Inhibition of Assembly of Bacterial Cell Division Protein FtsZ by the Hydrophobic Dye 5,5′-Bis-(8-anilino-1-naphthalenesulfonate)*

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To gain further insight into the structural relatedness of tubulin and FtsZ, the tubulin-like prokaryotic cell division protein, we tested the effect of tubulin assembly inhibitors on FtsZ assembly. Common tubulin inhibitors, such as colchicine, colcemid, benomyl, and vinblastine, had no effect on Ca2+-promoted GTP-dependent assembly of FtsZ into polymers. However, the hydrophobic probe 5,5′-bis-(8-anilino-1-naphthalenesulfonate) (bis-ANS) inhibited FtsZ assembly. The potential mechanisms for inhibition are discussed. Titrations of FtsZ with bis-ANS indicated that FtsZ has one high affinity binding site and multiple low affinity binding sites. ANS (8-anilino-1-naphthalenesulfonate), a hydrophobic probe similar to bis-ANS, had no inhibitory effect on FtsZ assembly. Because tubulin assembly has also been shown to be inhibited by bis-ANS but not by ANS, it supports the idea that FtsZ and tubulin share similar conformational properties. Ca2+, which promotes GTP-dependent FtsZ assembly, stimulated binding of bis-ANS or ANS to FtsZ, suggesting that Ca2+ binding induces changes in the hydrophobic conformation of the protein. Interestingly, depletion of bound Ca2+ with EGTA further enhanced bis-ANS fluorescence. These findings suggest that both binding and dissociation of Ca2+ are capable of inducing FtsZ conformational changes, and these changes could promote the GTP-dependent assembly of FtsZ.

The key bacterial cell division protein FtsZ is widespread among all prokaryotes. It is essential for cell division and assembles into a ring-like structure at the site of cytokinesis (1–8). Despite its actin-like behavior in the cell, FtsZ is biochemically quite similar to tubulin. FtsZ proteins share limited primary sequence homology with tubulin, but more importantly, they can bind and hydrolyze GTP and assemble into polymers, such as sheets of tubulin-like protofilaments and minirings as determined by electron microscopic analysis (9–13). FtsZ polymers have not yet been visualized in vivo, making it impossible to verify the physiological relevance of such structures. Secondary structural prediction suggests that FtsZ and tubulin share 70–80% similarity, with the exception of their short carboxyl termini (14). Therefore, the overall three-dimensional structures of FtsZ monomers and tubulin are likely to be very similar.

Because many inhibitors of tubulin assembly exist, investigating the effects of tubulin inhibitors on FtsZ assembly should provide important information for FtsZ structure and function. Tubulin inhibitors have been classified into four categories: (i) colchicine and its structural analogues, such as colcemid and podophyllotoxin; (ii) vinblastine and its analogues vincristine and maytansine; (iii) the metal ions Ca2+, Cu2+, and Hg2+ (15, 16); and (iv) aminonaphthalenes, such as bis-ANS1† (17). Different inhibitors bind to tubulin at different sites and presumably arrest tubulin assembly by different mechanisms (17).

We previously developed a simple in vitro assay for FtsZ assembly using a FtsZ-green fluorescent protein fusion (FtsZ-GFP) (18). By using this assay, we demonstrated that FtsZ is capable of microtubule-like dynamic assembly and can self-assemble into structures that are similar to microtubule asters. This assembly is strictly dependent on GTP. In addition, there is a striking difference in the effects of Ca2+ on FtsZ and tubulin assembly. Whereas tubulin assembly into microtubules is strongly inhibited by Ca2+, millimolar concentrations of Ca2+ specifically promote assembly of FtsZ into polymer networks. The polymers are composed of bundles of protofilaments that are structurally similar to those observed by electron microscopic analysis, and their ability to grow and interconnect in a GTP-dependent manner suggests that they are physiologically relevant structures. The stimulatory effect of Ca2+ suggests that Ca2+ interacts with FtsZ, but with different effects on protein conformation than with tubulin. The mechanism of Ca2+ stimulation of FtsZ assembly is unknown, as is any possible role of Ca2+ for FtsZ assembly in the cell.

Here, we use the fluorescent assembly assay and other analytical techniques to test the effects of some tubulin inhibitors on FtsZ assembly. We show that colchicine, colcemid, benomyl, and vinblastine have no effect on Ca2+-induced FtsZ polymerization, suggesting that FtsZ interacts with these drugs in a manner distinct from that of tubulin. However, we show that another tubulin inhibitor, bis-ANS, effectively inhibits FtsZ polymerization, and its possible mechanism of action is investigated and discussed. Because bis-ANS is a fluorescent probe that measures protein hydrophobic surface properties, we used bis-ANS and a related compound, ANS, to probe directly for FtsZ conformational changes induced by Ca2+ binding and dissociation.

EXPERIMENTAL PROCEDURES

Reagents—Bis-ANS was obtained from Molecular Probes, Inc. ANS, colchicine, colcemid, vinblastine, and GTP were purchased from Sigma, and radiolabeled GTP was from Amersham Pharmacia Biotech. Benomyl was obtained from DuPont. Other chemicals were of analytical grade or better. Molecular mass markers were from Life Technologies, Inc. The concentrations of ANS and bis-ANS in stock solution (dissolved in H2O) were determined using ε350 = 4900 and ε350 = 16,790 M−1 cm−1, respectively.

Protein Purification—FtsZ and FtsZ-GFP proteins were overexpressed and purified from strains WM688 and WM617, respectively, as
described previously (18). The glycerol and EDTA in the protein preparation were removed by loading the protein onto a small DEAE-Sephasel column; washing with 20 volumes of 50 mM Tris, pH 7.5, 0.1 M KCl; and eluting with the same buffer containing 1 M NaCl. The protein peak was then pooled and dialyzed against the above buffer.

Protein concentration was determined with the bicinchoninic acid method (19) using bovine serum albumin as a standard. The protein concentration of the same FtsZ sample was also determined by quantitative amino acid analysis on an Applied Biosystems 420A analyzer.

The measured amino acid composition is consistent with the composition predicted from the DNA sequence. When two protein dilutions were measured in duplicate assays, the bicinchoninic acid method gave a concentration of 0.997 ± 0.002 mg/ml, whereas the quantitative amino acid analysis gave a concentration of 1.14 ± 0.03 mg/ml. Therefore, a factor of 1.14 was used to calibrate the bicinchoninic acid assay.

FtsZ Assembly—The fluorescent microscopic assay for FtsZ polymerization was described previously (18). Briefly, FtsZ-GFP or a mix of FtsZ and FtsZ-GFP at 5.7 μM was incubated in assembly buffer (50 mM Tris, pH 7.5, 1 mM MgCl₂, 1 mM GTP, 10 mM CaCl₂) at 37 °C for 5–10 min. In some cases, the GTP concentrations were varied. Four μM of sample were then loaded onto a glass slide and visualized with an Olympus BX60 fluorescence microscope equipped with a ×100 oil immersion plan fluorite objective (numerical aperture = 1.3), a 100-W mercury lamp, a standard fluorescein isothiocyanate filter set, and an Ocean Optics DHR-750 cooled video camera. Images were digitized with a Scion LG3 video card, manipulated with Adobe Photoshop, and printed on a Tektronix Phaser 400 dye sublimation printer. For light scattering assays, 5.7 μM FtsZ in 1 ml of the same buffer without CaCl₂ was incubated at room temperature for 3 min, and then CaCl₂ was added to initiate the polymerization. Light scattering at 600 nm was recorded continuously for 10 min. To test the effect of tubulin inhibitors on FtsZ polymerization, the compounds at different concentrations were added to the polymerization buffer.

Binding of ANS and Bis-ANS to FtsZ—The binding of bis-ANS or ANS to FtsZ protein was monitored by bis-ANS or ANS fluorescence intensity and the shift of λmax. Unless otherwise specified, protein at 1.14–22.8 μM was incubated with different amounts of bis-ANS or ANS (up to 1 μM) in 50 mM Tris, pH 7.5, 0.1 mM KCl for 50 min. The bis-ANS or ANS fluorescence emission spectra were recorded on a Photon Technology International spectrophotometer. Both excitation and emission bandwidths were 2 nm. The excitation wavelengths for bis-ANS and ANS were 390 and 381 nm, respectively.

The stoichiometry and affinity of bis-ANS binding to FtsZ were determined using a double titration method (20, 21) and Scatchard Plot analysis (22). Briefly, bis-ANS at several fixed concentrations was titrated with different concentrations of FtsZ and vice versa. The common intersection points on the double reciprocal plots of the fluorescence intensities versus the concentration of the varied components gave the values of Kd/n or Kd, where n is the number of bis-ANS binding sites and Kd is the dissociation constant. The fluorescence emission was recorded at a fixed wavelength of 480 nm. The observed fluorescence intensities were corrected for the low background fluorescence of bis-ANS in buffer and for the inner filter effect of varying concentrations of bis-ANS (22). For Scatchard plot analysis, the concentration of bound bis-ANS was determined from the relationship [bis-ANS]B = F/Fmax where Fmax is the theoretical fluorescence of a molar solution if all of the ligand were in the form of FtsZ. Fmax was calculated by titration of 1 μM bis-ANS with different concentrations of FtsZ. The intercept of the double reciprocal plot of fluorescence intensity versus bis-ANS concentration gave the reciprocal of Fmax. The data thus generated were analyzed by the Scatchard method.

Photoincorporation Methods—Photoincorporation of bis-ANS to FtsZ was performed as described previously (23). Briefly, FtsZ at 1.14–5.7 μM was incubated with 10–50 μM bis-ANS in 50 mM Tris, pH 7.5, and 0.1 M KCl for 30 min on ice, 2 cm from a UV light source. Protein samples were then subjected to SDS-polyacrylamide gel electrophoresis. Fluorescent bands representing bis-ANS bound to FtsZ were photographed by an IS1000 digital imaging system (Alpha Innotech Corp.) with a UV light box. The UV cross-linking of GTP to FtsZ was performed as described previously (18).

RESULTS

Inhibition of FtsZ Polymerization by Bis-ANS—To study the structural and functional similarity between FtsZ and tubulin, we assessed the effect of tubulin inhibitors on FtsZ polymerization. Initially, the most common tubulin inhibitors, colchicine, colcemid, benzomyl, and vinblastine, were tested for their effects on Ca²⁺-promoted FtsZ assembly into protofilament bundle networks. These drugs, even at concentrations up to 500 μM or higher, had no significant effect on assembly under our conditions (data not shown). This suggests that the interaction of these drugs with FtsZ is distinct from their interaction with tubulin.

The effects on FtsZ assembly of another group of tubulin inhibitors, such as ANS and bis-ANS, was also examined. Whereas FtsZ formed polymer networks in the absence of bis-ANS (Fig. 1A), 50 μM bis-ANS was sufficient to prevent polymer formation (Fig. 1B). It should be noted that polymer networks shown in Fig. 1A were also typical of those observed after addition of the tubulin inhibitors tested above. Because of the complex topology of FtsZ polymers formed in this assay, the distribution of polymers in the glass slide is typically nonuniform. As a result, it is very difficult to use this technique quantitatively to evaluate inhibition of assembly. To circumvent this problem, we used the concentration of inhibitor required to block the formation of visible polymers under the fluorescence microscope as a standard to evaluate the inhibition. For bis-ANS, this concentration was determined to be 36 μM in the standard polymerization buffer.

To rule out the possibility that the inhibition by bis-ANS was due to the GFP tag on the FtsZ protein used for fluorescence detection, the effect of bis-ANS on FtsZ polymerization was also examined by light scattering. Fig. 2A shows the time course of FtsZ polymerization in the absence (trace 1) and presence (trace 2) of 50 μM bis-ANS as detected by light scattering at room temperature. The apparent velocity of the increase of light scattering in the presence of different concentrations of bis-ANS is shown in Fig. 2B. The IC₅₀ was around 14 μM under these conditions. The results suggest that bis-ANS is a specific inhibitor of FtsZ polymerization.

The inhibition of FtsZ polymer formation by bis-ANS prompted us to determine whether it could promote FtsZ depolymerization. Because assembly of FtsZ in our assay occurs dynamically in solution, it was possible to analyze the polymerization state in solution over time in order to address this question. FtsZ-GFP at 5 μM was incubated in assembly buffer at 37 °C for 10 min, and aliquots of the sample were checked by fluorescence microscopy to verify the formation of fluorescent polymers. Then, bis-ANS was added to the sample to make a final concentration of 50 μM. After a 20-min incubation, no polymers could be detected by fluorescence microscopy (data not shown). This result suggests that bis-ANS, in addition to inhibiting FtsZ polymerization, is also able to depolymerize FtsZ polymers. The possibility that bis-ANS can depolymerize microtubules has not been reported.

The bis-ANS analog ANS (8-anilino-1-naphthalenesulfonate) has been reported to be much less effective at inhibiting tubulin assembly than bis-ANS (17, 24). Using the fluorescence assay,
we found that ANS had no detectable effect on FtsZ polymerization, even when the ANS concentration was as high as 1.5 mM (data not shown). We can conclude that the effects of bis-ANS and ANS on FtsZ assembly are very similar to their respective effects on tubulin assembly, suggesting that FtsZ and tubulin may interact similarly with these two compounds.

**Binding of Bis-ANS to FtsZ**—Because bis-ANS specifically inhibits polymerization of both tubulin and FtsZ, it was reasonable to propose that FtsZ may have a hydrophobic surface arrangement similar to that of tubulin. It has been reported that tubulin has one high affinity bis-ANS binding site and 6 (20) to 40 (25, 26) low affinity binding sites. The high affinity binding site has been proposed to be responsible for the inhibition of tubulin assembly (24). To characterize the interaction between bis-ANS and FtsZ in more detail, we analyzed bis-ANS-FtsZ binding. Evaluation of multiple binding sites is extremely difficult using spectroscopic techniques, because the binding of bis-ANS at different sites may not have the same quantum yield (26). Nevertheless, we took advantage of the extensive studies of the binding of bis-ANS to tubulin (20, 24–26) to apply spectroscopic techniques to bis-ANS-FtsZ binding. As shown in Fig. 3A, the presence of FtsZ greatly enhances bis-ANS fluorescence (trace 2) over the baseline (trace 1) with a blue shift of $\lambda_{\text{max}}$ from 530 nm (data not shown) to 480 nm, suggesting strong binding of bis-ANS to FtsZ. Fig. 3B shows an example of a Scatchard plot for an FtsZ concentration of 2.28 $\mu$M. These data indicate that FtsZ has a high affinity binding site for bis-ANS with a $K_d$ of 1.33 $\mu$M and 3.59 low affinity binding sites, each with a $K_d$ of 22.92 $\mu$M. Interestingly, these $K_d$ values for both high and low affinity binding sites are similar to those previously reported for tubulin (20, 24–26). The number of low affinity binding sites for FtsZ is smaller than six, the smallest number reported for tubulin. To rule out the possibility that this difference was a result of the analytical method employed, the same double titration method as described by Prasad et al. (20) was applied to FtsZ. The data confirmed that the number of low affinity binding sites is 3.66 and the $K_d$ is 19.2 $\mu$M (Fig. 4). The data derived from the same method strongly suggest that the bis-ANS binding sites of FtsZ are very similar to those of tubulin, except that tubulin may have additional sites. It is reasonable to propose from this result that FtsZ and tubulin may have a similar pattern of hydrophobic patches on their surfaces.

**Ca$^{2+}$ Changes the Binding of Bis-ANS and ANS to FtsZ**—Ca$^{2+}$ is a key factor for FtsZ polymerization under our conditions. Because binding of Ca$^{2+}$ is likely to have an important role in conformational changes in FtsZ leading to its assembly,
and because bis-ANS is a probe for hydrophobic surface arrangement, it was logical to include Ca\(^{2+}\) in the study of bis-ANS-FtsZ interactions and to determine the effect of Ca\(^{2+}\) on the binding of bis-ANS to FtsZ. The data in Fig. 5 show a 30% increase in bis-ANS fluorescence upon addition of Ca\(^{2+}\) to FtsZ, suggesting that Ca\(^{2+}\) moderately increases bis-ANS binding.

Either a change in bis-ANS binding or a change in quantum yield of the bis-ANS-FtsZ complex could explain the changes in fluorescence. To rule out the second possibility, the binding of bis-ANS to FtsZ was also examined by photoincorporation of bis-ANS to FtsZ. In this assay, the bis-ANS was first cross-linked to FtsZ protein, followed by detection of photolabeled protein after denaturation and separation by SDS-polyacrylamide gel electrophoresis. As detected by UV cross-linking, Ca\(^{2+}\) appeared to increase the binding of bis-ANS (Fig. 6B). Although FtsZ protein was stable in the presence of bis-ANS (data not shown), UV-specific protein degradation was often observed in our experiments in the presence of bis-ANS, especially with longer UV light exposure and high bis-ANS concentrations. To address this problem, the Coomassie-stained protein bands and bis-ANS fluorescent bands from the same gel were quantified, and bis-ANS fluorescence was normalized to the amount of protein in the bands. The effect of different concentrations of Ca\(^{2+}\) on bis-ANS binding is summarized in Fig. 7A. Ca\(^{2+}\) concentrations between 5 and 10 mM enhanced bis-ANS binding by approximately 2-fold, as detected by UV

![Fig. 4. Double-reciprocal plots for the binding of bis-ANS to FtsZ.](image)

**A.** A plot of the inverse of the fluorescence intensity versus the inverse of bis-ANS concentration at various fixed concentrations of FtsZ. The various FtsZ concentrations were 2.28 (1), 5.7 (2), 11.4 (3), and 22.8 (4) μM. **B.** A plot of the inverse of the fluorescence intensity versus the inverse of FtsZ concentration at various fixed concentrations of bis-ANS. Each line corresponds to a fixed bis-ANS concentration of 2 (1), 3 (2), 5 (3), or 12 (4) μM. Excitation was at 390 nm, and emission was at 480 nm, with a bandwidth of 2 nm.

![Fig. 5. Effect of the binding and dissociation of Ca\(^{2+}\) on the enhancement of FtsZ-dependent bis-ANS and ANS fluorescence.](image)

**A.** Enhancement of bis-ANS fluorescence. Traces 1–4 represent 2.28 μM FtsZ and 5 μM bis-ANS in 50 mM Tris, pH 7.5, 0.1 M KCl in the presence of 0, 2.5, 5, and 20 mM CaCl\(_2\), respectively. Traces 5 and 6 represent the same samples as in trace 4 with the addition of 4 and 6 mM EGTA, respectively. **B.** Enhancement of ANS fluorescence. Traces 7–10 represent 6.84 μM FtsZ and 120 μM ANS in 50 mM Tris, pH 7.5, 0.1 M KCl in the presence of 0, 5, 20, and 40 mM CaCl\(_2\), respectively.

![Fig. 6. Effect of Ca\(^{2+}\) on the photoincorporation of bis-ANS into FtsZ.](image)

5.7 μM FtsZ was incubated with 16 μM bis-ANS in 50 mM Tris, pH 7.5, 0.1 M KCl containing different concentrations of CaCl\(_2\), 2 cm from a UV light for 30 min at 4 °C, and then subjected to SDS-polyacrylamide gel electrophoresis. Lane m contains a protein molecular mass ladder representing 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, and 20 kDa. Lanes 1–5 contain Coomassie Blue-stained FtsZ protein bands representing incubations with Ca\(^{2+}\) at concentrations of 0, 1.25, 2.5, 5, and 10 mM, respectively. **B** shows the same gel corresponding to lanes 1–5 in A, imaged for fluorescence prior to Coomassie Blue staining.
cross-linking. This effect was greater than that detected by bis-ANS fluorescence spectra (Fig. 5A) and could be attributed to a lower quantum yield of the additional exposed binding site(s) induced by Ca\(^{2+}\). Another explanation for this apparent increase is the predicted block of bis-ANS dissociation from FtsZ due to covalent cross-linking. Therefore, the photoincorporation method may give higher numbers for binding and as a result amplify the difference in binding affinities.

Because bis-ANS inhibits FtsZ assembly, bis-ANS itself is likely to induce FtsZ conformational changes. As a result, changes in bis-ANS fluorescence in the presence of Ca\(^{2+}\) may be due to a combination of both Ca\(^{2+}\) and bis-ANS effects on FtsZ conformation. To test whether Ca\(^{2+}\) alone induces changes of FtsZ hydrophobic surface properties in the absence of bis-ANS, we examined the effect of Ca\(^{2+}\) on the enhancement of ANS fluorescence by FtsZ. This was feasible because, as described earlier, ANS has no detectable effect on FtsZ polymers (27). The rapid binding of bis-ANS to the GTP interaction of FtsZ with GTP, has been proposed to form a hydrophobic pocket (27). The rapid binding of bis-ANS to the GTP site might also overlap with at least one bis-ANS binding site. Therefore, the photoincorporation method may give higher numbers for binding and as a result amplify the difference in binding affinities.

To test whether the effect of Ca\(^{2+}\) on bis-ANS binding to FtsZ was reversible, EGTA was added to the assembly reaction to chelate Ca\(^{2+}\). When the Ca\(^{2+}\) concentration was below 5 mM, the bis-ANS fluorescence decreased back to the level observed in the absence of Ca\(^{2+}\) if sufficient EGTA was added (data not shown). When the Ca\(^{2+}\) concentration was 7.5 mM or greater, addition of 4 mM EGTA slightly decreased the bis-ANS fluorescence, consistent with a decrease in available Ca\(^{2+}\) (Fig. 5A, trace 5). However, when EGTA concentration was increased to deplete Ca\(^{2+}\) from the solution, bis-ANS fluorescence did not decrease but instead increased to a level greater than reached during stimulation by Ca\(^{2+}\) (Fig. 5A, trace 6, and data not shown). This result is surprising, but it is consistent with the previous finding that a critical concentration of Ca\(^{2+}\) at 7 mM is required to trigger FtsZ assembly into visible fluorescent polymers (18). One possible explanation is that when Ca\(^{2+}\) in the solution reaches the critical concentration, the dissociation of Ca\(^{2+}\) from certain binding sites leads to a further exposure of hydrophobic surfaces, which may be essential for FtsZ to assemble in the presence of GTP. In a control experiment, EGTA itself had no effect on the fluorescence of the bis-ANS-FtsZ complex (data not shown) in the absence of Ca\(^{2+}\). In another control, Ca\(^{2+}\) and EGTA had no effect on the enhancement of bis-ANS fluorescence by bovine serum albumin. This suggests that the observed effects of depleting Ca\(^{2+}\) on bis-ANS fluorescence are specific under our conditions. Moreover, ANS fluorescence was also enhanced by EGTA-mediated depletion of Ca\(^{2+}\) (data not shown). This result supports the idea that additional EGTA-mediated FtsZ conformational changes were in fact due to Ca\(^{2+}\) dissociation and were not merely due to a specific effect of bis-ANS.

**GTP Inhibits Bis-ANS Binding to FtsZ**—Although it is well established that bis-ANS inhibits tubulin polymerization (24), the mechanism of inhibition is not understood. However, the similar binding behavior of bis-ANS to FtsZ and tubulin observed here suggested that its mechanism of inhibition of FtsZ and tubulin assembly might be similar. FtsZ polymerization under our conditions specifically requires GTP, although GDP is capable of supporting FtsZ polymerization mediated by DEAE-dextran and cationic lipid monolayers (11, 12). Because GTP is essential for both FtsZ and tubulin assembly, it was important to determine whether GTP binding could be affected by bis-ANS binding and vice versa.

Fig. 8A shows that preincubation of FtsZ with 1 mM GTP greatly decreased the binding of bis-ANS to FtsZ. The maximum decrease of fluorescence by increasing GTP concentration is about 50% of that in the absence of GTP, suggesting that GTP may inhibit bis-ANS binding to certain binding sites but not to other sites. This result was also consistent with the titration data described earlier, which demonstrated that FtsZ has multiple bis-ANS binding sites. When bis-ANS was preincubated with FtsZ for 1 min, the addition of GTP caused a slower decrease of bis-ANS fluorescence, with the apparent rate of fluorescence decrease dependent on GTP concentration (Figs. 8B). This suggests that the dissociation of bis-ANS from FtsZ is slower than the binding of bis-ANS to FtsZ. The tubulin signature sequence GGGTGTG, which is likely involved in the interaction of FtsZ with GTP, has been proposed to form a hydrophobic pocket (27). The rapid binding of bis-ANS to the GTP binding site and its slow dissociation is consistent with this model.

To investigate further the inhibition of bis-ANS binding by GTP, a photoincorporation experiment was performed. As in the Ca\(^{2+}\) experiment, GTP was also shown to inhibit photoincorporation of bis-ANS to FtsZ (Fig. 7B). The effect of GTP on bis-ANS binding seems to be slightly greater than that detected by fluorescent spectra. As suggested earlier, this phenomenon may be due to amplification of the difference in binding by UV cross-linking.

**Effects of Bis-ANS on GTP Binding**—The inhibition of bis-ANS binding by GTP binding suggested that the GTP binding site might also overlap with at least one bis-ANS binding site. The effect of bis-ANS on GTP binding is shown in Fig. 9A. At a low GTP concentration (1 \(\mu\)M), the IC\(_{50}\) of bis-ANS is approximately 4 \(\mu\)M, but when GTP concentration increases to 10 \(\mu\)M, the IC\(_{50}\) increases to approximately 17 \(\mu\)M. The inhibition of
GTP binding by bis-ANS and inhibition of bis-ANS binding by
GTP suggest that GTP and bis-ANS compete for the same site
on FtsZ.

To determine whether the inhibition of FtsZ polymerization
might be due to competitive binding of bis-ANS to the GTP
binding site, we determined the effect of 50 μM bis-ANS on GTP
binding to FtsZ at different GTP concentrations. Because a bis-
ANS concentration of 50 μM was sufficient to block the formation
of visible fluorescent polymers (Fig. 1), this low inhibitor concen-
tration was a good starting point to investigate its effect on GTP
binding as a function of GTP concentration. When the GTP
concentration was greater than 200 μM, 50 μM bis-ANS had little
effect on GTP binding (Fig. 9B). However, 50 μM bis-ANS inhib-
ited FtsZ assembly even when the GTP concentration was as
high as 2 mM (data not shown), suggesting that the bis-ANS-
mediated inhibition of FtsZ assembly is caused by noncompeti-
tive or uncompetitive inhibition of GTP binding.

Any competitive binding of GTP and bis-ANS to FtsZ would
be predicted to be most apparent at low GTP concentrations.
The bis-ANS concentration required to completely block the for-
mation of visible fluorescent FtsZ polymers decreased slightly,
from 36 to 30 μM, when the GTP concentration was reduced from 1 mM to 50 μM (data not shown). Therefore, it is

possible that at low GTP concentrations, competitive binding of
bis-ANS to the GTP binding site may have some role in the
inhibition of FtsZ assembly.

**DISCUSSION**

Recent direct and indirect evidence strongly suggests that
the FtsZ ring marks the plane of cytokinesis in all prokaryotic
cells, even chloroplasts (8, 28). Despite this apparently actin-
like role, FtsZ clearly has structural and biochemical similarity
to tubulin, including the ability to bind and hydrolyze GTP (9,
13, 29) and to self-assemble in vitro into protofilament bundles
that have dimensions similar to that of tubulin (11, 30) and
that have microtubule-like dynamic and morphological proper-
ties (18). Our aim is to establish a biochemical foundation for
FtsZ in order to clarify the differences and similarities between
FtsZ and tubulin. A deeper understanding of these key proteins
should help elucidate more about their evolutionary rela-
tionship and the precise function of FtsZ in prokaryotic cell divi-
sion. In addition, because tubulin is the target of such a wide
variety of inhibitors, FtsZ may also be a potentially good target
for antimicrobial compounds. Hence, understanding structural
differences between FtsZ and tubulin may eventually facilitate
design of FtsZ-based antimicrobials that are modeled on anti-
tubulin drugs.

In this paper, we report that common tubulin inhibitory
compounds did not inhibit FtsZ assembly but that the widely
used hydrophobic probe bis-ANS can both inhibit FtsZ assembly and also serve as a useful probe to measure FtsZ conformational changes. These findings are a first step in defining structural and functional differences between FtsZ and tubulin. One obvious difference between the two proteins is the C-terminal domain, both in primary sequence and in predicted secondary structure (14). This difference may be responsible for the different Ca\(^{2+}\) effects, because a C-terminal truncation of tubulin is Ca\(^{2+}\)-resistant (31, 32). Interestingly, when FtsZ and tubulins are aligned, it can be seen that FtsZ is missing the region in Neurospora crassa tubulin that contains the mutation for benomyl resistance (33). This could explain why colchicine, colcemid, and benomyl have no effect on FtsZ polymerization.

The similar inhibition of FtsZ and tubulin assembly by bis-ANS suggests that bis-ANS interacts similarly with both proteins. The titrations of FtsZ with bis-ANS and vice versa, using the same methods that were previously applied to tubulin, suggest that FtsZ has a high affinity bis-ANS binding site and multiple low affinity binding sites, with \(K_d\) values similar to those of tubulin. This analysis implies that the hydrophobic surface properties of FtsZ and tubulin are similar. The inhibition of bis-ANS binding by GTP binding, and vice versa, suggests that GTP binding sites and bis-ANS binding sites overlap. Because bis-ANS binds selectively to protein hydrophobic surfaces, this result provides evidence that the GTP binding site is hydrophobic, in support of a previous proposal based on epitope mapping (27). At low GTP concentrations, competition for GTP binding by bis-ANS could play a role in its inhibition of FtsZ assembly. Because the inhibition of GTP binding by bis-ANS can be overcome by increasing GTP concentration, whereas the inhibition of FtsZ assembly cannot, it is likely that noncompetitive or uncompetitive binding by bis-ANS is also responsible for its inhibitory effect.

Hydrophobic interactions have been implicated in tubulin assembly, and our evidence is consistent with an analogous role for such interactions in FtsZ assembly. The fact that Ca\(^{2+}\) increases bis-ANS and ANS binding to FtsZ strongly suggests that Ca\(^{2+}\) induces FtsZ conformational changes. Based on our results, we propose that bis-ANS binding inhibits FtsZ assembly by blocking FtsZ intermolecular hydrophobic interactions. We further propose that Ca\(^{2+}\) binding may induce stronger intermolecular hydrophobic interactions that result in the stimulation of FtsZ assembly. This idea is in accord with the GTP-dependent stimulation of FtsZ assembly by 7–20 mM Ca\(^{2+}\) and the GTP-independent aggregation of FtsZ at higher concentrations of Ca\(^{2+}\) (18). Such changes in hydrophobic properties, therefore, could be independent of GTP or could influence or be influenced by GTP binding. For example, GTP binding has been shown to influence the ability of tubulin to interact with hydrophobic substrates (34).

It is likely that changes in hydrophobic surface properties of FtsZ are involved in the interaction between FtsZ and its natural protein inhibitors MinC and SulA. There is good evidence that SulA interacts directly with FtsZ, and it was proposed that this interaction prevents a GTP-induced conformational change that normally leads to polymerization (35). This is consistent with the dispersal throughout the ftsZ gene of mutations that result in SulA resistance and the variable effects of these mutations on GTP binding and hydrolysis (36). These findings are completely consistent with the idea proposed here that hydrophobic interactions drive FtsZ polymerization. In fact, it is tempting to speculate that the inhibition mechanisms of bis-ANS and SulA may be similar. Future in-depth comparisons of bis-ANS and SulA inhibition of FtsZ assembly, as well as investigation of the effects of bis-ANS on SulA-resistant FtsZ proteins, should prove fruitful in understanding the molecular details underlying FtsZ assembly.

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