PPIase domain of trigger factor acts as auxiliary chaperone site to assist the folding of protein substrates bound to the crevice of trigger factor

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\textbf{A R T I C L E ~ I N F O}

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\textbf{A B S T R A C T}

Trigger factor (TF) is the first chaperone encountered by nascent chains in bacteria, which consists of two modules: peptidyl-prolyl-cis\textendash/trans-isomerase (PPIase) domain and a crevice built by both N- and C-terminal domains. While the crevice is suggested to provide a protective space over the peptide exit site of ribosome for nascent polypeptides to fold, it remains unclear whether PPIase domain is directly involved in assisting protein folding. Here, we introduced structural change into different regions of TF, and investigated their influence on the chaperone function of TF in assisting the folding of various substrate proteins, including oligomeric glyceroldehyde\textendash3-phosphate dehydrogenase (GAPDH) and monomeric carbonic anhydrase II (CA II) and lysozyme. Results showed that structural disturbances by site-specific mutations in the PPIase active site or by deletion of the PPIase domain from TF affected the chaperone activity of TF toward CA II and GAPDH but had no effect on TF-assisted lysozyme refolding, suggesting PPIase domain is involved in assisting the folding of substrates larger than lysozyme. Mutants with the structural disturbances in the crevice totally lost the chaperone activity toward all the substrates we used in this investigation. These results provide further evidence to confirm that the crevice is the major chaperone site of TF, and the hydrophobic pocket in PPIase domain acts as an auxiliary site to assist the folding of substrate proteins bound to the crevice in a substrate-dependent manner, which is beneficial for TF to provide appropriate assistance for protein folding by changing protective space and binding affinity.

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1. Introduction

The folding of newly synthesized proteins in the cell occurs with high efficiency and precision because of the activity of a network of molecular chaperones. In the \textit{Escherichia coli} cytosol, trigger factor (TF) binds to ribosome at the exit site of the peptide tunnel and acts as the first chaperone encountered by nascent chains (Hesterkamp et al., 1996; Valent et al., 1995). After interaction with TF, most proteins fold rapidly upon completion of synthesis, and only 20\textendash35\% of polypeptide chains require the further assistance of other chaperones (Deuerling et al., 1999; Teter et al., 1999). Deletion of TF results in an increase in protein aggregation in the cytosol (Deuerling et al., 1999, 2003), whereas overproduction of TF depresses the formation of inclusion bodies and increases the productive yield of aggregation-prone proteins (Li et al., 2001; Nishihara et al., 2000; Zeng et al., 2006). TF has overlapping chaperone function with DnaK, and only if both are deleted is it lethal for bacteria (Deuerling et al., 1999; Teter et al., 1999). TF has been shown to have a preference for random coil or loosely structured substrates (Huang et al., 2000a,b; Scholz et al., 1997), and the binding motif of TF has been identified as a short sequence enriched in hydrophobic and basic amino acids (Patzelt et al., 2001).

Most ribosomes exist in a 1:1 complex with TF, consistent with the role of TF in co-translational protein folding (Ferbitz et al., 2004; Kramer et al., 2002). However, TF is present in a 2\textendash3-fold molar excess over ribosomes in the cell, with an equilibrium between monomeric and dimeric states (Patzelt et al., 2002). The dimer and monomer of TF show different substrate binding properties (Liu et al., 2005b; Liu and Zhou, 2004; Martinez-Hackert and Hendrickson, 2009).

TF contains three distinct domains and displays an unusual extended conformation (Ferbitz et al., 2004; Ludlam et al., 2004).
The N-terminal domain is located at an end of the molecule and mediates ribosome docking via interaction with the L23 protein of the ribosome (Kramer et al., 2002). The C-terminal domain resides in the middle of the molecule and builds a crevice together with N-terminal domain. Co-crystal structure of TF bound to the ribosome shows that the crevice is positioned above the tunnel exit and provides a protective space for nascent polypeptides to fold (Hoffmann et al., 2006).

Peptidyl-prolyl-cis/trans-isomerase (PPIase) domain, the middle domain in primary sequence, is structural homology to FK506-binding proteins (FKBPs) and carries PPIase activity (Hesterkamp et al., 1996; Stoller et al., 1995). It is connected with the N-terminal domain via a long extension and positioned at another end of molecule in crystal structure (Ferbitz et al., 2004; Ludlam et al., 2004). While the PPIase activity of TF is able to accelerate the refolding of proteins containing proline in vitro (Liu and Zhou, 2004; Scholz et al., 1997; Zarnz et al., 1997), it is demonstrated that PPIase activity is dispensable for the chaperone function of TF in vivo and in vitro (Kramer et al., 2004a; Li et al., 2001; Liu and Zhou, 2004). However, the PPIase domain is able to be crosslinked to nascent peptides (Kaiser et al., 2006; Lakshmipathy et al., 2007). Therefore, whether the PPIase domain is involved in chaperone function remains enigmatic. Here, we investigated the role of the PPIase domain in the chaperone activity of TF in vitro. Our results suggest that the hydrophobic pocket inside the PPIase domain is an auxiliary chaperone site to assist the folding of protein substrates bound to the crevice in a substrate-dependent manner.

2. Materials and methods

2.1. Materials

1-Anilininonaphthalene-8-sulfonate (ANS), p-nitrophenyl acetate (p-NPA), bovine carbonic anhydrase II (CA II) and Micrococcus lysodeikticus dried cells were purchased from Sigma. Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was from Peptide Institute Inc. (Osaka, Japan). Guanidine hydrochloride (GdnHCl) was from ICN. Disuccinimidyl suberate (DSS) was from Pierce. Anti-TF polyclonal antibody was prepared by Shenzhen BioinfoBody Inc. (Shenzhen, China). All other chemicals were local products of analytical grade.

2.2. Preparation of proteins

Plasmid pQE60 containing the tig gene that encodes E. coli TF was donated by Professor G. Fischer. E. coli mutants: I195G, Y221G, F233G, F233Y, NP (1–251), PC (146–432), NC (Δ146–251), TF419 (1–419), TF389 (1–389) and TF360 (1–360) were generated using GeneEditor in vitro Site-Directed Mutagenesis System (Promega) and PCR. TF species were expressed in E. coli JM109 and purified as described (Stoller et al., 1995). Protein extinction coefficients were calculated according to the method of Gill and von Hippel (1989). PPIase activity was assayed by the chymotrypsin-coupled method using succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as the substrate (Fischer et al., 1984). Purification of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was as described previously (Liang et al., 1990). Cyclophilin was prepared from porcine kidney according to Kofron et al. (1991).

2.3. Spectroscopic measurements

Trp fluorescence measurements were carried out on a Hitachi F4500 fluorescence spectrophotometer. Trp fluorescence emission spectra were recorded between 300 and 400 nm with excitation at 295 nm. For ANS fluorescence, excitation was set at 370 nm and emission spectra were recorded from 400 to 600 nm.

2.4. Equilibrium measurements

All experiments were carried out at 25 °C in 0.1 M Tris-HCl (pH 7.8). GdnHCl induced equilibrium denaturation experiments were performed after 18 h incubation of protein in buffer containing different GdnHCl concentrations. Equilibrium refolding was performed by diluting unfolded protein (12 h in 3.0 M GdnHCl) to the same conditions as described for unfolding experiments. Spectroscopic measurements were performed as above. Data were fitted to 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.

2.5. TF-assisted reactivation of GAPDH

As described previously (Huang et al., 2000b), GAPDH was denatured in 3 M GdnHCl for 12 h at 25 °C. Reactivation was initiated by rapid 50-fold dilution to a final GAPDH concentration of 2.73 μM, with 0.1 M phosphate buffer, pH 7.5, containing 5 mM dithiothreitol and different concentrations of TF variants. The reactivation mixture was kept at 4 °C for 30 min and then for a further 3 h at 25 °C to allow reactivation to go to completion before the reactivation yield of GAPDH was determined. Apparent rate constant was obtained by fitting the time course of GAPDH reactivation to a single-exponential function. GAPDH aggregation was monitored at 25 °C by 90° light scattering at 488 nm in a Hitachi F4500 fluorescence spectrophotometer.

2.6. TF-assisted reactivation of CA II

As described previously (Liu and Zhou, 2004), CA II was denatured in 5 M GdnHCl for 12 h at 20 °C. Reactivation was initiated by a rapid 100-fold dilution with 50 mM Tris sulfate buffer, pH 7.5, at 20 °C to a final CA II concentration of 4 μM. The buffer contained different concentrations of TF variants. Recovery of activity was complete 4 h after dilution and no further change was observed for at least 24 h. Reactivation yields were determined by following the esterase activity of CA II toward p-NPA. CA II aggregation was monitored at 20 °C by 90° light scattering at 488 nm in a Hitachi F4500 fluorescence spectrophotometer.

2.7. TF-assisted reactivation of lysozyme

Lysozyme was denatured as described previously (Huang et al., 2002). Refolding of denatured lysozyme was achieved by dilution into 0.1 M phosphate buffer, pH 7.5, containing 2 mM EDTA, or 0.1 M Hepes buffer, pH 7.0, containing 2 mM EDTA, 5 mM MgCl2 and 20 mM NaCl. The refolding buffers contained 1 mM oxidized glutathione, 2 mM reduced glutathione and different concentrations of TF variants. 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 Micrococcus lysodeikticus (Goldberg et al., 1991). Lysozyme aggregation was monitored at 25 °C by 90° light scattering at 488 nm or by absorption at 600 nm.

2.8. Crosslinking and electrophoresis

As described previously (Liu et al., 2005b), reactivation of denatured GAPDH was carried out in 15 mM phosphate buffer, pH 7.5, at 4 °C; the final concentrations of GAPDH and TF variants were 3.6 and 36 μM, respectively. After proteins were incubated for
Fig. 1. Design of TF variants. (A) Multiple sequence alignment of TF sequences from different species. Sequences are taken from GenBank with the following accession numbers: E. coli P22257; H. influenzae P44837; V. cholerae P80698; B. subtilis P80698; M. genitalium P74880; human FKBP12 P62942. Conserved residues are in black, and semi-conserved residues are in gray. (B) Schematic representation of TF constructs used in this study. C-terminal truncation variants were truncated at the positions indicated in upper panel. Replacements of three conserved residues chosen are shown in middle panel. TF fragments with domain deletion are shown in lower panel.

30 min at 4°C, DSS dissolved in DMSO was added, and the final concentration of crosslinker was 6 mM. The crosslinking reaction was stopped after 2 h with 150 mM Tris–HCl, pH 7.0. After desalted using Sephadex G–25 columns, samples were analyzed by gradient SDS-PAGE and detected by silver staining and western-blot using anti-TF polyclonal antibody.

3. Results

3.1. Properties of wild-type TF and site-specific mutants

The site-specific mutagenesis experiments with the PPlase domain of E. coli TF were based on a result of multiple sequence alignment of TF sequences from different species and human FKBP12, and three conserved residues: Ile195, Tyr221 and Phe233 were chosen as mutagenesis sites, which generated four full-length mutants: Ile195Gly (I195G), Tyr221Gly (Y221G), Phe233Gly (F233G) and Phe233Tyr (F233Y) (Fig. 1). These mutants showed very low PPlase activity. The substrate specificity of wild-type TF for succinyl-Ala-Ala-Pro-Phe–p-nitroanilide was 264.58 mM$^{-1}$s$^{-1}$ and those of I195G, Y221G, F233G and F233Y were 2.26, 2.49, 1.48 and 1.18 mM$^{-1}$s$^{-1}$, respectively. These results suggest that these conserved residues of TF are crucial in PPlase catalysis.

Trp151, the sole Trp in E. coli TF, is located in the PPlase domain, whose fluorescence spectrum change would reflect the structural change of the PPlase domain. While other site-specific mutants showed Trp fluorescence spectra similar to wild-type TF, the Trp fluorescence of F233G showed a red shift of maximum emission wavelength from 337 to 341 nm (Fig. 2A). Likewise, ANS binding fluorescence intensity of F233G was dramatically lower than those of the other full-length TF species (Fig. 2B). These results indicate that replacement of Phe233 by Gly leads to structure loosening and hydrophobic inner surface exposing in the PPlase domain, while the other residue substitutions in the PPlase domain fail to cause structure disturbance enough to be detected by Trp fluorescence and ANS binding fluorescence.

To further investigate the effect of mutations on the PPlase domain, stability of wild-type TF and the mutants was probed by GdnHCl induced equilibrium unfolding and refolding with the probe of Trp151 fluorescence. The thermodynamic parameters obtained are shown in Table 1. The transition curves of Trp151 fluorescence of wild-type TF and the mutants followed a three-state model (Fig. 3). Early studies show that the first transition of Trp151 fluorescence reflects the structural stability of the PPlase domain (Liu et al., 2005a). The fitting results showed that the free energy changes of the mutants in the first transition were 5–10 kJ/mol lower than that of wild-type TF, indicating the residue substitutions resulted in destabilization of the PPlase domain (Table 1).

α-Helical structure predominates in both terminal domains of TF while the PPlase domain is composed essentially of β-sheet structure (Ferbitz et al., 2004; Ludlam et al., 2004). Therefore, change in the far-UV CD spectrum principally reflects the structure change of terminal domains (Liu et al., 2005a; Zarnt et al., 1997). It was found that the CD spectra and GdnHCl induced transitions monitored by far-UV CD at 222 nm of the four site-specific mutants showed no significant difference from wild-type TF (data...
Fig. 2. Spectral properties of wild-type TF (solid line), F233Y (dashed line), F233G (dot line), Y221G (dash-dot line) and I195G (dash-dot-dot line). (A) Trp fluorescence spectra of 5.2 μM protein in 0.1 M Tris-HCl buffer (pH 7.8). (B) Different fluorescence spectra of 52 μM ANS binding to 1.04 μM protein in 0.1 M Tris-HCl buffer (pH 7.8).

Table 1
Thermodynamic parameters for the equilibrium unfolding and refolding of wild-type TF and mutants.

<table>
<thead>
<tr>
<th>Method</th>
<th>Protein</th>
<th>ΔG&lt;sub&gt;U-N&lt;/sub&gt; (kJ mol&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>m&lt;sub&gt;N&lt;/sub&gt; (kJ mol&lt;sup&gt;−1&lt;/sup&gt; M&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>[D]&lt;sub&gt;I-N&lt;/sub&gt;/2 (M)</th>
<th>ΔG&lt;sub&gt;I-U&lt;/sub&gt; (kJ mol&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>m&lt;sub&gt;I&lt;/sub&gt; (kJ mol&lt;sup&gt;−1&lt;/sup&gt; M&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>[D]&lt;sub&gt;I-U&lt;/sub&gt;/2 (M)</th>
<th>ΔG&lt;sub&gt;U-N&lt;/sub&gt; (kJ mol&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Max em-wavelength of intermediate&lt;sup&gt;a&lt;/sup&gt; (nm)</th>
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</thead>
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<tr>
<td>Intensity of Trp fluorescence</td>
<td>Wild-type TF</td>
<td>25.6 ± 2.3</td>
<td>45.7 ± 4.0</td>
<td>0.56</td>
<td>6.6 ± 2.2</td>
<td>5.6 ± 1.2</td>
<td>1.18</td>
<td>32.2 ± 2.3</td>
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<td></td>
<td>F233Y</td>
<td>19.5 ± 1.3</td>
<td>37.0 ± 2.1</td>
<td>0.53</td>
<td>7.2 ± 2.7</td>
<td>6.2 ± 1.5</td>
<td>1.16</td>
<td>26.7 ± 2.7</td>
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<tr>
<td></td>
<td>F233G</td>
<td>16.3 ± 2.6</td>
<td>34.3 ± 5.0</td>
<td>0.48</td>
<td>7.0 ± 1.1</td>
<td>6.0 ± 0.6</td>
<td>1.16</td>
<td>23.3 ± 2.6</td>
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<tr>
<td></td>
<td>Y221G</td>
<td>16.3 ± 1.5</td>
<td>34.1 ± 2.6</td>
<td>0.48</td>
<td>7.7 ± 3.1</td>
<td>6.6 ± 1.8</td>
<td>1.17</td>
<td>23.9 ± 3.1</td>
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<tr>
<td></td>
<td>I195G</td>
<td>14.5 ± 2.1</td>
<td>33.6 ± 3.7</td>
<td>0.43</td>
<td>5.1 ± 3.6</td>
<td>5.1 ± 1.8</td>
<td>0.99</td>
<td>19.5 ± 3.6</td>
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<tr>
<td>Wavelength of Trp fluorescence</td>
<td>Wild-type TF</td>
<td>26.0 ± 2.3</td>
<td>45.6 ± 4.0</td>
<td>0.57</td>
<td>5.6 ± 1.9</td>
<td>4.9 ± 0.9</td>
<td>1.14</td>
<td>31.7 ± 2.3</td>
<td>342.0</td>
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<td>F233Y</td>
<td>19.7 ± 0.9</td>
<td>37.1 ± 1.7</td>
<td>0.53</td>
<td>7.0 ± 2.3</td>
<td>5.1 ± 1.2</td>
<td>1.38</td>
<td>26.8 ± 2.3</td>
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<td>F233G&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.9 ± 0.4</td>
<td>6.8 ± 0.3</td>
<td>0.31</td>
<td>8.9 ± 0.4</td>
<td>6.8 ± 0.3</td>
<td>1.31</td>
<td>343.9</td>
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<tr>
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<td>Y221G</td>
<td>15.3 ± 0.5</td>
<td>35.1 ± 1.0</td>
<td>0.45</td>
<td>7.0 ± 2.0</td>
<td>5.1 ± 1.1</td>
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<td>22.9 ± 2.0</td>
<td>347.1</td>
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<td></td>
<td>I195G</td>
<td>14.3 ± 0.6</td>
<td>35.2 ± 1.4</td>
<td>0.41</td>
<td>6.1 ± 1.3</td>
<td>5.3 ± 0.8</td>
<td>1.15</td>
<td>20.4 ± 1.3</td>
<td>345.0</td>
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</table>

ΔG<sup>U</sup> is the free energy of unfolding extrapolated to zero denaturant concentration, m is the denaturant dependence (or slope), and [D]<sub>I-N</sub>/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, ΔG<sub>I-N</sub> + ΔG<sub>I-U</sub> = ΔG<sub>U-N</sub>.

<sup>a</sup> Data were acquired from the two-state or three-state fitting function.

<sup>b</sup> The transition curve was analyzed with two-state model, I ↔ U, because the transition from N to I is too fast to be fitted.

not shown). In addition, the fluorescence spectrum of mutant I195G with excitation at 280 nm was identical to that of wild-type TF, indicating environments surrounding all aromatic residues over whole TF molecule (∼2/3 of aromatic residues in terminal domains) were unchanged in I195G (data not shown). These results indicate that the structure of terminal domains (N and C) is not affected by the residue substitutions in the PPIase domain, which is far from terminal domains.

Fig. 3. GdnHCl induced equilibrium unfolding (solid) and refolding (empty) of wild-type TF (square), I195G (down-triangle) and F233G (diamond). The fit to a three-state or two-state model is shown as line. (A) Change of Trp fluorescence intensity monitored at 327 nm with excitation at 295 nm. (B) Shift of the maximum emission wavelength of Trp fluorescence with excitation at 295 nm.
3.2. Effects of mutations of PPIase domain on TF-assisted GAPDH reactivation

As shown in Fig. 4A, the reactivation yield of denatured GAPDH increased with increasing TF concentration in the presence of low concentrations of wild-type TF, and reached 55% at 10 μM wild-type TF. With a further increase in wild-type TF concentration, the reactivation yield decreased, to around 20% in the presence of 36 μM wild-type TF. However, in the presence of NC fragment, a TF variant that lacks the PPIase domain, but comprises the intact crevice structure, the shape of the GAPDH refolding curve differed from that of the wild-type TF. The reactivation yield of denatured GAPDH gradually increased with increasing NC concentration, and reached 47% at 36 μM NC fragment (Fig. 4A). Likewise, NC fragment displayed a significantly weaker chaperone activity in preventing GAPDH aggregation than wild-type TF (Fig. 4C). This is consistent with Kramer’s early results (Kramer et al., 2004b).

To verify whether the PPIase domain is involved in TF-assisted GAPDH reactivation further, GAPDH reactivation was investigated in the presence of TF variants with single residue substitution in the PPIase domain. It was found that the profile of GAPDH reactivation assisted by F233Y or Y221G was similar to that assisted by wild-type TF. However, the reactivation yield of GAPDH in the presence of F233G or I195G was always higher than that in the presence of wild-type TF, at the same concentration of assisting proteins. The maximum reactivation yield of GAPDH reached 70% at 14 μM I195G or F233G. As control, BSA and cyclophilin, a PPIase, showed no effect on GAPDH reactivation.

We also investigated the effects of wild-type TF and the full-length mutants on the reactivation kinetics of GAPDH (Fig. 4B). It was found that the apparent first-order rate constant of GAPDH reactivation decreased with increasing concentrations of wild-type TF or mutants. The mutants could also be divided into two groups, according to their effects on the reactivation kinetics of GAPDH. One group included F233Y and Y221G, which showed ability in lowering the refolding rate of GAPDH similar to wild-type TF, but stronger than another group including F233G and I195G. Retarding the refolding rate of substrates is a characteristic of proteins behaving as molecular chaperone, because the binding of folding-competent intermediates to chaperone would decrease the efficient concentration of the intermediates. The stronger ability in lowering the refolding rate of substrates indicates stronger interaction between chaperone and substrates and a more stable complex of chaperone with substrates. Our results show that single residue substitution has no or slight effect on the ability of F233Y and Y221G binding to substrates, but dramatically reduces the binding affinity of F233G and I195G toward substrates. As we have known that the tetrameric GAPDH is the active form, our previous studies show that TF is able to bind and hold the dimeric folding intermediates of GAPDH (Huang et al., 2000b; Liu et al., 2005b). Thus, the reduction in the interaction between TF and GAPDH intermediates contributes to the release of GAPDH intermediates from TF and so as to accelerate the following folding and association of GAPDH intermediates into the active tetramer of GAPDH, in consistent with early observation that addition of ethylene glycol is able to reduce the interaction between TF and GAPDH intermediates and consequently increase the reactivation yield of GAPDH (Huang et al., 2000b). As a result, the reactivation yield of GAPDH in the presence of F233G or I195G was higher than that in the presence of wild-type TF, F233Y or Y221G.

While the reduction in the binding affinity of TF to folding intermediates contributes to increasing the reactivation yield of GAPDH, an adverse result could be predicted that GAPDH aggregation in the presence of I195G or F233G would be more severe than that in the presence of wild-type TF, F233Y or Y221G, because weakening the ability of chaperone binding to substrates would decrease

Fig. 4. Effects of TF variants on GAPDH reactivation. 2.73 μM denatured GAPDH was refolded in the presence of various amounts of TF variants. Reactivation yield (A), apparent reactivation rate constant (kapp) (B) and aggregation (C) were then determined (see Section 2). Solid square, circle, diamond, up-triangle, down-triangle and cross symbols represent wild-type TF, F233Y, F233G, Y221G, I195G and NC fragment, respectively. Effects of NP, PC, TF419, TF389 and TF360 variants are shown as open square, circle, diamond, up-triangle and down-triangle symbols, respectively. BSA (open asterisk) and cyclophilin (solid asterisk) were used as control. All data are the mean values for at least three samples. Standard error bars are not shown for data points in which the width of the error bar is less than the width of the symbol. The inset in (B) shows the time course of GAPDH reactivation in the presence of 0 μM TF (curve 1), 3 μM TF (curve 2), 6 μM TF (curve 3) or 14 μM TF (curve 4).
the protection provided by chaperone for aggregation-prone intermediates. To verify this, GAPDH aggregation in the presence of wild-type TF and the mutants was investigated. As shown in Fig. 4C, the aggregation extent declined with increasing concentrations of wild-type TF or the mutants. However, I195G and F233G showed a weaker ability in preventing GAPDH aggregation than wild-type TF, F233Y and Y221G, as suggested above.

3.3. Effects of N- and C-terminal truncation on TF-assisted GAPDH reactivation

Distinct from the PPlase domain with a compact structure, N- and C-domains of TF form a crevice with an unusual extended conformation (Ferbitz et al., 2004; Ludlam et al., 2004). Sequence alignment shows that residues on the inner surface of the crevice of TF are less conserved (data not shown) (Hesterkamp and Bukau, 1996; Martinez-Hackert and Hendrickson, 2007). Thus, it is difficult for residue substitution to cause significant structure perturbation in the crevice and thereby to change its function providing a protective space for substrates. Instead, we studied the importance of the crevice on the chaperone function using truncation variants with the incomplete crevice structure. These truncation variants include TF fragments lacking either terminal domain: NP and PC fragments and C-terminal truncation variants with different length: TF419, TF389 and TF360, in which the C-terminal 13, 43 and 72 residues were deleted, respectively (Fig. 1B). As shown in Fig. 4A, distinct from all of the full-length TF species with the intact crevice, truncation variants showed negligible chaperone activity in assisting the refolding of GAPDH. Likewise, all of the truncation variants failed in preventing the aggregation of GAPDH intermediates (Fig. 4C). These results suggest that the integrity of the crevice is indispensable for the chaperone activity of TF, in agreement with early studies (Kramer et al., 2004b; Merz et al., 2006; Zarnit et al., 1997).

It is rational to speculate that loss of chaperone activity result from weakening of the binding of TF to substrates caused by truncation in terminal domains. Thus, we investigated the interaction of truncation variants with GAPDH intermediates by DSS crosslinking. As shown in Fig. 5, when denatured GAPDH was refolded in the presence of wild-type TF, a new crosslinked product with molecular mass of around 200 kDa was observed, which was not detected when native GAPDH was crosslinked with DSS in the presence of wild-type TF. The site-specific mutants in the PPlase domain showed the same crosslinking property as wild-type TF (data not shown). Early results show that the formation of the new crosslinked complex arises from tight binding of TF to the folding intermediates of GAPDH (Liu et al., 2005b). However, no crosslinked products between the truncation variants of TF and GAPDH intermediates were detected by silver staining and western-blot after the same treatments (Fig. 5). These results showed that the truncation variants failed in binding to GAPDH intermediates and consequently preventing their aggregation. As a result, the aggregation-prone intermediates of GAPDH formed insoluble aggregates and were excluded by centrifugation before loaded onto SDS-PAGE.

3.4. TF-assisted CA II reactivation

To exclude possible artificial effect from characteristics of GAPDH itself, another substrate, CA II, was chosen to verify the effect of the mutations on the chaperone activity of TF. CA II is a monomeric enzyme, whose pathways of folding and aggregation are different from those of tetrameric GAPDH (Huang et al., 2002; Liu and Zhou, 2004). Although CA II is rich in proline, the reactivation yield and aggregation of CA II are not changed by the PPlase activity of TF (Liu and Zhou, 2004). As shown in Fig. 6A, the aggregation extent of CA II declined markedly with increasing concentrations of wild-type TF or the site-specific mutants. Interestingly, these full-length TF species could also be divided into two groups according to the ability in preventing CA II aggregation. One group, including wild-type TF, F233Y and Y221G, showed stronger ability in preventing CA II aggregation than another group, including F233G and I195G. It further confirms that residue substitution reduces the binding affinity of I195G and F233G to aggregation-prone substrate but has no effect on that of F233Y and Y221G.

Effects of wild-type TF and the mutants on the reactivation yield of CA II were also investigated. As shown in Fig. 6B, the spontaneous reactivation yield of CA II was around 32% under the experimental conditions used here. With increasing wild-type TF concentration, the reactivation yield of CA II increased gradually, reaching a plateau of around 42% in the presence of 24 μM wild-type TF. In contrast to TF-assisted reactivation of GAPDH, the reactivation yield of CA II in the presence of I195G or F233G was lower than that in the presence of wild-type TF, Y221G or F233Y, though all full-length mutants were active in promoting the reactivation yield of CA II.

CA II is a monomeric protein, in whose refolding pathway no subunit association is involved. Thus, binding of CA II intermediates to TF and consequently preventing the off-pathway association of CA II intermediates are favored in increasing the reactivation yield and inhibiting aggregation. Compared with I195G and F233G, wild-type TF, F233Y and Y221G showed a stronger binding affinity to CA II intermediates, resulting in a distribution between native species and aggregates of CA II shifted to the former.

Similar to TF-assisted refolding of GAPDH, terminal truncation variants of TF failed in increasing the reactivation yield and preventing aggregation of CA II, which further confirmed the indispensable role of the crevice in the chaperone function of TF. Noticeably, NC fragment retained partial chaperone activity in preventing aggregation of CA II, but lost the ability to promote the reactivation yield (Fig. 6). It indicates that deletion of the PPlase domain has different impacts on the chaperone activity of TF toward the folding-competent and aggregation-prone intermediates of CA II.

3.5. Chaperone activity of TF toward lysozyme reactivation in phosphate buffer

Previous results show that nascent peptides are able to be crosslinked to the PPlase domain of ribosome-associated TF in a length-dependent manner (Kaiser et al., 2006; Lakshmipathy et al., 2007; Merz et al., 2008). Therefore, a question was raised of whether the involvement of the PPlase domain in the chaperone function of TF is related to the size of substrates. Since crystal structure results show that the crevice in TF is large enough to accommodate a globular protein up to a molecular weight of approximately 15 kDa (Ferbitz et al., 2004), lysozyme of 14 kDa was chosen as substrate to investigate the role of the PPlase domain in the chaperone function of TF further, besides CA II of 30 kDa and GAPDH, monomer of which is 36 kDa.

As described previously (Huang et al., 2002), denatured lysozyme aggregated rapidly and to a significant degree upon dilution in phosphate buffer. However, lysozyme aggregation was inhibited markedly by increasing wild-type TF concentration (Fig. 7A). Meanwhile, the reactivation yield of denatured lysozyme increased gradually with increasing wild-type TF concentration, reaching a maximum of around 20% in the presence of 20 μM wild-type TF, after which no further increase was observed (Fig. 7B). Unexpectedly, all of the site-specific mutants showed chaperone activity identical to wild-type TF in both preventing aggregation and increasing the reactivation yield of lysozyme, indicating that residue substitutions in the PPlase domain had no effect on the binding of TF to lysozyme intermediates, namely that the PPlase domain was not involved in TF-assisted lysozyme refolding. To verify this, lysozyme refolding was investigated in the presence of NC.
Fig. 5. Crosslinking of TF variants with GAPDH. Denatured GAPDH was refolded in the presence of TF variants. Proteins were then crosslinked with DSS (see Section 2). Samples were separated by gradient SDS-PAGE and detected by silver staining (A) or western-blot using anti-TF polyclonal antibody (B). The sizes of the molecular mass marker (M) and the positions of the bands corresponding to TF dimer (Δ) and TF-GAPDH complex (*) are indicated.

Fig. 6. Effects of TF variants on CA II reactivation. 4 μM denatured CA II was refolded in the presence of various amounts of TF variants. Aggregation of CA II in the presence of various amount of TF variants (A) and reactivation yields in the presence of 24 μM TF variants (B) were then determined (see Section 2). All data are the mean values for at least three samples. Standard error bars are not shown for data points in which the width of the error bar is less than the width of the symbol. Solid square, circle, diamond, up-triangle, down-triangle and cross symbols represent wild-type TF, F233Y, F233G, Y221G, I195G and NC fragment, respectively. Effects of NP, PC, TF419, TF389 and TF360 variants are shown as open square, circle, diamond, up-triangle and down-triangle symbols, respectively. Ovalbumin (open asterisk) was used as control. The insert in (B) shows the reactivation of 4 μM denatured CA II in the presence of various amounts of wild-type TF, I195G or ovalbumin.
Fig. 7. Effects of TF variants on lysozyme reactivation. In the presence of various amounts of TF variants, 10 μM denatured lysozyme was refolded in phosphate buffer (A and B) or Hepes buffer (C and D). Aggregation of lysozyme in the presence of various amount of TF variants (A and C) and reactivation yield in the presence of 25 μM TF variants (B) or in the presence of various amount of TF variants (D) were then determined (see Section 2). All data are the mean values for at least three samples. Standard error bars are not shown for data points in which the width of the error bar is less than the width of the symbol. Solid square, circle, diamond, up-triangle, down-triangle and cross symbols represent wild-type TF, F233Y, F233G, Y221G, I195G and NC fragment, respectively. Effects of NP, PC, TF419, TF389 and TF360 variants are shown as open square, circle, diamond, up-triangle and down-triangle symbols, respectively. Ovalbumin (open asterisk) and cyclophilin (solid asterisk) were used as control. The insert in (B) shows the reactivation of 10 μM denatured reduced lysozyme in the presence of various amounts of wild-type TF, I195G or ovalbumin.

fragment. As expected, NC fragment showed chaperone activity similar to all of the full-length TF species in both preventing aggregation and increasing the reactivation yield of lysozyme (Fig. 7A and B).

Similar to TF-assisted refolding of GAPDH and CA II, all of the terminal truncation variants of TF still displayed negligible chaperone activity in assisting lysozyme refolding.

3.6. Antichaperone activity of TF toward lysozyme reactivation in Hepes buffer

Although acting as the chaperone to assist protein folding in most cases, TF is able to reduce the reactivation yield and facilitate the formation of aggregates under some conditions, which has been described as antichaperone activity (Huang et al., 2002). As shown in Fig. 7D, the reactivation yield of lysozyme decreased significantly with increasing wild-type TF concentration in the presence of low concentrations of wild-type TF, when lysozyme refolded in Hepes buffer. The reactivation yield of lysozyme decreased from 41% in spontaneous reactivation to 11% in the presence of 4 μM wild-type TF. When the concentration of wild-type TF was greater than 4 μM, the reactivation curve of lysozyme showed a slow upward turn, nevertheless the reactivation yields were still much lower than that of spontaneous refolding. In the meanwhile, the extent of aggregation was found to increase initially with increasing wild-type TF concentration, but then decrease when the concentration of wild-type TF was above 4 μM (Fig. 7C). As a control, cyclophilin had no influence on either recovery of native lysozyme or the extent of aggregation (Fig. 7C and D). These results indicate that in TF-assisted lysozyme reactivation in Hepes buffer, the antichaperone activity of TF dominates in the presence of low concentrations of TF, while chaperone activity begins to counteract the influence of antichaperone activity in the presence of a high concentration of TF.

Early studies suggest that antichaperone activity is an alternative behavior of the intrinsic chaperone activity under specified conditions, and essentially depends on the substrate-binding site(s) for chaperone activity (Huang et al., 2002; Song et al., 1997). Our results showed that NC fragment displayed less antichaperone activity in converting denatured lysozyme into large aggregates and decreasing the recovery of natural lysozyme in the presence of lower concentrations of NC fragment (Fig. 7C and D), indicating that the PPlase domain was involved in interacting with aggregation-prone intermediates of lysozyme to facilitate the formation of aggregates. Noticeably, NC fragment, unlike wild-type TF, did not show a considerable chaperone activity to counteract the antichaperone activity in higher concentrations. It further suggests that the PPlase domain of TF is involved in the chaperone function of TF.
Compared with NC fragment, TF389 showed less antichaperone activity toward lysozyme refolding in Hepes buffer (Fig. 7C and D). It indicates that the crevice is not only indispensable for chaperone function of TF, but also is the major substrate-binding site for the antichaperone activity of TF.

4. Discussion

4.1. Chaperone site in PPIase domain is assigned to hydrophobic pocket

The PPIase domain of TF consists of two parts: one is a hydrophobic pocket composed by a α-helix and β-strands, another is a loop which is named as flap and located upside the hydrophobic pocket (Fig. 8A) (Ferbitz et al., 2004; Ludlam et al., 2004; Vogtherr et al., 2002). To exactly map the site(s) related to chaperone function, three conserved residues were chosen as mutation sites in different regions within the PPIase domain. While Tyr221 locates in the flap, Phe233 and Ile195 are both in the hydrophobic pocket. Phe233 is on the hydrophobic surface of β-sheet consisting of antiparallel β-strands, against Ile195 at the end of the α-helix (Fig. 8A).

Our results showed that replacement of these conserved hydrophobic residues by Gly disturbed the structure of the hydrophobic pocket or the flap, resulting in destabilization of the PPIase domain (Fig. 3 and Table 1). Especially in mutant F233G, the breakage of a backbone β strand even resulted in the structure loosening of TF in native state (Fig. 2). Unexpectedly, when Phe233 was replaced by an aromatic analog, Tyr, to produce mutant F233Y, the PPIase domain was also destabilized. Considering aromatic ring of Phe233 pointing toward the cavity (Fig. 8A), destabilization of the PPIase domain in F233Y would result from introduction of a polar hydroxyl into the hydrophobic pocket, rather than the volume difference between Tyr and Phe.

Besides involvement in structure and stability of the PPIase domain, these conserved residues are thought to be involved in interaction with the ligands and tetrapeptide substrates (DeCenzo et al., 1996). Our results showed that substitution of these residues resulted in loss of PPIase activity toward tetrapeptide substrates, suggesting that both the hydrophobic pocket and the flap in the PPIase domain are indispensable for TF to carry out PPIase catalysis. For many PPlases, PPIase site has been demonstrated to be involved in many important physiological functions unrelated to PPIase activity, such as signal transduction (Huang et al., 2006; Lopez-Ilasaca et al., 1998) and chaperone function (Ramm and Pluckthun, 2001; Tremmel and Tropschug, 2007). Our results showed that structure disturbance by site-specific mutation in the PPIase active site was able to change the chaperone activity of TF toward various substrate proteins whether monomeric CA II or oligomeric GAPDH, indicating the influence of structure disturbance in the PPIase domain on the chaperone function of TF is general rather than specific.

Our results showed that when replacement of larger residue by Gly was in the hydrophobic pocket, whether in β-sheet or at the end of α-helix, the binding affinity of TF toward target proteins was decreased (Figs. 4 and 6). However, mutant Y221G with residue substitution in the flap showed chaperone activity similar to wild-type TF. These results suggest that the hydrophobic pocket, rather than the flap, is involved in the chaperone function of TF, though both of them are crucial for the PPIase activity of TF.

4.2. Hydrophobic pocket within PPlase domain acts as an auxiliary chaperone site

After identifying the hydrophobic pocket as a chaperone site in TF, we met the next question of what relationship is between the two chaperone sites in TF molecule: the hydrophobic pocket and the crevice. Although recent results from in vitro transcription/translation coupled crosslinking show that the N-terminus of long-length nascent peptides, rather than short chains, are able to traverse through the interior of ribosome-associated TF in a sequential manner from the crevice to the PPIase domain (Merz et al., 2008), it is unclear whether the substrate recognition of the more abundant non-ribosome-associated TF molecules follows similar rule, due to lack of the spatial constraints from ribosome to direct polypeptide chains through a defined path in the TF-ribosome complex. In this study, we found that while the structure disturbance within the hydrophobic pocket was able to change the chaperone activity of TF toward large-size substrate GAPDH and CA II, whether the PPIase domain exists or not had no effect on TF-assisted reactivation of lysozyme, which may be accommodated in the crevice. These results suggest that the crevice is the major chaperone site of TF, and the hydrophobic pocket in PPIase domain acts as an auxiliary chaperone site to assist protein folding when the size of substrate is over the volume of crevice. Furthermore, it was unexpectedly found that the structural damage of the crevice resulted in the failure of TF in assisting the folding of proteins no matter what size and structure, including GAPDH, CA II and lysozyme. It suggests that the structural integrity of the crevice is not only necessary for the chaperone activity of the crevice, but also is the prerequisite for the hydrophobic pocket to perform chaperone function, in consistent with early findings that the occupancy of the crevice with bis-ANS completely inhibited the chaperone activity of TF in assisting GAPDH folding (Shi et al., 2007).

It was found that all of the truncation variants with the intact PPlase domain, even PPlase domain alone, showed PPlase activity toward tetrapeptide substrates similar to wild-type TF (data not shown), indicating the structure of the PPlase active site was unchanged. Therefore, the dependence of chaperone function of the hydrophobic pocket on the structure integrity of the crevice does not arise from the mutual structural interaction of these chaperone sites. It was observed that TF variants with truncation in terminal domains not only showed negligible chaperone activity, but also failed in dimerization (Fig. 5). However, mutations in the PPIase domain were able to significantly change the affinity of TF toward intermediates of GAPDH or CA II even at very low concentration of TF, the major of which is in the monomeric state (Figs. 4 and 6). It indicates that dimerization mediated by the crevice is unrelated to, or at least is not the prerequisite for the chaperone function of the hydrophobic pocket.

We speculated that the interaction of protein substrates with the crevice was required for the chaperone function of the hydrophobic pocket toward substrates. To verify this, we investigated the binding of protein substrates to TF variants by crosslinking, and found that TF variants with the imperfect crevice lost the ability of binding to protein substrates (Fig. 5). Early studies showed that the PPIase domain displayed approximately 10% affinity of wild-type TF to 13meric peptides (Patzelt et al., 2001). These results indicate that the hydrophobic pocket alone fails in providing chaperone assistance for protein folding, due to its low binding affinity toward protein substrates. If the size of substrates is over the volume of the crevice, however, the high binding affinity of the crevice to protein substrates can provide benefit for the hydrophobic pocket to act as auxiliary chaperone site toward protein substrates bound to crevice.

4.3. Hydrophobic pocket enlarges shielding space provided by TF

TF is a promiscuous chaperone, whose substrates range in size from several kDa to over 100 kDa (Martinez-Hackert and Hendrickson, 2009). However, the crevice of TF can only accom-
moderate 15 kDa global protein (Ferbitz et al., 2004), which raises a question of how TF provides enough chaperone protection for larger proteins. While the functional cooperation of TF and DnaK molecules is required for the folding of large multidomain proteins associated with the ribosome (Agashe et al., 2004), recent results suggest that large cytosolic proteins can be encapsulated in the hydrophobic cage formed by dimerization of TF (Martinez-Hackert and Hendrickson, 2009).

Here, our results suggest there is a third manner whereby TF itself may provide protein substrates with the larger protective space rather than only the crevice as suggested previously. Since the hydrophobic pocket in the PPlase domain is involved in assisting the folding of protein substrates bound to the crevice as shown in this study, it indicates that the protective space provided by TF could be expedited from the crevice to the hydrophobic pocket. Crystal structure of TF shows that the PPlase domain, arm1 and the upper back form a miniature of clamp-like structure (Fig. 8B), which is a universal structural feature of many chaperones including prefoldin, Skp, Hsp40 and Hsp90. The substrate gripping function conferred by multivalent binding sites distributed at individual clamp arms, and/or the shielding space provided by the clamp-like architecture are crucial for these chaperones to stabilize non-native polypeptides and facilitate their transition to the native state (Stirling et al., 2006). In the clamp-like structure formed by the PPlase domain, arm1 and the upper back of TF, while the hydrophobic pocket as substrate-binding site is located at one clamp arm, it is worth noting that Phe322 together with Leu332, Leu336 and Phe337 forms a conserved hydrophobic patch on the surface of arm1, another clamp arm of the semi-closed structure. This hydrophobic patch is not located on the interior surface of the crevice, but is unusually exposed on the external side of the crevice, directly opposite to the hydrophobic pocket (Fig. 8B). Therefore, we suggest that the hydrophobic pocket together with the conserved hydrophobic patch on arm1 and other hydrophobic patches in the clamp-like structure may provide multivalent hydrophobic binding to facilitate the chaperone function of TF. This clamp-like structure can provide an extra protection space for the folding of protein substrates to avoid off-pathway reactions, if these substrates are too large to be completely accommodated by the crevice, which enable TF to be a chaperone with large-scale substrate spectrum in vitro, as shown in this study, and in vivo (Deuerling et al., 1999, 2003; Martinez-Hackert and Hendrickson, 2009; Teter et al., 1999).

4.4. Substrate-dependent cooperation of two chaperone sites contributes to chaperone function of TF

Chaperones may be classified into ATP-dependent and ATP-independent groups. While ATP-dependent chaperones, including DnaK and GroEL, can actively assist protein folding by undergoing ATP-regulated conformation change, ATP-independent chaperones are considered to change folding pathway and suppress aggregation of substrate proteins in a passive manner of binding-release (Deuerling and Bukau, 2004; Hartl and Hayer-Hartl, 2002; Haslbeck et al., 2005; Huang et al., 2000b; Sun and MacRae, 2005). However, recent studies show that some ATP-independent chaperones are able to modulate their chaperone function according to substrate and environment, thereby provide optimized chaperone assistance for substrates. For example, sHsps may shift between multiple oligomeric states with different chaperone activity, in response to temperature, pH, ionic strength and identity of substrate protein (Haslbeck et al., 2005; Sun and MacRae, 2005).

Our results show that the hydrophobic pocket of TF is able to be involved in assisting protein folding in a substrate-dependant manner. This substrate-dependant cooperation between the crevice and the hydrophobic pocket not only improves the chaperone capacity of TF, but also enables TF to provide different shielding space and binding affinity for different protein substrates according to their size or structure, thereby reducing the possibility of adverse events caused by dimensional mismatching between substrate and the shielding space provided by chaperone. It indicates that just like sHsps, TF, an ATP-independent chaperone, is able to modulate its chaperone function according to the properties of substrate proteins, rather than to passively assist protein folding by a simple binding-release cycle as suggested previously (Huang et al., 2000b).

Unless be arranged in a specific topology such as in chaperonin cylinder, would coexistence of multiple substrate-binding sites in one molecule/complex be unfavorable to assisting protein folding under certain circumstances, because multivalent binding of the aggregation-prone substrate intermediates to the chaperone molecule will specifically facilitate substrate aggregation, the process of which is similar to immunoprecipitation of a multivalent antigen by bivalent antibodies (Sideraki and Gilbert, 2000). As expectedly, we found that TF displayed antichaperone activity under certain circumstances, and the hydrophobic pocket was able to promote the formation of aggregates (Fig. 7C and D). However, we suggest that the adverse antichaperone effect arising from coex-
istence of multiple chaperone sites would be diminished by the low affinity of the hydrophobic pocket and the substrate-dependent manner of interaction between the hydrophobic pocket and protein substrates, while cooperation of the hydrophobic pocket and the crevice significantly improves chaperone function of TF. Noticeably, the antichaperone activity of TF dominates only when TF is less or is highly overloaded by substrate proteins. With increasing TF concentration, the antichaperone activity of TF can be counteracted by the chaperone activity of TF (Fig. 7). Taking into account that TF is a very abundant protein (approximately 50 μM) in the E. coli cytosol (Teter et al., 1999), and works together with many chaperones including DnaK, GroEL/ES, HtpG, HtpAB to assist protein folding and prevent aggregation, we suggest that TF predominantly displays chaperone activity in physiological condition, while antichaperone activity is negligible.

4.5. Concluding remarks

We have shown in this study that besides the crevice consisting of terminal domains as the major chaperone site, the hydrophobic pocket within the PPlase domain of TF can act as auxiliary chaperone site to assist the folding of protein substrates bound to the crevice in a substrate-dependent manner. These in vitro results suggest that TF can modulate its chaperone function according to the properties of substrate and thereby provide appropriate protective space and binding affinity for the folding of protein substrates, rather than to passively assist protein folding by a simple binding-release cycle as suggested previously.

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