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Effect of C-terminal truncation on the molecular chaperone function and dimerization of *Escherichia coli* trigger factor

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Abstract

To examine the role of the C-terminal domain in the chaperone function of trigger factor (TF), a number of truncation mutants were constructed, namely: TF419, TF389, TF380, TF360, TF344, and TF251, in which the C-terminal 13, 43, 52, 72, 88 residues or the entire C-domain were deleted, respectively. Co-expression of mutant chicken adenylate kinase (AK) with TF and the C-terminal truncation mutants was achieved using a plasmid pBVAT that allows expression of TF and AK from a single plasmid. The results show that truncation of the C-terminus of TF has only minor effect on its ability to assist AK refolding in vivo. Further, ribosome-binding experiments indicate that C-terminal truncation mutants can still bind to the ribosome and the presence of the C-terminus may in fact lower the affinity of TF for the ribosome in vivo. This indicates that the C-domain of trigger factor may not be essential for the ribosome-associated molecular chaperone function of TF. However, the purified TF C-terminal truncation mutants had a dramatically reduced ability to assist rabbit muscle GAPDH refolding in vitro and a reduced tendency to dimerize. This shows that the structural integrity of the C-terminus contributes to both the chaperone function of TF and the stability of the dimeric form.

Keywords: Chaperone; Co-expression; C-terminal truncation; Dimerization; Cross-linking; Trigger factor

1. Introduction

Escherichia coli trigger factor (TF) is an important molecular chaperone in nascent peptide folding [1–4]. TF binds at the ribosome exit tunnel through the interaction of the highly flexible loop in its N-terminal domain with the L23 and L29 proteins of 50S ribosome subunit [5,6]. Thus TF is the first molecular chaperone that nascent polypeptides encounter [1,2]. TF is a peptidyl–prolyl cis–trans isomerase (PPIase) [1,3] belonging to the FKBP family [7–9]. TF is effective as a molecular chaperone and its chaperone activity is distinct from its PPIase activity both in vitro and in vivo [10–12]. Consistent with its chaperone function, TF has been shown to have a preference for random coil or loosely structured substrates [4,12,13] and an apparent anti-chaperone phenomenon has been observed [14]. The extraordinary efficiency of TF as a folding catalyst has been attributed to its modular structure and its ability to bind substrates with high affinity [4,15]. *E. coli* TF is 432 residues in length and contains three domains with distinct structural and functional properties (Fig. 1A). The N-terminal domain (1–144) of TF is important for ribosome-binding [16] and the M-domain (145–247) carries the PPIase activity [17,18]. However, the function of the C-terminal domain (248–432) remains unclear and the residues or regions directly involved in substrate binding have not yet been identified. The crystal structure of TF (Fig. 1B) shows that it folds into a unique shape resembling a crouching dragon with the N-terminal domain forming the "tail", the M-domain forming the "head", the C-terminal forming the "arms" and the connecting regions building up the "back" [19,20].

Abbreviations: AK, adenylate kinase; DSS, disuccinimidyl suberate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KJE, DnaK–DnaJ–GrpE; TF, trigger factor.

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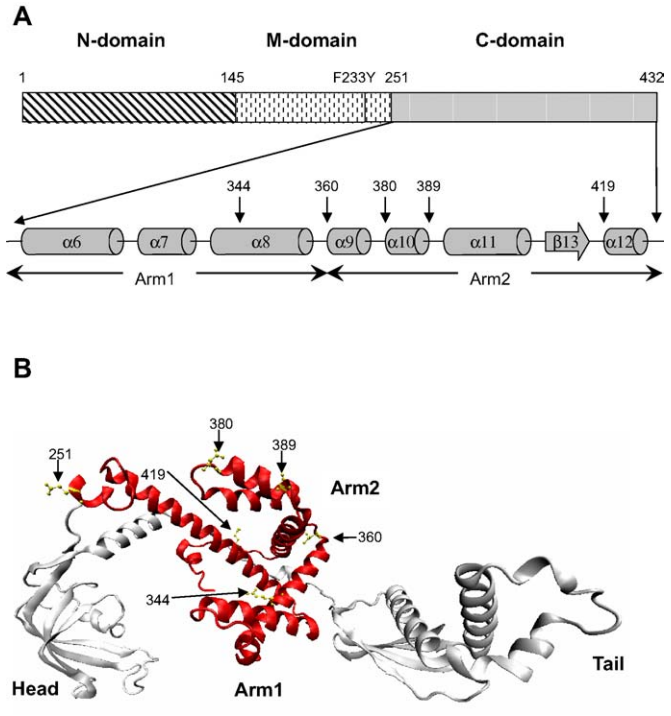

Fig. 1. Domain structure of TF and C-terminal truncation mutants.
The secondary structural elements (A) and the arm structures of the C-terminal domain (B) are shown [20]. The C-terminal region was truncated at the positions indicated.

The structure suggests a potential role of the C-domain in substrate binding [20,21].

The trigger factor gene, *tig*, is universally conserved in eubacteria and is also retained in *Mycoplasma* species which have lost the genes for all other PPIases [22]. The N-terminal domain and M-domain of TF are well conserved but the C-terminal domain varies in different species. To further examine the chaperone function of trigger factor and its relationship with the C-terminal domain, a number of C-terminal truncation mutants were constructed, each containing a mutation (F233Y) in the PPIase domain which causes the PPIase activity to be lost towards peptide substrates [10,11,23]. Co-expression of mutant chicken AK with TF was achieved using a plasmid pBVAT, that yields approximately equal amounts of TF and AK when heat shocked, and excess of TF over AK when heat shocked in the presence of IPTG [11]. This system makes it possible to evaluate the abilities of different mutants of TF in assisting protein folding in *E. coli*, by measuring the amount of soluble AK produced during co-expression with TF. Co-expression of mutant AK with TF and C-terminal truncation mutants resulted in an increase in the amount of soluble AK. However, no obvious differences were observed among these different mutants when introduced into the co-expression system, which suggests that the C-terminal domain is not essential for molecular chaperone function of TF in vivo. Detailed comparison of the in vitro chaperone activity of the TF mutants, as well as the relationship between their in vivo and in vitro chaperone activities, provides information about the role of the C-domain in TF-assisted protein folding.

2. Materials and methods

2.1. Materials

DL-Glyceraldehyde-3-phosphate, dithiothreitol, disuccinimidyl suberate (DSS), NADH, and bovine serum albumin (fraction V) were purchased from Sigma. Anti-TF polyclonal antibody was prepared by Shenzhen Bioinforbody Inc. (Shenzhen, China). Anti-AK monoclonal antibody was prepared in this laboratory [24]. All other chemicals were local products of analytical grade.

2.2. Bacterial strain and plasmids

E. coli strain JM109 was used throughout this study. An expression vector (pBVAK, derived from pBV220) for chicken AK mutant P17G was donated by Dr. X.R. Sheng. Expression of AK was induced by heat shock at 42 °C. Plasmid pQE60 containing the wild-type *tig* gene that encodes TF was donated by Professor G. Fischer. $pQE60_{F233Y}$ containing TF mutant F233Y was obtained by site-directed mutagenesis as described in [23].

2.3. Construction of TF C-terminal truncation mutants and coexpression vector

Based on the sequence of the TF mutant F233Y, PCR 3′-end primers were designed to construct TF C-terminal truncation mutants (Fig. 1) as follows: TF251-5′-cgcggatcctcacgccgaaacgtttgatgaa-3′; TF344-5′-cgcggatcctcagcggcgtttagcctgtt-3′; TF360-5′-cgcggatcctcacagctcgttggtgcgg-3′; TF380-5′-cgcggatcctcaatcttcgtacgcagaagc-3′; TF389-5′-cgcggatcctcagctgtagaactcgataactt-3′; TF419-5′-cgcggatcctcattcagtcactttcgctttcg-3′.

These primers, paired with the 5′-end primer: 5′-cgcggatcctgactcgagaaa tcataaaaaattt-3′ which anneals with pQE60 upstream of the T5 promoter and SD sequence, were designed to amplify the DNA sequence corresponding to: T5 promoter—SD sequence—respective TF C-terminal truncation mutant.

The vector for mutant chicken AK and TF co-expression under control of different promoters was constructed as follows: using $pQE60_{F233Y}$ as the template, a pair of PCR primers was designed to amplify the corresponding DNA sequence and to introduce a *Bam*H1 restriction site at both 5′ and 3′ ends. The resulting PCR product was inserted into pBVAK at the *Bam*H1 site, downstream of the mutant AK gene. The desired clones were selected and sequenced. The resulting plasmid, named pBVAT, yields approximately equal amounts of plasmid-borne TF and AK when heat shocked and excess of TF over AK when heat shocked in the presence of IPTG.

2.4. AK production during co-expression with TF

Co-expression of AK with TF and its C-terminal truncation mutants was carried out exactly according to the protocol described previously [11]. The AK bands were confirmed with

anti-AK McAb3D3 [25] and AP-conjugated goat anti-mouse IgG. TF bands were confirmed by anti-TF multi-clonal antibody and HRP-conjugated anti-chicken IgY.

2.5. Quantification of AK and TF

The AK activity in the supernatants or 6 M urea-solubilized inclusion bodies was measured as described previously [11,26]. The TF/AK ratio was calculated by analyzing the images of Coomassie blue stained gels as described in [11], using the average result of at least three independent gels.

2.6. Isolation of ribosome-bound TF and AK

JM109 strains harboring the different plasmids were cultured and cells were harvested as described [16]. Ribosomal pellets were resuspended in SDS-PAGE sample buffer, and aliquots of ribosomes and post-ribosomal supernatant (50% of the corresponding amount of ribosomal pellets) were analyzed on 13.5% SDS-PAGE gels in duplicate. Gels were blotted onto nitrocellulose, one was then probed with anti-TF polyclonal antibody and HRP-conjugated anti-chicken IgY, and the other one was probed using anti-AK monoclonal antibody and AP-conjugated goat anti-mouse IgG. The relative percentage of ribosome bound WT or mutant TF in each experiment, as a proportion of the total amount of TF in that experiment, was calculated by densitometry using the program TotalLab v2003.03.

2.7. Purification of TF and GAPDH

Full-length trigger factor and C-terminal truncation mutants were purified according to the method of Stoller et al. [3] and quantified by the Bradford method using bovine serum albumin as a standard. Purification and activity determination of rabbit muscle GAPDH were performed as described previously [27]. An absorbance coefficient of $\varepsilon_{280\ nm} = 144,000\ M^{-1}\ cm^{-1}$ was used for holoenzyme concentration determination.

2.8. TF-assisted GAPDH refolding

GAPDH denaturation and TF-assisted GAPDH refolding or aggregation was performed as described [12].

2.9. Cross-linking and electrophoresis

The cross-linking reactions were carried out in 15 mM sodium phosphate buffer, pH 7.5, containing different concentrations of wild-type or mutant TF and 1 mM DSS at 20 °C. After 30 min incubation, the reaction was quenched by the addition of the same volume of 2 × loading buffer for SDS-PAGE. Cross-linking products were analyzed by 6–15% gradient SDS-PAGE and visualized by Coomassie brilliant blue staining.

3. Results

3.1. Construction of TF C-terminal truncation mutants

To investigate the effect of the C-terminal domain on the molecular chaperone function of TF, a number of C-terminal truncation mutants were designed. On the basis of secondary structure prediction [28], and more recently using the published crystal structure of TF [19,20], we designed a series of PCR primers so as to retain the integrity of the α-helical structure in the TF C-terminal domain, namely: TF419, TF389, TF380, TF360, TF344 and TF251, in which the C-terminal 13, 43, 52, 72, 88 residues or the entire C-terminal domain have been deleted, respectively (Fig. 1A). Fig. 1B shows the relationship between the truncated regions and the two ‘arm’ structures in the C-terminal domain.

3.2. Co-expression with full-length or C-terminally truncated TF increases the yield of soluble AK

The in vivo molecular chaperone activity of TF in assisting AK refolding was investigated by evaluating the relative amounts of soluble mutant chicken AK produced in the co-expression system. As shown in Fig. 2, when mutant AK was induced by heat shock at 42 °C of the strain harboring pBVAK (which contains only the mutant AK gene), less than 15% of the expressed AK was found in the supernatant. When mutant AK was co-expressed with wild-type TF using the strain harboring pBVAT (which contains both AK and TF genes) by heat shock at 42 °C or by heat shock plus 0.1 mM IPTG, the production of soluble AK was increased to greater than 40% (Fig. 2). Interestingly, when AK was co-expressed with the C-terminally truncated TF, the result was similar to full-length TF, and the percentage of soluble AK was much

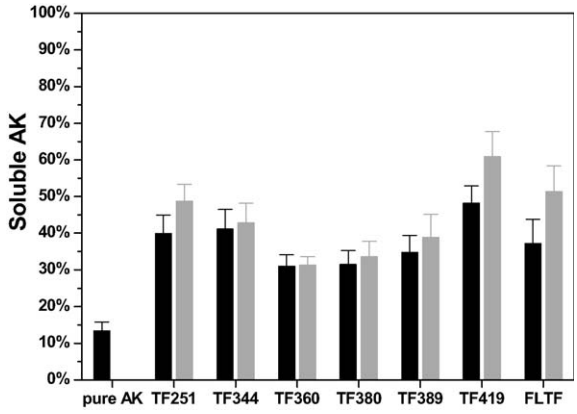

Fig. 2. The amounts of soluble AK formed in different co-expression systems. Two sets of data corresponding to two inducing conditions for co-expression of AK and TF, namely 42 °C heat (black) and 42 °C heat plus 100 μM IPTG (gray), are illustrated. The percentage of soluble AK was the average value quantified from the AK activity and SDS-PAGE (see Section 2).

higher than when AK was expressed alone (Fig. 2). The percentages of soluble AK formed when co-expressed with different C-terminal truncated mutants ranged from 30–50%, showing that all of the C-terminal truncation mutants were able to promote the folding of newly synthesized AK. There is negligible difference between the effect of co-expression with TF251, in which the entire C-domain has been truncated, compared with TF419, in which only 13 C-terminal residues have been truncated, or with full-length TF. It seems that C-terminal truncation has little effect on TF-assisted AK refolding, indicating that the C-domain of trigger factor may not be essential for the in vivo molecular chaperone function of TF. This is consistent with the previous observation that the N domain is sufficient to partially substitute for TF in vivo [29].

3.3. C-terminally truncated TF can bind to the ribosome

It has been previously reported that the N-terminal 118 amino acids of *E. coli* trigger factor constitutes a domain that is necessary and sufficient for binding to the ribosome [16] and the N-terminal is also sufficient to assist nascent peptide folding in vivo [29]. To further investigate the role of the C-domain, we examined whether C-terminally truncated TF is able to bind to the ribosome and whether AK can also bind to the ribosome through its interaction with TF. For this purpose, we used the method described by Hesterkamp et al. [16] to detect ribosome-bound TF and AK by Western blot using polyclonal anti-TF and monoclonal anti-AK antibodies. As shown in Fig. 3A (lanes 5, 7 and 9), C-terminally truncated TF, such as TF251 and TF344, shows the ability similar to full-length TF to bind to ribosomes. (The additional band visible in lanes 2–7 corresponding to full-length TF is due to constitutive expression of the intrinsic TF gene in the *E. coli* cell.) We analyzed the bands shown in Fig. 3A quantitatively. The results show that the relative percentages of the ribosome-bound TF were 17.5%, 19.8% and 30% for full-length TF, TF344 and TF251, respectively (taking the sum of TF in ribosome-bound and supernatant as 100% in each individual experiment). These results provide further experimental evidence to confirm that TF binds to the ribosome through the N-terminal domain [29] and suggest that the C-terminal residues may in fact reduce the binding affinity of TF for the ribosome.

To test whether AK itself binds to the ribosome, we did the same experiment but using an anti-AK monoclonal antibody. As shown in Fig. 3B, for the plasmid pBVAK, most AK was in the post-ribosomal supernatant and only a very small amount of AK can be detected in the pellets (Fig. 3B, lines 2 and 3). This indicates that any direct interaction between AK and the ribosome is not sufficiently strong to be detected under the experimental conditions used here. When AK was co-expressed with TF or C-terminally truncated mutants, the total level of AK expression (including both soluble and aggregated fractions) was usually ~ 30% lower than in the system expressing AK alone. Despite this, much more AK was found in the ribosome pellets when TF was co-expressed with AK (Fig. 3B, lines 5, 7 and 9). This shows that AK binds to ribosome-bound TF.

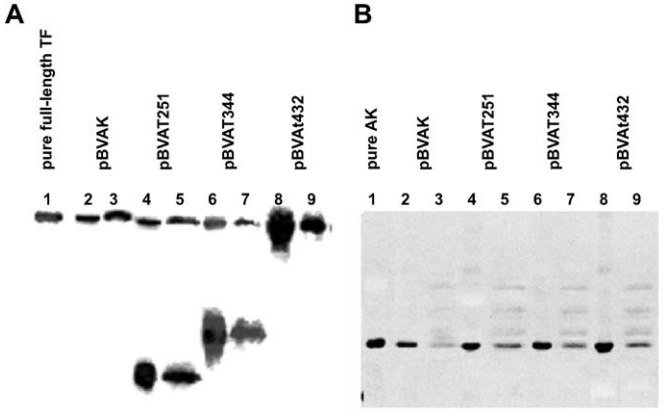

Fig. 3. The Western blot results for TF (A) and AK (B) in different co-expression systems, as indicated.
Lanes 2, 4, 6 and 8 show the post-ribosome supernatant. Lanes 3, 5, 7 and 9 show the ribosome pellets.

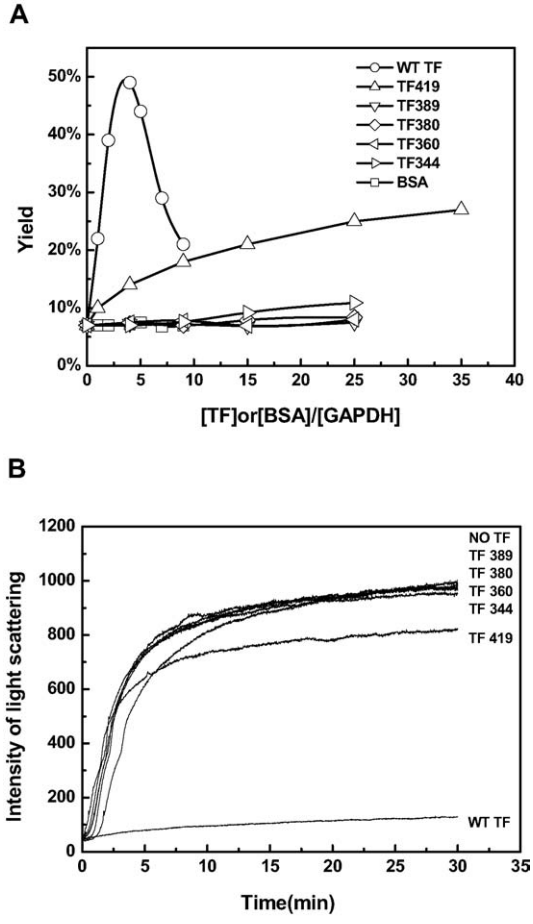

Fig. 4. Effect of wild-type TF and C-terminal truncation mutants on the reactivation (A) and aggregation (B) of GAPDH. BSA was used as a control. The concentration of GAPDH was 2.8 μM.

3.4. The in vitro chaperone function of C-terminally truncated TF

The in vitro chaperone function of C-terminally truncated TF was compared with full-length TF in assisting refolding of guanidine denatured rabbit muscle GAPDH [12]. As shown in Fig. 4A, the spontaneous refolding yield of GAPDH is very low (≈10%). When denatured GAPDH was diluted into buffer containing full-length TF, the reactivation yield of GAPDH increased with increasing TF concentration, reaching a maximum (≈50%) at a TF/GAPDH ratio of 5. However, with further increase in the TF concentration, the reactivation yield of GAPDH decreased, falling to 22% at a TF/GAPDH ratio of 9. This has previously been attributed to the formation of a stable complex between GAPDH folding intermediates and dimeric TF [30]. When denatured GAPDH was diluted into the buffer containing TF419, unlike in the presence of the full-length TF, the reactivation yield of GAPDH increased slowly and continuously with increasing concentration of TF419, finally reaching a refolding yield of about 25% at the extremely high TF419 to GAPDH molecular ratio of about 35. The deletion of the C-terminal 13 amino acids thus causes a dramatic difference in the extent and mode of TF-assisted GAPDH refolding. Surprisingly, under the same conditions, TF389, TF380, TF360 and TF344 showed almost no ability to assist GAPDH refolding (Fig. 4A) or to suppress GAPDH aggregation (Fig. 4B). This is quite different from the in vivo results, where the degree of C-terminal deletion had little effect on the ability of TF to act as a chaperone to assist AK refolding. These results indicate that the C-terminal structure of TF is important for its chaperone activity towards GAPDH refolding in vitro.

3.5. Effect of C-terminal truncation on the dimerization of TF

Earlier studies detected formation of a TF dimer by treatment with the cross-linker DSS, consistent with a monomer–dimer equilibrium of TF with a K_d of 20 μM [31]. DSS cross-linking was therefore used to test the effect of C-terminal truncation on the dimerization of TF. As shown in Fig. 5, wild-type TF showed a strong tendency to dimerize, while all of the C-terminal truncation mutants showed a reduced ability to dimerize. The dimerization capability of TF is substantially reduced even by deletion of only the C-terminal 13 amino acids. This indicates that those residues are important in dimer formation, either due to direct disruption of the dimer interface or to perturbation of the overall conformation. The effect of C-terminal truncation on dimerization of TF therefore correlates with the loss of binding chaperone function towards GAPDH (Fig. 4A). This provides further support that the 'binding chaperone' or 'holdase' function of TF is a property of dimeric TF [30,32], and is distinct from the chaperone function observed in the monomeric ribosome-bound state.

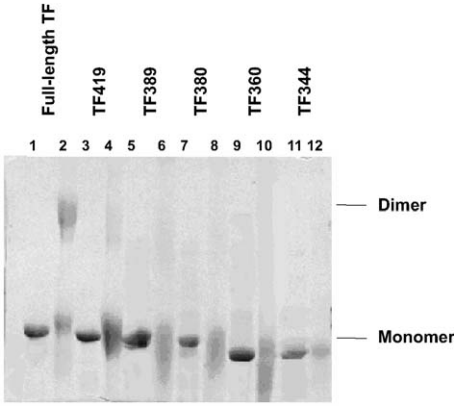

Fig. 5. Cross-linking of full-length TF and C-terminal truncation mutants, as indicated, using DSS. Lanes 1, 3, 5, 7, 9 and 11 show the controls without cross-linking.
Lanes 2, 4, 6, 8, 10 and 12 show the DSS cross-linked products. The concentrations of TF and C-terminal truncation mutants were 40 μM. The concentration of DSS was 5 mM.

4. Discussion

The modular structure of TF and the functional contributions of its individual domains have been investigated extensively both in vivo and in vitro [15–18,29]. While the N-domain is required for ribosome binding, and the M-domain carries PPIase activity, the function of the C-terminal domain has not yet been determined. The recently solved crystal structure of *E. coli* TF shows an interesting arrangement of the domains, likened to a 'crouching dragon', where the N-domain 'tail' and C-domain 'arms' are brought together, while the middle PPIase domain protrudes from the structure as the 'head' [20]. In the ribosome-bound form, the dragon is anchored by its N-domain tail and 'hunches' over the exit of the ribosome tunnel, providing a protected folding environment or 'cradle' for emerging nascent peptides [20]. The belly of the dragon (or inner surface of the cradle) presents a hydrophobic surface to the nascent peptide, formed by residues from the N- and C-domains. Thus, in the ribosome-bound form, the C-domain has a potential role in peptide binding [20,21].

One limitation in investigating the function of the TF C-domain is that it cannot form a stable folded unit in isolation, and so has only been studied in conjunction with the N-terminal and/or PPIase domains [15,29]. In this study we constructed a series of C-terminal truncation mutants of TF and introduced them into a co-expression system with an aggregation-prone mutant of chicken AK. This then allowed us to investigate the contribution of the C-terminal residues to TF chaperone function in vivo. This represents a complementary approach to the recent study describing the ability of the various TF domains to complement a Δ*tig*Δ*dnak* strain of *E. coli* [29]. We found that the C-terminal truncation mutants had about the same ability as full-length TF to assist refolding of AK when the two proteins were co-expressed in approximately stoichiometric amounts (Fig. 2). All of the C-terminal truncation mutants of TF could still bind to the ribosome (Fig. 3). Further, the proportion of TF bound to the ribosome was found to increase from 17% for full-length TF to 30% for TF251, suggesting that the C-terminal residues may in fact

reduce the affinity of TF for the ribosome. The recently reported crystal structure of the N-terminal domain (TFa) bound to the large ribosomal subunit from eubacteria reveals a significant conformational rearrangement of the N-terminal domain upon ribosome binding [33]. It is therefore possible that the C-terminal residues of TF stabilize the N-domain structure and so reduce the affinity of binding to the ribosome. The results presented here are consistent with the finding that the presence of the N-domain is necessary and sufficient to complement the synthetic lethality of Δ*tig*Δ*dnak*, to prevent aggregation of proteins (< 40 kDa), and to allow cross-linking of TF with nascent peptides [29]. These results therefore confirm that the C-terminal residues of TF are not essential for ribosome binding. Further, they indicate that the structural integrity of the C-domain is not essential for chaperone function in vivo, at least for proteins of moderate size, such as AK (21 kDa). Further investigation of the effect of C-terminal truncation on the structure and ribosome-binding ability of TF may provide additional insights into the functional role of the TF C-domain.

Given the limited effect of C-terminal truncation on TF function in vivo, it was surprising to find that even small deletions in the C-terminus had a dramatic effect on the ability of TF to assist GAPDH refolding in vitro (Fig. 4). The loss of chaperone activity was accompanied by a decreased tendency of the TF mutants to dimerize (Fig. 5). The TF-assisted renaturation of GAPDH is independent of its PPIase activity, but requires the chaperone function [12]. It has been found that the removal of the entire 181-residue C-domain renders TF inactive as a chaperone towards GAPDH [29]. According to the crystal structure of the non-ribosome-bound state, truncation of the C-terminal 44 residues causes one of the 'arms' to become disordered, but otherwise the structure is essentially identical to that of the full-length protein [19–21]. Further, TF forms a dimer even when the C-terminal 44 residues are truncated, by interactions between the N-terminal and C-terminal domains. It is therefore interesting that deletion of even 13 C-terminal residues (TF419) is sufficient to dramatically reduce the chaperoning ability of TF towards GAPDH, and deletion of 43 residues (TF389) removes this chaperoning ability altogether (Fig. 4). These results suggest that truncation of the C-terminus destabilizes conformational features important for chaperone activity and/or removes residues crucial for substrate binding. Concomitant with this, partial disruption of the dimer interface may reduce the stability of the dimeric form and hence perturb the monomer–dimer equilibrium. This indicates that while the structural integrity of the TF C-domain is not always required to assist nascent protein folding at the ribosome, the integrity of the C-terminus is crucial for its function as a chaperone towards GAPDH.

The majority of ribosomes are predicted to exist in a 1:1 complex with TF; however, TF is present in 2–3-fold molar excess over ribosomes in *E. coli* cells, with the majority of free TF present as a dimer, raising the possibility of a functional role for the dimeric form [32]. We recently found that a stable complex is formed between folding intermediates of GAPDH and dimeric TF, and that these bound intermediates can be rescued by the DnaK–DnaJ–GrpE system in an ATP-dependent manner [30]. We suggested that dimeric TF in the *E. coli* cytosol may serve as a 'binding chaperone', to maintain non-native proteins in a refolding competent conformation and cooperate with downstream molecular chaperones to facilitate post-translational or post-stress protein folding. Consistent with this hypothesis, the N-domain of TF shows structural similarity to the *E. coli* chaperone Hsp33 [34], which also shows binding chaperone or 'holdase' activity in its dimeric form, and cooperates with DnaK–DnaJ–GrpE to allow release and refolding of bound intermediates [35,36].

The results presented here support the conclusion that neither the presence of the TF C-domain, nor the PPIase activity of its M-domain, are necessary for ribosome binding or for prevention of aggregation during protein synthesis in vivo. However, the structural integrity of the C-domain is crucial for manifestation of chaperone activity towards GAPDH in vitro and also contributes significantly to the stability of the TF dimer. This study highlights that the chaperoning properties of ribosome-bound and ribosome-free TF are distinct, supporting the suggestion that the two forms may have distinct physiological roles.

Acknowledgements

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