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Two distinct intermediates of trigger factor are populated during guanidine denaturation

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Abstract

Trigger factor (TF) is an important catalyst of nascent peptide folding and possesses both peptidyl–prolyl *cis–trans* isomerase (PPIase) and chaperone activities. TF has a modular structure, containing three domains with distinct structural and functional properties. The guanidine hydrochloride (GuHCl) induced unfolding of TF was investigated by monitoring Trp fluorescence, far-UV CD, second-derivative UV absorption, enzymatic and chaperone activities, chemical crosslinking and binding of the hydrophobic dye, 1-anilinonaphthalene-8-sulfonate (ANS); and was compared to the urea induced unfolding. The native state of TF was found to bind ANS in 1:1 stoichiometry with a K_d of 84 μM. A native-like state, N′, is stable around 0.5 M GuHCl, and shows increased ANS binding, while retaining PPIase activity and most secondary and tertiary structure, but loses chaperone and dimerization activities, consistent with slight conformational rearrangement. A compact denatured state, I, is populated around 1.0 M GuHCl, is inactive and does not show significant binding to ANS. The data suggest that TF unfolds in a stepwise manner, consistent with its modular structure. The ability of TF to undergo structural rearrangement to maintain enzymatic activity while reducing chaperone and dimerization abilities may be related to the physiological function of TF.

Keywords: Chaperone; Folding intermediate; Pre-molten globule; Prolyl isomerase; Protein folding; Trigger factor

1. Introduction

Escherichia coli trigger factor (TF) is an important chaperone in nascent peptide folding [1–4]. TF is a peptidyl–prolyl *cis–trans* isomerase (PPIase) [1,3] belonging to the FKBP family [5–7]. Consistent with its chaperone function, TF has been shown to have a preference for random coil or loosely structured substrates [4,8,9] and an apparent antichaperone phenomenon has been observed [10]. The extraordinary efficiency of TF as a folding catalyst has been attributed to its modular structure and its ability to bind substrates with high-affinity [4,5,11]. Limited proteolysis indicates that TF contains three distinct domains from N-terminal to C-terminal, defined as the N domain (1–145), M domain (146–261) and C domain (262–432) [11,12]. The N-terminal domain of *E. coli* TF is important for ribosome binding [13] and the M domain carries the PPIase activity [12,14]. The isolated M domain fragment retains PPIase activity towards small substrate peptides [12,14], with a preference for hydrophobic or aromatic residues [15]. However, both the terminal domains are required for efficient binding of protein substrates [4]. The residues or regions directly involved in high-affinity substrate binding have not yet been identified.

Understanding the determinants of protein folding remains a major challenge in molecular biology [16]. Determining the mechanism of protein folding requires structural characterization of the native and unfolded states as well as any partially folded states formed during folding. Multi-domain and multi-subunit proteins are generally observed to fold by stepwise or parallel folding pathways, via one or more partially folded intermediates [17]. In some cases, such intermediates are stable under the conditions of equilibrium experiments, which facilitates study of their structural properties [18–20]. The multi-domain structure of TF makes it an interesting and challenging candidate for folding studies.

Abbreviations: ANS, 1-anilininonaphthalene-8-sulfonate; DSS, disuccinimidyl suberate; GuHCl, guanidine hydrochloride; MG, molten globule; PPIase, peptidyl–prolyl *cis/trans* isomerase; TF, trigger factor.

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Urea denaturation of TF and its fragments suggests that the three structural domains may fold relatively independently [11]. However, no intermediates have previously been characterized. In order to further understand the relationship between structure, function and folding for TF, we investigated its guanidine hydrochloride (GuHCl) induced unfolding and refolding by multiple structural probes. We identified and characterized two distinct intermediates that are populated during equilibrium denaturation in GuHCl. Their structural properties and possible physiological significance are discussed.

2. Materials and methods

2.1. Materials

GuHCl and urea (ultra pure) were from Fluka; chymotrypsin (TLCK treated), bovine serum albumin (fraction V), chicken egg albumin, *Micrococus lysodeikticus* dried cells and 1-anilininonaphthalene-8-sulfonate (ANS) were from Sigma; reduced and oxidized glutathione were from Amresco; hen egg white lysozyme was from Serva; disuccinimidyl suberate (DSS) was from Pierce. Other reagents were local products of analytical grade. Double-deionized water was used throughout. Solutions were made volumetrically.

TF was purified following the method of Stoller et al. [1] and a value for $\epsilon_{280\ nm}$ of 15,930 M^{-1} cm^{-1} (0.3317 (mg ml)$^{-1}$ cm^{-1}) was calculated using the procedure of Gill and von Hippel [21]. PPIase activity of TF was assayed by the chymotrypsin-coupled method [22] using the tetrapeptide (succinyl-Ala-Ala-Pro-Phe-4-nitroanilide; Peptide Institute Inc., Japan) as substrate. Chymotrypsin remains functional over the GuHCl concentration range used here for activity measurements (0–2 M) [23].

2.2. Equilibrium measurements

All experiments were carried out at 20 °C in 0.1 M Tris–HCl pH 7.8. GuHCl or urea induced equilibrium denaturation experiments were performed as described [24] after 18 h incubation of protein in buffer containing different concentrations of denaturant. Equilibrium refolding was performed by diluting unfolded protein (12 h in 3.0 M GuHCl or 6 M urea, 0.1 M Tris–HCl, pH 7.8 at 20 °C) to the same conditions as described for unfolding experiments. Spectroscopic measurements were performed as described [25] or as outlined below.

2.3. Intrinsic fluorescence

Equilibrium measurements were monitored using a Hitachi F4500 fluorescence spectrophotometer. Intrinsic Trp fluorescence emission spectra were recorded between 300 and 400 nm with excitation at 295 nm in a 1 cm pathlength cell. Both excitation and emission slits were set to 5 nm.

2.4. ANS binding

Equilibrium experiments were performed as described above, but excitation was set at 370 nm and emission spectra were recorded from 400 to 600 nm. Binding fluorescence was monitored at 490 nm. The stoichiometry of binding of ANS to TF was determined using Scatchard analysis as described [16].

2.5. Far-UV circular dichroism

The CD signal at 222 nm was measured with a Jasco J-720 spectropolarimeter. Equilibrium measurements were performed in a 0.1 cm cell.

2.6. Second-derivative UV absorption

TF contains a single Trp and eight Tyr residues: five in the N-terminal domain, one in the M domain and two in the C-terminal domain. A significant change in the UV absorbance difference spectrum is observed on unfolding of TF, implying that some or all of these residues are buried in the native state and are exposed on unfolding, making this a useful parameter to measure global changes in protein conformation [25,26]. Spectra were measured between 270 and 300 nm on a Beckman DU-7500 UV/VIS spectrophotometer. To obtain the second-derivative difference spectra, the spectrum of the native enzyme was subtracted from that of the denatured enzyme for each concentration of GuHCl. The height difference between the peak at 288 nm and the trough at 284 nm was measured. The results of independent processing of the same data, or of separate experiments measured under the same conditions, were highly reproducible. The protein concentration used was 1.5 mg ml^{-1}.

2.7. Analysis of equilibrium data

Activity data were fitted to a two-state model as described [24]. Where two transitions were observed, the curves were analyzed using a three-state model, $N \leftrightarrow I \leftrightarrow U$, where N, I, and U are native, intermediate, and unfolded states, respectively, and the fractions, f, of the respective species are given as $f_N + f_I + f_U = 1$ [24 and references therein]. As no discernable effect of TF concentration on the denaturation transition was observed (see Results), the simplest (i.e. monomeric) unfolding model was used.

2.8. Chaperone activity

Preparation and refolding of reduced and denatured lysozyme was performed as described previously [10]. Lysozyme at 20 mg ml^{-1} was completely reduced and denatured by incubation at room temperature for 4 h in 0.1 M sodium phosphate buffer, pH 8.0, containing 8 M GuHCl and 150 mM dithiothreitol. The reaction mixture was adjusted to pH 2.0 with 6 M HCl, and then dialyzed at 4 °C, first against

10 mM HCl and then against 100 mM acetic acid. The 200 μM reduced and denatured lysozyme was divided into aliquots and stored at –20 °C. Refolding of denatured lysozyme was achieved by dilution into 0.1 M phosphate buffer pH 7.5, containing 2 mM EDTA, 1 mM oxidized glutathione, 2 mM reduced glutathione and TF (or BSA or Ovalbumin) equilibrated in different concentrations of GuHCl. The final concentration of lysozyme in the refolding assay was 10 μM. Recovery of lysozyme activity was complete 5 h after dilution, and monitoring for a further 24 h detected no further change. Activity of lysozyme was determined at 30 °C by following the lysis of *M. lysodeikticus* dried cells [27,28]. The decrease in A_{450} of a 0.25 mg ml^{-1} cell suspension in 67 mM sodium phosphate buffer pH 6.2, containing a final concentration of 100 mM GuHCl was measured in a Shimadzu UV-1601 spectrophotometer. The yield of activity recovered was defined as a percentage of the activity of native lysozyme measured under the same conditions.

2.9. Crosslinking and electrophoresis

TF at different concentrations was crosslinked at 20 °C using 6 mM DSS in 15 mM sodium phosphate buffer pH 7.5, containing different concentrations of GuHCl or NaCl. After 30 min, the crosslinking reaction was quenched with 150 mM Tris–HCl pH 7.0. Cross-linking with glutaraldehyde was carried out in the same way as for DSS, except that the concentration of glutaraldehyde was 0.08% and the crosslinking time was 20 min.

The products of crosslinking were analyzed by gradient SDS-PAGE and visualized by Coomassie Brilliant Blue staining. The relative population of uncrosslinked and crosslinked TF was analyzed using the gel analysis software Totallab™ V1.01 (Nonlinear Dynamics Ltd.), in order to obtain the fraction of crosslinked product at each given TF concentration, $f_{crosslinked}$. The results were then analyzed as follows. TF exists in an equilibrium of monomer and dimer in solution [29], and so:

$$K_d = [M]^2/[D] \quad (1)$$

where K_d is the dissociation constant, while [M] and [D] represent the concentrations of monomer and dimer, respectively. The total TF concentration can be expressed as:

$$C = [M] + 2 \times [D] \quad (2)$$

Combining Eqs. (1) and (2), Eq. (3) can be obtained:

$$[D] = \frac{4 \times C - \sqrt{K_d^2 + 8 \times C \times K_d} + K_d}{8} \quad (3)$$

Where the crosslinking reaction does not proceed to completion, the efficiency of the crosslinking reaction, r, can be expressed as:

$$r = [D]_{crosslinked}/[D]_{total} \quad (4)$$

The fraction of crosslinked TF, $f_{crosslinked}$, can then be expressed as:

$$f_{crosslinked} = 2 \times [D]_{crosslinked}/C = 2 \times r \times [D]/C = \left(1 - \frac{\sqrt{K_d^2 + 8 \times C \times K_d} - K_d}{4 \times C}\right) \times r \quad (5)$$

By fitting the experimental data to Eq. (5), the values for K_d and r can be obtained.

3. Results

3.1. GuHCl-induced unfolding of TF

As shown in Figs. 1 and 2, the GuHCl-induced equilibrium unfolding and refolding of *E. coli* TF was monitored by five different structural probes: far-UV circular dichroism (CD) at 222 nm; second-derivative UV absorbance; intrinsic fluorescence of Trp 151; PPIase activity towards a tetrapeptide substrate; and binding of the hydrophobic dye, ANS. In each case, unfolding and refolding curves were superimposable for a given probe, demonstrating that GuHCl denaturation of TF is fully reversible. The data were fitted to a two or three-state model, as appropriate (see Section 2) and the thermodynamic parameters obtained are displayed in Table 1.

Far-UV CD, a probe of secondary structure, and second-derivative UV absorbance, a probe of global changes in protein conformation, show approximately superimposable denaturation transition curves (Fig. 1A, B). There is an initial baseline region, then a sharp transition beginning at around 0.6 M GuHCl, which is followed by a more gradual transition, before the protein becomes fully-denatured above 2.0 M GuHCl. Trp fluorescence (Fig. 1C) shows a similar unfolding profile with a clearly biphasic transition, although the initial baseline and transition region shows changes at lower GuHCl concentration than is observed by far-UV CD. In each case, the biphasic curves fit well to a three-state model in which a partially unfolded intermediate, I, is maximally populated at 0.9–1.0 M GuHCl. Comparison with the changes in PPIase activity (Fig. 1D) and ANS binding (Fig. 2A) suggest that I is inactive and has little capacity to bind ANS. The intermediate I appears to be both energetically (Table 1) and structurally (Table 2) closer to the fully-denatured state than the native state and thus may best be described as a compact denatured state. The second, more gradual transition is less marked in urea denaturation (Ref. [11] and Fig. 3C). It is, therefore, possible that population of the compact denatured state, I, is favored by the combined effects of GuHCl as a denaturant and as a salt.

3.2. Identification of a native-like state, N′

While far-UV CD and second-derivative UV absorbance do not detect any significant structural changes below 0.6 M

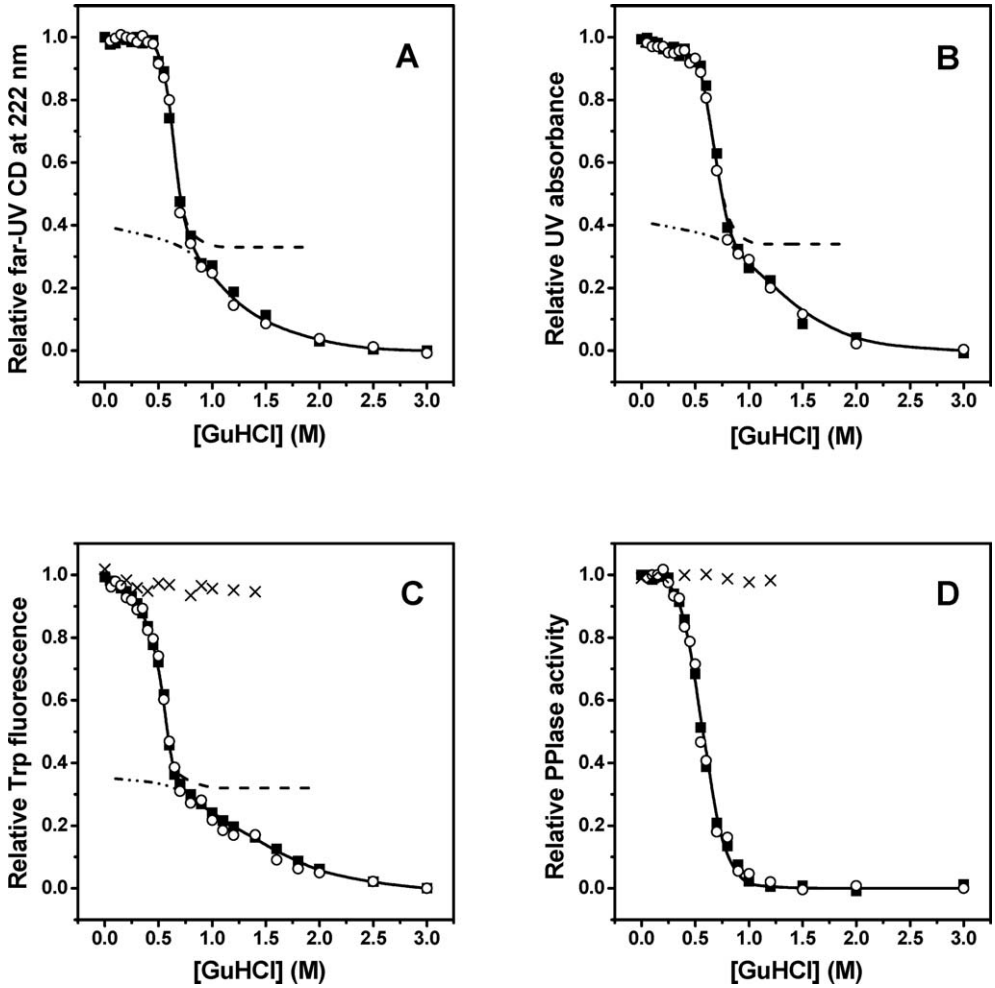

Fig. 1. GuHCl induced equilibrium unfolding (filled square) and refolding (empty circle) of TF. The fit to a three-state (A–C) or two-state (D) model is shown (solid line). Deconvolution of the first transition (dashed line) and second transition (dashed–dotted line) observed by the spectroscopic probes is also shown (A–C). (A) Far-UV CD at 222 nm. (B) Second-derivative UV absorbance. (C) Intrinsic fluorescence of Trp151 monitored at 327 nm after excitation at 295 nm. (D) PPIase activity. (C, D) The effect of equivalent concentrations of NaCl (crosses) to GuHCl is also shown.

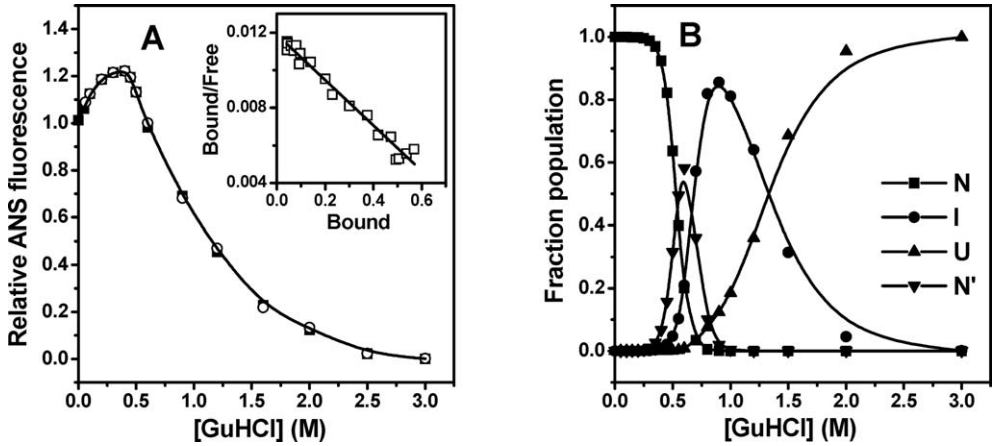

Fig. 2. Identification of a native-like intermediate, N′. (A) ANS binding fluorescence monitored at 490 nm after excitation at 370 nm. The insert shows a Scatchard plot for binding of ANS to native TF. (B) Fraction population of a native-like intermediate (N′), which accounts for the apparent difference in the fraction population of I observed by Trp fluorescence (Fig. 1C) compared to far-UV CD (Fig. 1A). N′ represents the non-coincidence in the N to I transition when measured by different structural probes (see text).

Table 1
Thermodynamic parameters for GuHCl denaturation of TF

Parameter / Method	ΔG_{IN} (kJ mol^{-1})	ΔG_{UI} (kJ mol^{-1})	ΔG_{UN} (kJ mol^{-1})	m_{IN} (kJ mol^{-1} M^{-1})	m_{UI} (kJ mol M^{-1})	$[D]_{1/2, IN}$ (M)	$[D]_{1/2, UI}$ (M)
Trp fluorescence	–24.9 ± 3.1	–7.2 ± 2.9	–32.1 ± 4.2	46.3 ± 5.1	5.8 ± 1.6	0.54	1.24
CD 222 nm	–30.3 ± 3.1	–8.8 ± 6.4	–39.1 ± 7.1	47.3 ± 5.1	8.6 ± 3.8	0.64	1.02
Second-derivative UV absorbance	–27.8 ± 3.0	–14.6 ± 7.5	–42.4 ± 8.1	41.0 ± 4.6	11.0 ± 4.8	0.68	1.33
PPIase activity [a]	–13.8 ± 1.1 [a]			24.7 ± 1.9 [a]		0.56 [a]	

ΔG is the free energy of unfolding extrapolated to zero denaturant concentration, m is the denaturant dependence (or slope), and $[D]_{1/2}$ is the denaturant mid-point of the transition. N, I and U indicate native, intermediate and fully-unfolded states, respectively. For the three-state unfolding model, $\Delta G_{IN} + \Delta G_{UI} = \Delta G_{UN}$ (see also Section 2).
[a] Fitted to a two-state model.

GuHCl, intrinsic fluorescence (Fig. 1C), PPIase activity (Fig. 1D) and ANS binding (Fig. 2A) all suggest that slight structural changes may occur at lower GuHCl concentrations. Intrinsic fluorescence and PPIase activity both show a sharp transition between 0.3 and 0.6 M GuHCl. Control experiments with NaCl demonstrate that the non-coincidence with other optical probes is not simply due to a salt effect on the Trp solvent environment, protein compactness or substrate binding (Fig. 1C, D). The ANS binding fluorescence was observed to increase at low GuHCl concentration, peaking at around 0.5–0.6 M GuHCl (Fig. 2A), suggesting population of an intermediate in this region. Loss of ANS binding is concomitant with loss of secondary and tertiary structure, as detected by far-UV CD and second-derivative UV absorbance. In general, a lack of coincidence between different structural probes suggests population of an intermediate within the non-coincident region [24]. The difference in population of the intermediate, I, calculated from the far-UV CD data (Fig. 1A) and the Trp fluorescence data (Fig. 1C), is plotted in Fig. 2B, and labeled as N′. This peak (N′), which represents the non-coincidence between different structural probes, coincides with the position of the peak observed by ANS binding. This then suggests that the changes in Trp fluorescence at low GuHCl concentration reflect the same conformation changes that lead to increased ANS binding. Taken together, these observations are consistent with the non-coincidence observed for different structural probes in urea denaturation [11], and suggest population of an additional conformational state in 0.5–0.6 M GuHCl with properties distinct from both the native state and the compact denatured state I. The structural and spectroscopic properties of N′ are summarized in Table 2. The lack of structural changes below 0.6 M GuHCl detected by far-UV CD and second-derivative UV absorbance, together with the high residual PPIase activity under these conditions, suggests that N′ is highly native like, and the structural rearrangement involved may be localized in the immediate environment of the single Trp residue, Trp151.

3.3. Scatchard analysis of ANS binding

To investigate further the structural properties of N′, we investigated ANS binding under native conditions and in 0.5 M GuHCl. The results are shown in Fig. 2A and Table 2. Scatchard analysis of ANS binding to native TF indicates a dissociation constant, K_d, of 84 (± 1) μM and a stoichiometry of 0.98 (± 0.04) ANS per TF molecule. In the presence of 0.5 M GuHCl, the dissociation constant is the same within error, but the stoichiometry is increased (Table 2). Under these conditions, a mixture of species are likely to be present and their relative proportions can only be estimated. The maximum emission wavelength of ANS binding fluorescence showed no change between 0 and 0.5 M GuHCl (data not shown). These results imply that the observed increase in ANS binding fluorescence on addition of low concentrations of GuHCl is due to an increase in the available exposed hydrophobic surface, rather than a change in the nature of the binding site(s) [30]. Thus the native-like intermediate, N′, which is maximally populated in 0.5 M GuHCl, shows an increase in hydrophobic binding area compared to the native protein.

Table 2
Comparison of structural properties of different conformation states of TF

Conformational state	GuHCl (M)	Secondary structure [a] (%)	Tyr/Trp exposure [b] (%)	Trp Fluorescence		ANS fluorescence		Activity (%)		
				Intensity (%)	λ_{max} (nm)	K_d (μM)	Binding number	PPIase	Chaperone	Dimerization
N (native state)	0	100	0	100	336	84 ± 1	0.98 ± 0.04	100	100	100
N′ (native-like intermediate)	~0.5	~90	<15	~30	~341	87 ± 2	Increased	≥50	0	0
I (compact denatured state)	~0.9	~25	~65	~30	~344	–	–	0	0	0
U (unfolded state)	2.5	0	100	0	350	–	0	0	0	0

The values for ANS binding were measured in 0 or 0.5 M GuHCl, as indicated. The K_d and binding number in 0.5 M GuHCl thus reflects a mixture of species. Values for the other structural probes reflect the specific properties of the native (N), unfolded (U), or intermediate states (N′ and I), estimated by comparison with Fig. 2B, under conditions where the species is maximally populated, as indicated.
[a] Monitored by far-UV CD.
[b] Monitored by second-deriviative UV absorbance.

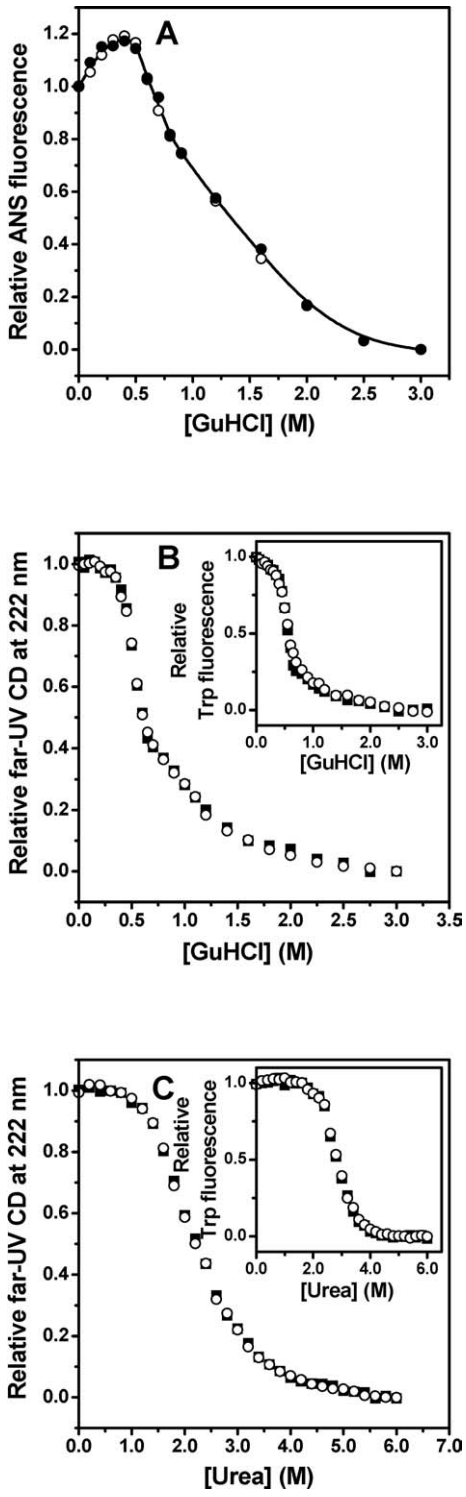

Fig. 3. Protein concentration dependence of the unfolding profile of TF. Fractional changes of GuHCl and urea induced equilibrium unfolding of different concentrations of TF were monitored in 100 mM Tris–HCl buffer, pH 7.8, at 20 °C. (A) ANS binding fluorescence. Concentrations of TF were 5 μM (filled symbols) and 50 μM (empty symbols). The ANS concentration was 100 μM. (B) Far-UV CD changes at 222 nm. Concentrations of TF were 5 μM (filled symbols) and 50 μM (empty symbols). Insert shows intrinsic fluorescence changes monitored at 327 nm after excitation at 295 nm. (C) Far-UV CD changes at 222 nm denatured by urea. Concentrations of TF were 4 μM (filled symbols) and 40 μM (empty symbols). Insert: intrinsic fluorescence changes monitored at 327 nm after excitation at 295 nm.

3.4. Concentration dependence of TF unfolding

A recent study has shown that TF exists in solution in a monomer–dimer equilibrium, with a dissociation constant of approximately 18 μM [29]. In general, the mid-point of a denaturation transition for an oligomeric protein should show protein concentration dependence, where that transition involves dissociation of the oligomer [31,32]. In order to establish whether population of the native-like intermediate, N′, is related to the monomer–dimer equilibrium of TF, we measured the effect of TF concentration on the GuHCl and urea induced denaturation profiles, monitored by intrinsic fluorescence, far-UV CD and ANS binding fluorescence. As shown in Fig. 3A–C, the unfolding transitions at low TF concentration (4–5 μM, in which about 25% of TF is dimeric) and high concentration (40–50 μM, in which about 65% of TF is dimeric) are superimposable. This implies either that the monitored structural changes do not involve dissociation of TF, or that these structural characteristics are identical for the monomer and dimer of TF. Therefore, the relationship between N′ and the monomer–dimer equilibrium of TF could not be determined from these experiments.

3.5. Effect of GuHCl on TF dimerization

As an independent approach, we performed DSS crosslinking to determine whether the N′ state of TF is able to dimerise. Under the experimental conditions used here, native TF can be crosslinked by DSS, and the extent of crosslinking increases with increasing TF concentration (Fig. 4A, D). The value of K_d obtained from these data (see Section 2) was 20 μM, which is in good agreement with the previously published value of 18 μM [29]. At a constant TF concentration, the fraction population of crosslinked TF was found to decrease with increasing GuHCl concentration, with a sharp transition over the range of 0.2–0.7 M GuHCl (Fig. 4B, E). Glutaraldehyde crosslinking gave the same result, except that the fraction population of crosslinked TF was higher (Fig. 4C, F). In contrast, NaCl has no effect on crosslinking (Fig. 4E, F). The GuHCl-induced decrease in TF dimerization is thus coincident with the change in the fraction population of the native state as N′ is formed (Fig. 2B). This suggests that only native TF forms a dimer in solution, whereas N′ is mainly present as a monomer.

3.6. Effect of GuHCl on TF-assisted lysozyme refolding

In order to discover whether the native-like intermediate, N′, retains the ability to assist protein folding, we investigated the effect of GuHCl concentration on the chaperone activity of TF, using reduced denatured lysozyme as a substrate. Reduced denatured lysozyme tends to aggregate at neutral pH and there is competition between productive folding and aggregation, resulting in a substantial reduction in the efficiency of refolding. However, the reactivation yield of 20 μM denatured lysozyme markedly increases with increas-

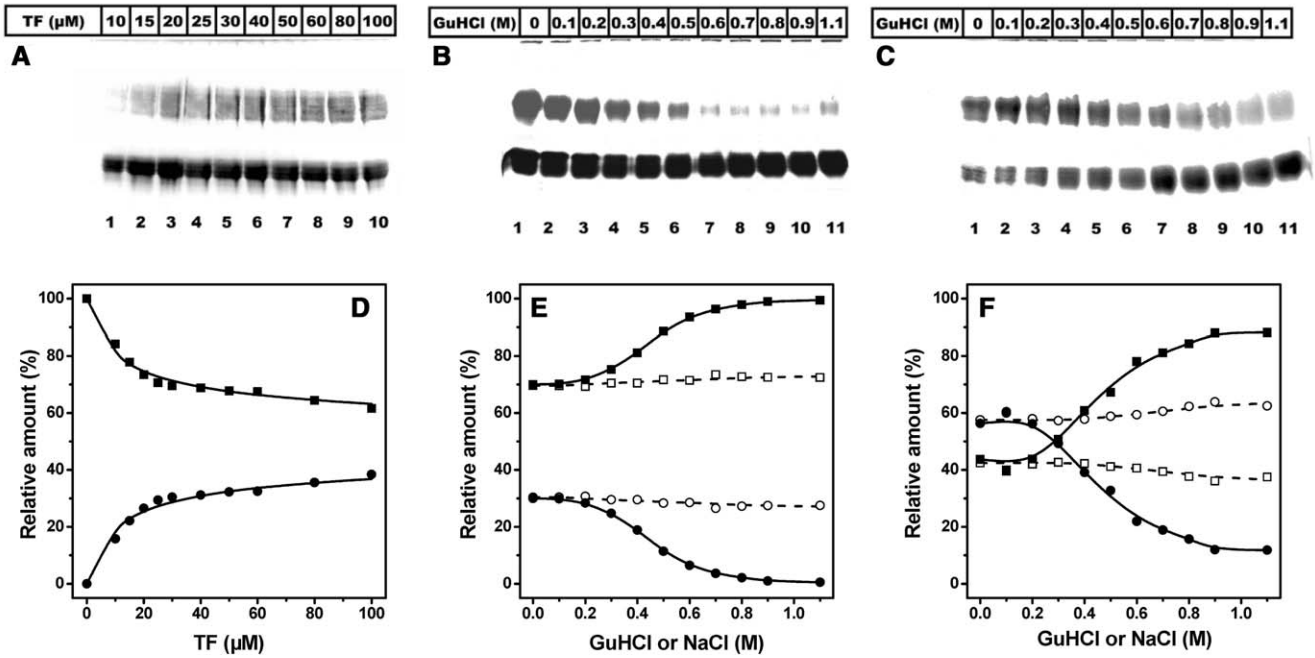

Fig. 4. Effect of GuHCl concentration on TF dimerization. TF was crosslinked using DSS (dissolved in DMSO) for 30 min (A, B) or glutaraldehyde (C) for 20 min in 15 mM sodium phosphate buffer, pH 7.8, at 20 °C. Proteins were separated by gradient SDS-PAGE (8–15%) and the relative amounts of uncrosslinked (squares) and crosslinked (circles) species were analyzed. (A, D) Crosslinking of different concentrations of TF, as indicated. (B, E), 40 μM TF was crosslinked using 6 mM DSS in the presence of different concentrations of GuHCl (filled symbols) or NaCl (empty symbols). (C, F) 40 μM TF was crosslinked using 0.08% glutaraldehyde in the presence of different concentrations of GuHCl (filled symbols) or NaCl (empty symbols).

ing concentration of TF, reaching a plateau at about 20% of native activity when the ratio of TF to lysozyme is greater than 1.5 [10] (Fig. 5 insert). We, therefore, investigated the effect of GuHCl on the chaperone activity of TF by monitoring the reactivation of reduced denatured lysozyme with increasing GuHCl concentration in the presence of a constant concentration of TF. As shown in Fig. 5, after excluding the contribution of denaturant to the reactivation yield of lysozyme refolding, the TF-assisted reactivation yield of lysozyme was clearly decreased in the presence of GuHCl. The sharp decrease observed between 0.2 and 0.7 M GuHCl correlates with the GuHCl-induced formation of N′. Bovine serum albumin or hen egg white albumin showed no effect on lysozyme reactivation over the range of 0–1 M GuHCl, indicating that this effect on refolding yield of lysozyme is not a result of non-specific protein-protein interactions in the refolding system. Previous studies have shown that lysozyme structure is not dramatically altered below 3 M GuHCl [33]. This suggests that the reduction in the TF-assisted reactivation yield of lysozyme in the presence of low concentrations of GuHCl is not due to denaturation of refolded lysozyme, but is due to reduced chaperone activity of TF. This loss of chaperone function of TF coincides with population of the intermediate state, N′, indicating that the structural integrity of TF is important for chaperone function.

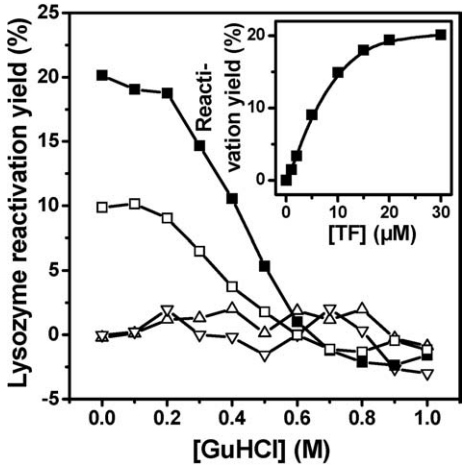

Fig. 5. Effect of GuHCl on TF-assisted lysozyme refolding. The reactivation yield was calculated from the difference in lysozyme activity in the presence of different concentrations of GuHCl and TF (20 μM, filled squares; 5 μM, empty squares), BSA (20 μM; up triangles) or chicken egg albumin (20 μM, down triangles). The insert shows the reactivation of 10 μM denatured reduced lysozyme in the presence of various amounts of TF.

4. Discussion

TF is a multi-domain, multi-functional molecular chaperone involved in many aspects of the quality control system in *E. coli*, such as nascent peptide folding [34–36], protein secretion [37], prevention of protein aggregation [38,39] and regulation of the activity of GroEL [40–42].

In this study we have applied a variety of structural probes to investigate the stability and folding of *E. coli* TF. We find that two intermediates, I and N′, are significantly populated during equilibrium denaturation in GuHCl, allowing their structural properties to be characterized.

Study of equilibrium and kinetic intermediates of a large number of proteins has lead to a view that multi-domain proteins fold by a hierarchical or step-wise process, involving ensembles of closely related structures for every detectable 'state' [16,17]. A commonly observed class of intermediate is termed a 'molten globule' (MG) state and is defined as having substantial secondary structure, a native-like tertiary fold, but lacking tight side-chain packing [18–20]. The loss of native side-chain packing is expected to render a MG state enzymatically inactive. The MG has hydrophobic surface that is accessible to solvent, making ANS binding a useful method of detection. The partially structured intermediate of TF, I, that is the predominant species in 1.0 M GuHCl, is clearly less structured than a MG. It may thus correspond to a 'pre-molten globule' or other compact denatured state, which, like the MG, are thought to be representative of denatured states formed under physiological conditions [16,18–20,43].

The native-like intermediate, N′, likewise does not fit the classic description of a MG. The estimated residual PPIase activity of N′ is at least 50% (Table 2), which is comparable to the 60–80% activity observed for M domain fragments [11]. Consistent with this, its secondary and tertiary structures are almost indistinguishable from the native state, except by intrinsic Trp fluorescence of Trp151, which is located in the hinge region between the N-terminal and M domains. From the recently published TF crystal structure [44,45] we can see that Trp151 is partially exposed on the surface and surrounded by Arg163, Thr150, Glu241, Arg243 and Glu242. No direct interaction of Trp151 with the N or C domains is apparent. However, a hinge-like movement of the M domain away from the N-terminal domain might result in increased exposure of Trp151, while maintaining the integrity of structure required for catalytic function.

As shown in Fig. 5, N′ has lost the ability to assist lysozyme refolding, indicating that the chaperone activity of TF is sensitive to structural fluctuations. Earlier studies have shown that chaperone function of TF results from the cooperation of all three domains [11], and the dimer and monomer of TF show different substrate binding properties [46,47]. In particular, the dimeric form of TF has the ability to stably bind and hold folding intermediates in a folding competent state until rescued by a folding chaperone such as the Hsp 70 system [47]. In this study we show that a minor environmental perturbation results in the loss of chaperone function, possibly involving a hinge-like movement of the domains, suggesting that the relative orientation of the TF domains is crucial for chaperone function. Similarly, dimerization of TF is also sensitive to this minor conformational rearrangement and the intermediate N′ is predominantly monomeric. This suggests that TF could cycle between dimeric, monomeric and native-like intermediate forms in cell, allowing modulation of its substrate binding and chaperoning properties (Scheme 1). The possible role of N′ in the substrate binding-release cycle, and the factors that control this process, is an area worthy of further investigation.

$$N_2 \leftrightarrow 2N \leftrightarrow 2N' \leftrightarrow (2I) \leftrightarrow 2U$$

Scheme 1. Reaction scheme for equilibrium denaturation of TF. The intermediate N′ is native-like in structure, predominantly monomeric, retains PPIase activity, but lacks chaperone activity. The intermediate I is largely denatured and is observed during GuHCl denaturation, but is not significantly populated during urea denaturation.

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