Polymerization of FtsZ, a Bacterial Homolog of Tubulin

IS ASSEMBLY COOPERATIVE?*

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FtsZ is a bacterial homolog of tubulin that is essential for prokaryotic cytokinesis. In vitro, GTP induces FtsZ to assemble into straight, 5-nm-wide polymers. Here we show that the polymerization of these FtsZ filaments most closely resembles noncooperative (or “isodesmic”) assembly; the polymers are single-stranded and assemble with no evidence of a nucleation phase and without a critical concentration. We have developed a model for the isodesmic polymerization that includes GTP hydrolysis in the scheme. The model can account for the lengths of the FtsZ polymers and their maximum steady state nucleotide hydrolysis rates. It predicts that unlike microtubules, FtsZ protofilaments consist of GTP-bound FtsZ subunits that hydrolyze their nucleotide only slowly and are connected by high affinity longitudinal bonds with a nanomolar $K_p$.

FtsZ and tubulin are homologs that share identical folds (4) and assemble into polymers with many of the same properties. Like tubulin, FtsZ polymerizes in the presence of GTP (5, 6) and can form straight protofilaments that are ~5 nm wide, with subunits spaced 4 nm apart (7, 8). The longitudinal bonds that connect the subunits in a tubulin protofilament are understood at atomic resolution, and the bonds between subunits in an FtsZ protofilament are likely to be very similar (9). Residues on both sides of the longitudinal protein interface are conserved, and the GTP binds to one side of the interface and is necessary for formation of a protofilament. In return, hydrolysis of the GTP occurs only after formation of the longitudinal bond, when residues from the adjoining subunit contact the nucleotide (4).

GTP hydrolysis causes both FtsZ and tubulin filaments to adopt a curved conformation (7, 10) and become more labile (11). Curved GDP-FtsZ filaments have been visualized when stabilized by various polycations and are half the diameter of GDP-tubulin rings, indicating that the angle of curvature between GDP-FtsZ subunits is the same as that between the $\alpha\beta$ heterodimers in tubulin rings (12). For both proteins, assembly with GDP requires magnesium and is relatively weak ($K_p = 20–50 \mu M$ (13, 14)).

In contrast to the conserved longitudinal contacts within tubulin and FtsZ protofilaments, the lateral contacts between protofilaments in a microtubule wall involve protein surfaces that are not conserved in FtsZ (4). Because FtsZ polymers have not yet been visualized with high resolution in vivo, it is not known how the protofilaments in the Z ring are associated. Under many conditions in vitro, GTP induces FtsZ to form individual 5-nm-wide polymers that are stable without additional lateral interactions (5, 10, 11, 15). Nonetheless, even these thin FtsZ polymers could consist of two protofilaments joined along the narrowest 3 nm axis of the protein, and there is a precedent for such a 6 nm double filament forming in the presence of $Ca^{2+}$ (8). FtsZ can form several types of lateral bonds when exogenous cations are added, resulting in a variety of paired protofilaments, bundles, double layered sheets, and tubes (6–8, 10, 16). For tubulin, aberrant lateral bonds can result in the formation of zinc sheets (17) and hooked and S-shaped polymers (18). It is unclear for FtsZ whether any of the lateral contacts that form in vitro are relevant in vivo.

Polymers that are multistranded assemble cooperatively (19). Cooperative assembly has three distinctive characteristics (see Fig. 1A) as follows: there is a critical concentration for assembly; there are kinetic lags in polymerization at low protein concentrations; and at equilibrium, subunits are distributed into two distinct populations, monomers and very long polymers. These properties derive from the two different phases of assembly, an unfavorable nucleation phase followed by a more favorable growth phase. Nucleation is unfavorable because individual bonds between monomers are weak, and therefore initiation of the polymer is difficult. However, once a stable nucleus has assembled, each new subunit can form multiple bonds to the growing polymer. Subunit addition becomes favorable and polymer growth rapid. The critical concentration is the minimum protein concentration at which any polymer forms; at all concentrations above this, subunits assemble until the concentration of monomer left in solution has fallen to the critical concentration.

Most biological polymers are multistranded and assemble...
Single-stranded, isodesmic polymers are less common in biology than cooperative, multistranded polymers, and none so far has been shown to be the major form of a protein in vivo. Isodesmic assembly was first analyzed by studying \(\beta\)-lactoglobulin (21) and glutamate dehydrogenase (22), but both these proteins function in vivo as monomers. The Drosophila septins can assemble in vitro both isodesmically, forming short, single-stranded polymers (23), and cooperatively, forming multi-stranded filaments, depending on which subunits are combined. Relevant to the present study, tubulin-GDP can assemble isodesmically into single, curved protofilaments that eventually close to form tubulin rings (13). Similarly, GDP-FtsZ assembly is noncooperative and deviates only slightly from isodesmic polymerization (14).

The two basic mechanisms of assembly described above are often complicated by nucleotide hydrolysis or conformational changes within a subunit. In actin and tubulin, these complications lead to phenomena such as treadmilling and dynamic instability (24, 25). Prion fiber assembly also shows characteristics that do not fall neatly into either mechanism described above. Prion assembly shows lag phases that are concentration-independent, and monomer, small oligomer, and polymer coexist in assembly reactions (26). A conformational change is thought to be necessary to activate the prion protein before it can assemble into multistranded fibers, and preformed fibers may accelerate the rate of this conformational change. For FtsZ, cycles of GTP hydrolysis and conformational changes in the subunit may also introduce complexities into its polymerization mechanism.

The straight 5-nm-wide FtsZ polymers that form in GTP are probably the building blocks of any larger structure that forms in vivo. In the cell, controlled nucleation followed by favorable growth would be an economic explanation of the FtsZ's tight localization and its ability to span the 3-\(\mu\)m circumference of an E. coli cell. However, if FtsZ polymers have the structure of an isolated tubulin protofilament, they would be single-stranded and would be expected to show isodesmic assembly. Our goal was to determine whether the assembly of these protofilaments occurs via isodesmic or cooperative assembly. Therefore, we determined whether these filaments are in fact single-stranded, whether there is a concentration-dependent lag phase during assembly indicative of nucleation, and whether there is a critical concentration for assembly. We found that unlike microtubules, the assembly of these FtsZ-GTP protofilaments appears isodesmic.

**Materials and Methods**

**FtsZ Purification**—E. coli FtsZ was overexpressed from a pET11b vector (Novagen) and purified largely as in Lu et al. (27) with a few modifications. Cells were grown in 500 ml of LB at 37 °C to \(A_{600} = 1\), induced with 0.5 mM isopropyl-\(\beta\)-galactopyranoside, and grown for an additional 2 h. Cells were sedimented and resuspended in 10 ml of 50 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM 4\((\alpha\)-aminoethyl)benzenesulfonfyl fluoride and frozen at –80 °C. Cells were thawed, and lysosome was added to 0.1 mg/ml and incubated for 10 min, and magnesium acetate was added to 10 mM. Cells were sonicated, and DNase was added to 10 \(\mu\)g/ml, and the extract was incubated for 1 h. Lysate was spun for 30 min at 220,000 \(\times\) g for 4 °C. The supernatant was mixed with 0.25 volume saturated (room temperature) ammonium sulfate, incubated on ice for 20 min and spun at 80,000 \(\times\) g for 10 min at 4 °C. The supernatant was removed, and 1/2 volume ammonium sulfate was added. This was spun again at 80,000 \(\times\) g for 10 min at 4 °C. The pellet was resuspended in 5 ml of polymerization buffer (50 mM NaMES, pH 6.5, 2.5 mM magnesium acetate, 1 mM EDTA) and spun again at 160,000 \(\times\) g for 10 min at 4 °C. The final supernatant was brought to 10% glycerol and 50 mM GTP, aliquoted, snap-frozen in liquid nitrogen, and stored at –80 °C. For these analyses, the FtsZ was used with the GTP concentration in the range of 5 to 20 mM.

**Prion Fiber Assembly**—To assess whether FtsZ can assemble isodesmically, the protein was subjected to conditions that are known to induce isodesmic fibers. The PrP106-126 sequence (28) was overexpressed from a pET11b vector as a C-terminally tagged protein. The protein was purified as above and analyzed by scanning transmission electron microscopy.

1 M. Glotzer, personal communication.
2 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; GMPCPP, guanylyl-(\(\alpha\),\(\beta\))-methylenediphosphonate; STEM, scanning transmission electron microscopy.
liquid nitrogen, and stored at −80 °C.

**Protein Concentration Determinations and Assembly Reaction Conditions**—Protein concentrations were measured using the BCA assay (Pierce) with bovine serum albumin as a standard, calibrated for the 0.75 color ratio of FtsZ/bovine serum albumin (27). All assembly reactions were carried out in the above polymerization buffer (50 mM NaMES, pH 6.5, 2.5 mM magnesium acetate, 1 mM EGTA). Reactions were supplemented with magnesium acetate so that the total magnesium concentration was 2 mM in excess of any nucleotide. Assembly reactions were performed at room temperature unless otherwise indicated.

**FtsZ Cycling**—On the day the protein was to be assayed, the FtsZ was put through a cycle of calcium-aided assembly and disassembly (based on Ref. 16) to select for active, nonaggregated protein. Thawed protein was resuspended in a TLA100 rotor for 15 min at 350,000 × g at 4 °C. The protein was then diluted 5-fold in polymerization buffer to lower the glycerol concentration (final protein concentration −1 mg/ml) and brought to 10 mM CaCl2 and 2 mM GTP. After incubation for 3 min at room temperature, polymer was sedimented for 15 min at 350,000 × g at 20 °C. The pelleted was resuspended well in polymerization buffer and incubated on ice for 30 min. A final spin (15 min, 350,000 × g, 4 °C) removed any remaining aggregated protein.

**Electron Microscopy and Filament Length Measurements**—Negative stain electron microscopy was used to visualize FtsZ filaments. Carbon-coated 300-Mesh, Electron Microscopy Sciences) were glow discharged for 5 s before use. For some samples, before applying the FtsZ a drop of 0.2 mg/ml cytochrome c was pipetted onto the carbon, incubated for 30 s, and then blotted with filter paper. This procedure aided good staining at low FtsZ concentrations but did not visibly affect filament number or length. Cycled protein was diluted to the desired concentration in polymerization buffer and nucleotide was added, and the reaction was incubated for several minutes at room temperature. A drop of FtsZ solution was then applied to the carbon and incubated for 10 s before the excess was blotted. The grid was immediately rinsed with 3−4 drops 2% uranyl acetate, blotted, and air-dried. Filaments were visualized and photographed using a Philips 301 electron microscope at ×50,000 magnification.

For length determinations, 0.75−3.5 μm FtsZ was assembled with either 2 mM GTP or 0.5 mM GMPCPP and visualized by negative stain. Image quality improved significantly at the higher protein concentrations, although overlapping filaments made lengths more difficult to measure above 2 μm FtsZ. Three fields on a grid were photographed from 10 different reactions with each nucleotide. The negatives were scanned into a computer at 600 dpi. Filaments were traced using NIH image, and the length distributions for a given grid were pooled and plotted.

The polymer length distributions were largely exponential. However, because the shortest polymers were more likely to be obscured in the noise of an image or overlooked during measurement, the frequency of observing these polymers was lower than expected for an exponential distribution. The peak polymer length correlated with image quality, with increasing exponential length distributions down to 8 subunits (32 nm). To avoid biasing the average filament length because of incomplete counting of short filaments, the average filament length for each grid was mathematically calculated from an exponential fitting to the data, and only data from filaments that were longer than 25 subunits were included in the fittings. Changing the cut-off from 25 to 50 subunits did not alter the best fit curve. Because the average length did not change significantly between the FtsZ concentrations tested, the data from all experiments were pooled to determine the final average length reported for each nucleotide.

**GTPase Assays**—A malachite green-sodium molybdate assay was used to measure production of inorganic phosphate (28). Protein was diluted to the desired concentration in polymerization buffer at room temperature, and GTP was added to initiate the reaction. At four time intervals, reactions were stopped by addition of a 1× volume of cold 0.6 M perchloric acid. The samples were stored on ice until all time points were collected, at which time a 2× volume of filtered malachite green solution (0.15 g of malachite green, 1 g of sodium molybdate, 0.25 g of Triton X-100 in 0.7 M HCl) was added. The samples were then incubated at room temperature for 30 min, and the A400 was measured. NaPO4 buffer created a standard curve, and the reaction was normalized by including a control without FtsZ.

**STEM Analysis**—FtsZ was assembled in polymerization buffer plus either 2 mM GTP/5 mM EDTA or 0.5 mM GMPCPP. A 3-μl sample of the reaction mix was injected into a drop of buffer on a thin (2−3 nm) carbon film supported by a thick holey film over a titanium grid to which tobacco mosaic virus had previously been applied as an internal standard. The grid was washed first 5 times with injection buffer, then 10 times with 20 mM ammonium acetate, blotted to a thin layer, plunged into liquid nitrogen slush, and freeze-dried overnight under vacuum before being transferred to the microscope. The grids were visualized using a custom built microscope (STEM1) at the Brookhaven National Laboratory using a modified TLA100 rotor with a Dr. El.. A precision stage with an annular detectors collect nearly all the scattered electrons. Because the FtsZ filaments were radiation-sensitive, it was necessary to keep the electron dose low to have meaningful measurements. With a high dose, the mass/length measurements could be lower by 50% (data not shown). However, at lower doses, results became reproducible.

Areas with relatively clean backgrounds and an adequate number of filaments were chosen for analysis. A digital image was saved consisting of 512 × 512 pixels, each of which shows the number of scattered electrons, which is directly proportional to the mass thickness in that pixel. Relatively straight, short (usually ~30 nm) segments were chosen along a filament for mass measurements. Because there were significant numbers of unpolymerized molecules in the background, it was necessary to mask these molecules before the background was computed and subtracted from the intensity summed over the filaments. The microscope calibration factor was checked against that of tobacco mosaic virus, and the summed intensities (minus the background) multiplied by the calibration factor gave the mass values for the specimen.

**Light Scattering Assays**—Cycled FtsZ was diluted to the desired concentration in polymerization buffer and introduced into a quartz cuvette in a FluoroMax fluorimeter with a custom-made temperature-controlled cuvette holder. The cuvette was illuminated with 310 nm light, and the 90° light scattering was detected. The slit widths were set at 0.5 nm and the signal was integrated once a second. A base line of scattering for the unpolymerized protein was established and then polymerization was initiated by the addition of 1 mM GTP or GDP or 0.25 mM GMPCPP. The nucleotide was introduced into the solution on the end of a custom-made plunger that fit snugly into the cuvette with a 1-mm clearance around all sides. The plunger was inserted into the cuvette and rapidly withdrawn to mix the contents. Typical dead times were 2−3 s. The noise in the signal increased when using this plunger. Typical dead times were 2−3 s. The noise in the signal increased when using this plunger.

**Sedimentation Assays**—Pre-cycled protein was diluted to the desired concentrations with polymerization buffer. GTP was added to a final concentration of 5−10 mM, GDP to 2 mM, or GMPCPP to 0.5 mM, along with additional magnesium acetate so that there was a 2 mM excess of magnesium over nucleotide. 75 μl of each reaction was added to a pre-cycled tube and centrifuged in the TLA100 rotor in a TL100 ultracentrifuge (Beckman) for 15 min at 350,000 × g at 20 °C. Removing the supernatant and rinsing the supernatant and pellet gave poorly reproducible results, so we removed and assayed only the upper two-thirds of the supernatant, leaving the pellet undisturbed. The upper 50 μl of supernatant was immediately removed from the tube, and the concentration of protein in the original sample and the supernatant was measured. To limit GTP depletion and diffusion of the pellet from the tube walls after sedimentation, no more than seven reactions were sedimented simultaneously to reduce the handling time. Results were identical using 5 or 10 mM GTP, confirming that the nucleotide is not being significantly depleted during the 25 min required to complete an assay. Each protein concentration was tested 2−4 times per experiment, and 2−4 separate experiments were combined and averaged to obtain accurate data.

To calibrate the assay, seven proteins with known S values between 4.5 and 30 were sedimented in the same manner. The resulting curve was linear up to 19 S, at which point 94% of the protein sedimented. The resulting standard curve indicated that the percent protein sedimented = 3.6 × 10−3 × S (R = 0.97). FtsZ monomers are 3.4 S (14), and the expected S values of single-stranded FtsZ polymers of a given length were calculated from the theory of Kirkwood (29). The above calculations predict that 85% of a 3.4 S FtsZ monomer should remain in the supernatant after centrifugation; 5-mers, which should be 8 S, would be 40% sedimented, and 40-subunit filaments, which should be 14.5 S, would be 70% sedimented.

**THEORY**

The theory of isodesmic polymer assembly at equilibrium can be used to predict an apparent association constant for assembly of FtsZ into protofilaments. Here we have extended this theory to include a step of GTP hydrolysis followed by rapid subunit dissociation. The modified theory fits the observed
maximum hydrolysis rates and length distributions of FtsZ and allows the prediction of a true association constant for the addition of GTP subunits.

**Estimating K_{A,app} from Polymer Lengths**—The average length of an isodesmic filament is determined by the affinity constant for assembly and the total protein concentration. To use length measurements to estimate an apparent affinity constant for GTP- and GMPCPP-FtsZ polymerization, we derived the following Equations 1–4, based on previous models for isodesmic assembly (19, 22, 30):

\[
C_i = C_{i-1} + 2K_A = (2K_A)^{i-1}C_i^i \quad \text{(Eq. 1)}
\]

\[
C_i = C_i(1 - 2K_AC_i)^2 \quad \text{(Eq. 2)}
\]

where \(C_i\) is the concentration of polymers of length \(i\); \(C_1\) is the monomer concentration; \(C_i\) is the total concentration of subunits in all polymers; and \(K_A\) is the association constant for adding a subunit. These equations are the same as those found in Oosawa and Kasai (19), except that \(K_A\) is multiplied by a factor of 2 because the \(i + 1\) subunit can be added to either end of a growing polymer.

\[
C_1 = \frac{4K_AC_i + 1 - (8K_AC_i + 1)^{1/2}}{8K_AC_i} \quad \text{(Eq. 3)}
\]

Equation 3 is the quadratic solution of Equation 2,

\[
L_n = \frac{\Sigma C_i}{C_1} = \frac{4K_AC_i}{1 + \sqrt{8K_AC_i + 1}} \quad \text{(Eq. 4)}
\]

Equation 4 is the number average length of the polymers (\(L_n\)).

At total FtsZ concentrations of \(-2\) \(\mu\)M, the average lengths of GTP and GMPCPP filaments were 23 and 38 subunits, respectively. According to Equation 4, the apparent affinities needed to produce filaments of such lengths are \(K_{A,app} = 1.25 \times 10^6\) and \(3.3 \times 10^8\) M\(^{-1}\), corresponding to dissociation constants of 8 and 3 nM. These apparent affinity constants underestimate the actual affinity at a GTP-FtsZ interface because in addition to dissociation of GTP-bound FtsZ subunits, filaments fragment due to GTP hydrolysis, as described below.

**A Model for Isodesmic Polymerization with GTP Hydrolysis**—The following polymerization Model 1 accounts for the on-off reactions of subunits in both the GTP and GDP states, and the rate of GTP hydrolysis (see also Fig. 7, which will be addressed under “Discussion”):

\[
\begin{align*}
{k_{on}(\text{GTP-FtsZ})} & \quad \text{PT-F} & \quad \text{FT} \quad \Rightarrow & \quad \text{FD-F} & \quad {k_{on}(\text{GTP-FtsZ})} \\
{k_{off}(\text{GTP-FtsZ})} & \quad \text{PT-F} & \quad \text{FT} \quad \Leftarrow & \quad \text{FD-F} & \quad {k_{off}(\text{GTP-FtsZ})} \\
& \quad \text{K_{exchange}} & \quad \text{exchange} & \quad \text{K_{exchange}} & \quad {k_{exchange}}
\end{align*}
\]

**MODEL 1**

FT = FtsZ bound to GTP; FD = FtsZ bound to GDP; and F = another subunit of any nucleotide state. The rates are assumed to be identical whether monomers or the ends of long polymers are interacting. In reality, the entropy and diffusion of long polymers will be different from that of monomers, but these deviations from isodesmic assembly are likely to be less than 2-fold, as was found by Rivas et al. (14).

The association of protein subunits was assumed to be diffusion-limited (\(k_{on} \approx 5 \times 10^5\) M\(^{-1}\) s\(^{-1}\)) (31) for both GTP and GDP-FtsZ. Nucleotide exchange is likely to be very rapid (\(k_{nucleotide} \approx 35\) s\(^{-1}\)), based on the \(7\) \(\mu\)M \(K_D\) of GDP for FtsZ monomer (32) and a diffusion-limited rate for nucleotide binding (\(\approx 5 \times 10^6\)). Dissociation of GDP-FtsZ is also likely to be very rapid; with a diffusion-limited \(k_{off}(\text{GDP-FtsZ})\) and a \(K_D = 20\) \(\mu\)M (14), \(k_{off}(\text{GDP-FtsZ})\) would be 100/s.

In contrast to the rapid dissociation at a GDP-FtsZ interface, the dissociation at a GTP-FtsZ interface would be slow, giving rise to the stability of the protofilaments. The 3 nM apparent affinity constant calculated for GMPCPP filaments can be used as a first approximation for the affinity at GTP-FtsZ interfaces. A diffusion-limited on rate then predicts \(k_{off}(\text{GTP-FtsZ}) = 0.014/s\).

The GTP hydrolysis rate can be shown to be on the same order as this dissociation rate of GTP-FtsZ. The overall nucleotide turnover rates are known from experimental measurements (in the present study 1.5/min or 0.025/s for GTP, 0.23/min or 0.0038/s for GMPCPP). The model assumes that hydrolysis occurs only after subunit association, and that all subunits in the polymer hydrolyze GTP at a rate characterized by the first order rate constant \(k_{hydrolysis}\). At high protein concentrations, these overall turnover rates are valid estimates of \(k_{hydrolysis}\). This is because subunit association is no longer rate-limiting, and there is only a small fraction protein that is either monomer or at a polymer end and so not contributing to the turnover rate.

We postulate that the subunits in the protofilaments are largely bound to GTP and that the \(K_{A,app}\) estimated above is determined by three parameters. \(k_{on}(\text{GTP-FtsZ})\) gives the rate of addition of GTP subunits (or annealing of polymers with GTP at their ends). The off rate includes both dissociation of the GTP-FtsZ interface (\(k_{off}(\text{GTP-FtsZ})\)) and hydrolysis followed by rapid breakage of the GDP-Fts-Z bonds. Because these two off rates affect all subunit interfaces stochastically, they can simply be added to give the total off rate. The isodesmic assembly can therefore be modeled with the following Equation 5:

\[
K_{A,app} = \frac{k_{off}(\text{GTP-FtsZ}) + k_{hydrolysis}}{k_{on}(\text{GTP-FtsZ})} \quad \text{(Eq. 5)}
\]

As described above, the length distributions led us to \(K_{A,app} = 1.25 \times 10^6\) and \(3.3 \times 10^8\) M\(^{-1}\), corresponding to dissociation constants of 8 and 3 nM. These apparent affinity constants underestimate the actual affinity at a GTP-FtsZ interface because in addition to dissociation of GTP-bound FtsZ subunits, filaments fragment due to GTP hydrolysis, as described below.

**Estimating the Percentage of Polymers >5 Subunits Long**—To estimate what should be seen when visualizing FtsZ-GDP by electron microscopy, the concentration of polymers of different lengths was calculated based on Equations 1 and 2 above. The concentration of protein in polymers longer than five subunits was \(C_i - (i = 1-4)\Sigma C_i\). If the \(K_D = 20\) \(\mu\)M, the value reported for FtsZ-GDP (14), and the \(C_i = 12.5\) \(\mu\)M, then 5% of the protein should be in polymers longer than five subunits. This corresponds approximately to what is seen experimentally for the FtsZ-GDP assembly (Fig. 2A).

**Estimating the GTP Hydrolysis Rate**—GTP hydrolysis is likely to be activated only when a longitudinal interface is formed (4, 33). Assuming that \(k_{hydrolysis}\) is the same for all subunits except those at the plus end of a protofilament (where the GTP is exposed), the rate of hydrolysis would then be predicted to be proportional to the total concentration of FtsZ subunits, minus the concentration of monomers and polymer plus ends. Therefore (see Equation 6),

\[
\frac{\text{hydrolysis}}{\text{FtsZ}} = \frac{C_i - (C_i - C_1)(1 - 2K_AC_1)}{C_i} = k_{hydrolysis} \cdot (1 - K_AC_1)\text{C}_i(1 - 2K_AC_1) \quad \text{(Eq. 6)}
\]
This prediction does not match experimental observation (see below), suggesting that $k_{\text{hydrolysis}}$ may not be identical for all subunits in all polymers. Alternatively, an inactive form of FtsZ may exist in equilibrium with an assembly competent form, raising the concentration of monomer above that expected for isodesmic polymerization. (see “Discussion”).

RESULTS

Assembly of Polymers with GDP, GTP, and GMPCPP—FtsZ was assembled in the presence of various guanine nucleotides to characterize the polymers that form under each condition. We first assembled FtsZ in the presence of GDP and examined it by negative stain electron microscopy. The protein concentration used was a compromise between the high concentrations that favor GDP-FtsZ assembly and the low concentrations that allow good images to be obtained. At 12.5 μM FtsZ, arcs and rings were visible in the middle of abundant unassembled protein (Fig. 2, A and E). The diameter of these rings (24 ± 2 nm) is consistent with previous observations of curved FtsZ-polymers formed in the presence of cationic lipids or DEAE-
Polymers were initially long individual filaments (Fig. 2B). Age FtsZ in the low potassium assembly buffer used here (averages, the polymers were of uniform width (4.6 ± 0.7 nm) and had a broad length distribution. Polymers as short as 40 nm (10 subunits) or 23 subunits. The scatter in the data between different experiments was too large to see a significant change in polymer length over the small range in protein concentrations at which measurements was taken. At 2.5 μM FtsZ, most of the GMPCPP filaments were individual 5 nm wide polymers. They remained largely unpaired for the first 10 min after GMPCPP-induced assembly, but after 30 min, a network of partially bundled filaments formed (Fig. 2D). At concentrations greater than 3.5 μM FtsZ, paired or partially paired filaments could be seen as early as several minutes after initiation of polymerization. The GMPCPP polymers therefore seem to pair or bundle somewhat more readily than GTP polymers do.

**Number of Strands in the 5-nm-wide Filaments**—At low protein concentrations (<2 μM), FtsZ polymer consists entirely of unpaired 5-nm-wide straight filaments. Scanning transmission electron microscopy (STEM) was used to determine whether these basic FtsZ polymers are a single protofilament or whether they are made from two protofilament strands packed tightly along their narrowest axis, similar to what has been seen previously in Ca²⁺-stimulated sheets (8). STEM was used because it involves direct visualization of individual biological molecules without staining, fixing, or shadowing, and so allows the mass in a given area to be determined accurately. The unstained filaments (Fig. 4) were very similar in structure to those seen by negative stain. We assembled FtsZ in either GTP or GMPCPP and determined the mass of protein per length of filament. The average polymer mass per 4 nm (the length of a protofilament) was 39 ± 4 and 41 ± 6 kDa for GTP and GMPCPP filaments, respectively. This corresponds exactly to the mass density expected for a single-stranded polymer. These measurements confirm that the basic FtsZ polymer is a single protofilament.

**FtsZ Assembles Rapidly without a Concentration-dependent Lag Phase**—To determine whether FtsZ polymerization exhibits a lag phase during which unstable nuclei are being formed, kinetic measurements of assembly were made using 90° light scattering (15). Light scattering has high time resolution and could detect initial polymerization kinetics when the dead time due to mixing was sufficiently reduced. The assay has the disadvantage, however, that when the polymers are shorter than the wavelength of light, the scattering is not linear with respect to polymer mass (37) (in our experiments, polymer ~100 nm, wavelength = 310 nm). As a result, at early time points or at low protein concentrations, when the polymers are the shortest, the signal is expected to underreport the amount of polymer actually present. Nonetheless, the assay gives an indication of the relative rate and extent of an assembly reaction and should not obscure any lags that existed due to nucleation.

Unpolymerized FtsZ was placed in a fluorimeter, and a baseline scattering level was established. Nucleotide was then rapidly introduced, with a dead time of 2–3 s (Fig. 5A). If GDP was added to the protein, no increase in light scattering was seen. Instead, at higher FtsZ concentrations there was a drop of GTPase activity (up to 20%) above 2 μM FtsZ (Fig. 3B).

Guanyl-(α,β)-methylenediphosphonate (GMPCPP) is a GTP analog that supports microtubule assembly without nucleotide hydrolysis (36). We find that GMPCPP can in fact be hydrolyzed by FtsZ, although 3–10 times more slowly than GTP, depending on the FtsZ concentration (Fig. 3C). Experimental setup for three experiments = 0.23 ± 0.03 GMPCPP/min/FtsZ. Long straight polymers formed readily in the presence of GMPCPP (Fig. 2C) and appeared more abundant at low protein concentrations than those formed with GTP. They were similar in width and somewhat longer (152 nm or 38 subunits, R = 0.94). As with the GTP polymers, the average length did not change significantly over the protein concentration range examined.

At 2.5 μM FtsZ, most of the GMPCPP filaments were individual 5 nm wide polymers. They remained largely unpaired for the first 10 min after GMPCPP-induced assembly, but after 30 min, a network of partially bundled filaments formed (Fig. 2D). At concentrations greater than 3.5 μM FtsZ, paired or partially paired filaments could be seen as early as several minutes after initiation of polymerization. The GMPCPP polymers therefore seem to pair or bundle somewhat more readily than GTP polymers do.

**Hydrolysis of GTP and GMPCPP.** A. Phosphate release with time. The FtsZ concentration was 3 μM for both curves. B. Specific activity (nucleotide hydrolyzed/FtsZ/min) versus FtsZ concentration. A, GTP; O, GMPCPP. The polymerization buffer (MES, pH 6.5, 1 mM GTP; GMPCPP. The polymerization buffer (MES, pH 6.5, 1 mM EGTA) is potassium-free and contains 2 mM excess magnesium.

![Graph](https://example.com/graph.png)
in the scattering due to protein dilution. In contrast, following GTP addition the scattering signal rose, with the highest protein concentrations showing the greatest increases in scattering.

At 28 °C, FtsZ assembly was complete in ~10 s. FtsZ assembly was much more rapid than that of actin or microtubules, which at low concentrations can take up to an hour to reach a steady state polymer mass (38–40). Such rapid FtsZ assembly, which occurred even at very low protein concentrations, would not be expected if polymer initiation required an unfavorable kinetic step.

In the assays performed at 28 °C, no lag could be detected before the scattering signals began to rise. However, a short lag could be seen at 20 °C and became more pronounced at 7 °C, where FtsZ assembly still occurred (Fig. 5B). In several previous studies (27, 32, 34), GTPase assays with FtsZ exhibited lags of between 15 min and an hour, and these lags could be seen at 20 °C and became more pronounced at 7 °C, which at low concentrations can take up to an hour to reach a steady state polymer mass (38–40). Such rapid FtsZ assembly, which occurred even at very low protein concentrations, would not be expected if polymer initiation required an unfavorable kinetic step.

In our assays, however, the lags were quite short, and preincubating FtsZ with GDP did not alter their lengths (data not shown).

If these lags are due to nucleated assembly, the length of the lags should be dependent on FtsZ concentration. To determine the effect of FtsZ concentration, the time for polymerization to reach 1/10th completion was plotted versus the total protein concentration (Fig. 5C). At all temperatures, the lag in assembly was independent of FtsZ concentration. This suggests that after GTP has been added to a reaction, a zero order transition can take place at the initial protein concentrations showing the greatest increases in scattering.

Next, the long straight filaments that assemble in the presence of GTP were sedimented. As the total FtsZ concentration was increased, the unsedimented protein increased gradually to ~20 μM (~55% of the total protein) and did not plateau in the concentration range analyzed. There was no abrupt transition that would be indicative of a critical concentration.

Next, the long straight filaments that assemble in the presence of GTP were sedimented. As the total FtsZ concentration was increased, the FtsZ in the supernatant increased smoothly and continuously (Fig. 6B). Significantly less protein was required for efficient assembly in the presence of GTP than was required with GDP, and the curve leveled off with the soluble fraction near 1.5 μM FtsZ at 17 μM total FtsZ.

Sedimentation with GMPCPP was quite similar to that with GTP (Fig. 6C). However, the maximum concentration of soluble protein was slightly lower (~1 μM), indicating that GMPCPP-bound FtsZ has a greater tendency to assemble than GTP-FtsZ. In addition, the curve appeared to plateau somewhat more abruptly. If the flattening of the curve is significant, it may be an indication of partially cooperative assembly. This cooperativity could be the result of the protofilament pairing that occurs more readily when nucleotide hydrolysis is slowed, particularly at high protein concentrations or after long incubations (see...
DISCUSSION

FtsZ Protofilaments Do Not Assemble Cooperatively—Although there is a large evolutionary distance between prokaryotic temperature, it was not dependent on protein concentration. Theoretical curves for a cooperatively assembling polymer, assuming that all protein is monomer below the critical concentration, after which long polymer forms. A, FtsZ assembled in 2 mM GDP, critical concentration for curve fit = 20 μM. B, FtsZ assembled in 5–10 mM GTP, critical concentration for curve fit = 1.4 μM, C). FtsZ assembled in 0.5 mM GMPCPP, critical concentration for curve fit = 1 μM.

Fig. 2D). Such pairing would increase the affinity of the subunit for the polymer and so could flatten the curve and make it appear more cooperative at higher FtsZ concentrations.
otic FtsZ and eukaryotic tubulin, many of the properties of the two proteins have been conserved. One noteworthy property of tubulin is that its assembly into microtubules is cooperative. We examined whether FtsZ polymers also assemble cooperatively. We found that FtsZ can form single-stranded filaments that are 10–100 subunits long, in contrast to actin and microtubules, which grow to include thousands of subunits in each polymer (42, 43). In addition, FtsZ polymerization is extremely rapid without evidence of a nucleation phase. Finally, there is no critical concentration for assembly of the FtsZ protofilaments. These results indicate that unlike microtubule assembly, FtsZ protofilament polymerization is isodesmic.

Previous studies of FtsZ protofilament assembly have extrapolated data from high protein concentrations to estimate a critical concentration (11, 15). However, they did not address directly whether the transition was abrupt or gradual. Our study found that the transition was more gradual than expected for a cooperative assembly, although when nucleotide hydrolysis was slowed with GMPCPP, bundles of protofilaments formed and polymerization may have become partially cooperative. It was recently found that tuberculosis FtsZ assembles into small bundles of protofilaments (44), and this assembly also exhibited a sudden transition in polymerization, suggesting cooperative assembly. Such changes in FtsZ polymerization upon assembly into a multistranded polymer are consistent with the traditional explanation of cooperative polymerization (19).

In our assays, the appearance of the multistranded bundles was kinetically slow compared with the assembly of the single-stranded, isodesmic polymers. This may explain why it occurred more readily with GMPCPP polymers than with the more rapidly cycling GTP polymer. The slow kinetics of bundling may also explain why the partial cooperativity seen in the GMPCCP sediments (14). The electron microscopic images of GDP-FtsZ are a single subunit wide. However, tubulin-GDP protofilaments are 10–100 subunits long, allowing them to be visualized by electron microscopy.

When FtsZ was polymerized with GTP and then sedimented, the protein remaining in the supernatant dropped by at least 20-fold. Because the GTP is being hydrolyzed continually, the FtsZ in these reactions is actually cycling between GTP- and GDP-bound forms that each have different $K_D$ values for assembly. Therefore, the affinity is a balance between the $K_D$ of the two species, and the apparent 20-fold difference represents the minimum difference in affinity between the GTP and GDP-FtsZ species. In addition, sedimentation overestimates the affinity of bonds within FtsZ filaments because short FtsZ polymers are not predicted to be quantitatively depleted from the supernatant (see “Materials and Methods”).

An alternative estimate of the affinity of GTP-FtsZ subunits was obtained by comparing the experimentally measured lengths of the protofilaments with predictions from the equations for isodesmic assembly. This estimate implies a much higher affinity for GTP-FtsZ assembly, in the nanomolar rather than micromolar range. If the $K_D$ were 1 $\mu$m, at the FtsZ concentrations at which we measured polymer lengths ($2 \mu$m), the average polymer should be only a few subunits long (see “Theory” for calculations). However, we found that the average filament was 23 subunits long. Because of the frequent annealing and fragmentation that occurs in a single-stranded polymer, for the polymers to be this long, the apparent $K_D$ for isodesmic assembly must be 8 $\mu$m. Assembly in GMPCPP may more closely indicate the behavior of GTP-FtsZ subunits, and in this case the filaments were even longer (38 subunits on average), resulting in an apparent $K_D$ of 3 $n$m.

A Model for Isodesmic Assembly with GTP Hydrolysis—We have developed a model for FtsZ assembly that incorporates GTP hydrolysis into previously developed models for isodesmic polymerization (Fig. 7). In this scheme, equilibrium constants are assumed to be independent of whether an FtsZ subunit is monomeric or at the end of a polymer. The constants depend only on the nucleotide to which the FtsZ is bound ($K_D$ (GTP-FtsZ) or $K_D$ (GDP-FtsZ)). Interconversion between the two nucleotide states occurs through nucleotide hydrolysis ($k_{ohd}$) and nucleotide exchange ($k_{oach}$). Polymer assembly and annealing are assumed to be diffusion-limited, whereas polymer fragmentation can occur by one of two pathways as follows: (a) dissociation at a GTP-FtsZ interface or (b) hydrolysis of a GTP followed by rapid dissociation at the GDP-FtsZ interface. To estimate the values of each rate constant, we have incorporated data from our nucleotide turnover and polymer length measurements, in addition to data found elsewhere in the literature (see “Theory” for details).

The model predicts that even though hydrolysis is continually occurring throughout the polymer, it is occurring very slowly, and almost all of the subunits in a protofilament are bound to GTP. Following hydrolysis, the polymer will rapidly fragment, allowing exchange of the nucleotide back to a GTP-bound state. This is because the breakage of the GDP-FtsZ protein bonds ($k_{oahd}$ (GDP-FtsZ)) and the subsequent nucleotide exchange ($k_{oach}$) are both likely to occur at rates of tens per second compared with less than two per minute for the overall nucleotide turnover rate.

The length of the polymers is limited by the fragmentation of the filaments through nucleotide hydrolysis or dissociation of the GTP-bound subunits. Each pathway has a single limiting rate constant, $k_{oahd}$ and $k_{oach}$ (GTP-FtsZ), respectively. These rates are slow and comparable to each other in magnitude.
is closer to the overall nucleotide turnover rate (1.5 and 0.23/ 
min for GTP and GMPCPP, respectively). $k_{\text{off}}(\text{GTP-FtsZ})$ can be 
calculated using data from GTP- or GMPCPP-induced poly-
merization and in both cases equals $\sim 1/\text{min}$. This gives a value of 
$K_D(\text{GTP-FtsZ}) = 2$–3 nm. Therefore, in GTP-induced assembly, 
fragmentation occurs via both hydrolysis- and nonhydrolysis-
exchange. The key kinetic parameters are $k_{\text{hyd}}$ and $k_{\text{exch}}$, which 
are slow and comparable to each other in magnitude. The 
values assigned to the kinetic constants are based on a number of assumptions 
but are probably accurate within an order of magnitude.

Our model suggests that FtsZ protofilaments differ from micro-
tubules in two important aspects. First, the longitudinal bonds in a 
protofilament are several orders of magnitude stronger for FtsZ ($K_D$ 
$\sim 3$ nm, this work) than for tubulin ($K_D$ 500 nm to 500 $\mu$m). The 
affinity of an isolated GTP-tubulin longitudinal bond has not been 
directly measured but is likely to be 10–100-fold lower than the 
affinity of a subunit for the growing end of the microtubule; it can 
be derived (45) from estimates of the latter value, which range 
between 50 nm and 5 $\mu$m (46–50). Therefore, isolated FtsZ proto-
filaments up to 30–50 subunits long can exist at steady state, 
whereas tubulin protofilaments are only found when stabilized by 
additional factors in the cell that could inhibit 
dissociation.

Additionally, the model might be extended to 
include an inactive form of FtsZ in equilibrium with active 
monomer. The existence of an inactive form is also suggested 
by the concentration-independent lags seen in Fig. 5C. Preliminary 
analysis suggests that with this modification the model 
can match the concentration dependence of GTP hydrolysis 
while still allowing long filaments to form at micromolar FtsZ 
concentrations. The model will clearly need to be refined and 
verified by independent experimental approaches in the future.

Implications for the Assembly of the Z Ring in Vivo—The 
structure of the FtsZ polymers in the Z ring in vivo is unknown. However, FtsZ forms protofilaments with high affinity bonds 
under a wide variety of conditions, and these protofilaments are 
likely to be part of the Z ring in vivo. If the Z ring consisted of 
unconnected protofilaments, FtsZ polymerization could be isodesmic in vivo as well as in vitro. However, if the protofila-
ments are organized into a larger, multistranded structure, polymerization of the Z ring would become cooperative.

Several features of the Z ring in vivo might be more readily 
explained by cooperative than by isodesmic assembly. First, for 
localization of the Z ring to be due to FtsZ nucleation at the 
center of the cell, assembly must be cooperative. Initiating an 
isodesmic filament at the center of the cell would not favor 
growth there over growth from any other monomer in the cell. 
However, isodesmic filaments that polymerize throughout the 
cell might still be localized by preferential association with the 
membrane at the center of the cell. Second, the rapid appearance 
of complete Z rings with controlled timing in the cell cycle 
(55–57) could be explained by the synthesis of FtsZ passing a 
threshold critical concentration (58). For an isodesmic polymer, 
rapid assembly might instead require activation of previously 
synthesized protein. Finally, following GTP hydrolysis, a mul-
istranded structure would be more stable than individual GDP 
protofilaments. To maintain individual protofilaments in a Z 
ring, subunits would need to be continually replaced, or to be 
stabilized by additional factors in the cell that could inhibit 
disassembly or suppress hydrolysis.

How might FtsZ protofilaments be assembled into multi-
stranded complexes in vivo? First, localization of FtsZ to the 
membrane at the center of the cell would concentrate the fila-
ments and might allow the relatively weak lateral bonds to be 
sufficiently stable to organize larger structures. Cytoplasmic 
conditions might also alter the strength of the lateral bonds. Additionally, other proteins required for cell division might 
cross-link the FtsZ filaments. For example, ZipA has been 
found to cross-link FtsZ filaments in vitro (59, 60). In addition, 
several of the E. coli cell division proteins seem to increase the 
stability of the Z ring, since fewer Z rings are observed in vivo 
when they are mutated (61–67). In vitro polymerization studies 
have already provided many details on the assembly of pure 
FtsZ polymers; it will be important to determine what the 
structure of the Z ring is in vivo and how other cell division 
proteins affect FtsZ polymerization properties both in vivo and 
in vitro.

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