Trigger Factor Binding to Ribosomes with Nascent Peptide Chains of Varying Lengths and Sequences*

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Trigger factor (TF) is the first protein-folding chaperone to interact with a nascent peptide chain as it emerges from the ribosome. Here, we have used a spin down assay to estimate the affinities for the binding of TF to ribosome nascent chain complexes (RNCs) with peptides of varying lengths and sequences. An in vitro system for protein synthesis assembled from purified Escherichia coli components was used to produce RNCs stalled on truncated mRNAs. The affinity of TF to RNCs exposing RNA polymerase sequences increased with the length of the nascent peptides. TF bound to RNA polymerase RNCs with significantly higher affinity than to inner membrane protein leader peptidase and bacterioopsin RNCs. The latter two RNCs are substrates for signal recognition particle, suggesting complementary affinities of TF and signal recognition particle to nascent peptides targeted for cytoplasm and membrane.

The importance of correct protein folding in living cells is reflected in the many chaperone systems that have evolved throughout the three kingdoms. In Escherichia coli the main cytosolic protein-folding chaperones belong to the DnaK/DnaJ and the GroEL/ES systems. It has been suggested that SecB also possesses general chaperone activity in addition to its role as a post-translational targeting factor (1) and that the RNA of the large ribosomal subunit may function as a universal trans-acting chaperone (2). However, there is also an apparent need for universal co-translational folding of nascent peptide chains on the ribosome. In prokaryotes, this chaperone function is carried out by Trigger Factor (TF), and although TF lacks non-bacterial homologues, there exist ribosome-bound chaperones also in eukaryotes, which seem to carry out the same function as TF (3, 4).

TF is a 48-kDa protein with both chaperone function and peptidyl-prolyl cis/trans-isomerase (PPIase) activity. Removal of the PPIase activity of TF seems not to affect its chaperone activity (5). Although TF is present in ~2-fold excess over ribosomes in the cytoplasm of E. coli, deletion of its gene has little effect on bacterial growth. However, simultaneous deletion of the genes for TF and DnaK leads to growth impairment and extensive protein aggregation (6–8), suggesting overlapping substrate pools of TF and DnaK (7).

In a study of TF binding to the members of a 13-mer peptide library, it was shown that TF binds most peptide chains, that binding does not depend on the proline content of the peptides, and that TF preferentially binds to peptides enriched in aromatic and basic amino acid residues (9). The N-terminal domain of TF binds to a site on the surface of the 50S ribosomal subunit localized to ribosomal proteins L23/L29, close to the opening of the peptide exit tunnel (10), where TF shares binding vicinity with signal recognition particle (SRP) (11). L23 can be cross-linked to nascent peptides (12, 13), suggesting that both TF and SRP are strategically positioned for efficient interactions with growing amino acid chains protruding from the peptide exit tunnel.

Docking of the crystal structure of TF to the 50S subunit suggests that TF forms a “folding cave,” in which an extensive surface is available for interaction with the nascent polypeptide (14). TF binds to non-translating ribosomes and unfolded proteins with similar affinity (~1 μM) but with slow kinetics in the former and rapid kinetics in the latter case (15, 16).

How the length and sequence of nascent peptide chains affect the affinity of TF to its natural RNC substrate are important questions that, however, have remained unanswered. Here we have taken advantage of a ribosome spin down assay (17) and used [35S]-labeled TF to estimate dissociation equilibrium constants (KD) for the binding of TF to RNCs with nascent chains of varying length and sequence. An E. coli system for protein synthesis with components of high purity was used with truncated mRNAs to stall ribosomes with nascent peptide chains of RNA polymerase (RpoB), Leader Peptidase (Lep), and Bacterioopsin (Bop). We observed increasing TF affinity to RpoB RNCs with increasing nascent peptide chain lengths; there was, for instance, a 20-fold smaller KD value for the binding of TF to an RNC with a 133-amino acid-long peptide nascent chain from RpoB than to a naked ribosome. The affinity of TF to RNCs displayed great amino acid sequence variation in that the KD values for TF binding to Lep and Bop RNCs were several times higher than those of TF binding to RpoB RNCs of similar nascent peptide lengths. From the present observations and recent structural data on TF binding to the ribosome (14), we suggest a model where the growing nascent chain interacts with multiple sites in the peptide binding cavity of TF.

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‡ The abbreviations used are: TF, trigger factor; SRP, signal recognition particle; RNC, ribosome nascent chain complex; RpoB, RNA polymerase; Lep, leader peptidase; Bop, bacterioopsin; IMP, inner membrane protein.

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**TF Binding to Ribosomes with Nascent Peptide Chains**

**EXPERIMENTAL PROCEDURES**

*RpoB, Bop, and Lep Constructs*—To produce RpoB mRNAs, the DNA sequences coding for the N-terminal 60, 80, 103, and 133 amino acid residues were amplified from the plasmid pC4MetRpoB156 by PCR. To produce Lep and Bop mRNAs, the Lep100 DNA sequence for the N-terminal 98 amino acids of Lep was amplified from the pC4MetLep98 plasmid and the DNA sequences for Bop81, Bop98, and Bop136 were amplified from the pC4MetBop plasmid. The corresponding mRNAs were subsequently obtained from these DNA templates by T7

*In Vitro Protein Synthesis and Preparation of [35S]-TF*—E. coli 70S ribosomes (>70% active in tetra peptide formation) were purified from MRE600 cells by zonal centrifugation (18). Initiation factors (IFs) and fMet-tRNA\textsuperscript{fMet} were prepared as described (19). Elongation factors (EFs), tRNA bulk, aminoacyl-tRNA synthetases were purified as described (20). Pyruvate kinase and myokinase were from Sigma.

*In vitro* translation of mRNA was performed in polymix buffer (20). Initiation mixes to obtain mRNA-programmed ribosomes with fMet-tRNA\textsuperscript{fMet} contained (final concentrations stated): ribosomes 1.5 μM; initiation factors IF1, IF2, and IF3 1.5 μM each; mRNA 3 μM; fMet-tRNA\textsuperscript{fMet} 3 μM; GTP 1 mM; phosphoenolpyruvate 2 mM. Factor mixes containing elongation factors, tRNAs, and aminoacyl-tRNA synthetases were prepared separately as follows (final concentrations stated): amino acids 1 mM each, ATP 2 mM, phosphoenolpyruvate 2 mM; GTP 1 mM; EF-Tu 30 μM; EF-G 2 μM; EF-Ts 3 μM, bulk tRNA 100 μM; all aminoacyl-tRNA synthetases 0.15 units/μl each (1 unit is the amount of aminoacyl-tRNA synthetase that aminoacylates 1 pmol cognate tRNA/second at standard conditions); 0.1 μg/μl myokinase; 0.25 μg/μl pyruvate kinase. The initiation and factor mixes were preincubated for 15 min at 37 °C before mixing, and then the *in vitro* translation reactions were incubated for another 30 min. Ribosomes from a small aliquot of each master reaction containing [\textsuperscript{14}C]glycine were purified on an S-300 gel filtration column, after which the ribosome and [\textsuperscript{14}C]glycine concentrations were determined to estimate the fraction of ribosomes carrying a nascent chain.

His-tagged TF was expressed in *E. coli* in the presence of [\textsuperscript{35}S]methionine and was purified essentially as described before (17). The total concentration of TF was measured by the Bradford procedure.

*Equilibrium Binding Experiments*—Directly after *in vitro* mRNA translation, 200 μl of ribosome nascent chain complexes (50–150 nm final concentration) were incubated with varying concentrations of [\textsuperscript{35}S]-TF in polymix. After 20 min of incubation at 4 °C, the samples were spun down during 15 min at 225,000 × g in a microultracentrifuge (Sorvall) at the same temperature. The supernatants were rapidly removed, the pellets dissolved, and the radioactivity counted in a liquid scintillation counter (Beckmann LS6500). The background radioactivity was determined by parallel ultracentrifugation of various concentrations of TF in ribosome lacking translation mixes. The amount of spin down material was calculated from the radioactive-specific activity of [\textsuperscript{35}S]-TF.

**FIGURE 1**. High affinity binding of TF to ribosomes is nascent chain dependent. A, schematic representation of the ribosome nascent chain constructs used in this study. The boxed region represents the part of the nascent chain that is hidden inside the ribosomal tunnel (~35 amino acid residues). The approximate peptide length that is expected to be outside the ribosome and available for TF interaction is indicated. B, SDS-PAGE gel demonstrating homogeneity of nascent [\textsuperscript{35}S]-labeled Bop and Lep produced by *in vitro* translation. C, TF titration to RpoB103 RNC, before and after puromycin treatment to release the nascent chain. After puromycin-mediated nascent chain release, the K\textsubscript{d} increased to a value close to that observed for TF binding to naked ribosomes.

**Analysis of Equilibrium Binding Data**—The equilibrium relations for the TF binding reactions are given as shown in Equation 1 by the expression

\[
y = \frac{Rib \cdot f \cdot (x - y)}{K1 + x - y} + \frac{Rib \cdot (1 - f) \cdot (x - y)}{K2 + x - y}
\]  

(Eq. 1)

where, y is the concentration of TF in complex with ribosomes, x is the total concentration of TF, Rib is the total concentration of ribosomes, f is the fraction of ribosomes carrying a nascent chain, and K1 and K2 are the equilibrium binding constants for TF binding to RNCs and non-translating ribosomes, respectively. Because the concentration of TF in complex with ribosomes (y) is much smaller than K2 + y in these experiments the equation simplifies as shown in Equation 2.

\[
y = \frac{Rib \cdot f \cdot (x - y)}{K1 + x - y} + \frac{Rib \cdot (1 - f) \cdot (x - y)}{K2 + x}
\]  

(Eq. 2)

MAPLE\textsuperscript{7} software (Maple Waterloo Inc.) was used to solve the equation, and the data from at least three independent titra-
TABLE 1
The $K_D$ values for TF binding to RpoB, Bop, and Lep RNCs
The data from a minimum of three independent TF titration experiments were fitted simultaneously to a two-site binding model by non-linear regression in order to obtain each $K_D$ value.

| RNC   | Nascent chain | $K_D$ (nM) | %nM |
|-------|---------------|------------|-----|
| RpoB60 | 60            | 690 ± 77   |     |
| RpoB80 | 60            | 141 ± 13   |     |
| RpoB100| 60            | 71 ± 7     |     |
| RpoB133| 50            | 53 ± 9     |     |
| Bop81  | 50            | 670 ± 194  |     |
| Bop98  | 50            | 316 ± 66   |     |
| Bop136 | 50            | 422 ± 86   |     |
| Lep100 | 60            | 311 ± 113  |     |
| 70S    | 1100 ± 300    |            |     |

RESULTS
A cell-free system for protein synthesis with E. coli components was used to produce ribosomes that were stalled on truncated mRNAs (21) and displayed nascent RpoB and, for comparison, inner membrane Lep or Bop peptide chains of varying lengths (Fig. 1A). The present choice of nascent RpoB peptides as the main target for TF was motivated by the previous identification of RpoB as one of the aggregation-prone proteins in a TF/DnaK double deletion strain (7). The truncated mRNAs, encoding N-terminal polypeptides of RpoB, Bop, or Lep (Fig. 1A), were translated, and the stalled ribosome complex was in each case targeted by [35S]-TF in a spin down assay (17) to estimate the dissociation equilibrium constant ($K_D$). In this type of assay, TF in complex with a ribosome is rapidly pelleted in the microcentrifuge, whereas free TF remains in the supernatant. As long as the pellet does not “leak” TF, the binding of TF to the ribosome is monitored under authentic equilibrium conditions (17). The fraction of ribosomes active in nascent chain synthesis was in each case estimated in a parallel experiment in which the truncated mRNA was translated in the presence of [14C]glycine. The ribosomal complexes were subsequently separated from the other components of the system by gel filtration, and the active ribosome fraction was obtained from the specific activity of [14C]glycine and the OD estimated total ribosome concentration. In general, between 50 and 60% of the ribosomes carried a long nascent chain, and because TF also has affinity for naked ribosomes, a two-site binding model (see “Experimental Procedures”) was used to estimate the $K_D$ values. The homogeneity of those nascent chains that contained internal methionines was checked by in vitro translation in the presence of [35S]methionine and subsequent SDS-PAGE (Fig. 1B).

The Affinity of TF to Ribosomes with Nascent RpoB Chains of Varying Length—We have estimated the affinity of TF to ribosomes displaying N-terminal nascent RpoB peptides consisting of 60, 80, 103, or 133 amino acids. ~35 amino acids reside in the

FIGURE 2. TF binding to RpoB nascent chains. A, the affinity between TF and RpoB ribosome nascent chains complexes increases with displayed peptide length. The plots are representative examples of TF binding to RpoB60, 80, 103, and 133 complexes. RNCs were incubated with increasing concentrations of TF and subsequently separated from unbound TF by rapid ultracentrifugation. Because non-translating ribosomes are present in the reaction as well, the $K_D$ values for TF-RNC binding were determined by fitting the experimental data by non-linear regression using a two-site binding model (see “Experimental Procedures”). B, SDS gels showing the pelleted [35S]-TF in titration series with RpoB103 RNCs and, for comparison, naked 70S ribosomes.

FIGURE 3. TF binding to Bop and Lep nascent chains. The affinities between TF and ribosomes displaying nascent chains of inner membrane proteins Bop and Lep are in general low. The plots are representative examples of TF binding to Bop81, 98, and 136 and Lep100. RNCs were incubated with increasing concentrations of TF and subsequently separated from unbound TF by rapid ultracentrifugation. Because non-translating ribosomes are present in the reaction as well, the $K_D$ values for TF-RNC binding were determined by fitting the experimental data by non-linear regression using a two-site binding model (see “Experimental Procedures”).
peptide exit tunnel of the ribosome, implying that in these cases ~25, 45, 68, or 98 amino acid residues, respectively, were displayed on the outer surface of the 50S subunit, where they could interact with TF (Fig. 1A). The $K_D$ value for the binding of TF to non-translating (naked) ribosomes that had previously been estimated as ~1 µM (16, 17). We found that increasing RpoB nascent chain length correlated with increasing affinity (decreasing $K_D$ value) of TF to the ribosome (Table 1). Accordingly, we estimated the $K_D$ values for TF binding to naked ribosomes, to RNCs with RpoB60, RpoB80, RpoB103, and RpoB133 as 1100, 690, 141, 71, and 53 nM, respectively (Fig. 2 and Table 1). To further visualize the TF binding, we ran an SDS gel showing the pelleted [35S]-TF in titration series with RpoB103 and naked 70S ribosomes (Fig. 2B).

In control experiments, where puromycin was added to RpoB103 RNCs to release the nascent chains, $K_D$ increased to a value close to that observed for TF binding to naked ribosomes (Fig. 1C). This shows that the high affinity binding of TF to the RNCs related specifically to their nascent peptide chains.

**The Affinity of TF to Ribosomes with Lep and Bop Nascent Chains**— Lep is an *E. coli* inner membrane protein (IMP) that is targeted to the membrane by SRP. Bop is an IMP from *Halobacter halobium*, also recognized by the *E. coli* SRP (22). Cross-linking experiments suggest binding interactions between TF and nascent IMPs, but whether TF has different affinity to those nascent chains that are recognized by SRP has remained unknown. To clarify this issue, we estimated the $K_D$ values for TF binding to ribosomes carrying ~100-amino acid residue-long N-terminal peptides of Lep and Bop as 311 and 316 nM, respectively (Fig. 3 and Table 1). We found, in addition, that ribosomes carrying 81 and 136-amino acid residue-long nascent chains of Bop bound to TF with $K_D$ values of 670 and 422 nM, respectively (Fig. 3 and Table 1). Thus, for Bop the affinity increases ~2-fold with an increase in nascent chain length from 81 to 98.

These results imply that TF binds between 4.5 and 7 times more weakly to RNCs displaying nascent Lep and Bop than nascent RpoB chains of similar lengths (Fig. 4 and Table 1). From these results, we hypothesize that RNCs with nascent chains targeted by SRP have lower affinity to TF than RNCs with cytoplasm-targeted peptide chains. Comparison of those amino acid residues of RpoB, Bop, and Lep (Fig. 5A) that have emerged from the peptide exit tunnel and therefore are potentially available for TF interactions shows a slightly larger number of aromatic or basic residues in all the RpoB RNCs. Surprisingly, the number of negatively charged amino acids, suggested to disfavor TF binding (9), is actually larger in the RpoB than in the Bop and Lep RNCs. Furthermore, the nascent transmembrane domains of Lep and Bop are highly hydrophobic (see boxed regions in Fig. 5A), suggesting strong binding interaction with the hydrophobic surface of TF, in apparent contrast to what we actually observed. Thus, the amino acid sequences of the nascent RpoB, Bop, and Lep RNCs did not reveal why TF had comparatively high affinity to the RpoB RNCs, which could mean that structural differences between the nascent chains may account for the observed affinity differences.

**DISCUSSION**

We have observed that the affinity of TF to RpoB nascent chain ribosome complexes increases strongly with increasing RpoB peptide length (Table 1). This result is likely to be relevant for the function of TF, because successful folding of
the RpoB protein in a Δtg strain depleted of DnaK/DnaJ is compromised (7).

We estimated equilibrium dissociation constants ($K_D$ values) for the binding of TF to RNCs with RpoB chains containing 60, 80, 103, and 133 amino acid residues. For comparison, we also estimated the $K_D$ values for TF binding to RNCs with membrane protein Bop sequences of lengths of 81, 98, and 136 amino acids as well as to an RNC with a membrane protein Lep sequence of 100 amino acids. Although cross-links have been observed between TF and nascent peptides as short as 57 amino acids (23), TF bound to the RNC with the RpoB60 chain with an affinity only slightly higher than that found for TF binding to the naked ribosome. As the nascent chain became longer, the $K_D$ value decreased monotonically to a value of 53 nM for the RpoB133 chain. These results are in line with qualitative data showing that, although TF binding to naked ribosomasome peptide chains is low, complexes formed between TF and RNCs are resistant to high salt treatment (24, 25). Our data are also in line with a proposal, based on the crystal structure of TF from *E. coli* (14, 26), that the binding cavity of TF has a large surface that can simultaneously interact with several stretches of a long nascent peptide (Fig. 5B). It is interesting in this context that a study involving nascent pOmpA chains only showed cross-linking between TF and peptide chains longer than 89 amino acids and increasing cross-linking efficiency with increasing nascent chain length (13). Our data on TF binding to RpoB agree less well with a model in which TF interacts with only one short peptide stretch at the time, although it cannot be definitely excluded that an increasing nascent chain length could somehow make a putative single site on TF successively more available for nascent chain binding.

A structural corollary to these results regarding TF function comes from the crystal conformation of TF in combination with ribosome docking experiments (14). These suggest that TF from its attachment point on ribosomal protein L23 bends over the nascent peptide exit tunnel, thereby creating a large hydrophobic surface that protects the nascent chain from other hydrophobic interactions. These structural data are in complete accordance with our biochemical data showing that the affinity of TF to nascent chains increases with their length all the way up to 133 amino acids (Table 1).

In the present experiments the RNCs were stalled on truncated mRNAs and TF was added post-translationally, in contrast to the situation in the living cell where there may be no significant stalling and TF is present at all times. However, we believe that the substrate specificity revealed by our $K_D$ estimates (Table 1) may be functionally relevant and hence contribute significantly to clarification of the mechanism of action of TF.

Both SRP and TF are in contact with ribosomal protein L23 as they interact with nascent peptides, and an important question is whether the two factors have complementary specificity for membrane- and cytoplasm-targeted sequences. Here we have found that the nascent membrane proteins Bop and Lep, both substrates for SRP, have considerably lower affinity to TF than nascent RpoB chains of similar lengths. From this finding we hypothesize that nascent chain substrates for SRP bind poorly to TF and *vice versa*. That is, in addition to the high affinity of SRP to nascent IMPs (27), these chains also have low affinity to TF, which may further reduce target competition between SRP and TF. At the same time, TF is positioned close to the exit tunnel with significant affinity even to naked ribosomes, which readily explains the observed cross-links between TF and nascent IMPs (12).

Our preliminary search for differences in amino acid composition between the RpoB, Bop, and Lep nascent chains did not render any obvious explanation for why TF has lower affinity to Bop and Lep than to RpoB nascent chains. Accordingly, the observed affinity differences may originate in structural elements of the nascent chains rather than in their amino acid composition. This would be in line with the suggestion that nascent signal sequences bind to SRP in α-helical conformation as induced already in the exit tunnel (28), whereas TF may, to speculate further, prefer extended peptide sequences. Future systematic studies of TF binding to different types of RNCs will be required to decide whether, in general, SRP and TF have complementary affinities to nascent peptides targeted for membrane and cytoplasm.

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TF Binding to Ribosomes with Nascent Peptide Chains

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