Concerted Action of the Ribosome and the Associated Chaperone Trigger Factor Confines Nascent Polypeptide Folding

Anja Hoffmann,[1,3] Annemarie H. Becker,[1,3] Beate Zachmann-Brand,[1] Elke Deuerling,[2] Bernd Bukau,[1,*] and Günter Kramer[1,*]
[1]Center for Molecular Biology of the University of Heidelberg, German Cancer Research Center, DKFZ-ZMBH Alliance, 69120 Heidelberg, Germany
[2]Molecular Microbiology, Department of Biology, University of Konstanz, 78457 Konstanz, Germany
[3]These authors contributed equally to this work
*Correspondence: bukau@zmbh.uni-heidelberg.de (B.B.), g.kramer@zmbh.uni-heidelberg.de (G.K.)

SUMMARY

How nascent polypeptides emerging from ribosomes fold into functional structures is poorly understood. Here, we monitor disulfide bond formation, protease resistance, and enzymatic activity in nascent polypeptides to show that in close proximity to the ribosome, conformational space and kinetics of folding are restricted. Folding constraints decrease incrementally with distance from the ribosome surface. Upon ribosome binding, the chaperone Trigger Factor counters folding also of longer nascent chains, to extents varying between different chain segments. Trigger Factor even binds and unfolds pre-existing folded structures, the unfolding activity being limited by the thermodynamic stability of nascent chains. Folding retardation and unfolding activities are not shared by the DnaK chaperone assisting later folding steps. These ribosome- and Trigger Factor-specific activities together constitute an efficient mechanism to prevent or even revert premature folding, effectively limiting misfolded intermediates during protein synthesis.

INTRODUCTION

Our understanding of protein folding is derived mainly from in vitro data for the refolding of denatured full-length proteins. Folding of nascent polypeptide chains as they emerge from the ribosome is less clear. A prominent question is to what extent nascent polypeptides attain tertiary structure during synthesis, rather than after the entire protein sequence is available. In early stages of translation, folding is restricted (Kosolapov and Deutsch, 2009; O'Brien et al., 2010). Nascent chains may acquire some secondary structure, such as α-helical folds, during passage through the ribosomal exit tunnel (Wilson and Beckmann, 2011). Upon exposure at the ribosomal surface, and when given enough time (e.g., upon translational arrest), nascent chains are in principle capable of building elements of tertiary structure and will eventually form folded domains outside the exit tunnel (Zhang and Ignatova, 2011). Productive cotranslational folding, however, depends on the character of the nascent chain, on translation speed and interactions with molecular chaperones (Hartl and Hayer-Hartl, 2009; Kramer et al., 2009).

Two groups of chaperones assist de novo folding: (1) ribosome-associated chaperones that interact early with nascent chains, and (2) chaperones that do not associate with ribosomes and act later during translation or after polypeptide release (Hartl and Hayer-Hartl, 2009; Hoffmann et al., 2010). In bacteria, the chaperone Trigger Factor (TF) cycles on and off ribosomes (Kaiser et al., 2006; Rutkowska et al., 2008), employing ribosomal protein L23 as the main docking site (Baram et al., 2005; Ferbitz et al., 2004; Kramer et al., 2002; Schlunzen et al., 2005). Ribosome association is crucial for the cotranslational interaction of TF with its broad substrate spectrum (Oh et al., 2011). Recruitment of TF to ribosome-nascent chain complexes is controlled by length and identity of the nascent polypeptide and occurs in vivo only after on average 100 amino acids (aa) have been synthesized (Oh et al., 2011). TF can also stay associated with a subset of nascent proteins after these have left the ribosome (Kaiser et al., 2006; Lakshmipathy et al., 2010).

The dragon-shaped TF (Ferbitz et al., 2004) consists of three domains: the N-terminal ribosome binding domain, the peptidyl-prolyl isomerase (PPIase) domain with PPIase activity (Hesterkamp and Bukau, 1996; Scholz et al., 1997), and the C-terminal domain, which is located between the N and PPIase domains in the TF structure. Both N- and C-terminal domains provide large surfaces for substrate interactions (Lakshmipathy et al., 2007; Martinez-Hackert and Hendrickson, 2009; Merz et al., 2008) and form an open cavity, which accommodates unfolded nascent chains as well as small folded proteins (Hoffmann et al., 2006; Martinez-Hackert and Hendrickson, 2009; Merz et al., 2008; Tomic et al., 2006). Together these two domains harbor the main chaperone activity of TF, while the PPIase domain may constitute an auxiliary substrate binding site (Genevaux et al., 2004; Gupta et al., 2010; Kramer et al., 2004; Lakshmipathy et al., 2007; Merz et al., 2006).

Following TF, the DnaK chaperone, in concert with the DnaJ and GrpE cochaperones, assists de novo folding of cytosolic proteins, both co- and posttranslationally (Mayer and Bukau,

2005). DnaK and TF have overlapping functions, and their combined deletion is lethal above ~30°C, causing aggregation of several hundred different cytosolic proteins (Deuerling et al., 1999, 2003; Genevaux et al., 2004; Martinez-Hackert and Hendrickson, 2009; Teter et al., 1999).

How chaperones affect folding of nascent chains, however, is still poorly understood. Chaperones may either facilitate cotranslational folding or keep nascent chains unfolded to prevent premature folding that may lead to energetically trapped or misfolded proteins. Both modes of action have been proposed for TF. Activity measurements of multidomain firefly luciferase and β-galactosidase showed that TF (also DnaK) delays folding relative to translation and improves posttranslational folding yields (Agashe et al., 2004), presumably by binding to strongly hydrophobic segments of nascent chains (Kaiser et al., 2006; Lakshmipathy et al., 2010; Rüdiger et al., 1997). Other studies demonstrate that TF promotes folding of denatured proteins in vitro (Huang et al., 2000; Kramer et al., 2004; Merz et al., 2006) and may bind and accommodate folded domains both in solution or exposed at the ribosomal exit tunnel (Ferbitz et al., 2004; Martinez-Hackert and Hendrickson, 2009; Merz et al., 2008). The crystal structure of *Thermotoga maritima* TF in complex with the folded single-domain protein S7 revealed TF-substrate interactions of high affinity, involving predominantly hydrophilic contacts. These data suggest that TF accelerates folding by promoting the burial of hydrophobic residues in an Anfinsen-cage-like chamber (Martinez-Hackert and Hendrickson, 2009). These contradictory models raise questions as to how TF affects structure formation of its large heterogeneous substrate pool to promote efficient folding. Does TF action vary for individual substrates, or is there a general mechanism by which TF guides native folding? Does TF act on locally confined areas of the newly synthesized polypeptide? Does the mode of action of TF differ from that of DnaK?

Here, we determine how the ribosome, TF, and DnaK affect cotranslational folding of newly synthesized proteins. We show that ribosome proximity and TF constrain cotranslational sampling and native folding for multiple substrates with diverse characteristics. In contrast to DnaK, TF binds and unfolds pre-existing folded domains, with the unfoldase activity apparently limited by the thermodynamic stability of nascent chains. In summary, we show that the ribosome and TF each uniquely affect the folding landscape of nascent polypeptides to prevent or reverse early misfolds as long as important folding information is still missing and the nascent chain is not released from the ribosome.

RESULTS

Model Substrates for Monitoring De Novo Folding

We selected model substrates of different categories with respect to size, domain composition, hydrophobicity, and folding speed. As rather hydrophilic single-domain constructs (hydropathy plots in Figure 1) we used the small β sheet Src-homology 3 (SH3) domain of α-spectrin (~7 kDa, Figure 1A) and its folding-deficient point mutant m10 (Blanco et al., 1999). In addition, we investigated the αβ-single domain protein barnase (~12 kDa) (Figure 1B) and its folding-deficient truncation barnase95 (Neira and Fersht, 1999).

As multidomain model protein we mainly utilized periplasmic *E. coli* TEM1 β-lactamase (~29 kDa), which carries a single disulfide bond (S-S), acquires a stable fold in the absence of its signal sequence, and allows activity measurements during in vitro synthesis (McCarthy et al., 1998) (Figure 1C). While denatured SH3 and barnase fold within milliseconds (Serrano et al., 1992; Viguera et al., 1994), β-lactamase refolds more slowly in the minutes range (Vanhove et al., 1995). Further multidomain model proteins were *E. coli* isocitrate dehydrogenase (ICDH, ~46 kDa), a natural substrate of TF (Deuerling et al., 2003) (see Figure S1A available online), and *E. coli* dihydrofolate reductase (DHFR, ~18 kDa, Figure S1B).

Disulfide Bond Formation as Measure of Conformational Sampling Processes

We investigated cotranslational folding by measuring the kinetics of S-S formation in nascent polypeptides each containing a single cysteine pair. Therefore, we developed an oxidizing, nonsynchronized in vitro transcription/translation (t/t) system based on an extract from cells lacking TF (Moser et al., 2007). ^{35}S-methionine-labeled translation products appeared after ~2–3 min of synthesis, and amounts increased linearly for about 12 min. S-S formation occurred spontaneously in the t/t system and was measured by quantification of free thiols using a pegylated maleimide (Figure S2A). Within the first 10 min of translation, modification occurred at a constant level of 80%–85% (Figure S2B), leading to a background level of nonmodified substrates of 15%–20% (Figure S2C). At later time points of synthesis, modification efficiencies dropped considerably to 60%–70% after 30 min (Figure S2B). Absolute modification efficiencies varied from day to day but were highly reproducible within single experiments. We therefore compared only data originating from the same experiment.

Overall, we analyzed intramolecular S-S formation of two different types of cysteine pairs: (1) cysteine pairs shown or predicted (Dombkowski, 2003) to form S-S bonds in the native fold (hereafter termed "native" cysteines), and (2) control cysteine pairs which do not form S-S bonds in the native structure ("control" cysteines, S-S_{con}). Some control cysteines were positioned in too large a distance from each other to form S-S bonds in the folded protein (e.g., in different domains in case of β-lact$[77–278]_{con}$) (Figure 1). Others (e.g., SH3$[7–33]_{con}$) were designed to come in close proximity in the native protein but were experimentally verified to form no S-S bonds in the native structure.

To determine whether S-S formation in the t/t system occurs mainly during or after de novo folding, we analyzed selected model constructs: wild-type β-lactamase with its native cysteines (β-lact[77–123]), cysteine mutants of β-lactamase and SH3 harboring control cysteine pairs (β-lact$[77–278]_{con}$, SH3$[7–33]_{con}$), and the random coil m10 mutant of SH3 (m10 $[7–33]_{con}$). If S-S formation occurs predominantly after folding, we would expect low levels of S-S bonds for control cysteine pairs. This was the case for the control position in SH3 (SH3 $[7–33]_{con}$), which did not show any S-S formation above background (Figure S2C). This indicates that in the fast-folding substrate SH3 S-S bonds form only after a native-like structure has been attained.

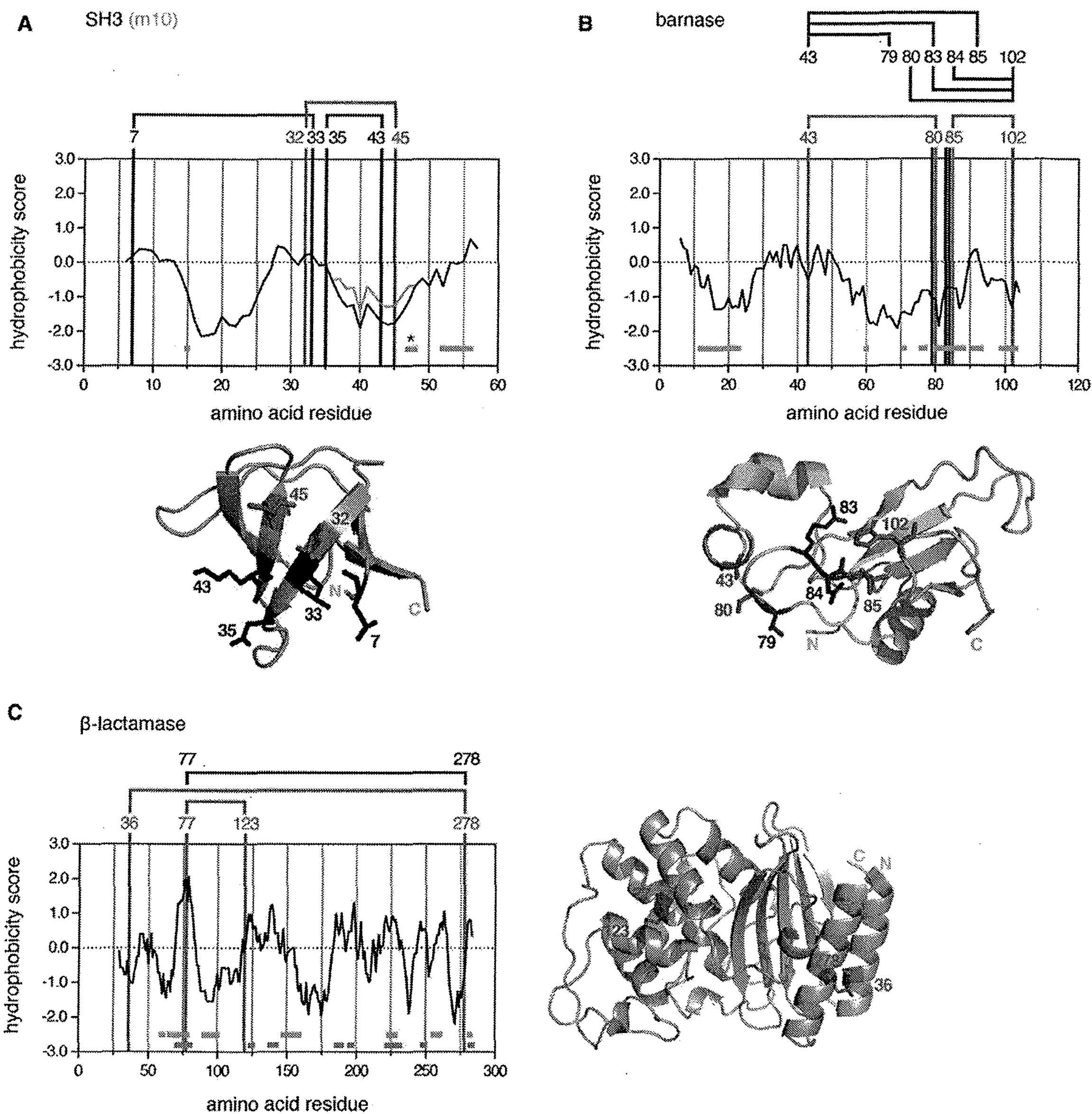

Figure 1. Characteristics of Selected Model Substrates
Hydropathy plots (according to Kyte and Doolittle [1982]; window size 11) for SH3 (black) and m10 (gray) (A), barnase (B), and β-lactamase (C). Cysteine pairs known or predicted to form a S-S bond in the native structure are marked in red. Control cysteine pairs are in black. Predicted linear TF-binding motifs according to published algorithms are displayed at the bottom of each hydrophobicity plot in orange (Patzelt et al., 2001) and green (Kaiser et al., 2006). *, binding site present only in SH3, not in m10. Crystal structures are from PDB files 1SHG (SH3), 1A2P (barnase), and 1BTL (β-lactamase).

In contrast, this cysteine pair ($[7–33]_{con}$) in the unfolded m10 built S-S bonds above background (Figure S2C). Control cysteines also formed S-S bonds in slow-folding β-lactamase (β-lact $[77–278]_{con}$), albeit to a lesser degree than the native position (77–123) (Figure S2D). These results suggest that S-S bonds can form during de novo folding if the nascent polypeptide spends enough time in an unfolded conformation to allow extended conformational sampling, which can be monitored in the oxidizing t/t system.

Ribosome Vicinity Reduces Flexibility and Folding Capability of Nascent Chains

To investigate solely cotranslational folding, we generated ribosome-arrested constructs by genetically fusing a C-terminal linker (Hoffmann et al., 2006) containing the SecM stalling sequence (Nakatogawa and Ito, 2002) to model substrates. Ribosome stalling via this SecM linker was not completely stable in the t/t system, and arrested constructs were released from ribosomes over time. However, arrested and released

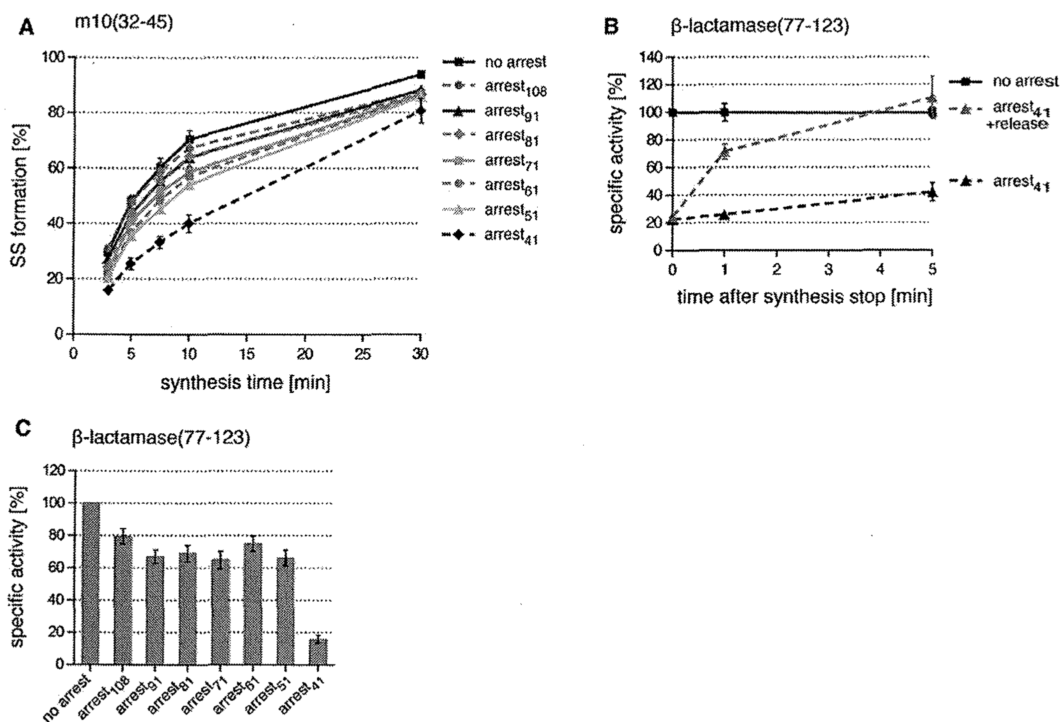

Figure 2. Ribosomal Tethering Constrains Disulfide Formation and Folding

(A) Kinetics of S-S formation in m10 with increasing distance to the ribosome. Unfolded m10 was synthesized in an oxidizing in vitro t/t system lacking TF, either as ribosome-released construct ("no arrest") or connected to the peptidyl-tRNA by a flexible linker of 41 to 108 aa. At different times of synthesis, intramolecular S-S formation at position 32–45 was quantified (for details, see Figure S2) (SEM of two experiments).

(B and C) Specific enzymatic activity of ribosome-stalled or released β-lactamase in the absence of TF. (B) β-lactamase (77–123) was synthesized in vitro for 7.5 min. Zero, one, and five minutes after translation, stop β-lactamase activities were measured and normalized to the amount of synthesized protein. Where indicated, arrested β-lactamase was released from the ribosome by treatment with RNase A and EDTA (SEM of four experiments). (C) Normalized activities of arrested β-lactamase constructs with increasing linker lengths and nonarrested β-lactamase after 7.5 min of synthesis (SEM of 13 experiments).

constructs were easily separable by size (Figure S2E), which allowed quantification of exclusively arrested constructs.

First we determined the minimal length of the arrest linker required to span the ribosomal exit tunnel and to allow folding of stalled proteins. Proteolysis assays revealed that SH3 and barnase acquire a protease-stable fold at linker lengths of 32 and 25 aa, respectively (Figures S2F and S2G), and the Sumo-specific Ulp1 protease cleaves ribosome-arrested SUMO at 37 aa linker length (data not shown). Thus, 37 aa suffice to span the exit tunnel and to fully expose folded proteins.

To analyze early conformational sampling processes of unfolded nascent chains, we monitored S-S formation in m10(32–45). When m10 was stalled at the ribosome with a 41 aa long linker ($arrest_{41}$), S-S bonds formed considerably more slowly than in nonarrested m10 (Figure 2A). Thus, the ribosome seems to impair the flexibility of m10 even outside the exit tunnel.

To explore whether these conformational restraints get relieved upon further translation, we gradually increased the distance of m10 to the ribosome by extending the linker in steps of 10 aa up to 91 aa. To ensure linker flexibility, we used sequences of m10, which according to prediction algorithms do not form stable secondary structure. In addition, we generated a flexible linker of 108 aa by inserting a second m10 between the m10 carrying the cysteine pair and the 41 aa linker ($arrest_{108}$). Elongating the linker from 41 to 51 aa significantly accelerated S-S formation in m10 (Figure 2A). Further linker lengthening gradually advanced S-S formation until kinetics similar to released m10 were obtained for the longest arrested construct. These results suggest that during translation m10 gains its full conformational freedom only when sufficiently distanced from the ribosome, either by a flexible linker of around 100 residues or upon release.

To investigate native folding, we expressed β-lactamase as ribosome-stalled or released construct, and measured specific activities. β-lactamase, arrested via the 41 aa linker, displayed an ~5-fold reduced specific enzymatic activity compared to the released form (Figure 2B). This arrested form only slowly gained activity over time but rapidly recovered activity upon ribosome release.

As for m10, we increased the distance of β-lactamase to the ribosome by gradual elongation of the arrest linker (Figure 2C). C-terminal arrest linkers of 51–108 aa significantly accelerated β-lactamase folding, resulting in specific activities of 60%–80% of released β-lactamase. Taken together, these

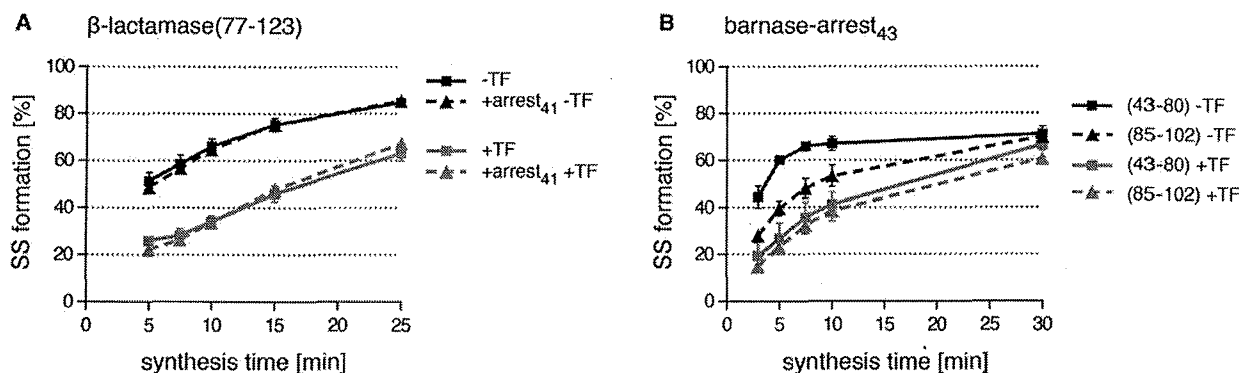

Figure 3. TF Decelerates Disulfide Formation
Model constructs with native S-S bonds (ribosome-arrested and -released β-lactamase [A], arrested barnase [B]) were synthesized in the absence or presence of TF (2 μM). To improve ^{35}S labeling, two methionines were fused to the C terminus of barnase, resulting in a 43 aa long arrest linker (SEM of three experiments).

results suggest that the ribosome confines conformational freedom and folding capability of nascent chains. The extent of this effect likely depends on size and specific folding properties of the nascent protein.

TF Constrains Cotranslational Folding More Than the Ribosome

To investigate whether TF constrains the folding process as well, we analyzed nonarrested, folding-competent proteins carrying native cysteines: wild-type β-lactamase (Figure 1C) and two double cysteine mutants of barnase with either a more N-terminal (barn[43–80]) or a more C-terminal (barn[85–102]) cysteine pair (Figure 1B) (Clarke and Fersht, 1993). We did not detect any effect of TF on the fast-folding barnase constructs (data not shown), but TF severely postponed S-S formation in the slowly folding β-lactamase (Figure 3A).

We next studied ribosome-stalled constructs. As with released β-lactamase, TF postponed S-S formation in the arrested protein, while the ribosome itself (in absence of TF) did not affect S-S formation of this N-terminal cysteine pair (Figure 3A). TF also decelerated S-S formation in both positions of ribosome-arrested barnase (Figure 3B), in contrast to released barnase. These results imply that TF confines nascent chain conformations more severely than the ribosome and also restricts cotranslational folding of proteins like barnase that fold independently of TF after their release from the ribosome.

TF Action Varies for Different Substrate Regions

To investigate whether TF uniformly postpones S-S formation at different cysteine positions, we analyzed sets of additional S-S mutants of various model substrates (Figure 1 and Figure S1). In the case of arrested barnase, we kept the position of one cysteine fixed (either position 43 or 102) and permutated the second cysteine in steps of one to five amino acids. TF equally delayed S-S formation of all cysteine pairs in region 43-X (Figure 4A) but restricted S-S formation at cysteine positions in region X-102 to varying degrees (Figure 4B). TF also postponed S-S formation with different efficiencies at three positions in arrested ICDH (Figure S3A). In β-lactamase (Figure 4C) and m10-arrest$_{41}$ (Figure S3B), TF decelerated S-S formation only at one of three positions. Furthermore, TF did not alter S-S formation at three positions each in *E. coli* MBP (maltose binding protein) and firefly luciferase (data not shown).

We considered the possibility that the observed differences are correlated to the presence of TF-binding motifs in our model substrates. Two algorithms predicting such motifs in the linear (unfolded) sequence were developed earlier: initially, TF-binding sites were predicted as stretches of 8 aa enriched in basic and aromatic residues with a positive net charge (Patzelt et al., 2001) (orange lines in Figure 1 and Figure S1). Later, TF was found to stay associated with nascent chains containing motifs of five or more consecutive amino acids of high mean hydrophobicity (Kaiser et al., 2006) (green lines in Figure 1 and Figure S1). β-lactamase (Figure 1C), ICDH (Figure S1A), MBP, and luciferase contain both types of linear binding motifs. TF restricted S-S formation of cysteines positioned within these motifs in β-lactamase (Figure 1C and Figure 4C, position 77–123) and ICDH (Figure S1Aand Figure S3A, e.g., position 52–375) but did not affect similar cysteines in MBP and luciferase (data not shown). Conversely, TF postponed S-S formation in barnase (Figure 1B and Figures 4A and 4B; all positions) and m10 (Figure 1A and Figure S3B; position 7–33), both of which lack linear TF-binding motifs of strong hydrophobicity (green lines in Figure 1). In short, the effects of TF varied for different substrate regions but based on the available data set do not appear to correlate with predicted linear binding motifs.

TF Activity Changes as Translation Proceeds

TF substrates include nascent chains of larger multidomain proteins (Deuerling et al., 2003; Oh et al., 2011). We were interested in how the impact of TF on folding changes upon elongation of the nascent polypeptide or presence of additional domains.

We assayed S-S formation in ribosome-arrested m10(32–45) constructs harboring linker lengths from 41 to 108 aa (Figure 4D). TF did not affect the shortest construct (m10*-arrest$_{41}$) but postponed S-S formation in all longer constructs to a similar extent. TF likewise delayed S-S formation in m10 when the

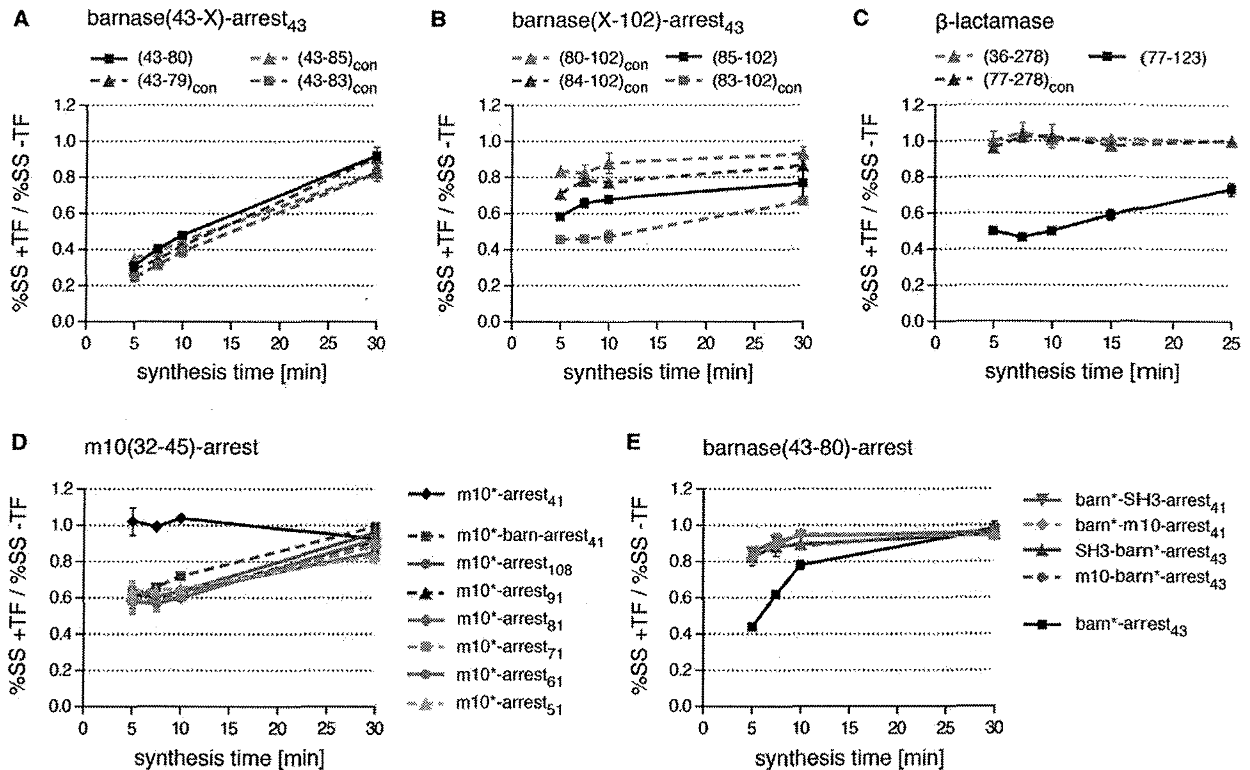

Figure 4. TF Affects Different Substrate Regions to Varying Extent

(A–C) Effect of TF on S-S formation at different cysteine positions in barnase (A and B) and β-lactamase (C) (SEM of two experiments). For each time point, the amount of S-S formed in the presence of TF was divided by the amount formed in the absence of TF.

(D) Effect of TF on ribosome-arrested m10 upon elongation of the arrest linker. * marks the domain (here m10) containing the cysteine pair (32–45); full-length barnase is abbreviated as "barn" (SEM of two to five experiments).

(E) Synthesis of additional N- or C-terminal domains reduces the influence of TF on arrested barnase. Depicted are quantifications for arrested N- or C-terminal fusions of barnase (43–80) to SH3 or m10 as representatives of multidomain proteins. * marks the domain (here barnase) containing the cysteine pair (43–80) (SEM of two experiments).

C-terminal linker was substituted by ribosome-arrested folding competent barnase (m10*-barn-arrest$_{41}$). Therefore, from linker lengths of 51 aa and more, TF restricted conformational freedom of m10, independent of length and folding potential of contiguous C-terminal sequences.

We moved the cysteine pair in the multidomain constructs from m10 to barnase. m10 fused to either the N or the C terminus of barnase reduced the impact of TF on S-S formation in barnase (43–80) compared to arrested barnase alone (Figure 4E). Replacing unfolded m10 with folding-competent SH3 gave the same result. Thus, the impact of TF on conformational sampling is influenced by the state of translation and neighboring domains in the nascent polypeptide.

TF Unfolds Nascent Chains

Previous results showed that TF transiently shields folding-deficient polypeptides from degradation by exogenously added proteases (Hoffmann et al., 2006; Tomic et al., 2006). We used limited proteolysis as another experimental approach to detect whether TF counteracts nascent chain folding. We screened for substrates that are highly protease resistant without TF, which allows monitoring a destabilization through TF. Barnase proved to be a suitable model construct.

In the absence of TF, ribosome-released and arrested barnase folded into a protease-stable conformation (Figure 5A). Cotranslational presence of TF did not alter the stability of released barnase but facilitated degradation of arrested barnase such that it was degraded with similar kinetics as the folding-deficient truncation mutant barnase95-arrest$_{43}$. This suggests TF precludes barnase from acquiring a stable fold during translation.

We considered that TF not only holds barnase in an unfolded conformation but also promotes unfolding of barnase upon rebinding to ribosome-nascent chain complexes. To test this, we synthesized arrested barnase in the absence of TF and added TF after barnase had acquired a protease-stable fold. Posttranslationally added TF destabilized barnase to the same extent as when present cotranslationally (Figure 5B). This implies that TF can at least partially reverse folding of ribosome-arrested barnase.

To support the concept of TF as a "nascent chain unfoldase," we asked whether intramolecular S-S bridges protect barnase

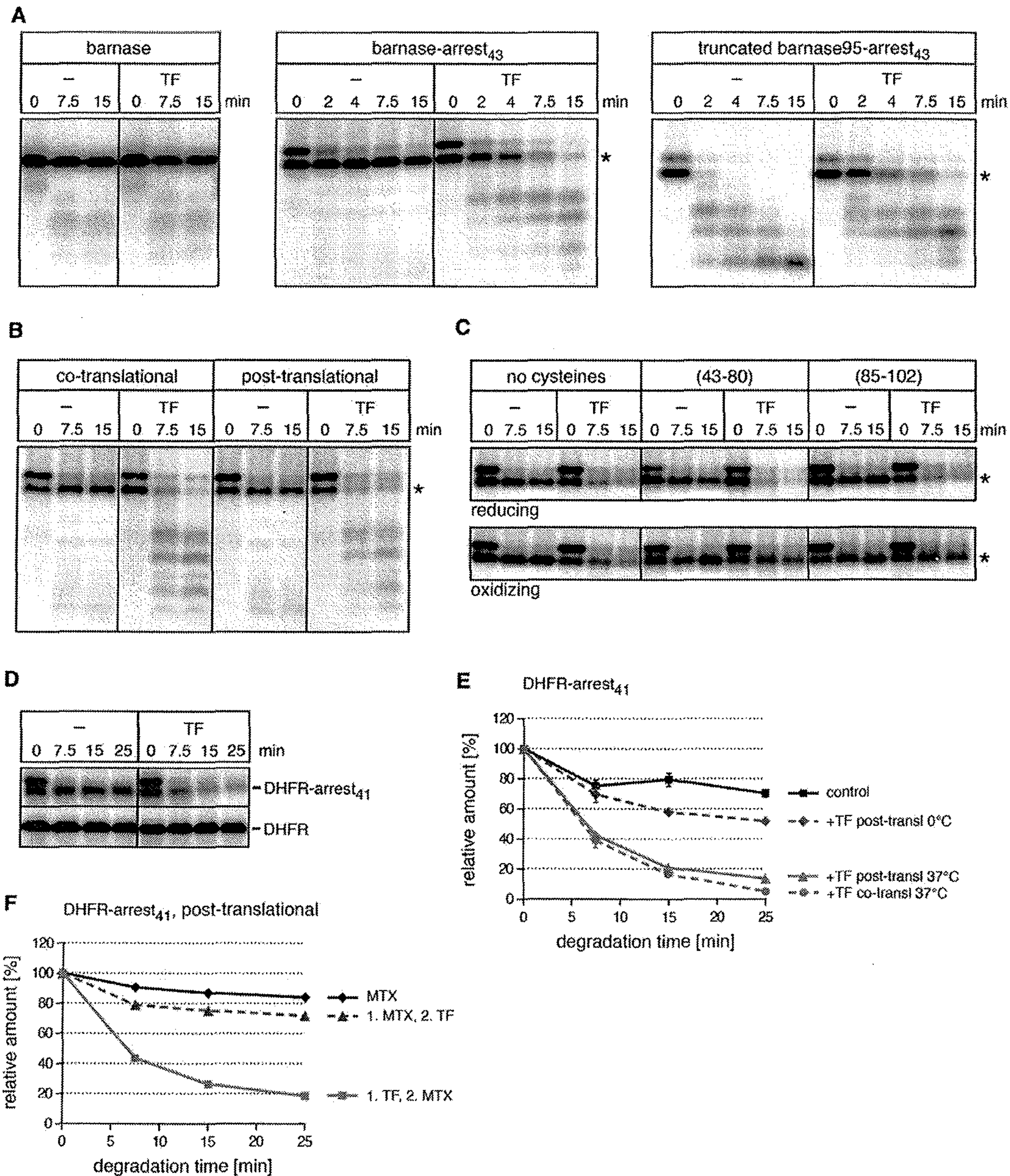

Figure 5. TF Unfolds Ribosome-Arrested Barnase and DHFR

(A) TF prevents cotranslational folding of barnase. ^{35}S-labeled polypeptides were synthesized under reducing conditions in the absence or presence of 2 μM TF. After stop of translation, Proteinase K was added (5 ng/μl), and remaining ribosome-nascent chain complexes were isolated after depicted times and visualized by autoradiography. * marks ribosome-arrested constructs.

(B) TF destabilizes arrested barnase when added after folding. TF was either present during translation ("cotranslational") or added after synthesis and folding ("posttranslational") of barnase. Proteinase K digests were performed as in (A).

(C) S-S formation in arrested barnase reduces TF-induced degradation. Barnase or barnase cysteine mutants were synthesized under reducing or oxidizing conditions. Following folding (and S-S formation under oxidizing conditions), TF was added and proteolysis assays were carried out as in (A).

(D) TF counteracts cotranslational folding of DHFR. Ribosome-arrested and -released DHFR were synthesized in the presence and absence of TF and subjected to proteolysis.

(E) TF destabilizes DHFR more efficiently at 37°C than at 0°C. Quantification of degradation assays of ribosome-arrested DHFR after co- or posttranslational addition of TF. TF was incubated with DHFR at either 0°C or 37°C prior to Proteinase K digest (at 0°C) (SEM of three to six experiments).

(F) Ribosome-arrested DHFR is more resistant against unfolding by TF upon binding of MTX. MTX (10 μM) and TF (2 μM) were added posttranslationally in the denoted order prior to Proteinase K (SEM of two to three experiments).

from destabilization through TF and performed degradation assays with two arrested S-S mutants (barn[43–80]-arrest$_{43}$ and barn[85–102]-arrest$_{43}$). Without TF, both mutants displayed wild-type-like stabilities (Figure 5C and Figures S4A and S4B). TF destabilized them similarly to the wild-type protein under reducing conditions, when no S-S bonds were formed. However, under oxidizing conditions, formation of S-S bonds reduced the ability of TF to destabilize barnase, with the position 85–102 stabilizing the protein more effectively.

To confirm the unfolding activity of TF, we selected DHFR as second model substrate. As for barnase, TF did not affect released DHFR but destabilized ribosome-arrested DHFR (Figure 5D). The extent of destabilization was indistinguishable between co- and posttranslational addition of TF (Figure 5E).

DHFR stability increases by binding the small ligand methotrexate (MTX) (Eilers and Schatz, 1986) (Figure S1B). During translation with or without MTX, TF prevented DHFR from acquiring a stable conformation (Figure S4C). When TF and MTX were added posttranslationally, order of addition determined whether TF destabilized DHFR. Added before MTX, TF rendered DHFR susceptible to degradation, but added after MTX, TF hardly destabilized the preformed DHFR-MTX complex (Figure 5F). Besides stabilization by MTX, lowering the temperature during incubation with TF from 37°C to 0°C also stabilized DHFR (Figure 5E). We conclude that TF not only keeps nascent barnase and DHFR in more unfolded conformations but also, after binding to ribosome-nascent chain complexes, destabilizes pre-existing folds.

TF Action Depends on Ribosome Binding, while the PPIase Domain of TF Is Dispensable

We investigated whether TF must bind to ribosomes to restrict folding of nascent polypeptides. We analyzed the ribosome-binding-deficient mutant TF-AAA (Kramer et al., 2002) in S-S and proteolysis assays. Compared to TF, TF-AAA hardly decelerated S-S formation in barnase-arrest$_{43}$ (Figure 6A) and β-lactamase (Figure 6B) and barely destabilized ribosome-arrested barnase (Figure 6C) and DHFR (Figure 6D). We conclude that ribosome binding is a prerequisite for TF action on nascent proteins.

Recent work showed that the PPIase domain of TF counteracts cotranslational folding of eukaryotic multidomain proteins (Gupta et al., 2010), so we examined the activity of the TF-NC fragment lacking the PPIase domain. TF-NC was nearly as efficient as full-length TF in decelerating S-S formation in barnase-arrest$_{43}$ (Figure 6A) and β-lactamase (Figure 6B) and destabilized barnase (Figure 6C) and DHFR (Figure 6D) similarly to full-length TF. These results show that TF does not require its PPIase domain for confining de novo folding of these substrates.

TF and DnaK Affect De Novo Folding Differently

Lack of TF activity in Δ*tig* mutant cells can be compensated by the DnaK-DnaJ-GrpE system (Deuerling et al., 1999; Teter et al., 1999). This raises the possibility that TF and DnaK are redundant chaperones with similar effects on de novo folding. We investigated how DnaK affects this process by using translation extracts from cells lacking both chaperones. DnaK alone neither decelerated S-S formation in ribosome-arrested barnase (Figure 6A) or β-lactamase (Figure 6B), nor affected protease sensitivity of ribosome-arrested barnase (Figure 6C) or DHFR (Figure 6D). Moreover, presence of DnaK negligibly modulated the influence of TF on nascent chain folding (Figures 6A–6D). Hence, DnaK and TF differ fundamentally in their effects on de novo folding.

DISCUSSION

This work investigates basic principles of cotranslational folding of proteins in a bacterial system, using a set of model proteins which differ in size, domain composition, hydrophobicity, and folding kinetics. We show that conformational freedom and folding speed of nascent polypeptides are restricted by the ribosome and the ribosome-associated chaperone TF. We propose a model describing the early folding environment of nascent polypeptides (Figure 7). Kinetic data on folding-impaired m10 revealed that ribosomal tethering constrains the conformational flexibility of nascent chains after they emerge from the ribosomal exit tunnel (e.g., carrying a 41 aa long C-terminal linker). These constraints are gradually relieved as chains elongate, until synthesis of around 100 C-terminal residues confers full conformational freedom for m10 comparable to that of the released polypeptide.

Restricting conformational space can in principle have opposing effects on the folding process. For the chaperonin GroEL, steric confinement of the unfolded protein within the Anfinsen cage was proposed to accelerate folding (Brinker et al., 2001), although this model has been challenged (Tyagi et al., 2011). For cotranslational folding of nascent polypeptides, however, we show that the conformational restrictions imposed by ribosomal tethering confine folding capacity and slow down native folding of newly synthesized chains, e.g., β-lactamase. Our data agree closely with recent single-molecule experiments showing that ribosome proximity decelerates native folding of T4 lysozyme carrying a linker of 41 aa and, to a lesser extent, for a linker of 61 aa (Kaiser et al., 2011).

How can these ribosome-induced folding constraints be explained? For T4 lysozyme it was suggested that electrostatic interactions of the ribosomal surface with charged residues in the nascent chain decelerate folding (Kaiser et al., 2011). Furthermore, transient interactions between nascent chains and the ribosomal surface were detected in NMR studies (Eichmann et al., 2010; Hsu et al., 2007), but the influence of these interactions on de novo folding remained unclear (Eichmann et al., 2010). Simulations of folding-unfolding transitions of ribosome-arrested polypeptides recently suggested that ribosome proximity destabilizes native conformations of nascent chains by increasing unfolded state entropy and decreasing native state entropy (O'Brien et al., 2011). In accordance with our experimental data, simulated folding rates rise for N-terminal sequences as chain length and distance to the ribosome increase, while ribosome-proximal regions remain limited in structure formation. This scenario may promote stepwise domain folding in multidomain proteins. Moreover, ribosome-induced folding constraints prolong the time frame during which the N termini of nascent chains remain accessible to the cytoplasm. This may facilitate cotranslational

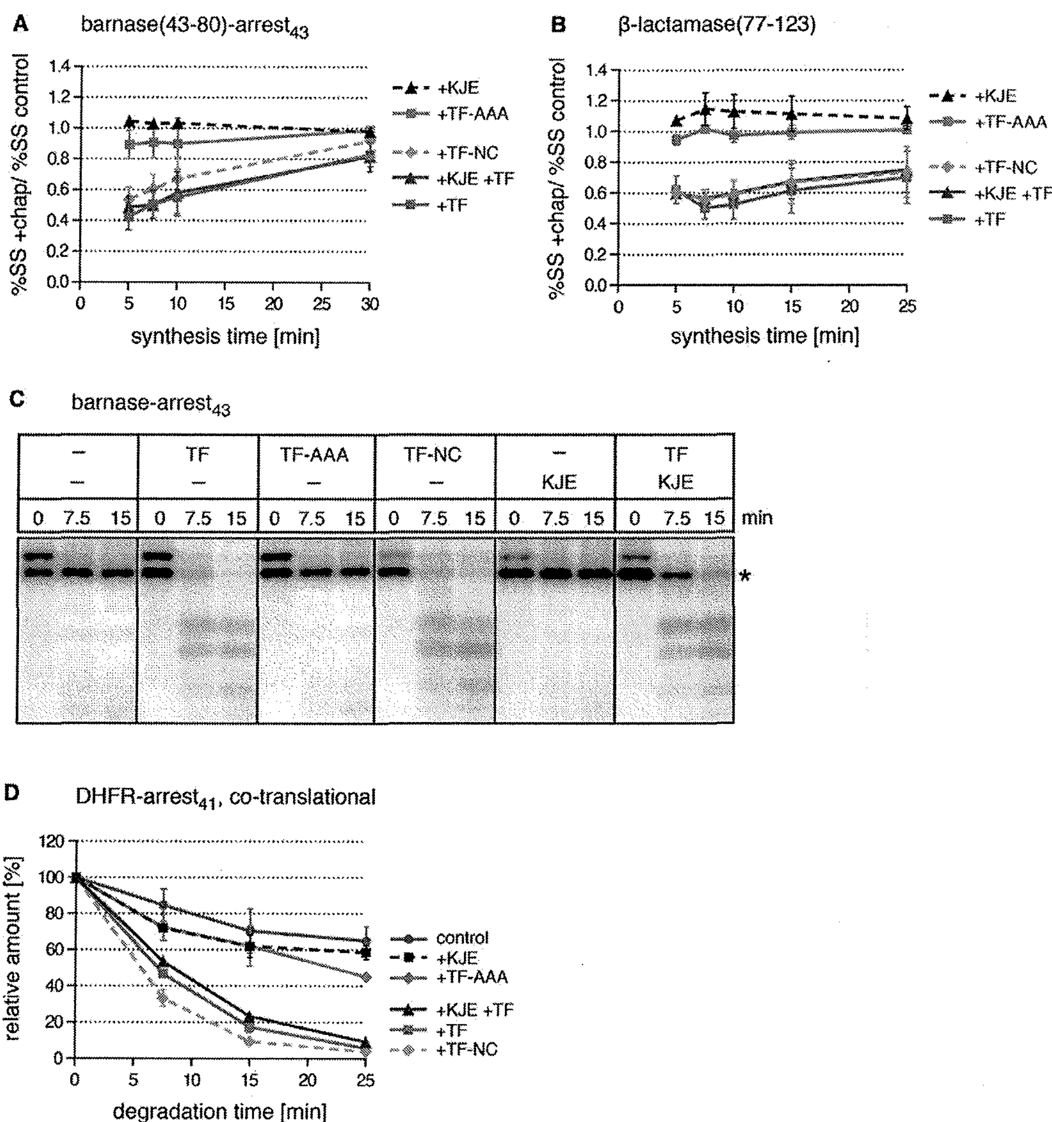

Figure 6. Different Chaperones Have Distinct Effects on De Novo Folding

(A and B) Ribosome-arrested barnase (A) and β-lactamase (B) were synthesized in the t/t system derived from *E. coli* Δ*tig* Δ*dnaK* cells in the absence or presence of readded chaperones: TF, TF-NC, TF-AAA (2 μM each), or DnaK-DnaJ-GrpE (2 μM/0.4 μM/0.2 μM). Ratios of S-S formation in the presence of the respective chaperones compared to control conditions are depicted (SEM of four [A] and two [B] experiments).

(C and D) Arrested barnase (C) and arrested DHFR (D) were synthesized in the presence or absence of indicated chaperones (concentrations as in A and B). Proteinase K digests were performed as before (SEM of two experiments).

action of essential ribosome-associated enzymes and targeting factors.

Our results do not contradict the formation of native-like structures observed in equilibrium measurements using NMR on ribosome-stalled immunoglobulin (Hsu et al., 2007), barnase (Rutkowska et al., 2009), or SH3 (Eichmann et al., 2010) domains. Instead our approach extends these findings by providing information on ribosome-induced constraints on nascent chain folding over time and relative to nascent chain length.

Our data indicate that TF confines the folding landscape of nascent chains, consistent with molecular simulation studies (O'Brien et al., 2012), and it does so to a greater extent than the ribosome alone (Figure 7). TF postpones S-S formation at both ribosome-proximal positions (e.g., the cysteine positions in barnase located 50–70 aa from the ribosomal P site) and ribosome-distal positions not subjected to ribosome-induced folding constraints (e.g., ~170 aa in m10 or ~250 aa in β-lactamase from the P site). TF decelerates native structure formation

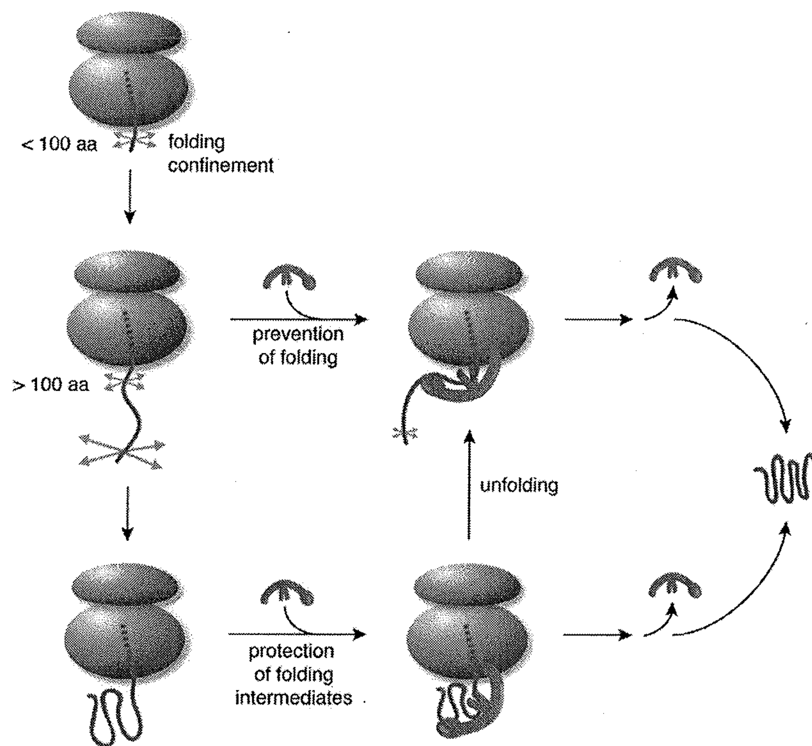

Figure 7. Model for Nascent Chain-Folding Landscapes

The ribosome limits conformational freedom and folding options of nascent polypeptide chains in its proximity. As chains lengthen, ribosomal influence decreases for more distant (N-terminal) regions, and some polypeptides may form premature structures. At an average chain length of 100 aa, TF binds the majority of polypeptides in several binding/release cycles (Oh et al., 2011) and limits conformational sampling and folding more efficiently than the ribosome while protecting folding intermediates from unwanted interactions or degradation. TF can also unfold preformed structures and reverse premature misfolds, giving nascent chains a new opportunity for productive folding. After leaving the ribosome, some polypeptides might not need further support to reach the native state, whereas others stay associated with TF or are transferred to downstream chaperone systems.

in unfolded nascent chains carrying linear motifs of strong hydrophobicity (such as β-lactamase and ICDH) but also, deviating from what was proposed based on the TF-S7 substrate complex (Martinez-Hackert and Hendrickson, 2009), in nascent chains with mainly hydrophilic character (e.g., barnase). By detecting local effects of TF, we show that TF postpones structure formation in different substrate regions to varying extents, yet the efficiency of TF does not correlate with the occurrence of linear TF-binding motifs. Furthermore, effects of TF on a nascent protein can change as translation proceeds and be influenced by adjacent N- or C-terminal domains irrespective of their intrinsic folding characteristics. These findings argue against TF acting solely by "sticking" to linear stretches of nascent chains. Instead the data suggest TF is a versatile chaperone, able to interact with diverse structural elements including hydrophobic surface features composed of discontinuous linear sequences (Lakshmipathy et al., 2010) or folded hydrophilic surfaces. We do not exclude the possibility that upon substrate binding TF might allow or facilitate the formation of local substructures while simultaneously preventing more distant interactions.

We show that in addition to postponing cotranslational folding, TF can unfold ribosome-arrested polypeptides which have acquired a stable, native-like structure before TF binding (Figure 7). We propose two mechanisms by which TF acts as an unfoldase for nascent chains. (1) Conformational breathing of nascent chains may allow TF to bind nonnative conformers and shift the equilibrium to the unfolded state. Such breathing may be locally enhanced in ribosome proximity where native conformations are less stable and unfolding rates are elevated (Kaiser et al., 2011; O'Brien et al., 2011). (2) Alternatively, in accordance with studies of TF bound to ribosome-arrested SH3 (Merz et al., 2008), TF may bind folded structures on ribosomes and provoke local destabilization. Stretching forces that trigger unfolding could be generated by the simultaneous binding of different substrate segments at multiple sites of TF, similar to GroEL in the absence of ATP (Sharma et al., 2008). N- and C-terminal domains of TF provide large surfaces for such multifaceted interactions (Hoffmann et al., 2010). In both models, unfolding is independent of substrate binding by the PPIase domain of TF, which is dispensable for destabilization of nascent chains.

Since TF cannot use ATP, we propose that TF unfolding activity is restricted by the intrinsic thermodynamic stability of the substrate. Several lines of evidence support this idea. First, TF is less effective in destabilizing nascent chains at 0°C versus 37°C. Second, TF does not destabilize released folded substrates, but only arrested substrates that most likely have a reduced stability due to ribosomal tethering. Finally, increasing the stability of ribosome-arrested constructs by introducing S-S bridges for barnase or binding of MTX for DHFR largely protects nascent chains from TF-induced unfolding. This suggests that the unfoldase activity of TF is restricted to nascent chains, controlled by chain thermodynamic stability, and likely causes partial rather than complete unfolding.

DnaK, with its cofactors DnaJ and GrpE, substitutes for the loss of TF in vivo (Deuerling et al., 1999; Teter et al., 1999) but affects cotranslational folding differently from TF. DnaK neither postpones nor reverts cotranslational structure formation in any of our model substrates. This contrasts with the folding delay reported previously for the DnaK system (Agashe et al., 2004) and suggests DnaK acts primarily posttranslationally, or on significantly longer nascent chains compared to TF. Hence, despite partially overlapping functions, TF and DnaK appear to

have mechanistically, spatially, and temporally distinct roles in de novo folding. Presumably the unfoldase activity of TF evolved to efficiently rescue kinetically trapped folding intermediates during ongoing translation.

Recent data describing TF engagement with nascent polypeptides in vivo underline the significance of the unfoldase activity reported here. Selective ribosome profiling reveals that TF engages nascent chains at an average length of around 100 aa (Oh et al., 2011). Our data show that the ribosome functions earlier during translation to confine premature folding of nascent chains before TF restricts folding further. At later stages of translation, cycles of TF binding and release may reset premature folding events that escaped the folding constraints conferred by the ribosome. We propose that the activity of TF as both holdase and unfoldase repeatedly resets untimely folding during translation, giving the growing polypeptide further scope for correct folding.

EXPERIMENTAL PROCEDURES

The Oxidizing In Vitro Transcription/Translation System

Preparation of translation extracts was performed essentially as described (Oh et al., 2011) (for details, see the Supplemental Experimental Procedures).

Disulfide Assays

Model constructs were synthesized at 37°C under oxidizing conditions. At different time points during synthesis, reactions were stopped through addition of trichloroacetic acid. Free cysteines were trapped with (methyl-$PEO_{12})_3$-PEO_4-maleimide and analyzed in autoradiography. Modification efficiencies were quantified as described in the Supplemental Experimental Procedures.

Proteinase K Digests

Except for the experiment in Figure 5C (lower panel, "oxidizing") and Figure S4B, model proteins were synthesized under reducing conditions. Post-translational Proteinase K digests were performed as described (Hoffmann et al., 2006) with slight modifications (see the Supplemental Experimental Procedures).

β-Lactamase Activity Assays

β-lactamase activities were measured after synthesis in the t/t system for 7.5 min by following the change in absorption at 486 nm upon hydrolysis of the substrate nitrocefin (200–500 μM). Synthesized β-lactamase was quantified to calculate specific activities (see the Supplemental Experimental Procedures).

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