SulA Inhibits Assembly of FtsZ by a Simple Sequestration Mechanism

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ABSTRACT: We have investigated the inhibition by SulA of the assembly of Escherichia coli FtsZ. Using quantitative GTPase and fluorescence assays, we found that SulA inhibition resulted in an increase in the apparent critical concentration for FtsZ assembly. The increase in apparent critical concentration was always less than the total amount of SulA added, suggesting that the association of SulA and FtsZ was of modest affinity. Isothermal titration calorimetry gave a value of 0.78 μM for the dissociation constant of the FtsZ–SulA complex, similar in magnitude to the 0.72 μM critical concentration of FtsZ protofilament assembly at steady state. We modeled the reaction as an equilibrium competition between (a) FtsZ subunits assembling onto protofilaments or (b) binding SulA. When FtsZ was assembled in GMPCPP or in EDTA, the inhibition of SulA was reduced. The reduced inhibition could be explained by a 3- and 10-fold weaker binding of SulA to FtsZ. The mutant D212G, which has no GTPase activity and therefore minimal subunit cycling, was shown here to assemble one-stranded protofilaments, and the assembly was blocked by SulA. We also assayed the SulA and FtsZ proteins from Pseudomonas. The SulA inhibition was stronger than with the E. coli proteins, and the model indicated a 5-fold higher affinity of Pseudomonas SulA for FtsZ.

FtsZ is a bacterial tubulin homologue and the major cytoskeletal protein involved in bacterial cell division. It assembles short, one-stranded protofilaments in vitro, and these are further assembled into a Z ring, which is tethered to the membrane at the site of cytokinesis. FtsZ provides not just the cytoskeletal framework, but also generates the constriction force, probably by a mechanism of protofilament bending.1–4 A dozen other proteins are involved in cytokinesis, most of them in remodeling the peptidoglycan layer. For a review of FtsZ see ref 4, and for a review of the accessory proteins and overall process see ref 5.

SulA is a small protein induced as part of the SOS response to DNA damage in Escherichia coli and related Gram-negative bacteria. SulA binds to FtsZ and blocks cell division until the DNA is repaired and SulA is proteolyzed.6–9 E. coli SulA protein is unstable in most in vitro conditions tested, but can be produced as a fusion with maltose binding protein (MBP).10 In this original study MBP–SulA was toxic to E. coli in vivo (demonstrating activity) and bound to FtsZ in vitro, but appeared not to inhibit FtsZ GTPase activity.10 Two later studies provided convincing evidence that SulA fusion proteins inhibited FtsZ GTPase, but only about 50% at a 1:1 stoichiometry.11,12

In contrast to the instability of E. coli SulA, SulA from Pseudomonas aeruginosa can be expressed and purified as a soluble protein. A crystal structure of the complex of PaFtsZ and SulA (for clarity we will prefix the FtsZ with Ec or Pa where needed) showed the SulA bound to the bottom of FtsZ.13 The SulA made contact with the NxD of the synergy/T7 loop (NxDxxD), which is buried in the longitudinal interface in the protofilament. The bound SulA would therefore sterically block assembly. This structure thus suggests a simple model for SulA inhibition: it sequesters the FtsZ monomers to which it is bound, and reduces the effective concentration of active FtsZ.

A recent study by Dajkovic et al.14 used a sedimentation assay to measure EcFtsZ polymer over a range of FtsZ concentrations and for various concentrations of MBP–SulA. They found that in the absence of SulA the critical concentration (Cc) for assembly was 0.9 μM, and the “apparent Cc” increased to 4.3 and 5.9 μM in the presence of 3.5 and 5.0 μM SulA. We will use the term “apparent Cc” (CcApp) to designate the elevated minimal concentration for assembly in the presence of SulA. As reported by Dajkovic et al.,14 the d2 reaction behaved as if the concentration of active FtsZ were equal to the total FtsZ minus the concentration of SulA. This is consistent with the sequestration mechanism, with one important assumption. The affinity for FtsZ binding SulA must be much greater than the affinity of FtsZ for adding to a d7 protofilament. The affinity of FtsZ for SulA has not been measured previously.

The sequestration mechanism was complicated by a remarkable discovery. Dajkovic et al.14 found that when FtsZ was assembled in GMPCPP instead of GTP, the assembly was no longer sensitive to SulA. GMPCPP is hydrolyzed very slowly, about 1/50 the rate of GTP.15 This suggested that GTP hydrolysis and associated cycling of subunits is required for inhibition by SulA. Consistent with this, the authors found that assembly was also insensitive to SulA when it was induced with GDP + AlF, which acts as a nonhydrolyzable GTP analogue, or in GTP plus EDTA, which chelates Mg and completely blocks GTP hydrolysis. This is not consistent with a simple

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sequestration mechanism. Dajkovic et al.\textsuperscript{14} concluded that inhibition by SulA only occurred when FtsZ was cycling subunits following GTP hydrolysis, and proposed a model based on two conformations of FtsZ. We have discussed potential problems with this model elsewhere.\textsuperscript{4}

The mutant D212G has almost no GTPase activity, and one would predict from the above conclusion that it would be insensitive to SulA inhibition. However, D212 (the last D in the NxDxxD sequence) is outside the FtsZ–SulA interface,\textsuperscript{13} and a yeast two-hybrid study,\textsuperscript{14} as well as an affinity binding column assay,\textsuperscript{12} showed that it can bind SulA. Trusca et al.\textsuperscript{12} reported that assembly of D212G, when stabilized by DEAE dextran, was not inhibited by SulA. It has been thought that D212G cannot assemble protofilaments without DEAE dextran or an excess of wild type FtsZ,\textsuperscript{11} so this mutant was not tested in the Dajkovic et al. study.\textsuperscript{14}

We have now re-examined this study using the sensitive and quantitative fluorescence assays we have recently developed.\textsuperscript{16–18} We also wanted to determine quantitatively the association constant for SulA binding FtsZ. The new data show that this binding is similar in affinity to that for FtsZ binding a protofilament. Our data can be explained by a new model,\textsuperscript{102} based on an equilibrium competition between free FtsZ subunits binding either protofilament ends or SulA.\textsuperscript{104}

\section*{EXPERIMENTAL PROCEDURES}

\textbf{Protein Purification.} Expression vectors for EcFtsZ and mutants were constructed using site-directed mutagenesis in the plasmid pET11b-FtsZ, and proteins were purified as described previously.\textsuperscript{16,17} Briefly, the soluble bacterially expressed protein was purified by 30% ammonium sulfate precipitation, followed by chromatography on a source Q 10/10 column (GE healthcare) with a linear gradient of 50–500 mM KCl in lysis buffer (50 mM Tris, pH 7.9, 1 mM EDTA, 10% glycerol). Peak fractions were identified by SDS–PAGE and stored at −80 °C.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{EM images of EcFtsZ without SulA and with the indicated addition of SulA. (a, b) In HMK buffer with GTP; (c, d, e) in HMK with GMPCPP; and (f, g) in MEK buffer with GTP.}
\end{figure}
The E. coli SulA expression vector, a pBAD vector that produces SulA fused at its N terminus to MBP, was a gift from Joe Lutkenhaus.11 The vector was transformed into E. coli strain DH5α and expression was induced by 0.2% arabinose for 3 h. The bacteria were suspended in lysis buffer, and the soluble MBP–SulA was bound to a column of Amylose resin (New England Biolabs, MA). The MBP–SulA was eluted with 10 mM maltose in lysis buffer, and applied to a source Q 10/10 column (GE healthcare) and eluted with a linear gradient of 50–500 mM KCl in lysis buffer. Peak fractions were identified by SDS–PAGE and stored at −80 °C.

The cDNA for PaFtsZ was obtained by PCR from genomic DNA and inserted into the plasmid pET-15b, which produces a His6-tag at the N-terminus. Overexpressed His-PaFtsZ protein in 50 mM Tris, pH 7.4, 500 mM KCl was bound to a column of TALON Metal Affinity Resin (Clontech Laboratories, Inc.). The Histag protein was eluted with 10 mM EDTA. After incubation with thrombin to remove the His-tag, the protein was further purified by chromatography on a source Q 10/10 column (GE healthcare), similar to EcFtsZ. PaSulA was prepared by similar cloning and expression and was purified by the talon column. The Histag PaSulA protein was insoluble at high concentration and was usually stored below 30 μM.

**FtsZ Assembly Assays.** Before each experiment, a cycle of calcium assembly–disassembly was done to remove any inactive protein. EcFtsZ protein was in lysis buffer plus 300 mM KAc, or in HMK buffer (50 mM HEPES, pH 7.7, 5 mM MgAc, 100 mM KAc). 10 mM CaCl2 and 2 mM GTP were added, and the mixture was incubated for 5 min at 37 °C to assemble protofilament bundles. The EcFtsZ polymer was collected by centrifugation at 45 000 rpm for 30 min (Beckman TLA100 rotor). The pellet was resuspended in the appropriate buffer and centrifuged again to remove any insoluble protein. Most experiments were done in HMK buffer, which is close to the physiological condition of bacterial cytoplasm. To test the assembly without Mg, we used MEK buffer (50 mM MES, pH 6.5, 1 mM EDTA, 100 mM KAc; the pH was lowered to 6.5 because EcFtsZ assembles very poorly at pH 7.7 without Mg). Most assays used the tryptophan-induced ATTO fluorescence quenching developed previously.18 ATTO fluorescence can be efficiently quenched by a tryptophan that is close enough to the trp. We labeled the cys with the fluorescent dye ATTO-655-maleimide (Fluka). For assembly experiments, the labeled EcFtsZ protein was diluted with a 9-fold excess of wild type FtsZ to avoid the formation of bundles. Assembly kinetics were measured at the ATTO peak emission 680 nm, with excitation at 650 nm. The ATTO fluorescence increased as EcFtsZ assembled, presumably due to a small conformational change in the subunits upon assembly.18 All fluorescence measurements were done in a thermostatically controlled cell at 25 °C.

**GTPase Activity Measurement.** GTPase activity was measured using a continuous, regenerative coupled GTPase assay.22,23 In this assay, all free GDP in solution is rapidly regenerated into GTP, and the GTP hydrolysis rate is measured by the decrease in absorption of NADH. Measurements were made in a thermostatically controlled cell at 25 °C.

![Figure 2](https://dx.doi.org/10.1021/bi201669d| Biochemistry XXX, XXX, XXX--XXX)

**Table 1. The FtsZ Apparent Critical Concentration As a Function of SulA, in Different Assembly Conditions**

<table>
<thead>
<tr>
<th>Buffer (Assay)</th>
<th>SulA (μM)</th>
<th>Capp (μM)</th>
<th>KDS (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMK-GTP (ITC)</td>
<td>0.72</td>
<td>0.72</td>
<td>0.78</td>
</tr>
<tr>
<td>HMK-GTP (GTPase)</td>
<td>1.54</td>
<td>1.54</td>
<td>(0.16)</td>
</tr>
<tr>
<td>HMK-GTP (ATTO)</td>
<td>2.26</td>
<td>2.26</td>
<td>0.68</td>
</tr>
<tr>
<td>HMK-GTP (ITC)</td>
<td>3.15</td>
<td>3.15</td>
<td>0.76</td>
</tr>
<tr>
<td>HMK-GTP (ATTO)</td>
<td>1.73</td>
<td>1.73</td>
<td>(0.07)</td>
</tr>
<tr>
<td>GMPCPP (ATTO)</td>
<td>2.62</td>
<td>2.62</td>
<td>0.53</td>
</tr>
<tr>
<td>GMPCPP (ATTO)</td>
<td>3.64</td>
<td>3.64</td>
<td>0.62</td>
</tr>
<tr>
<td>MEK (ATTO)</td>
<td>2.41</td>
<td>2.41</td>
<td>1.91</td>
</tr>
<tr>
<td>MEK (ATTO)</td>
<td>1.61</td>
<td>1.61</td>
<td>2.47</td>
</tr>
<tr>
<td>MEK (ATTO)</td>
<td>5.29</td>
<td>5.29</td>
<td>16.51</td>
</tr>
<tr>
<td>MEK (ATTO)</td>
<td>3.55</td>
<td>3.55</td>
<td>10.72</td>
</tr>
<tr>
<td>PaFtsZ (GTPase)</td>
<td>0.81</td>
<td>0.81</td>
<td>1.27</td>
</tr>
<tr>
<td>PaFtsZ (GTPase)</td>
<td>1.72</td>
<td>1.72</td>
<td>(0.08)</td>
</tr>
<tr>
<td>PaFtsZ (GTPase)</td>
<td>3.44</td>
<td>3.44</td>
<td>0.11</td>
</tr>
<tr>
<td>PaFtsZ (GTPase)</td>
<td>4.92</td>
<td>4.92</td>
<td>0.18</td>
</tr>
<tr>
<td>PaFtsZ (GTPase)</td>
<td>2.2</td>
<td>2.2</td>
<td>0.22</td>
</tr>
<tr>
<td>PaFtsZ (GTPase)</td>
<td>4.0</td>
<td>4.0</td>
<td>1.47</td>
</tr>
</tbody>
</table>

**Electron Microscopy.** FtsZ filaments were imaged by negative stain electron microscopy (EM). Approximately 10 μL
of sample in the appropriate buffer was incubated with GTP for 1–2 min, or with GMPCPP for 3–4 min and applied to a carbon-coated copper grid. Samples were stained with 2% uranyl acetate and imaged at 50000× magnification in a Philips 420 electron microscope.

Isothermal Titration Calorimetry (ITC). The FtsZ–SulA binding affinity was measured at 25 °C using a Microcal VP-ITC microcalorimeter (MicroCal Inc., MA). The samples were prepared in the HMK or MEK buffer and degassed using a Microcal ThermoVac. The sample cell contained 1.45 mL of 3 μM SulA, and each titration was 10 μL of 30 μM FtsZ. The titration data were fitted using the “one set of sites model” Microcal software package to calculate the binding affinity.

The 30 μM FtsZ-GDP in the syringe would be expected to assemble into short, isodesmic oligomers. When these are diluted into the sample chamber they will disassemble, and this will have its own heat of reaction. However, since the FtsZ is diluted into the sample chamber they will disassemble, and this will have its own heat of reaction. However, since the FtsZ is immediately sequestered by the excess SulA, the concentration of free FtsZ in the sample chamber will remain low and the heat from the disassembly of GDP-FtsZ oligomers will be identical for each injection. This can then be ignored in the titration of SulA.

Assembly and SulA Inhibition in DEAE Dextran. In HMK buffer, 5 μM wild type FtsZ or the mutant D212G was mixed with 0, 5, or 10 μM SulA, and 0.1 mg/mL DEAE dextran, and assembly was initiated by adding 1 mM GTP. After 2 min, the samples were centrifuged at 20000g for 15 min, and the supernatants and pellets were run on 10% SDS–PAGE.

■ RESULTS

Inhibition of FtsZ by SulA from E. coli. Figure 1 shows assembly of EcFtsZ assayed by EM with or without MBP–SulA protein. EcFtsZ assembled mostly one-stranded filaments in the presence of GTP (Figure 1a). When mixed with equimolar MBP–SulA filament, assembly was abolished (Figure 1b). Assembly in GMPCPP, a slowly hydrolyzable GTP analogue, produced longer one-stranded filaments and some filament bundles (Figure 1c). Assembly in GMPCPP was substantially inhibited, but not completely blocked, by an equimolar or 2-fold excess of MBP–SulA (Figure 1d,e). Assembly in MEK buffer, where Mg is replaced with EDTA, gave long, one-stranded filaments (Figure 1f). In this buffer MBP–SulA only partially inhibited assembly (Figure 1g). These results contradict the report of Dajkovic et al. that MBP–SulA did not inhibit assembly in GMPCPP or EDTA.

The assembly of FtsZ is accompanied by hydrolysis of GTP, and GTPase activity provides an alternative assay of assembly. To explore the inhibition more quantitatively, we assayed the GTPase for increasing concentrations of EcFtsZ, and in the presence of different amounts of MBP–SulA. In the absence of MBP–SulA, the GTPase showed a linear increase of 6.7 GTP hydrolyzed per minute per FtsZ above a Cc of 0.72 μM SulA.14 The report of Dajkovic et al.14 that MBP–SulA did not inhibit assembly in GMPCPP or EDTA.

Table 1). The primary effect of MBP–SulA was to increase the CcApp, to 1.54 μM at 1 μM SulA, and to 3.15 μM at 5 μM SulA, with minimal change in the slope of the line above the Cc of 0.72. Note that the increase in CcApp is somewhat less than the amount of MBP–SulA. This is also different from the report of Dajkovic et al, where the increase in CcApp was approximately equal to the amount of SulA.14

We confirmed these results using our recently developed assay based on quenching ATTO-655 fluorescence by a nearby trp.18 Note that in the previous study we found that the ATTO label promoted protofilament bundling. To avoid this complication there, as well as in the present study, we used the ATTO-labeled protein as a dilute label, with a 9-fold excess of wild type protein. Thus, the assembly is dominated by the wild type protein, and kinetics were identical to those of other trp mutants. Figure 3a,b shows the EcFtsZ assembly kinetics at different concentrations of EcFtsZ without or with 1 μM SulA. In Figure 3a the curve for 6 μM EcFtsZ shows an overshoot peak at ~10 s, declining to a plateau at ~20 s. We do not understand the structural basis for the overshoot. Figure 3b...
shows that SulA inhibited the assembly as assayed by ATTO fluorescence. Figure 3c plots the steady state assembly as a function of increasing concentration of EcFtsZ, and different concentrations of SulA. The results are in excellent agreement with the GTPase assay. The CcApp values for all assays are listed in Table 1.

The ATTO quenching assay provided a means to measure the inhibition of assembly by SulA in GMPCPP and in EDTA. In both cases the effect of SulA was primarily to shift the CcApp. In GMPCPP, the Cc App were also shifted by SulA, but the shifts were less than for assembly in GTP (Figure 3d). In MEK buffer (without Mg), the Cc without SulA was much higher, 2.47 μM, and the shifts in SulA were even smaller (Figure 3e). These results confirmed the EM observation, which showed a major inhibition of assembly in GTP, somewhat less inhibition of assembly in GMPCPP, and only a modest inhibition of assembly in EDTA. The reduced inhibition in MEK buffer was due to the absence of Mg, not the pH, since we found inhibition was similar at pH 7.7 and 6.5 in 5 mM Mg.

The experiments in Figure 3 measured the inhibition of assembly when FtsZ was premixed with SulA. We next assayed the ability of SulA to disassemble preassembled protofilaments. Figure 4 compares the disassembly induced by SulA, to that induced by excess GDP. Disassembly of the Mg-GTP polymers occurred in ~10 s (this is the time to 1/e of the final plateau, from an exponential fit), while disassembly of GMPCPP and EDTA polymers took ~20 s, consistent with their reduced subunit exchange. In all cases, the extent of disassembly was somewhat less for SulA than for GDP, and this difference was larger for EDTA, consistent with reduced inhibition seen by EM. Importantly, the kinetics of disassembly induced by GDP and SulA were identical in each case. We have previously interpreted GDP-induced disassembly to be a sequestration mechanism, 17,21 so this suggests a similar mechanism for SulA.

**Direct Measure of K_A for FtsZ–SulA Binding by ITC.** All of these results suggest that the mechanism of inhibition involves SulA binding to FtsZ monomers and blocking them from assembly. To understand the mechanism quantitatively, we need to know the association constant for SulA binding FtsZ. Below we will deduce this indirectly, but for a direct measure we turned to ITC. Figure 5 shows the data and the fitting, which gave a K_A of 1.29 μM⁻¹. We attempted to measure the K_A in MEK buffer but the association was much weaker, and we obtained no meaningful data at the maximum protein concentrations available.

**FtsZ and SulA from *P. aeruginosa.*** The binding of FtsZ and SulA is well characterized by a crystal structure of the FtsZ proteins from *P. aeruginosa,* 13 but the inhibition of FtsZ assembly by SulA has not been studied for the *Pseudomonas* proteins. We first checked the assembly properties of PaFtsZ. Interestingly, we found PaSulA assembled a novel helical structure without GTP (Figure 6c). Centrifugation analysis showed that these were only a small fraction of the total FtsZ. The helices had a diameter of about 15 nm and a pitch of about 19 nm. These helices are similar to but smaller than the helices assembled by EcFtsZ in DEAE-dextran, which had a diameter...
of 23 nm and a pitch of 21 or 28 nm. Also, these helical polymers assembled without stabilization by DEAE dextran. When GTP was added, the PaFtsZ assembled thin protofilaments very similar to those of EcFtsZ (Figure 6a). EM showed that PaSulA strongly inhibited the assembly of PaFtsZ, virtually eliminating the protofilaments (Figure 6b). Unlike EcFtsZ, PaFtsZ assembled poorly in the MEK buffer. EM also showed that PaFtsZ could assemble into long filaments in GMPCPP, and this assembly was also inhibited by a 2-fold excess of SulA, but not as strongly inhibited as in GTP.

We next assayed the GTPase activity of PaFtsZ in various concentrations of PaSulA. In the absence of SulA PaFtsZ hydrolyzed ~7.8 GTP per min (Figure 7a), slightly higher than the 6.7 GTP per min of EcFtsZ (each of these values is from the slope of the curve above the Cc). Increasing concentrations of SulA progressively inhibited the GTPase, primarily by increasing the CcApp, the same as for EcFtsZ. The shifts were larger than in the E. coli system, indicating a stronger binding of PaSulA to PaFtsZ.

We then used a FRET assay to determine the kinetics of filament disassembly by PaSulA. PaFtsZ has two cysteines, but we found that labeling either of them with a fluorophore blocked assembly. We therefore changed both cysteines to serines, and mutated V324 to cysteine. EM showed that this mutant PaFtsZV324C could assemble well both unlabeled and after labeling with fluorophores (data not shown). We labeled PaFtsZV324C with fluorescein maleimide as donor and tetramethylrhodamine maleimide as acceptor to set up the FRET assay, similar to the one we have used previously with EcFtsZ. Figure 7b shows a rapid drop in donor fluorescence following addition of GTP, corresponding to protofilament assembly followed by a slower decrease that may be due to filament bundling. Filament bundling was confirmed by EM and a significantly reduced steady-state GTPase activity. We therefore did not use this FRET assay for assembly and CcApp, but it was useful for disassembly studies. Addition of excess SulA or GDP caused a rapid rise in donor fluorescence, indicating rapid disassembly. The kinetics of disassembly were the same when induced by SulA or GDP, suggesting a similar sequestration mechanism for each.

**SulA Inhibits Filament Assembly of GTPase-Deficient FtsZ Mutant D212G.** In the presence of GTP, the EcFtsZ mutant D212G forms one-stranded protofilaments in HMK buffer (Figure 8a). When D212G was mixed with equimolar SulA, filament assembly was reduced (Figure 8b), and a 2-fold excess of SulA completely abolished assembly (Figure 8c).
The observation that SulA inhibits assembly of D212G proteofilaments, but not of tubes stabilized by DEAE dextran, is probably explained by our model of balanced equilibrium between (a) FtsZ assembly into polymer and (b) its binding to SulA, as developed in the next section. The resistance of the D212G-DEAE-dextran polymers suggests that assembly with D212G DEAE dextran is of higher affinity than without, tilting the balance toward assembly rather than sequestration by SulA.

Model of SulA Inhibition Used to Estimate the Binding Affinity of FtsZ–SulA. We will show here that a simple sequestration mechanism is consistent with our data, and that we can use the shift in apparent Cc to calculate the $K_D$ for FtsZ–SulA association. We will use the term Cc for the critical concentration in the absence of SulA. This is the reciprocal of the association constant for adding a subunit to a protein complex, and equals the concentration of free FtsZ in apparent equilibrium with filament ends at steady state. Cc is assumed not to change in the presence of SulA. SulA is assumed to cause an increase in the CcApp by sequestering the FtsZ to which it is bound: $Cc_{App} = Cc + [ZS]$, where $[ZS]$ is the concentration of the FtsZ–SulA complex at steady state. If SulA bound FtsZ with high affinity, we would expect the $Cc_{App}$ to be increased by exactly the amount of SulA added, since the equivalent amount of FtsZ would be sequestered. This was the result reported by Dajkovic et al.14 In our experiments the $Cc_{App}$ was increased by the addition of SulA, but the increase was always less than the total amount of SulA. This suggests that the binding of SulA to FtsZ is of modest affinity and sets up a competition between binding of FtsZ to the protofilament ends and binding to SulA.

$Z + Zp \leftrightharpoons Zp + 1 \Rightarrow [Z] = Cc$

$[Z]$ is the concentration of free FtsZ subunits at steady state, not bound to SulA, and is equal to Cc.

$Z + S \leftrightharpoons ZS \Rightarrow [ZS] = K_{AS}[Z][S]$

where $[ZS]$ is the concentration of the FtsZ–SulA complex, and $K_{AS}$ is the association constant for forming the complex.

The concentration of free SulA in solution, $[S]$, is equal to the total concentration $[S_t]$ minus the amount bound to FtsZ.

$[S] = [S_t] - [ZS]$

Inserting 1 and 3 into eq 2

$[ZS] = K_{AS}C_{c}[S_t] - [ZS]] + [ZS] = \frac{K_{AS}C_{c}S_t}{1 + K_{AS}C_{c}}$

We can now write

$C_{cApp} = Cc + [ZS] = Cc + \frac{K_{AS}C_{c}S_t}{1 + K_{AS}C_{c}}$

$K_{AS} = \frac{C_{cApp} - Cc}{C_{c} - C_{cApp}Cc + Cc^2}$

We can now use eq 6 to calculate $K_{AS}$ for each value of $C_{cApp}$ determined from the GTPase assays or assembly assay by ATTO fluorescence. The calculated values are given in Table 1, where we have used $K_{DS} = 1/K_{AS}$ in units $\mu M$. These $K_{DS}$ values can be compared directly with Cc, the dissociation constant for assembly of a subunit onto a 423 protofilament ($Cc = C_{cApp}$ for 0 SulA). The $K_{DS}$ for 1 $\mu M$ SulA (in parentheses) is considered less accurate because of the small shift. Values for 3 and 5 $\mu M$ SulA binding to wild type FtsZ are

Figure 7. (a) GTPase of PaFtsZ is inhibited by SulA, primarily as an increase in the apparent critical concentration. (b) Assembly of 5 $\mu M$ PaFtsZ assayed by FRET. Assembly results in a drop in donor fluorescence. Upon addition of SulA or GDP, there was a small drop in fluorescence due to the 10% dilution, and then the donor fluorescence increased, indicating disassembly. The rate of disassembly is the same for GDP and SulA.

![Figure 7](Figure7.png)
427 FtsZ, 0.53 and 0.62, are very close to the K_DS from the ITC 428 measurement, 0.78 μM. The values from polymer assay by 429 ATTO fluorescence are similar those from the GTPase assay. 430 We should also note that during our ITC measurement, no 431 GTP was added and FtsZ should be monomeric with a small 432 excess of GDP. This suggests that SulA binds to FtsZ with 433 similar affinity in the GDP and GTP bound states. The reduced inhibition by SulA for assembly in GMPCPP or 434 EDTA can be explained by a reduced affinity for binding SulA 435 in these conditions. The binding affinity of SulA for EcFtsZ 436 appears to be reduced 3–4 fold for assembly in GMPCPP and 437 for assembly of D212G, and is reduced more than 10-fold in 438 EDTA (Table 1). This is consistent with our inability to 439 measure a K_AS by ITC in EDTA and explains why SulA only 440 weakly inhibits FtsZ assembly in EDTA. Finally the affinity of 441 SulA for PaFtsZ is 3–5 fold higher than for EcFtsZ.

**DISCUSSION**

Our work is consistent with the model that SulA inhibits FtsZ 447 by binding to the assembly site of FtsZ subunits and 448 sequestering them from the assembly reaction. We add one 449 important parameter not previously considered—the 450 association constant K_AS for the reversible binding of SulA to 451 FtsZ. For EcFtsZ assembly in HMK with GTP, this is the same 452 order of magnitude as the binding of FtsZ subunits to 453 protofilament ends, so the assembly needs to be treated as a 454 competition of these two reactions. Importantly, the K_AS 455 measured by ITC is very close to that deduced from the 456 model for EcFtsZ assembly in GTP.

We show here by EM that D212G assembles one-stranded 457 protofilaments similar to wild type. These results contradict 458 earlier work by Mukherjee et al., who reported that D212G 459 did not polymerize except with an excess of wild type FtsZ. 458 However, they examined filament formation under different 459 conditions (50 mM Mes-NaOH pH 6.5, 10 mM MgCl2, and 460 200 mM KCl). Under these conditions, primarily due to the pH 461 6.5, versus our more physiological pH 7.7, we also could not 462 detect filament formation of D212G. The ATTO fluorescence 463 assay confirmed and quantitated the assembly. Assembly of 464 D212G was inhibited by SulA. When assembly was stabilized 465 by DEAE dextran, assembly of wild type FtsZ was largely 466 inhibited by a 2-fold excess of SulA, while D212G was only 467 slightly inhibited, in agreement with the previous study of 468 Trusca et al. Our data contradict the previous conclusion that assembly in 469 GMPCPP or in EDTA was not inhibited at all by SulA. Our 470 EM shows qualitative inhibition of assembly under both 471 conditions, and our ATTO fluorescence assay confirms the 472 inhibition with extensive quantitative data. The inhibition of 473 assembly in GMPCPP was less than the inhibition in GTP, in 474 qualitative agreement with Dajkovic et al. Our model 475 estimates a 3–4-fold reduced affinity of SulA for FtsZ- 476 GMPCPP, relative to FtsZ-GTP. This is a small change 477 (0.5 kcal/mol) and may reflect problems in the indirect calculation 478 from CcApp, rather than an actual change in K_D. A similar 479 fold reduction in assembly was calculated from the inhibition of 480 D212G, which also has minimal GTPase activity and reduced 481 subunit turnover. It is clear from Table 1 that the calculation 482
gives inconsistent results at low FtsZ concentrations, and it may also show variation for reduced subunit turnover.

The mutant D212G was originally isolated as dominant inhibitor of SulA and designated “resistant to SulA.” This would seem to contradict our finding that assembly of D212G is sensitive to SulA. However, the original isolation step and subsequent testing, the mutant D212G was expressed from a plasmid in the presence of wild type FtsZ from the genome. There are two ways a plasmid-expressed mutant FtsZ can generate resistance to SulA. One is that the mutation blocks the binding site for SulA. Then the plasmid-expressed mutant FtsZ is immune to SulA and can function for cell division. This happens for F268C, which is in the middle of the SulA-binding site. The other mechanism is that the mutant FtsZ can bind SulA and is expressed in sufficient quantity to sequester most of the SulA, leaving the wild type FtsZ free to continue cell division. This is apparently the case for D212G, which is outside the SulA-binding site.

We found that the inhibition of assembly by SulA was even weaker in EDTA, but still measurable. The weaker inhibition could be attributed to a >10-fold reduction in affinity of SulA for EcFtsZ in the absence of Mg. This suggests that binding of SulA to FtsZ might require binding of Mg; however, no Mg was seen in the crystal structure of PaFtsZ–SulA. The FtsZ subunit may have a different conformation in EDTA, since the dimer nucleus was much weaker relative to elongation in EDTA than in Mg.

Our model is simpler than the one suggested by Dajkovic et al. It has only two parameters: the Cc for association of FtsZ subunits onto the end of protofilaments, and the association constant \( K_{AS} \) for forming the SulA–FtsZ complex. The previous model had an implicit assumption that this association was much stronger than that of protofilament assembly, which fit the centrifugation data showing that CcApp is equal to Cc plus the total added SulA. Our quantitative GTPase assays and fluorescence assays show that CcApp is always less than this, and the difference can be used to measure \( K_{AS} \). In the one case we were able to measure \( K_{AS} \) by ITC, it was very close to the value deduced from the model (0.78 \( \mu \)M from ITC vs 0.68–0.78 \( \mu \)M for the model).

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## ABBREVIATIONS

- GMPCP, guanosine-5’-[(α,β)-methylene]triphosphate; Cc, critical concentration; CcApp, apparent critical concentration; FRET, Förster resonance energy transfer; ITC, isothermal titration calorimetry; EM, electron microscopy; EDTA, ethylenediaminetetraacetic acid


