Trigger Factor from *Thermus thermophilus* Is a Zn$^{2+}$-dependent Chaperone*

Received for publication, October 22, 2003, and in revised form, November 5, 2003
Published, JBC Papers in Press, November 5, 2003, DOI 10.1074/jbc.M311572200

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The ribosome-associated chaperone trigger factor (TF) of *Escherichia coli* interacts with a variety of newly synthesized polypeptides to assist their correct folding. Here, we report that the TF of thermophilic eubacterium, *Thermus thermophilus*, arrested spontaneous folding of green fluorescent protein by forming a 1:1 binary complex. The complex was isolable by gel-filtration but was shown to be dynamic because green fluorescent protein was released by α-casein in large excess. Unexpectedly, EDTA completely abolished the folding-arrest activity of TF, and analysis revealed that the TF from our preparation contained ~0.5 mol Zn$^{2+}$/mol TF. The folding-arrest activity of TF that was saturated with Zn$^{2+}$ (~1 mol/mol TF) was twice as efficient as that of untreated TF. Thus, chaperone activity of thermophilic TF is Zn$^{2+}$-dependent.

Folding of the newly synthesized proteins to the native state is assisted by various chaperones such as trigger factor (TF), DnaK and GroEL systems in *Escherichia coli* (1–3). The first encounter of nascent polypeptides is with TF, because TF is a ribosome-associated chaperone, and cross-linking experiments of *E. coli* TF showed that it interacts with polypeptide chains emerging from the peptide exit tunnel of the ribosome (4–9). Although deletion of the TF gene does not impair growth of *E. coli*, deletion of both TF and DnaK causes synthetic lethality, suggesting functional overlapping and cooperation of TF with DnaK (10–12). TF is assumed to function alone as a ribosome-folding-motif (called TF signature; GFRXGXXP, X can be varied) (6), a central domain that displays peptidyl-prolyl cis-trans isomerase (PPlase), and chaperone-like activities in vitro, and a C-terminal domain of unknown function (5, 13–15).

TF has been shown to have a broad binding specificity and interact with both cytoplasmic and secretory nascent polypeptide chains which are as short as 57 residues (4). TF assists the folding of RNase T1 by catalyzing isomerization of the proline residues, suppresses aggregation of glyceraldehydes-3-phosphate dehydrogenase, and binds small peptides with low affinity (16–18). As demonstrated in experiments using permanently unfolded protein, the interaction of TF with unfolded protein is dynamic; TF binds and releases unfolded protein rapidly (19). The interaction of TF with substrate protein is independent of the presence of proline residues (17, 19, 20), and it has not been settled whether PPlase activity is required for the TF function in *vivo* (21, 22).

To gain insights into the mechanism of functions of TF, we have investigated the interactions between TF and denatured proteins by using TF from a thermophilic eubacterium, *Thermus thermophilus* (Tth-TF). Tth-TF arrests spontaneous folding of proteins, by forming a binary complex with denatured proteins, the complex can be isolated by gel-filtration. More importantly, formation of the binary complex is dependent on Zn$^{2+}$ inherent in TF.

**EXPERIMENTAL PROCEDURES**

Isolation of Tth-TF—Chromosomal DNA from *T. thermophilus* strain HB8 was isolated as described previously (23). A gene encoding Tth-TF was amplified by PCR to generate Ndel and EcoR I sites at the 5′ and 3′ ends, respectively, of the coding region of Tth-TF. The digested PCR fragment was inserted between Ndel and EcoR I sites of pET23c, creating pET23c-TTF. *E. coli* strain BL21(DE3) carrying plasmid pET23c-TTF, was cultured at 37 °C for 16 h, and collected by centrifugation. Cells were suspended in 50 mM HEPES-KOH, pH 7.5, and disrupted by a French press. The crude extract was incubated for 10 min at 60 °C and centrifuged at 120,000 g for 30 min at 4 °C. The supernatant was applied to a DEAE Toyopearl column (Tosoh, Tokyo), and eluted with 0–500 mM linear gradient of KCl in 50 mM HEPES-KOH, pH 7.5. Fractions containing Tth-TF were pooled and supplemented with ammonium sulfate (1.3 M in final concentration). The solution was applied to a Phenyl Toyopearl column (Tosoh, Tokyo) equilibrated in 50 mM HEPES-KOH, pH 7.5, containing 1.3 mM ammonium sulfate. The column was eluted with 1.3–0 M linear reverse gradient of ammonium sulfate in 50 mM HEPES-KOH, pH 7.5. Fractions of pure Tth-TF were dialyzed against 50 mM HEPES-KOH, pH 7.5, and stored at ~50 °C until use.

Other Proteins—A mutant GFP (F99S/M153T/V163A) that shows improved folding efficiency was used throughout this work and is termed GFP for simplicity. GFP and Tth-GroEL-GroES were purified as described previously (24, 25). RNase A and α-casein were purchased from Roche and Sigma, respectively.

GFP Folding Assay—GFP (10 μM) was denatured in 50 mM glycine-H$_2$SO$_4$, pH 2.0, at room temperature for 2 min and diluted 100-fold with 1.2 ml of 50 mM HEPES-KOH, pH 7.5, 200 mM KC1 containing Tth-TF at indicated concentrations, or other components indicated. Increase in fluorescence at 510 nm with excitation light at 395 nm was monitored continuously with a fluorometer (FP-6500, Josco, Tokyo, Japan). The reaction mixtures were stirred at 50 °C throughout the experiments.

**Gel Filtration Analysis**—Acid-denatured GFP (100 μM) was diluted...
Amino acid sequence of the trigger factor from *T. thermophilus* (upper line) aligned with that from *E. coli* (lower line). Boxes indicate identical residues in two sequences.

20-fold with 50 mM HEPES-KOH, pH 7.5, 200 mM KCl containing *Tth*-TF and incubated for 10 min at 50 °C. Final concentrations of *Tth*-TF in the mixtures were 10 μM (Fig. 3C) and 5 μM (Fig. 5B). The sample solutions (100 μl) were applied to a gel-filtration HPLC (Superdex™ 200HR10/300, ID 10 mm (Fig. 2A). An |element analysis of metal ions bound to Trigger Factor|—Element analysis of metal ions bound to *Tth*-TF was carried out with inductively coupled plasma spectrometry (ICPS). The 1-element standards (Merck) were used for calibration, and 50 mM HEPES-KOH buffer, pH 7.5, treated with Chilex-100 (BioRad), was used as a metal-free control. The amount of Zn²⁺ in the mixtures were 0.33 μM (Fig. 3B). The solution was dialyzed against 50 mM HEPES-KOH, pH 7.5, containing 1 mM EDTA at room temperature. The denatured TF solution supplemented with 1 mM Zn(CH₃COO)₂ was placed on ice for 20 min at 50 °C (Fig. 4A). The absorption at 280 nm and the GFP fluorescence (Ex 395 nm/Em 510 nm) were monitored.

The amount of Zn²⁺-requirement for Trigger Factor was calculated using the standard dilution (50% recovery of the native GFP) was taken as 100%.

Experimental Procedures.

**Other Methods**—Concentrations of *Tth*-TF and GFP were determined spectrophotometrically. Extinction coefficients at 280 nm used for *Tth*-TF and GFP were 64,000 and 20,600 M⁻¹ cm⁻¹, respectively. The value for *Tth*-TF was determined by the quantitative amino acid analysis. The value for GFP was calculated based on the amino acid
RESULTS AND DISCUSSION

Isolation of TF from T. thermophilus—We searched for trigger factor (TF) homolog in the whole genome information of Thermus thermophilus, and found a gene encoding a protein with 404 residues (GenBank™ accession number AB125633) whose amino acid sequence showed similarity to that of E. coli TF (21% identity) (Fig. 1). The N-terminal domain of the protein had a TF signature sequence (X2A9, 42GFRPGKAP48, underlined residues are conserved) that is essential for ribosome binding. Therefore, we concluded that this gene encoded the T. thermophilus TF (Tth-TF). We cloned the Tth-TF gene and overexpressed the protein in E. coli in the soluble fraction from which Tth-TF was purified to homogeneity. Amino acid sequencing of the purified Tth-TF gave the expected N-terminal sequence (MVAEILERSG). To confirm in vivo expression of Tth-TF in T. thermophilus, polyclonal antibody raised against the recombinant Tth-TF was used to estimate cellular contents of Tth-TF in the soluble fraction of T. thermophilus. Western blotting showed that about 0.2% of the soluble proteins were Tth-TF (data not shown).

Tth-TF Inhibits Spontaneous Folding of Denatured Proteins—To check whether Tth-TF interacts with denatured proteins, we investigated the effects of Tth-TF on spontaneous folding of GFP by monitoring appearance of fluorescence (28). When acid-denatured GFP, which has no fluorescence, was diluted into the buffer at neutral pH, the fluorescence was recovered spontaneously (Fig. 2A). At 50 °C, the yield of spontaneous folding was estimated to be ~50% from the magnitude of recovered fluorescence. When Tth-TF was present in the dilution buffer, the recovery of fluorescence was reduced (Fig. 2A). Degree of the reduction increased in parallel with the amount of added Tth-TF; the 3.3-fold molar excess of Tth-TF completely suppressed the spontaneous folding of GFP. The folding-arrest by Tth-TF was not restricted to GFP. Guanidine-HCl denatured isopropyl malate dehydrogenase from T. thermophilus folded spontaneously at 25 °C at ~50% yield (25). But in the case where 12-fold molar excess of Tth-TF was present in the buffer, the spontaneous folding of isopropyl malate dehydrogenase was completely arrested (data not shown).

Isolation of the Binary Complex of Tth-TF with Denatured GFP—We analyzed the folding mixture described in the previous section by gel-filtration HPLC (Fig. 3). The purified Tth-TF alone was eluted as a peak of ~60 kDa, likely corresponding to the monomeric state (Fig. 3A), and native GFP was eluted as a peak of ~30 kDa (Fig. 3B). When the mixture containing the folding-arrested GFP was analyzed, about half of Tth-TF was...
fluorescence at the ~100 kDa peak areas, suggesting that the GFP in the binary complex with Tth-TF was in a non-fluorescent denatured state. Taken together, we conclude that Tth-TF arrests the spontaneous folding of GFP by forming a binary complex with denatured GFP. The interaction between E. coli TF and denatured proteins have been reported (17–19, 29) but, as far as we know, the binary complex between TF and substrate protein was isolated for the first time.

**Dynamic Nature of the Binary Complex**—When excess amount of \( \alpha \)-casein, a permanently unfolded protein, was added to the mixtures containing the folding-arrested GFP, folding of GFP resumed (Fig. 2B). The folding rate increased as the amount of added \( \alpha \)-casein increased even though it was still slower than the rate of spontaneous folding when 187-fold molar excess of \( \alpha \)-casein (550 \( \mu \)g) was added. On the contrary, native protein, such as RNase A, did not relieve the folding-arrest at all. These results suggest that the binary complex is in dynamic equilibrium between associated and dissociated forms. In the presence of excess \( \alpha \)-casein, the binding site of Tth-TF might be filled with \( \alpha \)-casein more often than denatured GFP, and denatured GFP can stay longer in the bulk solution where it initiates folding. This contention was further examined by the effect of chaperonin GroEL-GroES on the binary complex of Tth-TF and denatured GFP. We previously demonstrated that chaperonin from *T. thermophilus* (Tth-GroEL-GroES) mediated the folding of GFP in an ATP-dependent manner. The addition of Tth-GroEL-GroES to the mixture containing the folding-arrested GFP folding did not induce GFP folding but subsequent addition of ATP triggered GFP folding (Fig. 2C). It appears that as soon as the denatured GFP dissociates from Tth-TF, it is immediately trapped by Tth-GroEL-GroES where it initiates folding. Dynamic nature of the binary complex might seem contradictory to the fact that the binary complex can be isolated by gel-filtration. However, if the association is much more rapid than the dissociation (a small dissociation constant) and the flow rate of elution as well as the sample volume relative to the column bed volume is large, then even the complex in dynamic equilibrium can be isolated by gel-filtration.

**Requirement of Divalent Cation for the Activity of Tth-TF**—During the course of the present study, we noticed that Tth-TF purified in the buffer containing EDTA had no or little folding-arrest activity (data not shown). Indeed, the folding of GFP resumed by addition of EDTA to the mixture in which folding of GFP had been arrested by Tth-TF (data not shown). These effects of EDTA strongly suggest requirement of divalent cation for the folding-arrest activity of Tth-TF. Therefore, we determined contents of divalent cations in our Tth-TF preparation by element analysis with the ICP-MS. The element analysis revealed that one mol of the purified Tth-TF contained 0.47 atoms of Zn. Other metals were negligible; 0.07 atoms of Cu, 0.02 atoms of Fe, undetectable levels of Mg, Mn, Ca, Co, and Cd. When O-phenanthroline, which preferentially chelates Zn\(^{2+}\) ion, was included in the assay mixture, Tth-TF failed to arrest the folding of GFP (data not shown). Half occupancy of Zn\(^{2+}\) in the purified Tth-TF prompted us to prepare Tth-TF that completely lost Zn\(^{2+}\). Tth-TF was denatured in 6 M guanidine-HCl and 1 mM EDTA, and refolded in a buffer containing EDTA. The resultant Zn\(^{2+}\)-depleted Tth-TF failed to arrest GFP folding; ~90% of GFP spontaneously folded in the presence of equal molar Zn\(^{2+}\)-depleted Tth-TF (Fig. 5A). There was a possibility that Zn\(^{2+}\) would be required for stabilization of protein conformation of Tth-TF at a high temperature, 50 °C, rather than for folding-arrest activity itself. However, this possibility was unlikely because circular dichroism spectra (195–350 nm) of Tth-TF at 50 °C were not affected by the presence

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### Fig. 5. Folding-arrest activity and binary complex formation of Zn\(^{2+}\)-saturated Tth-TF with denatured GFP

A. effect of Zn\(^{2+}\)-saturated Tth-TF and Zn\(^{2+}\)-depleted Tth-TF on the folding of GFP. The concentrations of Zn\(^{2+}\)-saturated Tth-TF, Zn\(^{2+}\)-depleted Tth-TF, and GFP in the mixtures were 0.12, 0.1, and 0.1 \( \mu \)M, respectively. Other experimental procedures were the same as described in the legend of Fig. IA. B, dependence of the degree of the GFP-folding arrest on the concentration of Zn\(^{2+}\)-saturated Tth-TF. Experimental procedures were the same as those to obtain each point described in A. Fluorescent intensities of the recovered GFP at 1000 s after addition of denatured GFP were plotted. The *solid line* shows a simulation curve assuming dissociation constant between Zn\(^{2+}\)-saturated Tth-TF and denatured GFP to be 9 \( \mu \)M. C, gel-filtration analysis of binary complex formation between Zn\(^{2+}\)-saturated Tth-TF and denatured GFP. The concentrations of Zn\(^{2+}\)-saturated Tth-TF and GFP in the mixtures of the arrested folding were 5 \( \mu \)M each. Other experimental procedures were the same as those described in the legend of Fig. 2C.

eluted as a new peak of ~100 kDa (Fig. 3C). This new peak might correspond to the binary complex between Tth-TF and denatured GFP and, to confirm this, the protein components of the eluted fractions were analyzed by SDS-PAGE. As expected, the ~100 kDa peak fractions contained both Tth-TF and GFP (Fig. 3C). Based on the staining intensities of the bands calibrated individually with those of standard amounts of Tth-TF and GFP, the molar ratio of Tth-TF and GFP in the complex was estimated to be 1.0:1. The in-line monitoring of fluorescence of the gel-filtration HPLC showed that there was no GFP
and absence of Zn$^{2+}$ (data not shown). In addition, the Zn$^{2+}$-dependent folding-arrest activity of Tth-TF was also observed at a lower temperature, 25 °C (data not shown).

1.1 Binding of Zn$^{2+}$ to Tth-TF—We also prepared Zn$^{2+}$-saturated Tth-TF. The Zn$^{2+}$-depleted Tth-TF was denatured, supplemented with 1 mM Zn$^{2+}$, refolded in a Zn$^{2+}$-containing buffer, and separated from free Zn$^{2+}$ by gel-filtration. The element analysis showed that the isolated Zn$^{2+}$-saturated Tth-TF contained 1.3 atoms of Zn per mol of TF. The spectroscopic titration of bound divalent cations with a chelate reagent, 4-(2-pyridylazo)resorcinol, confirmed the 1:1 binding of Zn$^{2+}$ to Tth-TF (Fig. 4). The isolated Zn$^{2+}$-saturated Tth-TF completely arrested the GFP folding at 1:2:1 (Tth-TF/GFP) molar ratio (Fig. 5A). Titration of the arrest yield of GFP folding versus various amount of Tth-TF gave a hyperbolic saturation curve with an apparent dissociation constant of the Tth-TF-GFP complex, 9 nM (Fig. 5B). The original untreated Tth-TF preparation required more than 2 moles of Tth-TF to achieve the complete folding-arrest of GFP (see Fig. 2A) and an apparent dissociation constant of 20 nM was obtained from the titration that was consistent with half occupancy of Zn$^{2+}$ in the original Tth-TF preparation. In contrast to Fig. 2C, gel-filtration analysis of the Zn$^{2+}$-saturated Tth-TF mixture of the arrested folding gave an elution pattern in which most Tth-TF was eluted as a binary complex with small tailing elution (Fig. 5C). Association of denatured GFP with Tth-TF at the peak fraction was confirmed by SDS-PAGE analysis and the Tth-TF:GFP molar ratio in the complex was estimated to be 1:0.8. These results lead to the conclusion that the folding-arrest activity of Tth-TF is dependent on Zn$^{2+}$ binding to Tth-TF.

Conclusion—In the present study, we have shown that Tth-TF arrests the spontaneous folding of denatured GFP and isopropyl malate dehydrogenase by forming a 1:1 binary complex, which can be isolated by gel-filtration but is in dynamic equilibrium between associated and dissociated forms. The stable but dynamic nature of the binary complex may be important for function of chaperones that do not utilize ATP in binding regulation (19). Stable nature helps TF to bind all the nascent substrate proteins at the exit of ribosome tunnel and dynamic nature enables TF to transfer the bound polypeptide to the next more efficient chaperones, which use ATP for binding regulation, such as GroEL. Another important message of this report is that the folding-arrest activity of Tth-TF is dependent on stoichiometric amount of bound Zn$^{2+}$. Typical Zn$^{2+}$-binding motif, such as Cys-rich zinc-finger motif in SecA, DnaJ, and Hsp93 or HEXXH motif in metallo proteinases (30–33), is not found in the sequence of Tth-TF and understanding how Zn$^{2+}$ binds to Tth-TF awaits further study. This kind of Zn$^{2+}$-requirement has not been reported for TFs from other sources and is worth testing.

Acknowledgments—We are grateful to Y. Watanabe, Y. Kato-Yamada, M. Masaki, F. Matsuura, and E. Muneyuki for valuable discussion, and S. Kuramitsu (Osaka University) for information on T. thermophilus genome. We also thank R. Mitamura for element analysis.

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*J. Biol. Chem.* 2004, 279:6380-6384.
doi: 10.1074/jbc.M311572200 originally published online November 5, 2003

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