Apparent Cooperative Assembly of the Bacterial Cell Division Protein FtsZ Demonstrated by Isothermal Titration Calorimetry*

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The assembly dynamics of FtsZ, a prokaryotic homolog of tubulin, are important for their role in bacterial cytokinesis. Here we used isothermal titration calorimetry (ITC) to measure the heat of FtsZ self-association under various conditions. The measurements were designed to test whether FtsZ protofilaments are assembled by an isodesmic (linear aggregates in which each bond has an identical equilibrium constant) or a cooperative (aggregates only become stable after forming a oligomeric nucleus) assembly process. The isodesmic model can fit the assembly in GDP closely but cannot fit the assembly in GTP. FtsZ-GTP without Mg2⁺ exhibits an apparent critical concentration, which is indicative of cooperative assembly, near 2.9 μM. With 2.5 mM Mg2⁺ (which allows FtsZ to hydrolyze GTP) the critical concentration is reduced 10-fold to −0.31 μM. Both with and without Mg2⁺ there is no evidence for assembly below the critical concentration, but there is an abrupt transition to full assembly above. The ITC data are highly suggestive of a cooperative assembly, although this is difficult to reconcile with the 1-subunit-thick protofilaments observed by electron microscopy.

FtsZ, a homolog of tubulin (1–3), is a cell division protein found in almost all bacteria, archaea, chloroplasts and some mitochondria (4–7). Light microscopy has shown that FtsZ assembles into a ring that constricts and divides the cell (8–10). The substructure of Z-ring has not been visualized by electron microscopy of bacteria, but much has been learned from in vitro assembly studies. FtsZ forms straight protofilaments with longitudinal bonds similar to those in microtubules (11, 12). Under many conditions the protofilaments tend to associate laterally into pairs or small bundles (13–15), but in most studies at least some polymers appear to be single protofilaments. Romberg et al. (16) used mass measurement by scanning transmission electron microscopy to demonstrate that protofilaments were indeed only 1 subunit thick. The existence of single protofilaments is quite different from tubulin, where protofilaments are stable only when assembled into parallel sheets in which the protofilaments are connected by lateral bonds (17). A single protofilament should have only a single type of longitudinal bond, and all bonds should be identical. This type of assembly is now referred to as isodesmic (16, 18).

Isodesmic assembly was demonstrated for glutamate dehydrogenase (19, 20) and β-lactoglobulin (21), and the thermodynamic principles of the assembly were developed. Tubulin has been interpreted to assemble isodesmically in the presence of GDP (18, 22). The assembly of glutamate dehydrogenase and β-lactoglobulin are probably not physiologically important, and the GDP-tubulin polymer is probably only transiently important during microtubule disassembly.

In contrast to isodesmic assembly, all physiologically relevant filaments studied so far, in particular tubulin and actin, assemble in a cooperative manner. The key distinction of cooperative assembly is that subunits are connected by two types of bonds to form a helical or two-dimensional polymer (17, 23). In addition to the longitudinal bonds within a protofilament, adjacent protofilaments are connected by lateral or diagonal bonds.

Cooperative assembly produces two features that are very important for cytoskeletal filaments like microtubules and actin. First, fragmentation in the middle is many orders of magnitude less favorable than removing a subunit from the end, and so the filament can last for long periods without breaking (23). Isodesmic assembly, in contrast, produces a population of relatively short filaments, in which fragmentation of every interface in the middle is equivalent to dissociation of a subunit from the end. Second, cooperative assembly exhibits a critical concentration (C) (24). This sharp transition at the C, coupled with unfavorable nucleation, results in a population of very long polymers, in equilibrium with monomers at a concentration C,. Assembly and disassembly at the ends can be finely controlled by the concentration of free subunits.

Several studies have suggested that FtsZ assembly is cooperative based on the observation of an apparent critical concentration. Mukherjee and Lutkenhaus (13) proposed a C, for Escherichia coli FtsZ of 1 μM using a centrifugation assay and 2 μM using light scattering. White et al. (14) proposed a C, of 3 μM for Mycobacterium tuberculosis FtsZ based on light scattering data. One of the most convincing indications of a C, was obtained from GTPase assays. GTPase, which is thought to be coupled to assembly, is highly dependent on FtsZ concentration. Relative specific activity was not measurable below 2 μM for Bacillus subtilis FtsZ and rose rapidly to a plateau at 5 μM (25). Similar curves were obtained for E. coli FtsZ, with activity beginning at an apparent C, of 0.5 μM (Ref. 16 and Footnote 2) and reaching a plateau at 2 μM.

Romberg et al. (16) proposed that FtsZ may assemble isodesmically. The primary basis for this proposal was an anal-

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1 The abbreviations used are: C, critical concentration; inj, injection; ITC, isothermal titration calorimetry; MES, 4-morpholineethanesulfonic acid.

2 S. Redick, personal communication.
ysis of protofilaments by scanning transmission electron microscopy, which determined the mass density to be that of a 1-subunit-thick protofilament. If the protofilaments are 1 subunit thick they could only have one type of bond, and hence assembly should be isodesmic. Romberg et al. (16) repeated centrifugation and light scattering assays, suggesting that might not support a critical concentration. A problem with these assays is that they under-report short protofilaments, which is particularly problematic at low FtsZ concentrations. Romberg et al. (16), however, did note that the GTPase assays provided strong evidence for a critical concentration and thus cooperative assembly. There was therefore a significant controversy as to whether FtsZ assembly is isodesmic or cooperative.

One of the most distinctive predictions of the isodesmic theory is that the association constant for each interface should be quite high, $K_A = 3.3 \times 10^8 \text{M}^{-1}$ ($K_A = 3 \text{ nm}^{-1}$). This high affinity bond was necessary to fit the observed filament lengths of 30 to 50 subunits. This leads to the prediction that short protofilaments should form above 3 nm FtsZ, and for FtsZ above 10–30 nm almost all of the subunits should be assembled (only the subunits at the ends of the protofilaments would not be involved in two interfaces). In contrast, in a cooperative assembly there will be no assembly until FtsZ exceeds the critical concentration, near 1 μM.

To test the theory further, we sought a technique that could quantify the extent of assembly at different protein concentrations, reporting the total number of interfaces formed without any bias from filament length. The formation of a protein-protein interface is typically associated with a release of heat, and isothermal titration calorimetry (ITC) can be used to measure the association. Because the heat generated by the association should be the same for each interface, ITC should meet the above criterion.

This application of ITC is different from the typical study of a heterodimeric association, where one subunit is placed in the reaction cell and the other in the syringe. In our case the entire FtsZ sample is initially in the syringe at high concentration and then is diluted by being injected into the reaction cell. In general the protein in the syringe will be assembled for the most part, and injection will result in disassembly, which is sometimes followed by reassembly. We found that informative data on FtsZ assembly can be obtained with this novel approach to ITC.

**MATERIALS AND METHODS**

**FtsZ Purification**—The following protocol was adapted from Romberg et al. (16). BL21 cells were transformed with Pet11b-wtFtsZ (wild type *E. coli* FtsZ). A 50-ml culture (LB with 100 μg/ml ampicillin) was grown overnight in a shaker at 37 °C, centrifuged to pellet the cells, resuspended in fresh LB, and added to two 500-ml cultures (LB with 100 g/ml lysozyme were added; the suspension was incubated on ice for 30 min to allow disassembly. Subsequent centrifugation at 15,000 × g for 5 min removed any particles unable to resolubilize. The concentration of the resultant solution was determined colorimetrically by BCA assay (Pierce) and corrected for the 25% color difference between FtsZ and the BSA standard (26).

**Sample Preparation**—For FtsZ samples containing no magnesium, the solution described above was brought to 10 mM EDTA (by adding 500 mM EDTA pH 8.0) and then diluted with buffer B (50 mM NaMES brought to pH 6.5 with KOH, 100 mM KCl, 1 mM EDTA) to the desired injectant concentration. For FtsZ samples containing magnesium, the solution from calcium cycling was diluted with buffer A. These samples were dialyzed against their appropriate buffers, buffer B and buffer A, respectively, at 4 °C. The buffer was changed after 1 h and again after a second hour; the last 50 ml of buffer was allowed to reach equilibrium overnight.

**Isothermal Titration Calorimetry**—ITC measurements were made in a MicroCal Systems ITC unit (MicroCal, Northampton, MA). The reaction cell was loaded (using a 5-ml gas-tight syringe, Hamilton Co., Reno, NV) with 5 ml of the FtsZ sample in buffer B (or buffer A with the GTP analog or other 100 μg/ml GDP or 1 mM GTP and degassed immediately before the experiment was run. The FtsZ solution was loaded into the injection syringe (250 μl capacity, 101.8 μl/inch) with no added nucleotide. MgCl₂ was the same concentration in the syringe as in the reaction cell. All FtsZ experiments were performed at 25 °C with data points taken every 2 s. For the GDP experiments (representative data shown in Fig. 1), 50 injections at 4.995 μl/injection and 12.56 s/injection were made after a 300-s initial delay and with 180 s between the start of each injection (reference offset = 10%). For the GTP experiments (represented in Figs. 2 and 3), 25 injections at 5.058 μl/injection and 12.72 s/injection were made after a 300-s initial delay, with 180 s between the start of each injection (reference offset = 10% for no magnesium, 20% for 2.5 mM magnesium).

The base line automatically generated by the MicroCal Systems analysis software (MicroCal Origin, version 5.0) was used except when it was obviously accepting an errant data point as reference, and then these base-line points were corrected by hand before further analysis. The software then integrated each peak to yield integrated heats; these were normalized to the moles of protein in the injectant. Because the base line for the FtsZ experiments with magnesium and GTP (Fig. 3) was highly variable, plots of raw data without base-line subtraction are presented to allow direct comparison (Figs. 1a, 2a, and 3). Three repetitions were performed to ensure reliability. However, only representative samples are shown in the figures.

**Modeling**—We used numerical modeling to test whether the isodesmic assembly model would match the experimental data. The goal of the calculation was to estimate the total number of FtsZ-FtsZ interfaces as a function of $C_T$, the total concentration of subunits in the reaction cell. The model is based on two equations from Romberg et al. (16).

$$C_T = \sum_{i=1}^{N} C_i$$

$$C_i = (2K_A)^{i-1} \cdot C$$

where $C_i$ is the concentration of protofilaments consisting of $i$ subunits, $K_A$ is the equilibrium association constant, $N$ is the longest protofilament modeled, and $C_T$ is the total concentration of FtsZ in the solution.
This differs from early formulations of isodesmic assembly (19, 20, 27) in the factor 2 in front of $K_A$ which accounts for the equal probability of adding a subunit at either end (16). When we present values of $K_A$ from previous studies, we will report the numbers as given in the original papers but will refer to them as $2K_A$ (or the equivalent $(1/2)K_A$).

To estimate the total number of interfaces, we used a numerical iteration. Taking FtsZ injected into buffer with GDP as an example, the sample calculation began with choosing $K_A$ arbitrarily. For the first calculated injection (concentration = 2.2 $\mu$m after injection), $C_1$ was chosen arbitrarily, $C_i$ values were calculated using Equation 1 (to $n$ = 50, at which the concentration is generally very low), and finally $C_n$ was calculated using Equation 2. The value of $C_1$ was changed, and the calculation was repeated until the $C_n$ value equaled 2.2 $\mu$m. The total moles of FtsZ-FtsZ interfaces ($N_{\text{INT},2.2\mu m}$) were calculated

$$N_{\text{INT},i} = V \sum_{i=1}^{N} (i-1)C_i \tag{Eq. 3}$$

where $V$ is the volume of the solution (2.5 ml in the case of the initial injection). Because an ITC data point consists of a change from the injectant state to the sample state, we also calculated the number of

$$\Delta H = \frac{\Delta H_{\text{INT},x\mu m} - \Delta H_{\text{INT},m\mu m}}{\text{mol inj}} \tag{Eq. 4}$$

where $\Delta H$ is the enthalpy of forming one FtsZ-FtsZ interface. We have now calculated the data point for the first injection.

Subsequent injections were calculated similarly. The desired $C_T$ value changes with each injection, and therefore the iteration of $C_1$ must vary accordingly. The only other change was in calculating the normalized heat of injection. This parameter and introduced a shift factor that was simply added to the calculated normalized heats of injection. The number of interfaces in the sample prior to injection and the number of interfaces in the injectant must both be subtracted from the number of interfaces in the sample after injection.

$$\text{Heat} = \frac{\Delta H_{\text{INT},x\mu m} - \Delta H_{\text{INT},m\mu m}}{\text{mol inj}} \tag{Eq. 5}$$

After an entire set of data points (2.2–110 $\mu$m) had been calculated, the square of the difference between the calculated points and the experimentally determined points was determined.

We found that the curve needed to be shifted down, and therefore we introduced a shift factor that was simply added to the calculated normalized heats of injection. This parameter and $\Delta H$ were optimized using the Solver tool in Microsoft Excel to minimize the sum of the squares of the differences between the calculated points and the experimental points. Finally, the shape of the curve depends on the value chosen for $K_A$. Changing this value required repeating the iteration of all $C_i$ values to achieve the desired $C_T$ for all injections; thus, this parameter was best-fit by trial and error, using several values and selecting the one that gave the best overall fit.

It is worth noting at this point that the isodesmic assembly model always predicts net disassembly from injectant to reaction cell, when the buffers are the same in both, and a curve in which amplitude always predicts net disassembly from injectant to reaction cell, when the buffers are the same in both, and a curve in which amplitude

FIG. 1. FtsZ injected into buffer with GDP. a, raw data are shown for an ITC experiment in which 1100 $\mu$m FtsZ was injected into buffer with 200 $\mu$m GDP. Each peak corresponds to one injection, and each injection increased the FtsZ concentration in the sample by 2.2 $\mu$m. The 50 injections shown here provide data for 2.2–110 $\mu$m. b, integrated heat/injection was calculated by subtracting the base line from the raw data and normalizing to the moles of FtsZ in each injection for each peak in panel a (diamonds). Calculations described under "Modeling" were used to obtain a best fit (solid line, $2K_A = 2.5 \times 10^5$ M$^{-1}$, $\Delta H = 13,645$ cal/mol, shift $= -1169$ cal/mol inj) and the closest fit with the literature value of $2K_A = 1.25 \times 10^5$ M$^{-1}$ (dashed line, $\Delta H = -9,821$ cal/mol, shift $= -1,719$ cal/mol inj).

varya with protofilament length. Because their $2K_A$ did not vary more than the error expected for our experiments, here we treated the Rivas et al. (27) model as isodesmic with a fixed $K_A$ derived from their Fig. 5a.

Because assembly in GDP is not complicated by hydrolysis, we decided first to establish the behavior of FtsZ when injected into a solution containing only buffer and GDP. FtsZ at 1100 $\mu$m, in buffer A, was injected into the chamber containing the same buffer with 200 $\mu$m GDP. The protein in the injection chamber is assumed to contain approximately equimolar amounts of FtsZ and bound GDP, and no free nucleotide. Fig. 1a shows the raw data (with no base-line subtraction to allow for direct comparison to Fig. 3) for an experiment in which the sample concentration was raised 2.2 $\mu$m by each injection. In Fig. 1b the data are replotted as integrated heats of mixing for each injection. The first heat of injection is the largest with heats decreasing monotonically as the FtsZ concentration in the sample increases. The interpretation is that at the 1100 $\mu$m concentration in the syringe, almost all of the FtsZ is associated into polymers. During the first injection a large fraction of these interfaces disassembled as the concentration dropped to 2.2 $\mu$m in the reaction cell; the second injection yielded a slightly smaller fraction of interface disassembly and thus a smaller heat of injection. The heat of injection continued decreasing until the fraction of disassembling interfaces
approached zero as the concentration in the chamber rose substantially above $K_D$.

To derive more information from these data, we calculated the response we would expect from an isodesmic disassembly of FtsZ protofilaments (Fig. 1b, solid and dashed lines). The curves were calculated as described under “Modeling” and were fit using three parameters: $K_D$, $\Delta H$, and a shift factor. In Fig. 1b the solid line shows the best fit (optimized $\Delta H$ and shift factor) using the value $2K_D = 2.50 \times 10^9$ M$^{-1}$ ($K_D = 8 \mu$M). The fit was only slightly worse when it was repeated with the $2K_D$ determined previously by Rivas et al. (27) (dashed line; $2K_D = 1.25 \times 10^9$) was extrapolated from their Fig. 5A for a magnesium concentration of 2.5 mM. Although there is no corroborating evidence for the $\Delta H$ value, the model does match the trend of the data, specifically the monotonic decrease in amplitude of the peaks. The best fit was obtained with $\Delta H = -13.6$ kcal/mol and a shift factor of $-1.17$ kcal/mol injectant for each calculated injection. Possible explanations for a shift factor are a difference between the temperature of the injectant and the reaction cell, a heat of mixing for dilution of the protein, or adding buffer.

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**Apparent Cooperative Assembly of FtsZ**—Because FtsZ has GTPase activity, we performed two experiments to determine the effects of GTP binding and GTP hydrolysis on assembly. The first experiment used a buffer with no magnesium and contained 1 mM EDTA to chelate any residual magnesium. In the absence of magnesium, E. coli FtsZ binds GTP and assembles into protofilaments, but hydrolysis of the nucleotide is blocked completely (16, 28). Fig. 2a shows the raw data for 98.7 $\mu$M FtsZ injected into buffer B with 1 mM GTP. The FtsZ in the syringe contained only its residual GDP. The key features of these data are the positive peaks that are obtained up to the 12th injection, at 3.2 $\mu$M. The 13th injection, at 3.4 $\mu$M, produces a distinctly smaller positive peak and initiates a sharp transition to negative peaks. This transition is complete by 4.5 $\mu$M, after which the peaks are uniformly negative. This can be seen more clearly in Fig. 2b, which shows the integrated heats of mixing for each peak. We believe the positive peaks are due to disassembly of FtsZ-GDP from the highly concentrated injectant state. We attribute the negative peaks to reassembly of subunits supported by GTP. The abruptness of the transition suggests a critical concentration of about 3.5 $\mu$M, with little to no assembly at concentrations below $C_C$ and almost complete assembly of all added FtsZ above $C_C$. This transition could not be modeled by an isodesmic assembly process regardless of the $K_D$, $\Delta H$ chosen. Therefore, we conclude that, in the presence of GTP, FtsZ assembles in an apparently cooperative manner. It is important to note that the switch to cooperative assembly requires only that FtsZ bind, not hydrolyze, GTP.

The addition of 2.5 mM magnesium acetate (and removing EDTA) allows FtsZ to hydrolyze GTP during the assembly process. Injecting FtsZ at 24.7 $\mu$M into buffer A containing 1 mM GTP yielded the results shown in Fig. 3. Qualitatively the base line begins straight (ITC base lines are rarely exactly horizontal) with a small positive peak at each injection. At about 0.4 $\mu$M, there are two abrupt changes; each injection now results in a substantial negative displacement, and this is maintained rather than returning to the base line, resulting in a continuous downward slope of the base line. This behavior may be attributed to continuous GTP hydrolysis at concentrations above 0.4 $\mu$M. This exothermic process should last at least 30 min to 1 h before the GTP is exhausted, similar to the time scale of the experiment. We had expected the base line to become more negative as more FtsZ was injected, leading to increased GTP hydrolysis, but instead the base-line slope remained constant for the remainder of the experiment. Because the GTP concentration is decreasing with time, we speculate that this behavior may be due to a balance of these factors. We attempted to test this hypothesis by adding a greater concentration of GTP (5 mM), but this created too large a mismatch
between the buffer and the injectant, resulting in large heats of mixing that masked the heats from FtsZ-FtsZ binding and GTP hydrolysis (data not shown).

These experiments were done three times each, with the transitions (beginning-ending) being 2.4–3.5 and 2.9–4.0 μM and 3.5–4.5 μM for assembly in GTP-EDTA. For assembly in GTP-Mg, the beginning of the transition was observed at 0.4, 0.26, and 0.26 μM. Taking the average of beginnings of the transition as the estimate of \( C_m \), we estimate \( C_m = 2.9 \) μM for GTP-EDTA and \( C_m = 0.31 \) μM for Mg-GTP.

**DISCUSSION**

One of the most distinctive predictions of the isodesmic assembly model is that the association constant for each interface should be very high (\( K_2 = 3.3 \times 10^7 \) M\(^{-1}\) (\( K_D = 3 \) mM)) for assembly in GTP (16). This implies that most FtsZ subunits should be associated into short protofilaments at concentrations 3–10 times above \( K_D \), ~10–30 nm. The ITC experiments reported here should report the total formation of interfaces, without the complication of underestimating short protofilaments. ITC experiments in GTP showed no evidence for assembly below micromolar concentrations and therefore do not support the isodesmic assembly model. On the contrary, they gave a clear indication of a critical concentration, which is characteristic of a cooperative assembly.

We first analyzed disassembly of FtsZ-GDP and found that it did fit the isodesmic assembly model, with a \( 2K_2 \) close to that derived from a previous study using sedimentation techniques (27). This \( 2K_2 = 2.5 \times 10^7 \) M\(^{-1}\) is 1000 times weaker than that predicted in the isodesmic model. Because the 1100 μM FtsZ in the syringe is well above the \( K_D \) (16 μM), it will be almost completely assembled. In the ITC experiments the first injections resulted in a dilution in the reaction cell that was well below \( K_D \), giving a positive peak corresponding to the disassembly of the polymer. As the concentration in the reaction cell approached \( K_D \), the peaks decreased, and at concentrations well above \( K_D \) the peaks approached zero.

The experiment was more complicated when FtsZ was injected into the reaction cell containing GTP. The concentration in the syringe was 98.7 and 24.7 μM for the assemblies in EDTA and Mg, respectively. In the experiment using Mg, the 24.7 μM concentration was less than twice the \( K_{1m} \); nevertheless, based upon the isodesmic assembly model, which seems to apply for FtsZ-GDP, we expect about two-thirds of the FtsZ to be assembled in the syringe. For the experiment using EDTA we cannot predict the \( K_D \), but because Rivas et al. (27) found the \( K_2 \) to be proportional to the Mg concentration, we can assume that in the absence of Mg the association will be very weak and the \( K_D \) high. In this experiment the FtsZ in the syringe should be largely unassembled, consistent with the much smaller positive peaks.

In both cases reassembly following the transition generates a substantial negative peak instead of the asymptote to zero seen in the dilution into GDP. This may be due to two factors. First, the disassembly applies only to the two-thirds or less of FtsZ that was assembled in the syringe, whereas reassembly following the transition may be close to 100%. Second, the bond formed in GTP is of higher affinity that that in GDP and may evolve more heat per bond. These two factors should explain why more heat is evolved in the reassembly in GTP than was lost upon the disassembly from GDP.

Assembly in magnesium differed from assembly in EDTA in two major respects: the critical concentration was about 10-fold lower, and once the transition occurred there was a continuous downward slope. In both cases the transitions were very sharp. In EDTA, as shown in Fig. 2, the transition began at 3.5 μM and was complete by 4.5 μM, which is only a 33% increase. In Mg the transition was perhaps even sharper, beginning at 0.4 μM and, although we cannot be exact because of the difficulty in integrating the heats of injection due to the changing base line, it seems the heat of injection reached a plateau after only two more injections (0.13 μM).

The abrupt transition to assembly at a critical concentration of 0.31 or 2.9 μM is characteristic of cooperative assembly. At present we do not know the mechanism for this apparent cooperativity. Cooperative assembly typically involves two types of bonds, one longitudinal and one lateral, to make a helical or two-dimensional polymer (23). Although protofilaments do seem to associate side-by-side later in assembly and at high protein concentrations (13–15), this association does not seem to be essential because the single subunit-wide protofilaments appear to be the basic polymer under the conditions used here (16). We have sought to explain how a single-stranded protofilament can show features of cooperativity, but several avenues of structural modeling and thermodynamics failed to provide a compelling mechanism. Understanding the mechanism for the apparent cooperativity of FtsZ assembly thus remains a major challenge.

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