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Equilibrium Unfolding and Dynamic Refolding of GFPuv

Mechanism of TF Assisted GFPuv Folding

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Trigger Factor Assisted Folding of Green Fluorescent Protein†

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ABSTRACT: Guanidine induced equilibrium and kinetic folding of a variant of green fluorescent protein (F99S/M153T/V163A, GFPuv) was studied. Using manual mixing and stopped-flow techniques, we combined different probes, including tryptophan fluorescence, chromophore fluorescence and reactivity with DTNB, to trace the spontaneous and TF-assisted folding of guanidine denatured GFPuv. We found that both unfolding and refolding of GFPuv occurred in a stepwise manner and a stable intermediate was populated under equilibrium conditions. The thermodynamic parameters obtained show that the intermediate state of GFPuv is quite compact compared to the denatured state and most of the green fluorescence is retained in this state. By studying GFPuv folding assisted by TF and a number of TF mutants, we found that wild-type TF catalyzes proline isomerization and accelerates the folding rate at low TF concentrations, but retards GFPuv folding and decelerates the folding rate at high TF concentrations. This reflects the two activities of TF, as an enzyme and as a chaperone. A general mechanism of TF assisted protein folding is discussed.

Green fluorescent protein (GFP†), originally isolated from the jellyfish Aequorea victoria, is a single domain globular protein of 238 amino acids, which autocatalytically generates a covalently attached chromophore when correctly folded and therefore fluoresces without external cofactors. GFP is remarkable for its structural stability and high fluorescence quantum yield. GFP and its analogues have been used extensively in biological and medical research as a marker of gene expression and protein localization (1); as an efficient tracer in physiological investigations (2); as an indicator of protein–protein interactions (3) or a reporter of the folding of fusion proteins (4, 5); and even as a biosensor (6). GFP is a typical β-barrel protein. Its mutant, GFPuv or c3–GFP (F99S/M153T/V163A), is cylindrical in shape and comprises 11 β-strands with one α-helix inside and short helical segments on the ends of the cylinder (Figure 1). The cylinder has a diameter of about 30 Å and a length of about 40 Å. The highly protected chromophore (Ser65/Tyr66/Gly67) is located on the central helix within a couple of angstroms of the geometric center of the cylinder (7). Although 42-fold more fluorescent than wild-type GFP, GFPuv has the same spectral characteristics, topology and overall structure as wild type, as well as similar folding and hydrogen-exchange behavior (8).

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† Abbreviations: GFP, green fluorescent protein; GFPuv, the GFP mutant, F99S/M153T/V163A; TF, trigger factor; PPhase, peptide-prolyl cis/trans isomerase; Tris, tris(hydroxymethyl)aminomethane; DNase I, deoxyribonuclease I; GuHCl, guanidine hydrochloride; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EG, ethylene glycol; BSA, bovine serum albumin (fraction V); FRET, fluorescence resonance energy transfer.

FIGURE 1: Ribbon diagram of the backbone structure of the GFP mutant, GFPuv (Protein Data Bank code 1b9c), with the chromophore (Ser65/Tyr66/Gly67) and the side chains of other residues: Cys48, Trp57, Cys70 and Pro89 (cis-configuration), displayed in ball and stick style. The β-strands are numbered from the N to the C terminus. The figure was created with the program YASARA (http://www.yasara.org); also using YASARA, the secondary structure content was analyzed giving 2.7% α-helix, 54.0% β-sheet and 22.8% turn and 20.5% coil.

The folding efficiency of GFP is known to limit its use in a number of applications. To date, many studies have already been carried out on the folding and unfolding kinetics of GFP (7–10). For example, the Kuwajima group has investigated the folding of GFPuv in detail by acid denaturation (11, 12); and the Jackson group found that GFP significantly populates an intermediate state under equilibrium conditions during chemical denaturation (13). Recently, a superfolder GFP has been engineered and characterized (14). The effect of a number of molecular chaperones on the folding of GFP has also been studied, such as trigger factor (TF) from...
**MATERIALS AND METHODS**

of the two functions of TF, as an enzyme and as a chaperone. The number of TF mutants, demonstrates clearly the involvement in the unfolding and refolding of GFPuv are gradual processes of guanidine denatured GFPuv. The results revealed that both fluorescence, chromophore fluorescence and the reactivity to protein substrates with loose tertiary structure (prolyl isomerase but also a chaperone. TF binds preferentially synthesized nascent chains which are at an early stage in translation protein folding. Most ribosomes exist in a 1:1 complex with TF, consistent with the role of TF in cotranslational protein folding (24, 30). However, TF is present in a 2–3-fold molar excess over ribosomes in the cell, with the majority of free TF present as a dimer (31), and the dynamic monomer–dimer equilibrium of TF contributes to the regulation of the accessibility of its chaperone sites (32, 33). TF is an efficient molecular chaperone in the catalysis of protein folding reactions which are rate-limited by isomerization of prolyl bonds (34). In *vivo* studies of TF-assisted GAPDH (35), bovine carbonic anhydrase II (36) and lysozyme (37) folding have revealed that TF is not only a prolyl isomerase but also a chaperone. TF binds preferentially to protein substrates with loose tertiary structure (35), which is consistent with the ability of TF to bind to newly synthesized nascent chains which are at an early stage in the folding process *in vivo*.

Here, we combined different probes, including tryptophan fluorescence, chromophore fluorescence and the reactivity with DTNB, to trace the spontaneous and TF-assisted folding of guanidine denatured GFPuv. The results revealed that both the unfolding and refolding of GFPuv are gradual processes and a stable intermediate is populated under equilibrium conditions. The folding of GFPuv, assisted by TF and a number of TF mutants, demonstrates clearly the involvement of the two functions of TF, as an enzyme and as a chaperone.

**MATERIALS AND METHODS**

**Reagents.** GuHCl (ultrapure) and Tris were from Fluka. L(+)-Arabinose, DNase I, DTT, the sulphydryl reagent DTNB and BSA were all from Sigma. Other chemicals were local products of analytical grade. The concentration of GuHCl solutions was determined using an Abbe refractometer (Atago, Japan) and calculated according to the formula

\[
[\text{GuHCl}] = 57.147(\Delta N) + 38.68(\Delta N^2) - 91.60(\Delta N^3)
\]

where \(\Delta N\) is the refractive index of the solution measured at 589 nm and 20 °C (38).

**Methods.** All equilibrium spectral measurements and kinetic unfolding and refolding measurements of GFPuv were performed at 25 °C. Concentrations of proteins were determined by UV absorption using extinction coefficients at 280 nm, as follows: GFPuv (2.00 × 10^4 M⁻¹ cm⁻¹), TF (1.59 × 10^4 M⁻¹ cm⁻¹) and TF mutants, F233Y-TF (1.72 × 10^4 M⁻¹ cm⁻¹), F233Y C389 (1.72 × 10^4 M⁻¹ cm⁻¹), NM (1.34 × 10^4 M⁻¹ cm⁻¹), MC (1.00 × 10^4 M⁻¹ cm⁻¹), NC (0.90 × 10^4 M⁻¹ cm⁻¹), calculated using the procedure of Gill and von Hippel (39).

(1) **Protein Expression and Purification.** GFPuv was expressed in *E. coli* HB101 transformed with the pGLO plasmid (BioRad) and cultured at ≤30 °C. GFPuv overproduction was induced by L(+)-arabinose addition. The bacterial lysate in buffer A (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) was centrifuged at 20000g for 1 h. The green fluorescent supernatant was collected and treated with DNase I for about 10 min. Protein purification was carried out as follows: (a) Differential ammonium sulfate precipitation. We mixed one volume ratio of binding buffer (buffer B (4.0 M (NH₄)₂SO₄) in buffer A) gently and slowly into the translucent solution at room temperature. The turbid mixture was centrifuged and the supernatant was collected. (b) Hydrophobic interaction chromatography. The sample was loaded on a Butyl-sepharose 4 Fast Flow column (Amersham Biosciences) and washed sequentially with equilibrium buffer (buffer C (2.0 M (NH₄)₂SO₄) in buffer A) and wash buffer (buffer D (1.3 M (NH₄)₂SO₄ in buffer A). The above operations were carried out at room temperature. The green fluorescent fractions were then eluted using buffer A and concentrated by ultracentrifugation at 4 °C. The concentrated sample was purified further by (c) gel filtration using a Sephadex G-75 column (Amersham Biosciences) and (d) ion-exchange chromatography using DEAE-Sepharose Fast Flow resin (Amersham Biosciences) with a gradient elution of 0–0.2 M NaCl in buffer A. Finally, the GFPuv peak with visible green fluorescence was collected, concentrated and stored at −80 °C.

Plasmid pQE60 containing the wild-type *tig* gene that encodes *E. coli* TF was donated by Professor G. Fischer. TF and domain deletion mutants NM, MC, NC were constructed with a 6×His tag on their N-terminus and were purified by elution with imidazole from a chelating Sepharose Fast Flow Ni-column (Amersham Biosciences); while other mutants F233Y-TF (25) and F233Y C389 (40) were purified according to the published protocols.

The purity of all proteins was confirmed by SDS–PAGE and the ratio of the absorbance at 397 nm to that at 280 nm for GFPuv was higher than 1.1, which is an indicator of the purity of GFP (41).

(2) **Monitoring of the Dynamic Unfolding of GFPuv by the Ellman Reaction.** The determination of thiol groups was carried out using DTNB as described by Ellman (42) with a UV-4802 spectrophotometer (UNIC, Shanghai) at 25 °C. To make a stock solution of Ellman’s reagent, DTNB was dissolved in 50 mM Tris buffer pH 7.5 to give a final concentration of 10 mM and the pH was adjusted again to pH 7.5 by adding NaAc or NaHCO₃; the solution was stored at 4 °C. In order to measure the number of reactive —SH groups, both the reference and sample cells contained 0.1 mM DTNB in 50 mM Tris buffer pH 7.5 with 10 μM native or 6 M guanidine denatured GFPuv added to the sample cell. The number of reactive —SH groups was evaluated using a molar absorbance coefficient of 13,600 M⁻¹ cm⁻¹ at 412 nm. In order to follow the time course of the change in
exposure of –SH groups during GFPuv unfolding, an aliquot of 10 μM GFPuv was unfolded in 6 M guanidine and aliquots were removed at different time intervals, added to the reaction system containing 0.1 mM DTNB and allowed to react for 30 s. The absorbance of the same solution without GFPuv was recorded at 412 nm as the reference. The change of absorbance at 412 nm during GFPuv unfolding in the absence of DTNB was also monitored.

(3) Fluorescence Measurements. All experiments were carried out in 50 mM Tris-HCl pH 7.5 containing 100 mM NaCl, 1 mM EDTA and 5 mM (or 1 mM) DTT with or without GuHCl, unless otherwise stated. Since the unfolded states of GFPuv are denaturant concentration and incubation time dependent, the refolding experiments were started from a stock solution of GFPuv denatured in 6.0 M GuHCl for 16 h.

GuHCl-induced GFPuv (1 μM) equilibrium unfolding experiments were performed as described (38). After 24 h incubation, the emission intensities of the protein in buffer containing different concentrations of GuHCl were measured at different excitation wavelengths.

For those kinetic unfolding and refolding measurements which were performed after 5 s manual mixing on a HITACHI F-4500 fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan), the slit widths were set at 2.5 nm for both excitation and emission unless otherwise stated. All the manual mixing renaturation reactions were initiated upon 100-fold dilution from 6.0 M GuHCl into renaturation buffer with or without additives, such as BSA, TF, TF mutants, GuHCl or ethylene glycol (EG). For renaturation systems with different pH values, 50 mM Tris, phosphate or sodium acetate buffer was prepared to give final pH values of 7.5, pH 7.0–6.0 or pH 5.6, respectively.

For those rapid kinetic folding measurements which were performed on a π*-180 PiStar stopped-flow apparatus (Applied Photophysics, Surrey, U.K.), both of the slit widths were set at 5 nm and the dead time of the standard 20 μL optical cell is 1 ms. Unfolding reactions were initiated by mixing the native protein with 6.6 M GuHCl in a volume ratio of 1:10, and the final concentration of GuHCl was 6.0 M. For the refolding reactions, in order to obtain a high degree of dilution (about 52-fold) we used the instrument in sequential multimixing mode (1:25 followed by 1:1 dilutions per phase, respectively).

(4) Data Analysis. (a) Analysis of Equilibrium Data. All fluorescence data were fitted to a two or three-state model as described (38, 43 and references therein). When two transitions were observed, the curve was analyzed using a three-state model, N ↔ I ↔ U, where N, I, and U are native, intermediate and unfolded states, respectively, and, the fractions, f, of the respective species are given as fN + fI + fU = 1. Since GFPuv is monomeric under the experimental concentrations used here, the simplest unfolding model was used.

(b) Kinetic Analysis. The unfolding and refolding kinetic data were fitted using the program SigmaPlot2000 according to the following equations (nonlinear regression):

\[ A(t) = A_0 + \sum \Delta A_i \exp(-k_it) \]  

for unfolding, where \( A(t) \) is the fluorescence intensity at time \( t \) and \( A_0 \) is the initial value, and

\[ A(t) = A_0 + \sum \Delta A_i (1 - \exp(-k_it)) \]  

for refolding, where \( A(t) \) and \( A_0 \) are the fluorescence intensities at time \( t \) and the starting time, respectively; and \( \Delta A_i \) and \( k_i \) are the amplitude and the apparent rate constant of the \( i \)th phase, respectively.

RESULTS

GFPuv Unfolding Is a Gradual Process and an Intermediate Is Maximally Populated around 3.5 M GuHCl. Guanidine induced unfolding of GFPuv was monitored by changes in intrinsic fluorescence, chromophore fluorescence and the exposure of cysteine residues. GFPuv shows maximum fluorescence emission at 508 nm when excited at either 397 or 280 nm (Figure 2a).

Figure 2b shows the comparison of equilibrium unfolding curves monitored by different fluorescence probes. The transition curves monitored by the three different probes clearly do not coincide. The Trp fluorescence was observed to decrease at lower guanidine concentration than the chromophore fluorescence: after equilibration for 24 h in 2.0 M GuHCl, Trp57 is significantly exposed, while the environment of the chromophore (Ser65/Tyr66/Gly67) is apparently unaffected. Only when the GuHCl concentration was higher than 3.0 M, where Trp57 has already become totally exposed to the solvent, was there a conspicuous drop in the chromophore fluorescence. There is a single Trp residue (Trp57) and eleven Tyr residues distributed over the GFPuv molecule; one Tyr residue is an integral part of chromophore. When following the changes in chromophore fluorescence after excitation at 280 nm, fluorescence resonance energy transfer (FRET) between Trp57 and the Ser65/Tyr66/Gly67 chromophore was observed. The parameters obtained by fitting the data to a two- or three-state model are shown in Table 1. The changes in chromophore or tryptophan fluorescence, when monitored independently, show a highly co-operative two-state transition. However, combined monitoring of the two probes by the observed FRET signal allows fitting to a three-state model and shows that, under equilibrium conditions, an intermediate (I) is maximally populated at around 3.5 M GuHCl (Figure 2b).

This intermediate state, the microenvironment around the chromophore is disturbed only partially although Trp57 is already extensively exposed. This conclusion can be corroborated by the results of the Kuwajima group showing that the unfolding transition for GFPuv measured by far-UV CD coincides with the one measured by green fluorescence (8), which means that the change in emitted green fluorescence directly reflects the change in secondary structure of GFPuv. Recently, a similar intermediate state, which is compact and stable with respect to the unfolded state, was identified by the Jackson group who monitored changes in structure by green fluorescence, tyrosine fluorescence and far-UV circular
dichroism \((I3)\). Taken together, this indicates that the intermediate is close in stability to the native state and maintains nativelike secondary structure, thus it may best be described as a native-like intermediate.

We used the two fluorescent probes to further monitor changes in the microenvironments of Trp57 and the chromophore during kinetic unfolding of GFPuv (Figure 3). When GFPuv was exposed to 6.0 M GuHCl, the fluorescence intensity of Trp57 decreased rapidly and the process could be fitted to three kinetic phases with rate constants of 14.85 ± 0.43 \(\text{s}^{-1}\), 3.78 ± 0.19 \(\text{s}^{-1}\) and 0.46 ± 0.03 \(\text{s}^{-1}\) (Figure 3a). However, the change in chromophore fluorescence was extremely slow, with an unfolding rate constant of 5.24 \(\times\) \(10^{-4}\) \(\text{s}^{-1}\) (Figure 3c). The very slow unfolding rate of GFPuv even at high chemical denaturant concentrations suggests that there is a high kinetic energy barrier for unfolding (Figure 3c). However, 6.0 M GuHCl is sufficient to completely denature GFPuv (Figure 2b) and the free energy of unfolding (45.2 kJ/mol) is not excessively high for a protein of this size (Table 1).

There are two cysteine residues in GFPuv, Cys48 and Cys70, which do not form a disulfide bond and are located nearly at opposite ends of the cylinder (Figure 1). The two Cys residues lie between \(\beta\)-strands 3 and 4, with Cys48 located within the \(\beta\)-sheet and Cys70 in a turn. Cys48 may be partially solvent accessible while Cys70 is buried within the core of the protein. We found here that native GFPuv reacts with DTNB with a stoichiometry of about 0.5 DTNB per GFPuv molecule, which can be ascribed to the contribution of Cys48; while fully denatured GFPuv reacts with about 2.0 DTNB molecules per GFPuv molecule, consistent with full exposure of both cysteine residues on unfolding of GFPuv. The rate of unfolding of GFPuv in 6.0 M GuHCl obtained by monitoring the exposure of cysteine residues ((3.8 ± 0.2) \(\times\) \(10^{-4}\) \(\text{s}^{-1}\)) (Figure 4) is around 3-fold slower than the rate constant ((10.8 ± 0.4) \(\times\) \(10^{-4}\) \(\text{s}^{-1}\)) obtained by monitoring chromophore fluorescence at 10 \(\mu\)M GFPuv concentration. This suggests that the \(\beta\)-barrel structure is at least partly maintained after the chromophore fluorescence has already been quenched.

We therefore conclude that the unfolding of GFPuv is a stepwise process in which exposure of Trp57 occurs first, followed by exposure of the chromophore, and then finally the whole \(\beta\)-barrel backbone unfolds.

The Refolding of GFPuv Involves Rapid Burial of the Tryptophan Residue Followed by Slow Acquisition and Even Slower Adjustment of the Microenvironment around the Chromophore. The changes in tryptophan fluorescence and chromophore fluorescence during refolding of GFPuv were also compared (Figure 3). When 6.0 M GuHCl denatured GFPuv was diluted with buffer to give a final GuHCl concentration of 0.12 M by sequential multimixing in a stopped-flow instrument (see methods), a burst phase increase in tryptophan fluorescence was observed within the dead time of the stopped-flow apparatus, and this was followed by a biphasic fast increase in the fluorescence with rate constants of 2.04 \(\text{s}^{-1}\) and 10.8 \(\times\) \(10^{-4}\) \(\text{s}^{-1}\) (Figure 3b). However, the increase in chromophore fluorescence during refolding is relatively slow (Figure 3c). The refolding curve when monitoring chromophore fluorescence was biphasic, and fitting to a double exponential yielded the rate constants \(k_1 = 5.29 \times 10^{-2}\) \(\text{s}^{-1}\) and \(k_2 = 0.91 \times 10^{-2}\) \(\text{s}^{-1}\), suggesting that the microenvironment around the chromophore is formed and then undergoes further adjustment. After about 100 s, we could observe a gradual quenching of the tryptophan fluorescence as the chromophore fluorescence was regained, suggesting a slow adjustment of the specific tertiary structure in the vicinity of the chromophore (Figure 3d). This slow adjustment could also be related to prolyl isomerization within the GFPuv molecule \((I1)\). Although in both cases a burst phase is observed, the change of tryptophan fluorescence of GFPuv refolding after guanidine denaturation (Figure 3b) is quite different from that after acid denaturation observed by Kuwajima group \((I1)\), in which the tryptophan fluorescence increased first and then decreased accompanying GFPuv refolding. The difference may come from the different denaturation methods used since fluorescence is an extremely sensitive technique and the tryptophan fluorescence of a protein depends strongly on the degree of tryptophan exposure and on the solvent conditions.

We further investigated the effect of residual guanidine concentration on GFPuv refolding. The refolding rate...
constant monitored by chromophore fluorescence was observed to decrease with increasing residual GuHCl concentration (Table 2) until, eventually, the refolding of GFPuv changed from biphasic to monophasic, although there was almost no change in the total amplitude when the GuHCl concentration was not higher than 0.25 M (data not shown). This is consistent with the previously reported results that the apparent rate constants are GuHCl concentration dependent and the fluorescence recoveries of GFPuv refolding are GuHCl concentration independent when the GuHCl concentration is not higher than 0.25 M (8).

**Low Concentrations of Trigger Factor Accelerate Folding of GFPuv.** TF assisted GFPuv refolding was investigated in detail. As shown in Figure 5a and Table 2, the refolding of GFPuv in buffer containing 0.18 M GuHCl monitored by chromophore fluorescence is biphasic, with rate constants of $k_1 = (3.57 \pm 0.07) \times 10^{-2} \text{ s}^{-1}$ and $k_2 = (0.71 \pm 0.10) \times 10^{-2} \text{ s}^{-1}$. These two processes are suggested to correlate with rapid recovery of the chromophore microenvironment followed by a further structural adjustment. There are 10 proline residues in GFPuv, one of which, Pro89, is in a cis-conformation in the native structure (Figure 1). During GFPuv refolding in the presence of TF, both $k_1$ and $k_2$ increased with increasing TF concentration for TF concentrations up to 0.4 $\mu$M (Figure 5b, inset). However, at the same low concentrations, the presence of BSA or F233Y-TF (a mutant of TF in which the PPIase activity has been impaired by mutation) showed no effect on the refolding rate constants (Table 3). (It should be noted that, to reduce the possible effect of residual guanidine concentration on the function of TF or TF mutants, the data in Table 3 was obtained in a refolding system in which the residual guanidine concentration was 0.06 M.) We also tested the effects on GFPuv refolding of other TF variants, NC, NM and MC, in which, respectively, the entire M, C or N domain of TF has been deleted. The results showed that the refolding of GFPuv was not affected by low concentrations of those TF variants (Table 3). Two of the truncated variants, NM and MC, show PPIase activity toward small peptides, but their reduced substrate binding ability significantly reduces their efficacy toward protein substrates (44). These results suggest that both the fast and slow phases of GFPuv folding are related to proline isomerization. Low concentrations of wild-type TF can catalyze proline isomerization during refolding, and so the refolding rate is increased.

**High Concentrations of Trigger Factor Retard Folding of GFPuv.** In contrast to low concentrations of TF, the refolding of GFPuv in the presence of high concentrations of TF was clearly retarded and the rate constants of refolding decreased with increasing TF concentration (Figures 5a and 5b). When the refolding of GFPuv was measured in the presence of 10 $\mu$M TF, refolding became monophasic and the rate constant decreased to $(1.00 \pm 0.01) \times 10^{-2} \text{ s}^{-1}$ (Figures 5a and 5b; Table 2). As a control, BSA (10 $\mu$M or 50 $\mu$M) had no detectable effect on the rates of GFPuv refolding (Figure 5b), suggesting a specific interaction between TF and GFPuv folding intermediates.

F233Y-TF shows about the same ability in retarding GFPuv refolding as wild-type TF at the high concentration even though it exhibits no PPIase activity toward GFPuv at the low concentration (Table 3), suggesting once again that the PPIase activity of TF is dispensable to its chaperone activity (25–27). A number of investigations have shown that the NC-domain segment of TF alone is sufficient for function as a molecular chaperone in vitro (27) and in vitro (28). However, the efficiency of NC assisted GAPDH refolding is much lower than that of the full length TF, so that a 10-fold-higher concentration of NC is required to prevent GAPDH aggregation to the same extent as full length TF (28). This can explain why in the presence of high concentrations of NC only mild deceleration of GFPuv refolding was observed in our case (Table 3). High concentrations of the NM or MC fragments show almost no effect on the GFPuv refolding (Table 3), which is consistent with the conclusion of Kramer and co-workers that the full chaperone activity of TF cannot be restored by single domain or domain combinations but needs the coordinated assembly within the entire TF molecule (28). In conclusion, the above results obtained using TF mutants suggest that TF function may depend on the structure of the target protein and that cooperation of the three domains of TF is required to assist GFPuv refolding.

**Effect of pH and Solution Hydrophobicity on TF Assisted Refolding of GFPuv.** As shown in Figure 6a, at pH values between 6.6 and 7.5, GFPuv refolding showed two phases and the rate constants did not show any obvious pH dependence; while in the presence of 10 $\mu$M TF, GFPuv refolding became monophasic and the rate constant also showed little pH dependence over this range. However, at pH values between 5.6 and 6.6, refolding of GFPuv in the presence or absence of TF was monophasic and the rate constants increased with increasing pH. The isoelectric points of GFPuv and TF are predicted to be 5.64 and 4.92, respectively (DNASIS v2.5), and so we did not investigate the refolding at pH lower than 5.6.

Ethylene glycol (EG) is a solvent that can change the hydrophobicity of the solution. When monitoring the chromophore fluorescence of GFPuv, the refolding rate constants, $k_1$ and $k_2$, did not show any obvious change with increasing EG concentrations up to 20% at pH 7.5 (Figure 6b). In the
presence of 6 μM TF but without EG, the refolding of GFPuv was retarded and became monophasic (Figure 6b); however, with increasing EG concentration, the refolding of GFPuv in the presence of 6 μM TF became biphasic once again and both phases were accelerated, suggesting that hydrophobic interactions provide the major force for interactions between TF and folding intermediates of GFPuv. Changing the hydrophobicity of the solvent perturbs the binding and release of target molecules by TF and so affects the refolding rate constants.

Like most other GFP variants (45, 46), the effect of pH on GFPuv can be detected by an intensity change in the chromophore fluorescence, although the positions of the peaks in its chromophore fluorescence spectra are not shifted; and for GFPuv, the highest fluorescence intensity can be obtained at about pH 7.5 (data not shown). This is consistent with a change in the protonation state of the chromophore phenolate (46). This then explains why the refolding rates of GFPuv are pH dependent below pH 6.6 (Figure 6a). However, in the presence of high concentrations of TF, the...
occurs with a half-life of around 64 s at a GuHCl concentration. The slowest observable phase in refolding of GFPuv unfolding and refolding are slow and quite complex (11–13, 49). Using guanidine as the denaturant, we likewise found that the folding of GFPuv occurs in a stepwise manner and intermediates can be detected under both equilibrium and kinetic folding conditions (Figures 2, 3 and 4; Tables 1 and 2). The formation of the backbone structure of GFPuv is essential but not sufficient to allow the chromophore to attain its native fluorescence, which suggests that a very slow adjustment of the microenvironment around the tripeptide chromophore motif occurs after the backbone has formed.

Discriminator is a nucleotide-independent molecular chaperone that combines two functions: chaperone activity and catalysis of prolyl isomerization. The binding affinity of TF for folding intermediates is lower than that of other chaperones, and the binding and release of folding intermediates by TF is a highly dynamic process, which may be related to its role in nascent peptide folding (37, 50). TF-assisted GFPuv refolding is quite different from TF-assisted folding of GAPDH (35), RCM-La (50), bovine carbonic anhydrase II (36) or lysozyme (37), in that the spontaneous refolding of GFPuv shows very high yields and almost no aggregation occurs during refolding. We therefore investigated how the folding rate constants are affected by different concentrations of TF. As shown in Figure 5 and Table 3, TF accelerates GFPuv folding at low concentrations and retards GFPuv folding at high concentrations. This phenomenon can be explained by competition between the two functions of TF, namely, catalysis and binding.

TF-assisted refolding of guanidine-denatured GFPuv is a complicated process involving a number of steps. The observed rate constant under a given set of conditions will reflect the slowest step under those conditions, which could be tertiary structure adjustment, prolyl isomerization, or formation of the GFPuv backbone. In addition, formation of intermolecular interactions, such as binding of GFPuv folding intermediates by TF, could also influence the rate of folding. In the presence of concentrations of TF lower than 0.4 μM, the rate of folding was increased, indicating that prolyl isomerization was previously limiting. However, proline isomerization can often be coupled to folding (36, 50, 51). With increasing TF concentration, the rate of folding was reduced as TF apparently binds and traps folding intermediates of GFPuv, as has been observed for other TF

### DISCUSSION

GFP is a molecule with properties that are quite distinct from most other small single domain proteins (47). Both its unfolding and refolding are slow and quite complex (8, 11, 12). The slowest observable phase in refolding of GFPuv occurs with a half-life of around 64 s at a GuHCl concentration of 0.06 M. This suggests high-energy barriers for both the unfolding and refolding reactions (48). A number of folding intermediate states have been detected under various denaturing conditions (11–13, 49). Using guanidine as the denaturant, we likewise found that the folding of GFPuv occurs in a stepwise manner and intermediates can be detected under both equilibrium and kinetic folding conditions (Figures 2, 3 and 4; Tables 1 and 2). The formation of the backbone structure of GFPuv is essential but not sufficient to allow the chromophore to attain its native fluorescence, which suggests that a very slow adjustment of the microenvironment around the tripeptide chromophore motif occurs after the backbone has formed.

Trigger factor is a nucleotide-independent molecular chaperone that combines two functions: chaperone activity and catalysis of prolyl isomerization. The binding affinity of TF for folding intermediates is lower than that of other chaperones, and the binding and release of folding intermediates by TF is a highly dynamic process, which may be related to its role in nascent peptide folding (37, 50). TF-assisted GFPuv refolding is quite different from TF-assisted folding of GAPDH (35), RCM-La (50), bovine carbonic anhydrase II (36) or lysozyme (37), in that the spontaneous refolding of GFPuv shows very high yields and almost no aggregation occurs during refolding. We therefore investigated how the folding rate constants are affected by different concentrations of TF. As shown in Figure 5 and Table 3, TF accelerates GFPuv folding at low concentrations and retards GFPuv folding at high concentrations. This phenomenon can be explained by competition between the two functions of TF, namely, catalysis and binding.

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Trigger Factor Assisted Folding of GFPuv

In conclusion, TF-assisted folding of GFPuv demonstrates spontaneously to the correct conformation (central pathway in Scheme 1).

In the presence of TF, the refolding yield of GFPuv does not change with increasing TF concentration. Instead, the refolding rate is affected by the TF concentration. In the presence of low concentrations of TF, the fast regain of Trp57 fluorescence and both fast recovery and slow adjustment phases of the chromophore fluorescence are accelerated, suggesting that those processes are all coupled to proline isomerization, since the presence of equivalent concentrations of BSA or a PPIase activity-impaired mutant of TF (F233Y-TF) did not show the same acceleration in rate. On the other hand, the lack of effect of TF truncation mutants NM and MC, which contain the PPIase domain but are defective in substrate binding (44), suggests that the binding (or chaperone) activity of TF may also contribute.

With increasing TF concentration, the rate constants for the rapid burial of Trp57 and for the chromophore fluorescence acquisition and adjustment are all decreased, ultimately leading to observation of a single slow phase (Table 3). These results may be best explained by competition between catalysis and binding by TF. It has been suggested previously that TF assisted protein folding requires repeated binding-and-release cycles between TF and folding intermediates (35); the higher the concentration of TF, the greater the chance of recapture of substrate intermediates by TF. This binding effect can lead to arrest of folding (35, 37, 50), so that GFPuv folding is no longer limited by proline isomerization. It has been suggested that the monomer–dimer equilibrium of TF is physiology relevant, either because dimeric TF represents a storage form ensuring saturation of ribosomes with TF or because the monomeric and dimeric forms have distinct functions (31, 32). The proportion of TF in the dimeric form will increase with the increase in TF concentration in our experiments, and so the very slow release of folding intermediates from the TF complex at high concentrations of TF could be attributed to the tight binding of intermediates by dimeric TF, since the TF dimer can bind with folding-competent intermediates stably in vitro (32).

In conclusion, TF-assisted folding of GFPuv demonstrates clearly how the two functions of the TF molecule can

substrates (35, 37, 50). In this scenario, the limiting step in folding may involve escape from TF to fold in solution, or could reflect the slow folding of GFPuv while bound to TF.

Scheme 1 presents a hypothetical mechanism for the events that could occur upon dilution of GuHCl denatured GFPuv in the presence of TF. In this model, upon sudden dilution of guanidine denatured GFPuv, rapid collapse of GFPuv occurs to form an intermediate in which Trp57 is buried. The chromophore microenvironment then gradually recovers, and proline isomerization may be coupled to folding. Refolding of GFPuv can reach maximum fluorescence at 508 nm without aggregation, suggesting that GFPuv can fold spontaneously to the correct conformation (central pathway in Scheme 1).

In the presence of TF, the refolding yield of GFPuv does not change with increasing TF concentration. Instead, the refolding rate is affected by the TF concentration. In the presence of low concentrations of TF, the fast regain of Trp57 fluorescence and both fast recovery and slow adjustment phases of the chromophore fluorescence are accelerated, suggesting that those processes are all coupled to proline isomerization, since the presence of equivalent concentrations of BSA or a PPIase activity-impaired mutant of TF (F233Y-TF) did not show the same acceleration in rate. On the other hand, the lack of effect of TF truncation mutants NM and MC, which contain the PPIase domain but are defective in substrate binding (44), suggests that the binding (or chaperone) activity of TF may also contribute.

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![Image of refolding curves of GFPuv](image-url)

**Figure 5:** (a) Representative refolding curves of GFPuv in the presence or absence of TF monitored by chromophore fluorescence. The refolding of GFPuv was performed by a jump of GuHCl concentration from 6.0 to 0.18 M in Tris buffer at pH 7.5. The refolding of GFPuv alone (−) and with 0.1 μM ( − −) or 10 μM (− − −) TF are shown. The curves of GFPuv refolding alone and in the presence of 0.1 μM TF fit well to double exponentials, whereas the refolding in the presence of 10 μM TF fits well to a single exponential. Plots of the residuals of the fits are shown in the lower panel. (b) Effect of TF concentration on the refolding rate constants of GFPuv, k1 ( ●) and k2 ( ●), corresponding to formation and release of intermediates by dimeric TF, since the TF dimer can bind with folding-competent intermediates stably in vitro (32). The concentrations of GFPuv and residual GuHCl were 0.2 μM and 0.18 M, respectively.

**Table 3:** The Effect of TF and TF Mutants on the Apparent Rate Constants of GFPuv Refolding Measured by Chromophore Fluorescence.

<table>
<thead>
<tr>
<th>concn of TF or TF mutants (μM)</th>
<th>k1 (s⁻¹)</th>
<th>k2 (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFPuv</td>
<td>5.42 ± 0.15</td>
<td>1.09 ± 0.04</td>
</tr>
<tr>
<td>GFPuv + BSA</td>
<td>5.72 ± 0.13</td>
<td>1.16 ± 0.10</td>
</tr>
<tr>
<td>6</td>
<td>5.72 ± 0.19</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>0.1</td>
<td>7.80 ± 0.15</td>
<td>1.44 ± 0.08</td>
</tr>
<tr>
<td>6</td>
<td>3.70 ± 0.11</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>0.1</td>
<td>5.85 ± 0.19</td>
<td>1.04 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>2.72 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>GFPuv + F233Y-TF</td>
<td>5.20 ± 0.16</td>
<td>1.10 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>4.78 ± 0.12</td>
<td>0.61 ± 0.07</td>
</tr>
<tr>
<td>GFPuv + NM</td>
<td>5.32 ± 0.18</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>6</td>
<td>5.43 ± 0.14</td>
<td>0.91 ± 0.09</td>
</tr>
<tr>
<td>GFPuv + MC</td>
<td>5.42 ± 0.05</td>
<td>0.80 ± 0.08</td>
</tr>
<tr>
<td>6</td>
<td>5.78 ± 0.13</td>
<td>0.81 ± 0.06</td>
</tr>
</tbody>
</table>

* The concentrations of GFPuv and residual GuHCl were 0.2 μM and 0.06 M, respectively. The data shown are the mean ± the standard error derived from at least 3 separate measurements.

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chaperone, TF binds and traps folding intermediates of GFPuv, which can lead to a retardation in the folding rate at high TF concentrations.

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REFERENCES


