was measured fluorometrically by excitation at 295 nm, employing FtsZ-His$_6$ standards. Concentration of FtsZ-W319Y-His$_6$ polymers was measured with the Bio-Rad assay (43) with FtsZ-W319Y-His$_6$ standards. Exchange of [α-32P]GTP into FtsZ-W319Y-His$_6$ or FtsZ-His$_6$ was measured employing a nitrocellulose filtration assay (17).

**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 MgCl$_2$, with a [GdmCl]$_{1/2}$ 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 ($T_m = 90.16 \pm 0.03$ °C, $\Delta H_D = 190 \pm 20$ kcal/mol) is further stabilized by GDP (100 μM), which increased the temperature of the transition by 10 °C ($T_m = 100.72 \pm 0.09$ °C, $\Delta H_D = 220 \pm 10$ kcal/mol). Magnesium (10 mM MgCl$_2$) does not significantly stabilize apoFtsZ ($T_m = 90.25 \pm 0.02$ °C, $\Delta H_D = 230 \pm 10$ kcal/mol) but apparently induces a destabilization of FtsZ-GDP (FtsZ-GDP-Mg$^{2+}$ $T_m = 96.2 \pm 0.7$ °C, $\Delta H_D = 190 \pm 10$ kcal/mol). The contribution of GDP dissociation to the denaturation enthalpy, $\Delta H_D$, 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-GTP (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

![FIGURE 1. A, DSC traces of ApoFtsZ (12.5 μM) with MgCl$_2$ (10 mM), with GDP (100 μM), and with GDP (100 μM) plus MgCl$_2$ (10 mM) in Pipes-KCl buffer. B and C, calorimetric titration (ITC) of FtsZ binding to GDP and GTP-Mg$^{2+}$, 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).](http://www.jbc.org/content/282/52/37518/F1.large.jpg)
TABLE 1

Energetics of the interactions of FtsZ with nucleotides determined by ITC at 25 °C

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_b$</th>
<th>$\Delta G_b$</th>
<th>$\Delta H_b$</th>
<th>$\Delta S_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$M</td>
<td>kcal mol$^{-1}$</td>
<td>kcal mol$^{-1}$</td>
<td>cal K$^{-1}$ mol$^{-1}$</td>
</tr>
<tr>
<td>GDP</td>
<td>50 ± 10</td>
<td>-10.5 ± 0.1</td>
<td>-6.80 ± 0.20</td>
<td>12.4 ± 0.3</td>
</tr>
<tr>
<td>GDP-Mg$^{2+}$</td>
<td>20 ± 8</td>
<td>-10.0 ± 0.2</td>
<td>-3.78 ± 0.09</td>
<td>20.9 ± 0.4</td>
</tr>
<tr>
<td>GTP</td>
<td>300 ± 100</td>
<td>-11.6 ± 0.2</td>
<td>-5.88 ± 0.08</td>
<td>19.1 ± 0.4</td>
</tr>
<tr>
<td>GTP-Mg$^{2+}$</td>
<td>30 ± 10</td>
<td>-10.2 ± 0.2</td>
<td>-3.31 ± 0.03</td>
<td>23.1 ± 0.6</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0.0001 ± 0.00003</td>
<td>-2.7 ± 0.2</td>
<td>4.2 ± 0.7</td>
<td>23 ± 2</td>
</tr>
</tbody>
</table>

ITC experiments were made with 0 or 10 mM MgCl$_2$ in both protein and GXP solutions (the titration with MgCl$_2$ was done without EDTA). The stoichiometry of the low affinity Mg$^{2+}$ binding cannot be measured from these experiments, and therefore, the enthalpy change is an estimate per mole of protein, not per mole of site. Titration of FtsZ-GTP (no EDTA) with equimolar Mg$^{2+}$ gave insignificant heat.

FtsZ-Nucleotide Interactions and Polymer Dynamics

![Graph showing FtsZ-Nucleotide Interactions and Polymer Dynamics](http://www.jbc.org/)

**FIGURE 2.** A, isotherms of binding of [8-3H]GTP to apoFtsZ. ApoFtsZ (500 nM) in Pipes-KCl buffer, in the absence (solid triangles) or presence of 10 mM MgCl$_2$ (solid circles) at 25 °C. Open symbols are [8-3H]GTP bound in the presence of an excess of GTP (200 μM). B and C, isotherms of binding of apoFtsZ and mant-GTP measured by fluorescence anisotropy of the ligand. B, titration of mant-GTP (50 nM) with apoFtsZ (fitted $\Delta r_{max} = 0.234 ± 0.005$ in this experiment). C, titration of apoFtsZ (500 nM) with mant-GTP. Open symbols are non-specific binding, measured with an added excess of GTP (250 μM). Lines in each case are the best fitted model isotherms (see “Experimental Procedures” for binding parameters).

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...
**FtsZ-Nucleotide Interactions and Polymer Dynamics**

**TABLE 2**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>[MgCl₂]</th>
<th>( K_a ) (25 °C)</th>
<th>( K_b ) (55 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mant-GDP</td>
<td>0.0</td>
<td>17 ± 2 (19 ± 2)</td>
<td>10 ± 1 (18 ± 3)</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>10 ± 3</td>
<td>11 ± 1</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>1.6 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Mant-GTP</td>
<td>0.0</td>
<td>18 ± 5 (32 ± 4)</td>
<td>35 ± 5 (21 ± 2)</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>5 ± 2</td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>4.2 ± 0.4</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

**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 \( K_a \) 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 mM) 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; measured at 25 or 55 °C can be explained by the small binding enthalpies of GTP and GDP measured by ITC.

Finally, affinities of binding of natural guanine nucleotides to FtsZ were measured by competition with mant-GTP in the presence of Mg²⁺ (Fig. 3A). \( K_b \) values for GTP, GDP, and GMP were 330 ± 80, 110 ± 40, and 0.022 ± 0.007 μM⁻¹, respectively. The \( K_b \) value of GTP determined by competition is 3.6-fold larger than the one determined directly by [8-³²P]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, \( k_{app} \), 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:

\[
\text{Eq. 1}
\]

We could determine the association rate constant, \( k_+ \), from the slope of the regression line, but not, with sufficient precision, the dissociation rate constant \( k_- \). 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. Calculation of equilibrium binding constants from the association and dissociation rate constants \( (K_b = k_+/k_-) \) gives values within a factor of 2 from the measured equilibrium values (Table 2). This supports the simple kinetic mechanism here proposed for binding of nucleotide to unassembled FtsZ. Under conditions for FtsZ polymerization (Mg²⁺, 55 °C), and depending on the protein concentration, both association and dissociation became multiphase; their kinetics are analyzed below.

**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-
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 concentra-

FIGURE 4. Kinetics of binding of mant-GDP and mant-GTP to ApoFtsZ. A and B, a 1 μM solution of mant-GDP was mixed with 10 μM ApoFtsZ in the stopped-flow instrument at 25 °C (final concentrations of 500 nM mant-GDP and 5 μM ApoFtsZ). Binding was followed by fluorescence intensity (A) and by anisotropy (B). Empty symbols in A and B are controls of mant-GDP mixed with buffer. Solid lines, best fits of a single exponential to experimental data (kₑ (intensity) = 101 ± 2 s⁻¹, ΔFₑ max = 2.00 ± 0.03; kₑ (anisotropy) = 110 ± 10 s⁻¹, Δrₑ max = 0.07 ± 0.005). C, dependence on the concentration of apoFtsZ of the observed rate constants of binding of mant-GDP (circles) and mant-GTP (squares) with 10 mM MgCl₂ (solid symbols) and without MgCl₂ (empty symbols) at 25 °C.

FIGURE 5. Kinetics of dissociation of mant-GTP and mant-GDP from unassembled FtsZ. The mant-nucleotide-FtsZ complex was formed by adding 2 μM ApoFtsZ to 1 μM mant-GXP, with or without 10 mM MgCl₂. At time 0, this solution was mixed 1:1 with 400 μM GTP. The reaction was followed by the change in fluorescence of the mant-GXP (gray traces). Data were fitted to single exponentials (black lines; see rate constant values in Table 4). Trace 1, mant-GTP, 25 °C; trace 2, mant-GDP, 25 °C; trace 3, mant-GDP with Mg²⁺, 25 °C; trace 4, mant-GTP with Mg²⁺, 25 °C; trace 5, mant-GTP, 55 °C; trace 6, mant-GDP, 25 °C; trace 7, mant-GTP alone. Note that each pair of mant-GTP/mant-GDP measurements is arbitrarily displaced on the y axis to facilitate comparison.

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TABLE 3
Kinetics of binding and dissociation of mant-nucleotides from FtsZ

Association and dissociation rate constants were determined from the fluorescence intensity change of mant-GXP in Pipes-KCl buffer; constants obtained from the anisotropy increment are indicated in parentheses. Predicted values of association and dissociation rate constants in magnesium at 55 °C, obtained by multiplying the values with magnesium at 25 °C by the ratio of values at 55 and 25 °C without magnesium, are indicated solely for the purpose of comparison with further measurements (Table 4).

<table>
<thead>
<tr>
<th>Rate constant</th>
<th>Mant-GTP</th>
<th>Mant-GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>55 °C</td>
<td>25 °C</td>
</tr>
<tr>
<td>$k_1$ (s$^{-1}$), no MgCl$_2$</td>
<td>$28 \pm 2 (26 \pm 5)$</td>
<td>$37 \pm 7$</td>
</tr>
<tr>
<td>$k_1$ (M$^{-1}$ s$^{-1}$), 10 mM MgCl$_2$</td>
<td>$12 \pm 1 (11 \pm 2)$</td>
<td>$6 \pm 1 (7 \pm 2)$</td>
</tr>
<tr>
<td>$k_2$ (s$^{-1}$), no MgCl$_2$</td>
<td>$0.82 \pm 0.01$</td>
<td>$2.25 \pm 0.01$</td>
</tr>
<tr>
<td>$k_2$ (s$^{-1}$), 10 mM MgCl$_2$</td>
<td>$6.30 \pm 0.02$</td>
<td>$17 \pm 0.03$</td>
</tr>
</tbody>
</table>

hydrolysis rate of mant-GTP (20 μM) to mant-GDP by polymers of FtsZ (12 μM) 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 μM, under the 7.0 μM Cr of polymer formation) or partially polymerized apoFtsZ (15 μM) and then mixing it with the ligand to the same final concentration (1.5 μM). 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 $0.42 \pm 0.05$ 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, $\sim 70$ and 4.6 s$^{-1}$; polymerized, $\sim 40$ and 4.3 s$^{-1}$. Their relative amplitudes were 10:1 in the unassembled sample and 0.4:1 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 $k_1 \approx 30$ μM$^{-1}$ 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, $k_2 \approx 1$ μM$^{-1}$ 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, $\sim 40 \text{s}^{-1}$, 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 $Cr_{20}$ for polymerization) took place with rate constants of $\sim 20$ and $\sim 50 \text{s}^{-1}$, 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 $0.20 \text{s}^{-1}$ (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 $\text{s}^{-1}$, 85% amplitude; 0.20 ± 0.01 $\text{s}^{-1}$, 15% amplitude). It was followed by partial depolymerization at a rate of 0.012 ± 0.001 $\text{s}^{-1}$ (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 $0.057 \text{s}^{-1}$ (which could not be fitted by a sum of exponentials) and was closely followed by depolymerization at $0.055 \text{s}^{-1}$ 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 $\text{s}^{-1}$ 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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TABLE 4  
Association kinetics of mant-GTP to apo-FtsZ under polymerization solution conditions  

Values in parenthesis are the average relative amplitude of each phase. Association values from apoFtsZ above Cr have been corrected by the fractions of unassembled and polymeric FtsZ in solution. Available mant-GDP data are roughly similar to the mant-GTP data (Fig. 7, B and C).  

<table>
<thead>
<tr>
<th>Initial [apoFtsZ]</th>
<th>( k_1 ) ( unassembled)</th>
<th>( k_{-1} ) ( unassembled)</th>
<th>( k_1 ) ( polymer)</th>
<th>( k_{-1} ) ( polymer)</th>
<th>( k_1 ) ( unknown)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Below Cr for polymerization</td>
<td>30 ± 6 (49 ± 8%)</td>
<td>38 ± 7</td>
<td>1 ± 2 (7 ± 3%)</td>
<td>0.42 ± 0.05 (44 ± 10%)</td>
<td></td>
</tr>
<tr>
<td>Above Cr for polymerization</td>
<td>35 ± 15 (24 ± 10%)</td>
<td>44 ± 20</td>
<td>1.1 ± 0.3 (20 ± 6%)</td>
<td>0.42 ± 0.05 (56 ± 8%)</td>
<td></td>
</tr>
</tbody>
</table>

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 MgCl₂ 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**  

<table>
<thead>
<tr>
<th>Conditions</th>
<th>( k_{-1} ) (GTP excess)</th>
<th>( k_{-1} ) (GDP excess)</th>
<th>( k_{-1} ) (GT excess)</th>
<th>( k_{-1} ) (GP excess)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unassembled mant-GTP-FtsZ (0.5 ( \mu )M FtsZ)</td>
<td>53 ± 8</td>
<td>NO</td>
<td>52 ± 9</td>
<td>NO</td>
</tr>
<tr>
<td>Unassembled mant-GTP-FtsZ (0.5 ( \mu )M FtsZ)</td>
<td>22 ± 2</td>
<td>NO</td>
<td>18 ± 3</td>
<td>NO</td>
</tr>
<tr>
<td>Mant-GDP-FtsZ oligomers (9 ( \mu )M FtsZ)</td>
<td>40 ± 10 (10%)</td>
<td>0.20 ± 0.05 (90%)</td>
<td>33 ± 10 (10%)</td>
<td>0.21 ± 0.05 (90%)</td>
</tr>
<tr>
<td>Mant-GTP-FtsZ polymers (9 ( \mu )M FtsZ)</td>
<td>NO</td>
<td>0.057 ± 0.005</td>
<td>0.20 ± 0.01 (15%)</td>
<td>0.018 ± 0.001 (85%)</td>
</tr>
</tbody>
</table>

**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 \( \text{Mg}^{2+} \) on FtsZ-GDP
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\(_{75}\) 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-

FIGURE 9. Interactions of nonhydrolyzing mutant FtsZ-W319Y-His\(_6\) with nucleotides. A, mant-GTP binding to polymers of FtsZ-W319Y-His\(_6\). Solid line, fluorescence emission spectrum of 12.5 \( \mu M \) mant-GTP in a solution of 12.5 \( \mu M \) polymerized apoFtsZ-W319Y-His\(_6\); dashed line, mant-GTP alone; dotted line, mant-GTP + polymerized FtsZ-W319Y-His\(_6\) + 1 \( mM \) GTP; dashed and dotted line, polymerized FtsZ-W319Y-His\(_6\) 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\(_6\)-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\(_6\) 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\(_6\) 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 \( \sim 0.1 \times 10^{-4} \) s\(^{-1}\) and an amplitude of 0.06 \( \pm 0.01 \) (marked fit); maximum incorporation was 0.05 \( \pm 0.003 \) (marked exp), determined by copolymerizing the FtsZ-WT-His\(_6\), together with FtsZ-W319Y-His\(_6\), D, time courses of exchange of \([\alpha-\overline{32P}]GTP into 12.5 \( \mu M \) FtsZ-W319Y-His\(_6\), (solid circles) or FtsZ-His\(_6\) (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\(_6\) polymers hydrolyze GTP, whereas FtsZ-W319Y-His\(_6\) ones do not.
**FtsZ-Nucleotide Interactions and Polymer Dynamics**

FtsZ would be compatible with a change in protonation upon Mg\(^{2+}\) binding or with the introduction of some strain by the binding of Mg\(^{2+}\) to the FtsZ-GTP complex.

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 Mg\(^{2+}\) binding are linked in αβ-tubulin (54). The nucleotide γ-phosphate and the coordinated Mg\(^{2+}\) ion bound at the functional GTP/GDP binding site of β-tubulin control microtubule stability, whereas the Mg\(^{2+}\) 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 Mg\(^{2+}\), which is coordinated to oxygen from the β- and γ-phosphates. However, the functional roles of the γ-phosphate and Mg\(^{2+}\) vary among different G-proteins. Thus, Ras and EF-Tu form tight GDP-Mg complexes, Mg\(^{2+}\) binding reduces the GDP off rate by 4 orders of magnitude, and GDP binds more tightly than GTP (56). As another example, Mg\(^{2+}\) is not required for GDP binding to eRF3 but strengthens GTP binding; no structural changes were observed for GTP-Mg\(^{2+}\) and GDP-Mg\(^{2+}\) binding to eRF3 (57). In Rho proteins, the Mg\(^{2+}\) 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 < \(k_a\) < 40 \(\mu\)M\(^{-1}\)s\(^{-1}\)) and dissociation rates (1 < \(k_d\) < 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 \(K_b\) values determined by competition and ITC methods, the ratio \(K_b(GTP-Mg^{2+})/K_b(GDP-Mg^{2+})\) determined with the competition method is 3 ± 2, which is comparable with the 3.2 ± 0.3 ratio determined with mant-GTP and GDP (Fig. 3B), with the 1.48 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.
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 Pi, 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. *M. jannaschii* FtsZ polymers hydrolyze GTP with a turnover of 0.10 s\(^{-1}\) (19), which is similar to the value of 0.07 s\(^{-1}\) reported for *E. coli* FtsZ, at lower temperature (21). *M. jannaschii* FtsZ polymers disassemble rapidly, with half-times of 0.6 s (FtsZ-GTP polymers) to 25 s (mant-GTP-FtsZ polymers); these values comprise the 5 s half-time for *E. coli* FtsZ polymer disassembly (in GDP excess) and the 7 s half-time of subunit exchange, reported under quite different conditions (25). GDP dissociation from FtsZ polymers, which is difficult to measure, is rate-limiting to the exchange of GTP into polymers. If it is faster than the rate of hydrolysis, the steady-state polymer may contain mainly GTP and a minor fraction of GDP-bound subunits, which will have a given probability of fragmenting the polymer. As long as the nucleotide exchange in the polymer is significantly faster than hydrolysis and disassembly, subunit turnover is expected to be independent of the GTPase rate. On the contrary, if GDP dissociation is slower than GTP hydrolysis, GDP-bound subunits will accumulate, and the polymer will disassemble. Subunits will then rapidly exchange nucleotide with the solution and recycle into new polymers (indicated by the circular arrow in Fig. 10B).

In this case, subunit turnover is expected to depend on the GTPase rate. A steady-state population of recycling FtsZ polymers will contain mainly GTP polymers and a small fraction of GDP-containing FtsZ polymers. An estimate for the dissociation rate of GDP-FtsZ polymers is provided by the observed GDP-induced dissociation rate constant (2.6 s\(^{-1}\)) of apoFtsZ polymers, assuming that the binding of GDP is not rate-limiting.

The observation that subunit exchange is very slow in polymers of the GTPase-deficient mutant FtsZ-W319Y-His\(_6\) (Fig. 9) compared with the fast disassembly of FtsZ-His\(_6\) active GTPase (19) 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, which has a slow GTPase in vitro (23, 24), (ii) FtsZ subunit exchange in a fluorescence resonance energy transfer assay is strongly reduced by the slowly hydrolyzable nucleotide GMP-CMP (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.

**Acknowledgments**—We thank Martin Alba for FtsZ purification and Dr. Jesús Mingorance for help with the [α-\(^{32}\)P]GTP filtration assay.
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REFERENCES

LEGENDS TO SUPPLEMENTARY FIGURES

Suppl. fig. 1. (A), CD spectra of FtsZ in Pipes-KCl buffer at 25 °C. Solid line, native FtsZ; dashed line, apo-FtsZ. (B), Equilibrium GdmCl unfolding-refolding curves of apo-FtsZ and (C) apo-FtsZ, 1 mM MgCl2, 50 µM GTP, in Pipes-KCl buffer at 25 °C. Closed circles, increasing GdmCl concentrations; open circles, reverse experiment made by dilution from 5.0 M GdmCl. The continuous lines are two state model fits to the data with half denaturant concentrations 3.1 M GdmCl.

Suppl. fig. 2. Isotherms of binding of apo-FtsZ to mant-GDP (A) and mant-GTP (B), in Pipes-KCl buffer, at 25 °C in the absence of magnesium (closed circles) and in the presence of 10 mM MgCl2 (closed triangles), measured by fluorescence anisotropy of the ligand. Lines in each case are the best-fitted model isotherms (see table 2 for the binding parameters).

Suppl. fig. 3. Kinetics of dissociation of mant-GTP and mant-GDP from FtsZ monomers and oligomers under polymerization solution conditions (Pipes-KCl buffer, 10 mM MgCl2, at 55 °C). (A), Dissociation of mant-GDP (line 1), and mant-GTP (line 2), from FtsZ monomers. 0.55 µM FtsZ-mant-GXP was diluted to 0.5 µM in 1 mM GDP (solid line) or in 1 mM GTP (dotted line). (B), Dissociation of mant-GDP from FtsZ oligomers. 10 µM FtsZ-mant-GDP was diluted to 9 µM in 1 mM GDP (solid line) or in 1 mM GTP (dotted line).
suppl figure 1
Suppl Figure 2

A

\[ \frac{[\text{FtsZ}]_{\text{bound}}}{[\text{mant-GDP}]_{\text{total}}} \]

against

\[ \log(\text{apo-FtsZ})_{\text{free}} \]

B

\[ \frac{[\text{FtsZ}]_{\text{bound}}}{[\text{mant-GTP}]_{\text{total}}} \]

against

\[ \log(\text{ApoFtsZ})_{\text{free}} \text{ (M)} \]
Suppl Figure 3

A

Fluorescence intensity (au)

0.00 0.05 0.10 0.15 0.20 0.25

time (s)

0.00 0.05 0.10 0.15 0.20 0.25

time (s)

B

Fluorescence intensity (au)

0 2 4 6 8 10

time (s)
The Interactions of Cell Division Protein FtsZ with Guanine Nucleotides
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