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 stable, 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 vitro, 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 in vivo, with 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 Z ring 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.

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A

**COOPERATIVE POLYMERS**

- **nucleation**
- **growth**
- **multi-stranded**
- **nucleated assembly with kinetic lags**
- **critical concentration for assembly**
- **long polymers**

B

**ISODESOMIC POLYMERS**

- **single stranged**
- **rapid stranged with no nuclei or lags**
- **no critical concentration for assembly**
- **short, easily fragmented polymers**

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**FIG. 1.** Properties of cooperative and isodesmic polymers. **A,** cooperative polymers are multistranded and assemble in two phases. During nucleation, only a single bond forms between subunits. After nucleation, growth is more favorable because two or more bonds form during subunit addition to the end of a multistranded polymer. **B,** isodesmic polymers are single stranged and so assemble through the formation of a single bond that is identical at all steps of polymerization. They therefore have no nucleation phase. The resultant characteristics of each type of polymer are listed. (After Oosawa and Kasai (19).)

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cooperatively with the properties described above. Cooperative polymers such as actin and microtubules may be common because they form filaments that are strong in the middle but can easily be remodeled by removing subunits from their ends (20). In addition, a cell can use cooperative assembly to control its spatial organization by localizing polymer nucleation sites and assembling long filaments that can span the cytoplasm.

In contrast to multistranded polymers, single-stranded polymers can be assembled with an identical bond at each step of polymerization (21, 22) (see Fig. 1B). For such isodesmic polymers, there is no separate nucleation and growth phase, and so there is no lag in the assembly kinetics. Instead, assembly is rapid because every monomer in solution can act as a start site for polymer growth. There is also no critical concentration for assembly, and as the total protein concentration rises, both polymer and monomer populations can increase simultaneously. Finally, fragmentation of a single-stranded polymer in the middle requires breaking only a single bond. Disassembly thus occurs not only at the ends of the polymers but also along their length, and isodesmic polymers remain relatively short.

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 β-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 multistranded 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-μm 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*~oo~ = 1, induced with 0.5 mM isopropyl-1-thio-β-D-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-(2-aminoethyl)benzenesulfonyl fluoride and frozen at −80 °C. Cells were thawed, and lysozyme 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 μg/ml, and the extract was incubated for 1 h. Lysate was spun for 5 min at 220,000 × 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 × g for 10 min at 4 °C. The supernatant was removed, and 1/12 volume ammonium sulfate was added. This was spun again at 80,000 × g for 10 min at 4 °C. The pellet was resuspended in 5 ml of polymerization buffer (50 mM NaMES, 2 pH 6.5, 2.5 mM magnesium acetate, 1 mM EGTA) and spun again at 160,000 × g for 10 min at 4 °C. The final supernatant was brought to 10% glycerol and 50 mM GDP, aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C.

1 M. Glotzer, personal communication.

2 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; GMPCPF, guanlyl-(α,β)-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 (Friche) 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 prespun 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 300,000 × g at 4 °C. The protein was then diluted 5-fold in polymerization buffer to lower the glycerol concentration (final protein concentration 4 °C. The protein was then diluted 5-fold in polymerization buffer to lower the glycerol concentration (final protein concentration 4 °C. The protein was then diluted 5-fold in polymerization buffer to lower the glycerol concentration (final protein concentration 4 °C. The protein was then diluted 5-fold in polymerization buffer to lower the glycerol concentration (final protein concentration 4 °C. 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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):

$$C_i = C_{i-1} \cdot 2K_A = (2K_A)^{i-1}C_i$$  
(Eq. 1)

$$C_i = C_i/(1 - 2K_A C_i)^2$$  
(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.

$$C_i = \frac{4K_A C_i + 1 - (8K_A C_i + 1)^{1/2}}{8K_A C_i}$$  
(Eq. 3)

Equation 3 is the quadratic solution of Equation 2.

$$L_n = \frac{\sum Ci}{2C_n} = \frac{4K_A C_i}{1 + \sqrt{8K_A C_i} + 1}$$  
(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 Eq. 4, the apparent affinities needed to produce filaments of such lengths are $K_{A, app} = 1.25 \times 10^6$ and $3.3 \times 10^6 M^{-1}$, 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”):

$$k_{on}(GTP-FtsZ)$$  
FT ≡ $k_{hydrolysis}$  
FD $\Rightarrow$  
$\uparrow \psi$  
$\downarrow \psi$  
$\Rightarrow$  
$\uparrow \psi$  
$\downarrow \psi$  
$F_{on}(GDP-FtsZ)$  
FT $\Rightarrow$  
$\Rightarrow$  
$F_{off}(GTP-FtsZ)$  
FT $\Rightarrow$  
$\Rightarrow$  
$F_{off}(GTP-FtsZ)$

**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 ($k_{on} \approx 5 \times 10^6 M^{-1} s^{-1}$) for both GTP and GDP-FtsZ. Nucleotide exchange is likely to be very rapid ($k_{(nucleotide)} \approx 35 s^{-1}$), based on the 7 μM $K_D$ 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 $k_{off}(GDP-FtsZ)$ and a $K_D = 20 \mu M (14), k_{off}(GDP-FtsZ)$ 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 $k_{on}(GTP-FtsZ) = 0.014/s$.

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. $k_{on}(GTP-FtsZ)$ 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 ($k_{off}(GTP-FtsZ)$) and hydrolysis followed by rapid breakage of the GDP-FtsZ 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:

$$K_{A, app} = k_{on}(GTP-RmZ) / (k_{off}(GTP-FtsZ) + k_{hydrolysis})$$  
(Eq. 5)

As described above, the length distributions led us to $K_{A, app} = 1.25 \times 10^6$ and $3.3 \times 10^6 M^{-1}$ for GTP and GMPCPP polymer, respectively. Assuming $k_{on} = 5 \times 10^6 M^{-1} s^{-1}$, and $k_{hydrolysis} = 0.025$ or 0.0038/s, then Equation 5 gives us the true $k_{on}(GTP-FtsZ) = 0.015/s$, or 0.01/s for GMPCPP-FtsZ. This results in a true assembly $K_D$ for FtsZ bound to unhydrolyzed nucleotide that is 2–3 nM. It is satisfying to note that the estimated $k_{on}$ is now approximately the same for the GTP and GMPCPP interfaces, as it should be if the GMPCPP is a true analog. Thus the longer polymers that assemble in GMPCPP can be explained by the experimentally measured decrease in the hydrolysis rate.

**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 $= C_i - (i = 1 - 4) \sum C_i$. If the $K_D$ is 20 μM, the value reported for FtsZ-GDP (14), and the $C_i$ is 12.5 μM, then 5% of the protein should be in polymers longer than five subunits. This corresponds approximatively 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),

$$\frac{\text{hydrolysis}}{\text{FtsZ}} = \frac{k_{hydrolysis} - (C_i - XC_i)C_i}{(1 - K_a C_i)(1 - 2K_a C_i)}$$  
(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 $\mu$M 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 ± 0.2 nm) and had a broad length distribution. Polymers as short as 40 nm (10 subunits) were frequently observed (Fig. 2A). 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+-assembled 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 subunit) 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 often a drop over in the bundles. In our experiments, the partial bundling at higher protein concentrations was accompanied by a decrease in GTPase activity (up to 20%) above 2 μM FtsZ (Fig. 3B).

Guanylyl-(α,β)-methylene-diphosphonate (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. 3, Vmax 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). In our experiments, the partial bundling at higher protein concentrations was accompanied by a decrease in light 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 often a drop

**Fig. 3.** 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; ○, GMPCPP. The polymerization buffer (MES, pH 6.5, 1 mM EGTA) is potassium-free and contains 2 mM excess magnesium.
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, where FtsZ assembly still occurred (Fig. 5B). In our assays, however, the lags were quite short, less than 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 and of nucleotide hydrolysis, they are consistent with an isodesmic rather than a cooperative assembly mechanism.

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 and of nucleotide hydrolysis, they are consistent with an isodesmic rather than a cooperative assembly mechanism.

The length of the lags also did not depend on whether GMPCPP or GTP was used, even though hydrolysis decreases the tendency of the subunits to assemble. Because the initial assembly kinetics are independent both of protein concentration and of nucleotide hydrolysis, they are consistent with an isodesmic rather than a cooperative assembly mechanism.

There Is No Critical Concentration for Assembly of FtsZ Protofilaments—To determine whether FtsZ has a critical concentration for assembly, we examined how polymer mass varied with total FtsZ concentration. For a cooperative polymer, as the protein concentration is increased, the protein would remain monomeric until it reached a critical concentration. Above this concentration, there would be a sharp transition, and polymer would form while the unassembled fraction would remain constant. In contrast, for an isodesmic polymer, as the protein concentration is increased, the unassembled fraction would continue to increase even after significant polymer began to form, and so the concentration of unassembled protein would only gradually approach a plateau.

FtsZ was sedimented to separate large polymers from soluble protein (11). Sedimentation has been used to quantitate microtubule and actin assembly, and it reliably separates these polymers from unassembled subunits. However, an isodesmic assembly reaction contains many short polymers whose S values are closer to that of monomer. By calibrating the assay using proteins with known S values, we predicted that monomers would remain largely soluble, tetramers would be 40% sedimented, and a typical 100-nm-long polymer would be >70% sedimented (see “Materials and Methods” for details). The amount of protein in the supernatant therefore overestimates the amount of protein that is monomeric or in very short polymers.

FtsZ was assembled with various guanine nucleotides and sedimented (Fig. 6). The FtsZ remained 90% soluble at the lowest protein concentration tested, indicating that the protein was not aggregated. The soluble fraction dropped to 9% at high FtsZ concentrations in the presence of GTP, indicating that the majority of the protein was active and assembled into polymers large enough to be pelleted. Although the GTP or GMPCPP in a reaction is continually being hydrolyzed, all protein concentrations used, >95% of the nucleotide is predicted to remain unhydrolyzed at the end of the assays.

Recently, Rivas et al. (14) performed extensive analytical ultracentrifugation on GDP-FtsZ and determined that it, like GDP-tubulin, assembles with a noncooperative mechanism that is close to isodesmic. Our sedimentation results with GDP-FtsZ agree with theirs (Fig. 6A). In our assays, as the total FtsZ concentration was increased, the un sedimented 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 total FtsZ concentration. For a cooperative polymer, as the protein concentration is increased, the protein would remain monomeric until it reached a critical concentration. Above this concentration, there would be a sharp transition, and polymer would form while the unassembled fraction would remain constant. In contrast, for an isodesmic polymer, as the protein concentration is increased, the unassembled fraction would continue to increase even after significant polymer began to form, and so the concentration of unassembled protein would only gradually approach a plateau.

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
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.

**DISCUSSION**

**FtsZ Protofilaments Do Not Assemble Cooperatively**—Although there is a large evolutionary distance between prokary-

temperature, it was not dependent on protein concentration. Theoretical curves for polymers with a nucleus of one (---) or two (—) subunits were plotted for comparison (on a log/log plot, slope = \( n + 1/2 \), \( n \) = nucleus size (40, 41)).

**Fig. 5.** Kinetics of FtsZ polymerization detected by light scattering. FtsZ polymerization was followed by 90° light scattering at 310 nm. Nucleotide was added at the arrow. —, GTP; —, GDP. Base-line scattering levels were normalized for graphing. A, scattering increased with FtsZ assembly and was dependent on protein concentration. 1 mM nucleotide was added to various concentrations of FtsZ at 28 °C. B, the length of the lag before polymerization began increased as the temperature was decreased. 1 mM GTP was added to 2.5 mM FtsZ at 28, 20, and 7 °C. At 20 °C, there was a 5-s lag before the trace began to rise; at 7 °C, the lag was 30 s. C, length of lag versus protein concentration. The time interval for a reaction to reach 1/10th its plateau was plotted against the FtsZ concentration for four conditions as follows: GTP addition at 28 (○), 20 (□), and 7 °C (○), and GMPCPP addition at 20 °C (▲). The length of the lag was different at each temperature, but at a given concentration, it was not dependent on protein concentration. Theoretical curves for polymers with a nucleus of one (—) or two (—) subunits were plotted for comparison (on a log/log plot, slope = \( n + 1/2 \), \( n \) = nucleus size (40, 41)).
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 than with the more rapidly cycling GTP polymer. The slow kinetics of bundling may also explain why the partial cooperativity seen in the GMPCPP sedimentation assay is not reflected as a concentration-dependent lag in the GMPCPP light scattering traces. Instead, in our light scattering assays, there was sometimes a slow rise in the light scattering signal over the course of 10 min or more (data not shown), possibly reflecting the formation of paired and bundled polymers.

Single-stranded FtsZ Polymers Are Stable Without Lateral Interactions—Both FtsZ and tubulin can form protofilaments that are a single subunit wide. However, tubulin-GTP protofilaments do not exist in isolation and assemble only when stabilized by lateral bonds in a two-dimensional microtubule wall. In contrast, our STEM results clearly show that FtsZ protofilaments are single-stranded polymers whose longitudinal bonds are stable at concentrations that do not favor lateral associations. Protofilament bundles do not become abundant until >5 μM FtsZ, despite the fact that two protofilaments lined side to side present the opportunity for tens of lateral bonds to form simultaneously. There must therefore be a large disparity between the relative strength of the FtsZ longitudinal and lateral interactions, with the lateral bonds much weaker than those in microtubules, and the longitudinal bonds orders of magnitude stronger than those for tubulin.

The Assembly Affinities of GDP- and GTP-FtsZ Protofilaments Are Micromolar and Nanomolar, Respectively—GDP-FtsZ polymers are curved protofilaments, as seen by electron microscopy. Our sedimentation experiments indicate that the assembly of these curved polymers is isodesmic, with a $K_D$ that is on the order of micromolar, consistent with the results of Rivas et al. (14). The electron microscopic images of GDP-FtsZ also support a $K_D$ in this range. The images show arcs and rings surrounded by large amounts of unassembled protein (see Fig. 2A). The average length of an isodesmic polymer depends only on its $K_D$ for association and the total protein concentration (see Theory for equations). For a polymer with a 20 μM $K_D$; one-third of the protein should be monomeric at 12.5 μM FtsZ, but 5% of the filaments should be greater than five 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 μM at the FtsZ concentrations at which we measured polymer lengths (∼2 μM), 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 nm. 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 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 ($k_{hyd}$) and nucleotide exchange ($k_{exch}$). 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 ($k_{af}(GDP-FtsZ)$) and the subsequent nucleotide exchange ($k_{exch}$) are both likely to occur at rates of tens per s compared with less than two per min 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, $k_{hyd}$ and $k_{af}(GTP-FtsZ)$, respectively. These rates are slow and comparable to each other in magnitude. $k_{hyd}$
is equal to the overall nucleotide turnover rate (1.5 and 0.23/ min for GTP and GMPCPP, respectively). $k_{off}(\text{GTP-FtsZ})$ can be calculated using data from GTP- or GMPCPP-induced polymerization and in both cases equals ~1/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-mediated pathways, whereas with GMPCPP, breakage of bonds containing unhydrolyzed nucleotide is the major factor limiting polymer length.

Our model suggests that FtsZ protofilaments differ from microtubules in two important aspects. First, the longitudinal bonds in a protofilament are bound to GTP. They can fragment through one of two pathways. The top pathway involves dissociation at an interface containing a GTP, without hydrolysis. The dissociation is slow because this interface has a very high affinity. The lower pathway involves hydrolysis of a GTP to GDP. This is followed by rapid dissociation at the GDP interface and nucleotide exchange. The key kinetic parameters are $k_{off}(\text{GTP-FtsZ})$ and $k_{on}(\text{GTP-FtsZ})$, 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.

FIG. 7. FtsZ polymerization model. This model incorporates GTP hydrolysis into an isodesmic polymerization mechanism. The model proposes that all the FtsZ subunits in a protofilament are bound to GTP. They can fragment through one of two pathways. The top pathway, involving dissociation at an interface containing a GTP, without hydrolysis, is the slowest and is the major factor limiting polymer length. The dissociation is slow because this interface has a very high affinity. The lower pathway involves hydrolysis of a GTP to GDP. This is followed by rapid dissociation at the GDP interface and nucleotide exchange. The key kinetic parameters are $k_{hyd}$ and $k_{exch}$, 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.

An inconsistency remains between our polymerization model and the data on GTP hydrolysis. A nanomolar affinity for FtsZ-GTP assembly implies that GTP hydrolysis should also approach saturation at very low FtsZ concentrations. If $K_{D, app} \text{ (GTP-FtsZ)} = 8$ nm, GTP hydrolysis should be 80% saturated at FtsZ concentration $= 80$ nm (see “Theory” for equations). However, the measured hydrolysis rate is extremely low below 0.5 μm FtsZ, and does not saturate until ~1–2 μm (Fig. 3B, see also Refs. 27, 53, and 54). One possible mechanism that could resolve the discrepancy would be for hydrolysis or the subsequent subunit dissociation to involve steps that are accelerated in longer protofilaments. Alternatively, 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 uncleaved protofilaments, FtsZ polymerization could be isodesmic in vivo as well as in vitro. However, if the protofilaments 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 multistranded 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 filaments 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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