Detection of an Intermediate during Unfolding of Bacterial Cell Division Protein FtsZ

LOSS OF FUNCTIONAL PROPERTIES PRECEDES THE GLOBAL UNFOLDING OF FtsZ

Received for publication, February 6, 2003, and in revised form, March 17, 2003
Published, JBC Papers in Press, March 20, 2003, DOI 10.1074/jbc.M301303200

Manas K. Santra‡ and Dulal Panda§
From the School of Biosciences and Bioengineering, Indian Institute of Technology, Bombay, Powai, Mumbai 400 076, India

Using environment-sensitive fluorescence of 1-anilinonaphthalene-8-sulfonic acid, polarization of fluorescein 5′-isothiocyanate-labeled FtsZ, and far-UV circular dichroism spectroscopy, the chemical unfolding of FtsZ was found to proceed through two steps. The first step of the urea-induced unfolding produced an intermediate, which then unfolded at higher concentrations of urea. The intermediate state contains native-like secondary structure and much less tertiary structure compared with the native state. It is distinct from the native state as well as from the unfolded state. Similar to urea-induced unfolding of FtsZ, thermal unfolding of FtsZ also occurs in two steps. The midpoints for the first and second thermal unfolding transitions were found to be 38 ± 4 and 77 ± 5 °C, respectively. Further, the functional properties of FtsZ are extremely sensitive to urea, guanidium chloride, and sodium dodecyl sulfate. For example, 50% inhibition of the FtsZ assembly and GTP hydrolysis occurred at 0.1 and 0.2 M of urea, respectively. FtsZ lost its functional properties before any significant perturbation in the secondary or tertiary structure was detected by using several fluorescence techniques and far UV-CD indicating preferential local unfolding of the functional region(s). In addition, the unfolded FtsZ regains its ability to polymerize fully upon removal of urea. The data taken together suggest that FtsZ unfolds reversibly through a multistep process, and local responses that inhibit functional properties precede the global transition of FtsZ to the unfolded state.

Equilibrium unfolding studies of globular proteins have provided significant understanding of a large number of local and long-range interactions that are essential for protein folding and stability (1, 2). Protein unfolding reactions have been shown to proceed through a variety of mechanisms (3, 4). The simplest mechanism is that of a two-state transition, i.e. a reaction that proceeds directly from the folded state to the unfolded state without the occurrence of any detectable intermediates. However, recent studies have demonstrated that the unfolding and unfolding processes of several proteins involve multiple steps that are associated with one or more productive/nonproductive intermediates (5–7). Several studies have led to insights into the conformational properties of partially folded intermediates and rate-limiting transition states (5–8). These partially folded intermediates are thought to be the early kinetic intermediates in the folding pathways of polypeptides. One of the well-characterized intermediates is a molten globule state, which contains native-like secondary structure but has less compact tertiary structure with an increase in hydrophobic surface (8–13). The molten globule state is induced in several proteins by mild denaturants, changing pH, ionic strength, and temperature (9–13).

FtsZ, a monomeric protein of single polypeptide chain of 383 residues, is a key cytoskeletal protein in bacteria that plays a major role in septum formation during bacterial cell division (14, 15). FtsZ is considered as a prokaryotic homolog of the eukaryotic cytoskeletal protein tubulin (16). Like tubulin, FtsZ displays GTPase activity and polymerizes in a GTP-dependent manner (17–24). FtsZ and tubulin have similar GTP binding motifs and the T7 loop, which is thought to be involved in the binding and hydrolysis of GTP, is conserved in both proteins (23, 24). In addition, hydrophobic interactions play a major role during polymerization of both FtsZ and tubulin (25–27). Further, monosodium glutamate induces polymerization of FtsZ and tubulin in a similar manner (28, 29). However, FtsZ and tubulin differ in many ways; for example, promoters of tubulin polymerization such as dimethyl sulfoxide, glycerol, and taxol fail to promote FtsZ polymerization (28–30). In addition, several drugs such as vinblastine and colchicine bind to tubulin and inhibit microtubule polymerization but most anti-tubulin agents do not bind to FtsZ or affect its polymerization (25, 26).

FtsZ has been overexpressed in bacteria in the soluble form in large quantities. However, overexpression of soluble tubulin has not yet been achieved. Several studies have indicated that the folding and unfolding mechanism of tubulin is an extremely complex process (31–35). Most recently, Andreu et al. (35) reported guanidium chloride-induced unfolding of FtsZ from Methanococcus jannaschii and Escherichia coli. This elegant study showed that FtsZ and tubulin display differential folding/unfolding behaviors. They used circular dichroism and the release of nucleotide to monitor unfolding and found that FtsZ released its bound nucleotide at lower denaturant concentrations where the effect of guanidium chloride on the circular dichroism spectra was not significant. In their work, the formation of an intermediate state during guanidium chloride-induced unfolding of FtsZ was not studied. In many proteins, although an intermediate forms during the unfolding process, the intermediate is often not detected due to the lack of an appropriate probe. It is believed that the detection of an intermediate under equilibrium conditions helps in understanding the mechanisms of protein unfolding and folding. In this study, we wanted to examine whether an intermediate is formed.

‡ Supported by a fellowship from the Council of Scientific and Industrial Research, Government of India.
§ To whom correspondence should be addressed. Tel.: 91-22-2572-2545 (ext. 7838); Fax: 91-22-2572-3480; E-mail: panda@btc.iitb.ac.in.

* This work was supported by a grant (to D. P.) from the Dept. of Science and Technology, Government of India. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is available on line at http://www.jbc.org
during the unfolding process of FtsZ. Intrinsic tryptophan residues of proteins have been routinely used as reporter groups for examining unfolding and folding processes. However, E. coli FtsZ does not contain a tryptophan residue that could be used to identify an intermediate during the unfolding process. To circumvent this problem, a fluorescent group was introduced in FtsZ by covalently modifying FtsZ with fluorescein 5'-isothiocyanate (FITC).1 With the idea that the covalently bound FITC will serve the role of an intrinsic protein reporter, we investigated the unfolding pathways of FtsZ in the presence of urea. The functional properties of FtsZ, such as polymerization of FtsZ, are extremely sensitive to low concentrations of denaturants. A variety of fluorescence techniques and circular dichroism spectroscopy were used to investigate the unfolding pathways of FtsZ in the presence of urea and at elevated temperatures. Urea-induced unfolding of FtsZ follows a two-step unfolding pathway; an intermediate is formed during the first unfolding transition in the presence of low concentrations of urea that unfolds completely during the second unfolding step at high concentrations of urea. Further, extremely low concentrations of urea inhibited polymerization and hydrolysis of GTP without significantly perturbing the secondary and tertiary structures of FtsZ, indicating that the loss of functional properties occurs prior to formation of the intermediate.

EXPERIMENTAL PROCEDURES

Materials—ANS was purchased from Molecular Probes. FITC, piperazin-1, 4-bis (2-ethanesulfonic acid) (Pipes), EDTA, GTP, guanidium chloride, sodium dodecyl sulfate (SDS), and urea were purchased from Sigma. DE-52 was purchased from Whatman International Ltd. All other reagents used were of analytical grade.

Protein Purification—Recombinant E. coli FtsZ protein was purified from E. coli BL21 strain using DE-52 ion exchange chromatography followed by a cycle of monosodium glutamate induced polymerization and depolymerization as described in our recent report (28). The purity of FtsZ was found to be >98% by analyzing a Coomassie Blue-stained SDS-PAGE of the protein (data not shown). The protein concentration was determined by the method of Bradford using bovine serum albumin as a standard. The protein was frozen and stored at -80 °C.

Spectroscopic Methods—The unfolding processes of FtsZ under different conditions were examined using several spectroscopic techniques. Fluorescence spectroscopic studies were performed using a JASCO FP-6500 spectrophotometer equipped with a constant temperature water-circulating bath. An appropriate blank spectrum was subtracted from the respective spectrum. The fluorescence spectra were measured using 1 μM FtsZ. The excitation and emission bandwidths were fixed at 5-nm each and a quartz cuvette of 0.3-cm pathlength was used for all measurements to reduce the inner filter effect. For the experiments involving ANS binding to FtsZ, 360 nm was used as the excitation wavelength and the emission spectrum was recorded over the range of 420–550 nm.

Circular dichroism studies were performed in a JASCO J810 spectropolarimeter equipped with a Peltier temperature controller. The secondary structure was monitored over the wavelength range of 200–260 nm using a 0.1-cm path length cuvette and the ellipticity was determined at 220 nm. A spectral bandwidth of 10 nm and time constant of 1 s were used for all measurements. Each spectrum was recorded using an average of 5 scans.

Preparation of Denaturant Solutions—Urea solutions were made in 50 mM sodium phosphate buffer pH 6.8. For determining the effects of urea, SDS, and guanidium chloride on FtsZ assembly and GTP hydrolysis, the desired concentrations of urea, SDS, and guanidium chloride were prepared in 50 mM Pipes buffer at pH 6.8. Finally, the desired pH of the urea solutions was achieved by adding HCl. Fresh urea solutions were used for each unfolding experiment.

1 The abbreviations used are: FITC, fluorescein 5'-isothiocyanate; ANS, 1-anilinonaphthalene-8-sulfonic acid; DE-52, diethylaminoethyl cellulose; Pipes, piperazine-1,4-bis(2-ethanesulfonic acid).
Detection of an Intermediate and Stepwise Unfolding of FtsZ

RESULTS

FtsZ Unfolding by Monitoring ANS Fluorescence—Tryptophan fluorescence is highly environment sensitive and the presence of tryptophan residues in a protein provides a convenient tool for analyzing the protein folding mechanism. However, FtsZ does not contain a tryptophan residue. Therefore, we used both covalent and noncovalent extrinsic probes for analyzing the unfolding processes of FtsZ. The environment-sensitive noncovalent probe ANS has been used extensively to analyze hydrophobic surface arrangements in proteins during unfolding and folding processes (8, 31, 32). Recently, ANS has been shown to bind to FtsZ (25). The binding of ANS to FtsZ increased ANS fluorescence 7-fold, and this increase occurred along with a significant blue shift (36 nm) of the emission maximum from 512 to 476 nm (data not shown). FtsZ (1 μM) was incubated with different concentrations of urea (0.1–8 M) for 30 min at 25°C in 50 mM sodium phosphate buffer, pH 6.8. Then, 50 μM ANS was added to the mixtures, which were then incubated for an additional 30 min at 25°C. All spectra were corrected by subtracting the appropriate blank (spectra containing 50 μM ANS in the presence of different concentrations of urea in the absence of FtsZ) from the original spectra. The excitation wavelength was 360 nm. Data were averages of five independent experiments. Error bars represent S.D.

was quenched at the desired time by addition of 10% (v/v) 7 M perchloric acid. The quenched reaction mixtures were then centrifuged for 5 min at 25°C to remove aggregated proteins. 15 μl of the supernatants were incubated with freshly prepared 900-μl solutions of filtered malachite green solution (0.045% malachite green, 4.2% ammonium molybdate, and 0.02% Triton X-100) at room temperature for 30 min, and the phosphate ions produced were determined by measuring the absorbance at 650 nm. The reaction was normalized including a control in the absence of FtsZ. A phosphate standard curve was prepared using sodium phosphate.

Detection of an Intermediate and Stepwise Unfolding of FtsZ

Fig. 1. Urea-induced unfolding of FtsZ was probed using FtsZ-bound ANS fluorescence intensity at 475 nm (○) and emission maximum (●). FtsZ (1 μM) was incubated with different concentrations of urea (0.1–8 M) for 30 min at 25°C in 50 mM sodium phosphate buffer, pH 6.8. Then, 50 μM ANS was added to the mixtures, which were then incubated for an additional 30 min at 25°C. All spectra were corrected by subtracting the appropriate blank (spectra containing 50 μM ANS in the presence of different concentrations of urea in the absence of FtsZ) from the original spectra. The excitation wavelength was 360 nm. Data were averages of five independent experiments. Error bars represent S.D.

The FtsZ-ANS complex showed an emission maximum at 476 nm in the absence of urea, and the emission maximum increased minimally (3 nm) in the presence of 1 μM urea (Fig. 1). However, the emission maxima increased sharply beyond 1.25 μM urea. For example, the emission maximum was 512 nm in the presence of 3 μM urea and the half-maximal increase in the emission maxima occurred at 1.5 μM urea. The red shift of the ANS-FtsZ spectra in the presence of increasing concentrations of urea indicated that ANS binding decreases with increasing urea concentration. We have also determined the effects of low concentrations of urea on the dissociation constant for the ANS and FtsZ interaction. Urea increased the dissociation constant of the ANS and FtsZ interaction in a concentration-dependent manner. For example, the dissociation constant was found to be 24 ± 7 μM in the absence of urea and 36 ± 7 μM, 43 ± 4 μM, and 57 ± 9 μM in the presence of 0.1, 0.25, and 0.5 μM urea, respectively. The modest increase in the dissociation constant for the ANS-FtsZ interaction indicated that low concentrations of urea induce a local conformational change in the protein, which causes a reduction in ANS binding.

Further, we determined whether low concentrations of urea could induce aggregation of FtsZ by monitoring 90° light scattering at 500 nm as described under “Experimental Procedures.” The light scattering intensity did not change detectably in the absence and presence of 0.1, 0.25, and 0.5 μM urea suggesting that low concentrations of urea do not induce aggregation of FtsZ (data not shown). Thus, the initial decrease in ANS fluorescence was not due to aggregation of FtsZ monomers but due to a reduction in the ANS-FtsZ binding interaction.

Unfolding of FtsZ by Monitoring FITC Polarization—We labeled lysine residues in FtsZ by FITC as described under “Experimental Procedures.” Under the conditions used, the incorporation ratio of FITC per FtsZ monomer was 0.6 ± 0.2. The unfolding equilibrium of FtsZ was monitored using polarization of covalently labeled fluorescent FtsZ (FITC-FtsZ) because the covalently labeled fluorescent probe could provide information, which is similar to intrinsic probes like tryptophan and different from the noncovalent hydrophobic probes such as ANS. Fig. 2 shows a polarization profile of the FITC-FtsZ in the presence of different concentrations of urea. The progressive decrease of polarization occurred in two stages with a plateau like region at 0.5 μM urea. The first transition was completed at 0.5 μM urea with a 29% decrease in the polarization value. There was only minimal decrease (−8%) in the polarization of FITC-FtsZ from 0.5 μM urea to 1.75 μM urea indicating that an intermediate was formed. A similar trend was also observed when the FtsZ-ANS fluorescence was used to monitor the urea-induced unfolding of FtsZ (Fig. 1). The second transition started at 1.75 μM urea and the polarization values decreased sharply with further increasing urea concentrations with half-maximal decrease in polarization occurring at 2.5 μM urea. A limiting polarization value for FITC-FtsZ was achieved at 4 μM urea.

Structural Transition as Monitored by Ellipticity Change—The secondary structural changes of FtsZ were followed by monitoring the far-UV circular dichroism spectra of FtsZ as a function of urea concentration (Fig. 3A). The variation of CD (mdeg) at 220 nm as a function of urea concentration is shown in the Fig. 3B. The secondary structure content of FtsZ increased minimally (−4%) in the presence of 0.5 μM urea compared with FtsZ in the absence of urea, and no change in the secondary structure of FtsZ was detected between 0.5 and 1 μM urea.
urea. Beyond 1.5 M urea, the CD values at 220 nm of FtsZ decreased strikingly with increasing urea concentration and the half-maximal decrease in the ellipticity occurred at 2.1 M urea. The secondary structure was completely lost at 4 M urea. Interestingly, even 0.5 M urea decreased the fluorescence intensity of FtsZ-ANS and the polarization of FITC-FtsZ by 35 and 29% respectively, suggesting that considerable loss of tertiary structure occurred at a low concentration of urea (Figs. 1 and 2). Further, the results obtained using both noncovalent and covalent fluorescence probes showed that the first unfolding transition was completed at 1 M urea (Figs. 1 and 2). In contrast to the loss of tertiary structure at low concentrations of urea, no significant change in the far UV-CD structure of FtsZ was detected up to 1 M urea (Fig. 3). Thus, the results demonstrate that the loss in the tertiary structure of FtsZ precede the loss of the secondary structure during the initial stages of urea-induced unfolding.

Does Low Concentration of Urea Perturb the Ability of FtsZ to Polymerize and Hydrolyze GTP?—We wanted to know whether urea could inhibit the functional properties of FtsZ in a concentration range in which it does not affect the secondary structure of FtsZ but does perturb the hydrophobic interactions. FtsZ (6 μM) was incubated with different concentrations of urea (0–0.5 M) for 30 min, and the calcium-induced polymerization of FtsZ was measured by monitoring light scattering at 500 nm (Fig. 4A). Urea inhibited FtsZ polymerization in a concentration-dependent manner (Fig. 4A, inset). Half-maximal inhibition occurred at 0.1 M urea, and ~90% inhibition was observed at 0.25 M urea. In addition, we have examined the effects of low concentrations (25–200 μM) of SDS and guanidium chloride (0.05–0.5 M) on the polymerization of FtsZ (Fig. 4B). Low concentrations of SDS reduced the rate and extent of FtsZ polymerization. For example, 25 μM SDS reduced the extent of polymerization by ~50%, and 100 μM SDS completely inhibited FtsZ polymerization. Similarly, low concentrations of guanidium chloride also exerted strong inhibitory effects on FtsZ polymerization. For example, 50% inhibition of FtsZ polymerization occurred in the presence of 0.1 M guanidium chloride (Fig. 4C). These results indicate that the region responsible for polymerization is exposed to the solvent and is extremely susceptible to denaturation. Interestingly, no changes in the ANS fluorescence and CD spectra of FtsZ were observed at the half-maximal polymerization inhibitory concentrations of the three denaturants (data not shown).

The polymerization of FtsZ requires hydrolysis of GTP and the ability to hydrolyze GTP is considered as one of the important functional characteristics of FtsZ (17–22). Urea (0–1 M) strongly suppressed the GTPase activity of FtsZ (Fig. 5). The results taken together demonstrated that functional properties of FtsZ were lost prior to the loss of its secondary or tertiary structure.

Polymerization of Refolded FtsZ—FtsZ (30 μM) was unfolded by incubating with 4 M urea for 1 h. The unfolded protein did...
not polymerize in the presence of 10 mM Ca\(^{2+}\) and 1 mM GTP.

The refolding of FtsZ was initiated by diluting the unfolded protein solution 20 times; the dilution reduced the concentration of urea to 0.2 M and the remaining urea was slowly removed by dialysis against Pipes buffer. The dialyzed protein solution was concentrated using Amicon concentrators. The concentrated protein was again diluted 3-fold to reduce the residual urea concentration. The polymerization of the refolded FtsZ (6 \(\mu\)M) was initiated by adding 10 mM Ca\(^{2+}\) and 1 mM GTP. The refolded protein showed similar polymerization ability as the native protein that was subjected to similar treatment in the absence of urea (Fig. 6). In addition, refolded FtsZ was found to completely regain its ability to bind to ANS after removal of urea (data not shown). The results of these refolding experiments suggest that the urea-induced unfolding of FtsZ is fully reversible.

**Thermal Denaturation of FtsZ as Monitored by Ellipticity Change**—We wanted to know the effects of increasing temperature on the secondary and tertiary structures of FtsZ. First, temperature induced unfolding of FtsZ was monitored by following the changes in far UV-CD spectra of FtsZ with increasing temperature as described under “Experimental Procedures” (Fig. 7A). The CD values at 220 nm did not change up to 30°C; however, it started decreasing beyond 30°C and reached a limiting value at 99°C (Fig. 7B). The thermal unfolding pattern of FtsZ followed a three state unfolding profile. The midpoint of the first transition \((T_M)\) was determined to be 38 ±
$4 \, ^\circ \text{C}$ and the $T_M$ for the second unfolding transition was determined to be $77 \pm 5 \, ^\circ \text{C}$ (Fig. 7B).

**Thermal Denaturation of FtsZ as Monitored by ANS Fluorescence**—Hydrophobic interactions play a major role in the protein folding and unfolding processes, and thermal energy may perturb the hydrophobic surface arrangements of a protein. Therefore, it is important to know how thermal energy changes the hydrophobic surface arrangement of FtsZ. The thermal denaturation of FtsZ was monitored using the hydrophobic probe ANS and the FtsZ-ANS fluorescence changed with increasing temperature in a complex manner (Fig. 8). Initially, the FtsZ-ANS fluorescence was found to increase by $19 \pm 3\%$ when the temperature was increased from $20$ to $30 \, ^\circ \text{C}$. However, increasing the temperature beyond $40 \, ^\circ \text{C}$ sharply reduced the FtsZ-ANS fluorescence and a limiting value was reached at $80 \, ^\circ \text{C}$. The $T_M$ for this transition was determined to be $48 \pm 3 \, ^\circ \text{C}$.

**DISCUSSION**

In this study, the thermal and chemical unfolding processes of FtsZ were found to proceed through multiple transitions. The experiments involving the noncovalent probe ANS (Fig. 1) and the covalent probe FITC (Fig. 2) showed that the urea-induced unfolding reaction of FtsZ occurred through a three-state transition pathway that involves the formation of an intermediate. The first unfolding transition was completed within $1 \, \text{mM}$ urea, and an intermediate was formed between $0.5 \, \text{mM}$ and $1.5 \, \text{mM}$ urea, which unfolded at higher concentrations of urea. Interestingly, the secondary structure of FtsZ was not altered up to $1.5 \, \text{mM}$ urea indicating that the urea-induced unfolding of FtsZ produces an intermediate, which has a native-like secondary structure (Fig. 3). Further, the polymerization of FtsZ was found to be extremely sensitive to denaturants. For example, the half-maximal inhibition of FtsZ polymerization occurred at $25 \, \mu\text{M}$ SDS, $0.1 \, \text{mM}$ guanidinium chloride, or $0.1 \, \text{mM}$ urea (Fig. 4). However, no significant change in the fluorescence intensity of FtsZ-ANS complex or molar ellipticity at $220 \, \text{nm}$ was observed under these conditions. The dissociation constant for the FtsZ-ANS interaction increased modestly in the presence of low concentrations of urea indicating subtle structural changes occurring in the protein under these conditions. Further, low concentrations of urea strongly suppressed the GTPase activity of FtsZ (Fig. 5). The effective concentration for the suppression of GTP hydrolysis paralleled those required for inhibition of FtsZ polymerization, with inhibition of GTP hydrolysis requiring somewhat higher urea concentrations than the inhibition of polymerization indicating that the polymerization reaction is relatively more sensitive to urea than the GTP hydrolysis. Taken together, the data demonstrated that the functional properties of FtsZ were lost in the presence of low concentrations of urea prior to the formation of the intermediate.

Hydrogen bonds and hydrophobic interactions play significant roles in determining the conformation and stability of the folded and unfolded structures of a protein (43). Further, these forces are also thought to dominate the non-covalent mutual interactions between two protein molecules. For example, hydrophobic interactions are considered to play major roles during FtsZ polymerization (24, 25). Urea ($0.5 \, \text{mM}$) reduced the
Detection of an Intermediate and Stepwise Unfolding of FtsZ

Fig. 8. Thermal unfolding of FtsZ was monitored by ANS fluorescence. First, FtsZ-ANS complex was formed by incubating FtsZ (1 μM) with 50 μM of ANS for 30 min at 20 °C. The FtsZ-ANS complex was heated in continuous fashion at the rate of 0.5 °C per min, and fluorescence was recorded at different temperatures after incubating the sample for 10 min. The excitation and emission wavelengths used were 360 and 475 nm, respectively.

ANS-FtsZ fluorescence by 35% and increased the dissociation constant of the ANS-FtsZ interaction by 2.4-fold suggesting that the hydrophobic surface interactions in FtsZ were perturbed under these mild denaturing conditions. Importantly, low concentrations (0.025–0.5 M) of urea exerted strong inhibitory effects on FtsZ polymerization and the polymerization ability of FtsZ was completely lost at 0.5 M urea. These findings are consistent with the idea that local hydrophobic interactions play a critical role during FtsZ polymerization. We suggest that urea perturbs monomer/monomer interfaces of FtsZ that inhibits the addition of new monomers by inducing conformational change in the monomeric FtsZ.

Urea at low concentrations strongly inhibited FtsZ polymerization and GTP hydrolysis without significantly perturbing FtsZ-ANS fluorescence, polarization of FITC-FtsZ, or the far UV-CD spectra, demonstrating that the urea-induced loss of functional properties of FtsZ preceded the global unfolding of the protein. The preferential loss of the functional properties of FtsZ was complete at 0.5 M urea. These findings are consistent with the idea that local hydrophobic interactions play a critical role during FtsZ polymerization. We suggest that urea perturbs monomer/monomer interfaces of FtsZ that inhibits the addition of new monomers by inducing conformational change in the monomeric FtsZ.

Urea at low concentrations strongly inhibited FtsZ polymerization and GTP hydrolysis without significantly perturbing FtsZ-ANS fluorescence, polarization of FITC-FtsZ, or the far UV-CD spectra, demonstrating that the urea-induced loss of functional properties of FtsZ preceded the global unfolding of the protein. The preferential loss of the functional properties of FtsZ in the presence of low concentrations of urea indicates that a functionally critical region of FtsZ such the GTP binding site is highly accessible to solvent and that this region is preferentially unfolded compared with other regions of FtsZ in the presence of low concentrations of urea. To test whether the GTP binding sequence is exposed to solvent, the average hydropathy of the GTP binding motif suggests that the GTP binding site supports the idea that the GTP binding site of FtsZ is exposed to the solvent. We suggest that the functionally critical region of FtsZ, which is highly accessible to denaturants, is the GTP binding motif.

The far-UV CD analysis of thermal unfolding of FtsZ showed that the temperature induced unfolding occurred in two steps with the formation of an intermediate (Fig. 7B). The midpoints for the first and second transitions were calculated to be 38 ± 4 °C and 77 ± 5 °C, respectively. The CD values at 220 nm of FtsZ were almost completely lost during urea-induced unfolding whereas −40% of the CD values at 220 nm were lost during temperature-induced unfolding. The reason for the differential residual structures obtained during chemical and thermal unfolding of FtsZ is not clear. The higher residual secondary structure in thermal unfolding may be due to hydrophobic clustering at higher temperatures. Further, the ANS-FtsZ fluorescence was reduced by 55% at 90 °C (Fig. 8) whereas it was reduced by 80% during urea-induced unfolding (Fig. 1) indicating that the hydrophobic patches were relatively more sensitive to urea than to temperature.

The polarization of FITC-FtsZ revealed that the mobility of FtsZ was considerably increased at low concentrations of urea indicating that the loosening of tertiary structure of FtsZ in the presence of low concentrations (≤0.5 M) of urea. Interestingly, the polarization of FITC-FtsZ did not change significantly between 0.5 and 1.75 M urea indicating that there was no appreciable increase in the mobility of the probe in this concentration range. However, the polarization of FITC-FtsZ reached a limiting value at 4 M urea. The polarization data suggested that the urea-induced unfolding of FtsZ proceeds from its native to unfolded state through an intermediate state with significantly decreased tertiary structure; however, the intermediate has almost native like secondary structure. Thus, the characteristics of the intermediate were somewhat similar to a molten globule like intermediate state. It has been thought that a protein will be semiflexible in nature at the molten globule state, which will increase the hydrophobicity of the protein surface by exposing the internal nonpolar groups to water. In support of the idea, several studies have demonstrated that the molten globule state of a protein binds to hydrophobic nonpolar molecules such as ANS more strongly than the native state of the protein (8, 47). However, the intermediate formed during urea-induced unfolding of FtsZ binds to ANS weakly compared with the native protein suggesting that the intermediate does not satisfy the increase of hydrophobic surface property of the molten globule state. Thus, although the intermediate is different from both the native and unfolded states of FtsZ, and the intermediate has a native like secondary structure with considerably less tertiary structure, the intermediate may not be considered as a molten globule state of the protein. Further

Fig. 9. The hydrophobicity plot of FtsZ was constructed according to the method of Kyte and Doolittle (44). The graph was generated using a window size of 6 amino acid residues.
Detection of an Intermediate and Stepwise Unfolding of FtsZ

studies will be required to describe the nature of the intermediate state.

We used mild denaturing conditions to examine the linkage between FtsZ unfolding and the loss of its functional properties. The half-maximal inhibition of calcium-induced FtsZ polymerization occurred at 0.1 M urea, 0.1 M guanidine chloride, and 25 μM SDS. It is possible that the low concentrations of denaturants inhibit FtsZ polymerization by increasing the solubility of the monomeric protein by a hydrophobic solvent effect. However, the critical concentration of micelle formation for SDS is nearly 830 μM (48), which is 33-fold higher than the concentration requires for inhibiting FtsZ assembly by 50%. In addition, low concentrations of urea (<0.5 M) decrease the polarization of FTTC-FtsZ (Fig. 2) and increase the dissociation constant of the ANS and FtsZ interaction, suggesting that local conformational change occurs in FtsZ under mild denaturing conditions.

FtsZ polymerization is not a nonspecific aggregation reaction. FtsZ polymerizes into filaments or rings and the FtsZ monomers are in equilibrium with its polymers (20–25) in contrast to tubulin, FtsZ completely regains its polymerization ability after removal of denaturants indicating that FtsZ folds productively in the absence of chaperones (34, 35). In contrast to tubulin, FtsZ completely regains its polymerization ability after removal of urea suggesting that FtsZ folds productively in the absence of chaperones (Fig. 6). These findings could possibly explain why overexpression of FtsZ in the soluble form has been successful in bacteria whereas overexpression of soluble tubulin has not yet been successful. Tubulin (31) and FtsZ both lose their functional properties before any significant perturbation in the secondary or tertiary structure is detected (Figs. 1–5). The loss of functional properties of FtsZ could be attributed to the preferential unfolding of the functional region, which is likely to be highly exposed to the solvent and the local unfolding that inhibits functional properties precede the global unfolding of FtsZ.

Acknowledgments—We thank Dr. H. P. Erickson for providing the FtsZ clone. We thank Drs. L. Wilson, A. K. Lala, T. R. S. Prasanna, D. Dasgupta, and S. Pathare for critical reading of the manuscript. We thank A. Banerjee for help and encouragement during the work.

REFERENCES

1. Baldwin, R. L. (2002) Science 295, 1657–1658
2. Klein-Soetharaman, J., Okawa, M., Grimsaw, S. B., Wirner, J., Dachhardt, E., Ueda, T., Imoto, T., Smith, L. J., Dobson, C. M., and Schwalbe, H. (2002) Science 295, 1719–1722
3. Daggett, V., and Fersht, A. R. (2003) Trends Biochem. Sci. 28, 18–25
4. Clarke, A. R., and Walillo, J. P. (1997)Curr. Opin. Biotechnol. 8, 400–410
5. Panda, M., Gorovits, B. M., and Horwitz, P. M. (2000) J. Biol. Chem. 275, 65–70
6. Nolting, B., Galhik, R., and Fersht, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10668–10672
7. Plaxco, K. W., and Dobson, C. M. (1996) Curr. Opin. Struct. Biol. 6, 630–636
8. Pitsyn, O. B. (1995) Adv. Protein. Chem. 47, 85–229
9. Kwajima, K. (1989) Proteins 6, 87–103
10. Pitsyn, O. B., Pain, R. H., Semisotnov, G. V., Zerovnik, E., and Razgulyaev, O. I. (1990) FEBS Lett. 262, 20–24
11. Ewbank, J. J., and Creighton, T. E. (1991) Nature 350, 518–520
12. Goto, Y., Takahashi, N., and Fink, A. L. (1990) Biochemistry 29, 3480–3488
13. Goto, Y., Calciano, L. J., and Fink, A. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 573–577
14. Yi, Q. M., and Lutkenhaus, J. (1985) Gene (Amst.) 36, 241–247
15. Bi, E., and Lutkenhaus, J. (1990) J. Bacteriol. 172, 2765–2768
16. van den End F., Amos, L., and Lowe, J. (2001) Curr. Opin. Microbiol. 4, 634–638
17. Nogales, E., Downing, K. H., Amos, L., and Lowe, J. (1996) Nat. Struct. Biol. 5, 451–458
18. de Boer, P., Crossley, R., and Rothfield, L. (1992) Nature 359, 254–256
19. Scheffers, D. J., and Driessen, A. J. (2000) Mol. Microbiol. 43, 1517–1521
20. Yu, X. C., and Margolin, W. (1997) EMBO J. 16, 5455–5462
21. Mukherjee, A., and Lutkenhaus, J. (1998) EMBO J. 17, 462–469
22. Mukherjee, A., and Lutkenhaus, J. (1994) J. Bacteriol. 176, 2754–2758
23. Dass, J. F., Kralicek, A., Mingeranzo, J., Palacios, J., Vicente, M., and Andreu, J. M. (2001) J. Biol. Chem. 276, 17307–17315
24. Scheffers, D. J., de Wit, J. G., den Blauwewen, T., and Driessen, A. J. (2002) Biochemistry 41, 521–529
25. Yu, X. C., and Margolin, W. (1998) J. Biol. Chem. 273, 10216–10222
26. Yu, X. C., Margolin, W., Gonzalez-Garay, M. L., and Cabral, F. (1999) J. Cell Sci. 121, 2301–2311
27. Prasad, A. R., Ludaeva, R. F., and Horwitz, P. M. (1986) Biochemistry 25, 739–742
28. Beuria, T. K., Krishnakumar, S. S., Sahar, S., Singh, N., Gupta, K., Meshrum, M., and Panda, D. (2002) J. Biol. Chem. 278, 3735–3741
29. D’Amato, R. J., Lin, C. M., Flynn, E., Folkman, J., and Havel, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3964–3968
30. Erickson, H. P., Taylor, D. W., Taylor, K. A., and Bramhill, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 519–523
31. Sackett, D. L., Bhattacharyya, B., and Wolff, J. (1994) Biochemistry 33, 12686–12678
32. Guda, S., and Bhattacharyya, B. (1995) Biochemistry 34, 6925–6931
33. Wolff, J., Knipling, L., and Sackett, D. L. (1996) Biochemistry 35, 5910–5920
34. Guda, S., and Bhattacharyya, B. (1997) Biochemistry 36, 12828–12833
35. Andrade, J. M., Oliveira, M. A., and Monasterio, O. (2002) J. Biol. Chem. 277, 43262–43270
36. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
37. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy 2nd Ed., pp. 291–320, Kluwer Academic/Plenum Publishers, New York
38. Panda, D., Roy, S., and Bhattacharyya, B. (1992) Biochemistry 31, 9709–9716
39. Gupta, K., and Dalal, P. (2002) Biochemistry 41, 13029–13038
40. Ward, J. L., and Edelman, G. M. (1971), Methods Enzymol. 117, 599–625
41. Mukherjee, A., and Lutkenhaus, J. (1999) J. Bacteriol. 181, 823–832
42. Geladopoulos, T. P., Sotiroudis, T. G., and Evangelopoulos, A. E. (1991) Anal. Biochem. 192, 112–116
43. Dill, K. A. (1990) Biochemistry 29, 7133–7155
44. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
45. Lowe, J., and Amos, L. (1998) Nature 39, 203–206
46. Lowe, J. (1998) J. Struct. Biol. 124, 235–243
47. Lala, A. K., and Kaul, P. (1995) J. Biol. Chem. 270, 19914–19918
48. Sia, S., Currell, D., Mazumder, S., and Mitra, S. (2002) Biophys. Chem. 98, 267–273

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
