RNA modification enzyme TruB is a tRNA chaperone

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Cellular RNAs are chemically modified by many RNA modification enzymes; however, often the functions of modifications remain unclear, such as for pseudouridine formation in the tRNA TΨC arm by the bacterial tRNA pseudouridine synthase TruB. Here we test the hypothesis that RNA modification enzymes also act as RNA chaperones. Using TruB as a model, we demonstrate that TruB folds tRNA independent of its catalytic activity, thus increasing the fraction of tRNA that can be aminoacylated. By rapid kinetic stopped-flow analysis, we identified the molecular mechanism of TruB's RNA chaperone activity: TruB binds and unfolds both misfolded and folded tRNAs thereby providing misfolded tRNAs a second chance at folding. Previously, it has been shown that a catalytically inactive TruB variant has no phenotype when expressed in an *Escherichia coli truB* KO strain [Gutgsell N, et al. (2000) *RNA* 6(12):1870–1881]. However, here we uncover that *E. coli* strains expressing a TruB variant impaired in tRNA binding and in in vitro tRNA folding cannot compete with WT *E. coli.* Consequently, the tRNA chaperone activity of TruB is critical for bacterial fitness. In conclusion, we prove the tRNA chaperone activity of the pseudouridine synthase TruB, reveal its molecular mechanism, and demonstrate its importance for cellular fitness. We discuss the likelihood that other RNA modification enzymes are also RNA chaperones.

RNA chaperone | RNA folding | RNA modification | pseudouridine | tRNA

Although there is a wealth of information on RNA structure, we are just beginning to understand the RNA folding process that is often assisted by RNA chaperones (1). In contrast to many protein chaperones, RNA chaperones are not ATPases, but instead facilitate unfolding and folding of RNA directly through their interactions with RNA. In addition, the vast majority of all RNAs, including mRNAs, are posttranscriptionally modified by a plethora of RNA modification enzymes (2). Very little is known about the interplay of RNA folding and modification, although it has been speculated that RNA modification enzymes may also act as RNA chaperones (3).

Despite the abundance of RNA modifications, their cellular functions are often unclear, including their possible contributions to RNA structure and stability (4). Interestingly, very few RNA modification enzymes are essential for the cell; however, many of these enzymes are conserved. The most abundant RNA modification is the conversion of uridines to pseudouridines that are found in almost all cellular RNAs (4–6). Pseudouridine formation is catalyzed by stand-alone pseudouridine synthases in all domains of life and in addition by H/ACA small ribonucleoproteins (H/ACA sRNPs) in eukaryotes and archaea (7). Remarkably, the only essential pseudouridine synthase is the eukaryotic enzyme Cbf5, the catalytic component of H/ACA sRNPs, whereas all known stand-alone pseudouridine synthases are nonessential (8). Indeed, deletion of most stand-alone pseudouridine synthases in *Escherichia coli* (9, 10) or *Saccharomyces cerevisiae* (11) does not impact cell growth under optimal conditions. Surprisingly, even an *S. cerevisiae* strain is viable that is expressing only a Cbf5 variant that is catalytically inactive (12). In accordance with this finding, the only essential H/ACA guide RNA is responsible for 18S pre-rRNA cleavage rather than pseudouridine formation, which is likely the reason why Cbf5 is essential (13). Together, all these findings raise the question why cells invest so much energy into seemingly dispensable pseudouridine formation.

Here, we use the tRNA pseudouridine synthase TruB as a model enzyme to identify the cellular function and mechanism of pseudouridine synthases. *E. coli* TruB catalyzes the modification of U55 in the TΨC arm of all elongator tRNAs (14) and is the homolog of the eukaryotic pseudouridine synthase Pus4 that modifies both tRNAs and mRNAs (5, 6, 15). Several crystal structures of TruB bound to a TΨC arm fragment have revealed that TruB gains access to the target base by flipping three nucleobases (positions 55–57) out of the T loop and into the active site (16, 17). Superimposing the T-arm bound to TruB onto full-length tRNA indicates that tRNA binding by TruB disrupts the tertiary interactions between the T- and D-arm of tRNA. Kinetic studies in our laboratory have shown that tRNA binds relatively quickly to TruB but that catalysis of pseudouridine formation is surprisingly slow and rate limiting (18). In fact, slow catalysis is a hallmark of all pseudouridine synthases studied in detail thus far (18–20). Deleting the *E. coli truB* gene does not impact growth under optimal conditions (10). However, the pseudouridylation activity of TruB is important for temperature adaptation in *E. coli* (21) and *Thermus thermophilus* (22). *truB* KO strains are outcompeted by WT *E. coli* in coculture at 37 °C, indicating that TruB does contribute to bacterial fitness (10). Surprisingly this fitness disadvantage of the *truB* KO strain can be rescued by expressing a catalytically inactive variant of TruB (10, 21). This finding suggests that the presence of the TruB protein itself is important for the cell, rather than pseudouridines that are formed by TruB. Therefore, Ofengand and coworkers speculated more than a decade ago that TruB might act as an RNA chaperone that facilitates tRNA folding (10); however, this hypothesis has not been experimentally tested.

Results

TruB Folds tRNA in Vitro Independent of Its Modification Activity. To assess whether the pseudouridine synthase TruB is able to promote tRNA folding, we used aminoacylation of tRNA as a readout for the successful folding of tRNA into a biologically active conformation. It is well established that folded tRNA is rapidly aminoacylated,

Significance

RNAs are intimately involved in numerous cellular functions such as gene expression. Typically, RNAs adopt a 3D fold, and their sequences may be chemically altered to contain modified nucleotides such as pseudouridines. There are many RNA modification enzymes, but often their cellular functions remain unknown. In this proof-of-principle study, we show that the tRNA pseudouridine synthase TruB is a tRNA chaperone. In contrast to its modification activity, the ability of TruB to fold tRNA is critical for cellular fitness. It is likely that other modification enzymes and RNA-binding proteins act as RNA chaperones increasing the efficiency of RNA maturation. This connection of RNA modification with RNA folding may have arisen during the evolution from an RNA to a ribonucleoprotein world.

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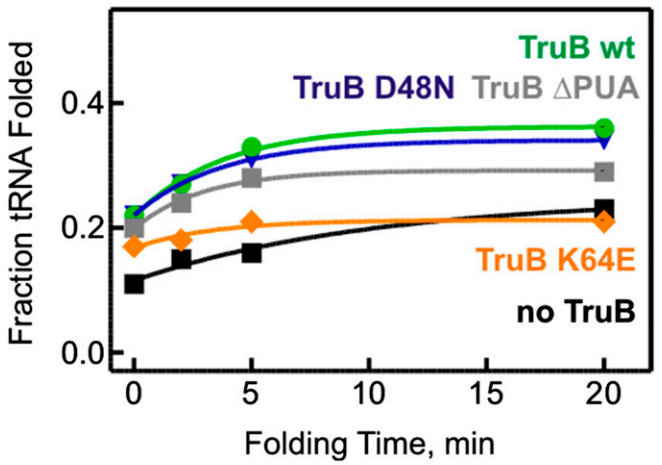

Fig. 1. In vitro tRNA folding in the presence and absence of TruB. tRNA was unfolded and then allowed to refold in the absence of TruB (black squares) or in presence of 200 nM each of TruB WT (green circles), catalytically inactive TruB D48N (blue triangles), TruB ΔPUA lacking the C-terminal PUA domain (gray squares), or TruB K64E that is severely impaired in tRNA binding (orange diamonds). At different times of the folding reactions, the aminoacylation reaction was started to determine the amount of folded tRNA that can be aminoacylated rapidly (*y* axis intercept of the aminoacylation time courses shown in Fig. S1). Here the fraction of folded tRNA is plotted over time. Fitting with an exponential equation revealed a higher rate of folding in the presence of TruB WT (0.26 ± 0.05 min^{-1}), TruB D48N (0.26 ± 0.05 min^{-1}), and TruB ΔPUA (0.34 ± 0.08 min^{-1}) in contrast to a rate of folding of 0.1 ± 0.07 min^{-1} in the absence of TruB, resulting in a significantly higher end level of folded tRNA under these conditions for TruB WT, TruB D48N, and TruB ΔPUA.

whereas un- or misfolded tRNA is only slowly aminoacylated (23). Using a published assay to monitor tRNA folding, we first unfolded unmodified in vitro-transcribed *E. coli* $tRNA^{Phe}$ through incubation at 65 °C and subsequently allowed tRNA to slowly fold at 0 °C in the presence or absence of TruB (23). At several time points, tRNA folding was analyzed by assessing aminoacylation of the tRNA (Fig. S1). The fraction of folded tRNA was determined by recording the fraction of instantaneously aminoacylated tRNA (23). In the presence of TruB WT, the rate of tRNA folding was increased about twofold, with twice as much tRNA folded at any given time point compared with the reaction without TruB (Fig. 1 and Fig. S1); thus, TruB assists in tRNA folding. Next, the experiment was repeated with the TruB D48N variant, which is inactive in pseudouridine formation but unimpaired in tRNA binding (18). The catalytically inactive TruB variant is able to fold tRNA at the same rate as TruB WT, demonstrating that TruB's tRNA chaperone activity is independent of its tRNA modification activity.

Molecular Mechanism of tRNA Interaction with TruB. To understand how TruB folds tRNA, we need to know how TruB binds tRNA, how it induces conformational changes in tRNA, and how it dissociates from tRNA. To separately detect and analyze these steps, we used a previously described $tRNA^{Phe}$ that is labeled at position 57 in the T-arm with a fluorescent base analog, 2-aminopurine (2AP) (24). Binding of the tRNA by TruB causes nucleotides 55–57 of the tRNA to flip into the catalytic pocket of the enzyme, which is reflected by an increase in 2AP fluorescence. Interaction with TruB WT results in a two-phase fluorescence increase reflecting tRNA binding and a subsequent conformational change followed by a slower fluorescence decrease due to tRNA dissociation after catalysis is completed (Fig. 2*A*). The first fluorescence increase likely reflects the encounter of TruB with tRNA, but it is fast and has a small amplitude, preventing a quantitative analysis. Therefore, we reduced the reaction rate by measuring TruB–tRNA interactions at a low temperature (5 °C) that does not affect the affinity of TruB for tRNA (dissociation constants of 0.8 ± 0.1 and 0.5 ± 0.1 μM at 5 °C and 20 °C, respectively; Fig. S2*A*).

The kinetic mechanism of tRNA interacting with TruB was investigated by titrating 2AP-labeled tRNA with TruB WT at both 20 °C and 5 °C in stopped-flow experiments (Fig. 2 and Fig. S2). This experimental system also allowed us to directly observe dissociation of tRNA from TruB in fluorescence chase experiments where a preformed complex of TruB with 2AP-tRNA is rapidly mixed with excess unlabeled tRNA. By using catalytically inactive TruB D48N, the dissociation of unmodified substrate RNA can be followed (Fig. 2*B*). Kinetic analyses of these time courses (Fig. S2) provide the rate constants for the initial encounter of tRNA and TruB (k_1 and k_{-1}), for the conformational change that TruB induces in tRNA to flip the target uridine into its active site (k_2 and k_{-2}), as well as for pseudouridine formation (k_ψ) as reported previously (Table 1). Comparing these rate constants reveals that tRNA binding is rapid and reversible. However, the subsequent disruption of the elbow region of tRNA to flip bases into TruB's active site is rather slow with a k_2 of only 1.3 s^{-1} at 20 °C, and it is readily reversible because k_{-2} is rather large with 1.9 s^{-1}. Importantly, reversal of the disruption of the tRNA elbow region is faster than actual pseudouridine formation ($k_{-2} = 1.9$ s^{-1} vs. $k_\psi = 0.2$ s^{-1} at 20 °C; Table 1). Therefore, after binding to TruB, the tRNA elbow region is repeatedly opened and then rapidly refolded before the tRNA is eventually slowly modified by TruB.

Because the previous experiments used folded WT tRNA (Fig. 2 *A* and *B*), we subsequently asked whether and how TruB interacts with a potentially mis- or partly unfolded tRNA. Therefore, we tested the interaction of TruB with 2AP-labeled tRNA harboring a G18A substitution in the D arm that affects the tertiary interaction of D and T arm (Fig. 2*C*). The affinity of TruB for this tRNA is unchanged, and the kinetics of the interaction between TruB and tRNA G18A are similar to WT tRNA (Fig. 2*C* and Fig. S2*F*). The most notable difference is an increase in the rate of base-flipping by about twofold (k_{app2}), which is consistent with a disturbed tertiary structure in the

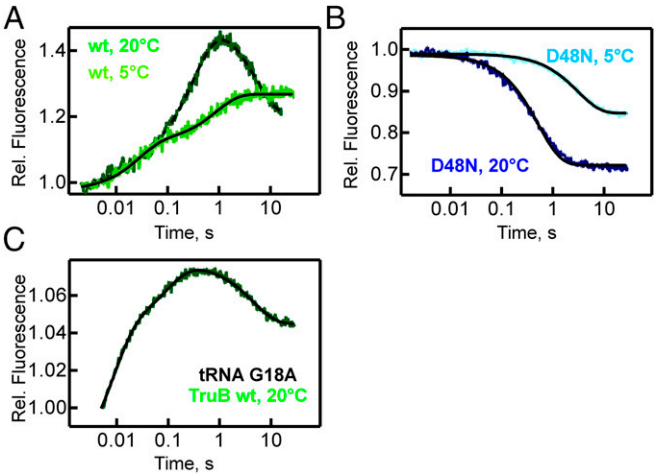

Fig. 2. Determining the kinetic mechanism of the TruB–tRNA interaction. (*A*) Time courses of TruB interacting with RNA^{Phe} containing a 2AP at position 57 were monitored using a stopped-flow apparatus; 2AP-tRNA (0.3 μM final concentration) was rapidly mixed with TruB WT (30 μM final concentration). Time courses were fitted with a two-exponential function (wt, 5 °C) or a three-exponential function (wt, 20 °C). (*B*) Dissociation of substrate tRNA from TruB was monitored by rapidly mixing TruB D48N in complex with 2AP-tRNA with an excess of unlabeled $tRNA^{Phe}$ (for dissociation rates, see Table 1). (*C*) Rapid-kinetic stopped-flow analysis of 2AP-labeled $tRNA^{Phe}$ G18A binding to TruB WT at 20 °C. Final concentrations were 1.5 μM tRNA and 5 μM enzyme. Fitting of the time course with a three-exponential function (gray line) yielded the following apparent rates: $k_{app1} = 123 \pm 3$ s^{-1}, $k_{app2} = 10.5 \pm 0.3$ s^{-1}, and $k_{app3} = 0.22 \pm 0.004$ s^{-1}.

Table 1. Summary of kinetic parameters for TruB WT and TruB ΔPUA at 5 °C, 20 °C, and 37 °C

	TruB WT			TruB ΔPUA		
Rate constant	5 °C	20 °C	37 °C	5 °C	20 °C	37 °C
k_1 (μM^{-1} s^{-1})	8 ± 1	ND	ND	2.0 ± 0.5	ND	ND
k_{-1} (s^{-1})	16 ± 5	ND	ND	ND	ND	ND
k_2 (s^{-1})	0.5 ± 0.5	1.3 ± 0.4	ND	(0.8 ± 0.3)*	(7.0 ± 0.5)*	ND
k_{-2} (s^{-1})	0.32 ± 0.04	1.9 ± 0.2	ND	ND	ND	ND
k_w (s^{-1})	ND	0.20 ± 0.02	0.5 ± 0.2†	ND	0.06 ± 0.03	0.02 ± 0.01
$k_{release}$ (s^{-1})	1.2 ± 0.1	10.6 ± 0.3	ND	0.9 ± 0.2	4.5 ± 1.2	ND

ND, not determined.
*Apparent rate (k_{app2}) for tRNA conformational change; k_2 for TruB ΔPUA will be slightly lower ($k_2 = k_{app} - k_{-2}$).
†Determined in ref. 18.

elbow region of tRNA that allows TruB to gain access to the target uridine more easily. In conclusion, TruB does not discriminate between folded and partially folded tRNAs but binds both folded and misfolded tRNAs followed by disruption of the tRNA elbow region.

tRNA Chaperone Activity of TruB Is Critical for Bacterial Fitness. The data in Figs. 1 and 2 show that TruB acts as a tRNA chaperone in vitro, but is TruB's tRNA chaperone activity important in vivo? If this were true, then bacterial fitness should be reduced when tRNA binding by TruB (but not its catalytic ability) is impaired. To test this hypothesis, single conserved, basic amino acid residues within the RNA binding surface of TruB (R40, K64, K130, and K176) were individually substituted with a negatively charged glutamate to impair tRNA binding (Fig. S3*A*). In multiple-turnover tritium release assays, all of the TruB variants were significantly slower in pseudouridylation than the WT (Fig. S3*B*). Single-turnover pseudouridylation experiments (Fig. S3*C*) revealed that the TruB K64E variant is most affected: it is 750-fold slower than the WT enzyme (Table S1) (17–19). Therefore, all further experiments were conducted with the TruB K64E variant. We determined the Michaelis constant (K_M) in single-turnover tritium release assays (Fig. 3*A* and Fig. S3*D*) because determination of a dissociation constant (K_D) for TruB K64E proved to be difficult as nitrocellulose filtration resulted in no signal change (Fig. 3*B*). Compared with TruB WT (K_D of 340 nM, K_M of 550 nM) (18), the K_M of TruB K64E is very high (28 ± 26 μM; Fig. 3*A*), indicating that the affinity of TruB K64E for tRNA is strongly reduced. We also assessed whether TruB K64E has tRNA chaperone activity in vitro (Fig. 1 and Fig. S1), revealing that the loss of RNA binding by this TruB variant is accompanied with a loss of tRNA folding by TruB K64E as expected. Next, we tested the impact of impaired tRNA binding by TruB K64E on bacterial fitness in coculture competition assays with WT (10, 21). Notably, the *truB* KO strain expressing TruB K64E was outcompeted by the *E. coli* WT strain in about 15 d (Fig. 3*C* and Fig. S4). This result confirms that tRNA binding by TruB and in turn its tRNA chaperone activity are critical for cellular fitness. In contrast, the pseudouridylation activity of TruB is not important for cellular fitness as shown previously with the catalytically inactive TruB variant TruB D48C (10). Notably, some pseudouridines were formed in tRNA in the strain expressing TruB K64E (roughly 50% of WT; Table S2), whereas essentially no pseudouridine was detected in presence of TruB D48C (Fig. 3*D* and Fig. S5). Residual pseudouridylation activity by TruB K64E is expected based on the low level of pseudouridylation activity observed in vitro and proves that the TruB K64E variant is expressed in the *truB* KO strain. This finding further supports the conclusion that tRNA binding and folding, in contrast to pseudouridylation, is the critical cellular function of TruB.

Functional Role of the PUA Domain of TruB for tRNA Interaction. The TruB family of pseudouridine synthases contains a conserved catalytic domain and a C-terminal pseudouridine synthase and archeosine transglycosylase (PUA) domain that is predicted to interact with RNA (16, 25). We hypothesized that the PUA domain is critical for tRNA binding and folding and in turn for cellular fitness. Initial studies with TruB ΔPUA lacking the PUA domain and tRNA labeled with fluorescein at the 3′CCA end suggested that the acceptor arm of tRNA interacts with the PUA domain of TruB (Fig. S6). Nitrocellulose filtration experiments with TruB ΔPUA revealed only a threefold reduced affinity for [^{3}H]-tRNA^{Phe}, with K_Ds of 9 ± 1 and 2.4 ± 0.3 μM for TruB ΔPUA and TruB WT, respectively (Fig. 3*B*). Single turnover pseudouridylation assays showed no concentration dependence, but a 25-fold reduced rate of pseudouridylation by TruB ΔPUA (Fig. 3*A*, Fig. S3*E*, and Table 1). Surprisingly, this suggests that the PUA domain is more important for catalysis than for tRNA binding, possibly by positioning the substrate tRNA onto the

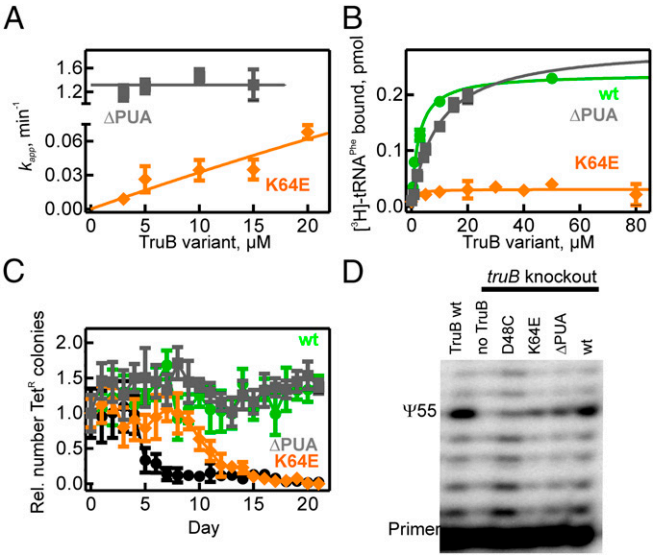

Fig. 3. Bacterial fitness depends on tRNA binding by TruB. (*A*) Apparent rates of pseudouridine formation (k_{app}) by TruB variants from single-turnover experiments (Fig. S1 *B* and *C*) were plotted against TruB concentration to determine the K_M. (*B*) Binding of tritium-labeled tRNA (10 nM) to TruB was determined through nitrocellulose filter binding. Hyperbolic fitting yielded the K_D: 2.4 ± 0.3 μM for TruB WT and 9 ± 1 μM for TruB ΔPUA. (*C*) Coculture competition assays between WT *E. coli* and the *E. coli truB* KO strain (black), the *truB* KO strain expressing TruB WT protein, TruB K64E, or TruB ΔPUA. (*D*) In vivo tRNA pseudouridine 55 formation in tRNA^{Phe} assessed by CMCT modification in *E. coli* WT and *truB* KO strains expressing TruB variants. Similar results were obtained with probing for pseudouridylation in tRNA^{Cys} (Fig. S5).

TruB enzyme. In accordance with the ability to bind tRNA, we also observe tRNA chaperone activity in vitro for TruB ΔPUA (Fig. 1 and Fig. S1).

Therefore, we used stopped-flow experiments with 2AP-tRNA to dissect the role of the PUA domain for the initial encounter vs. the disruption of tertiary interactions in the tRNA on binding to TruB (Fig. S7 and Table 1). The association rate constant (k_1) at 5 °C describing tRNA binding to TruB is fourfold less for TruB ΔPUA than for TruB WT (Table 1). In contrast, the opening of the tRNA elbow region (k_2) is minimally affected by the deletion of the PUA domain. The data indicate that deletion of TruB's PUA domain decelerates the initial encounter between tRNA and TruB, but does not strongly affect the subsequent disruption and refolding of the elbow region in tRNA. In conclusion, the PUA domain is not important for the tRNA chaperone function of TruB.

Last, we tested the cellular role of the PUA domain in coculture competition assays where expression of TruB ΔPUA was able to overcome the fitness disadvantage of the KO strain (Fig. 3*C*). However, we observed fewer pseudouridines in cellular $tRNA^{Phe}$ than in the WT strain (about 80%; Table S2, Fig. 3*D*, and Fig. S5), in agreement with the catalytic impairment of TruB ΔPUA. We conclude that the PUA domain of TruB contributes to tRNA binding, but is most important for accelerating pseudouridine formation. Notably, the PUA domain is not critical for TruB's tRNA chaperone activity. Accordingly, this domain is dispensable in vivo.

Discussion

We combined biochemical, biophysical, and cellular studies to verify the hypothesis that the model pseudouridine synthase TruB is a tRNA chaperone in vitro and in vivo. This discovery represents a proof-of-concept that during RNA maturation, the modification and folding of RNA are linked, synergistic processes. As such, it is likely that other RNA modification enzymes are also RNA chaperones.

Besides proving that TruB is a tRNA chaperone that enhances tRNA aminoacylation (Fig. 1), we characterized its molecular mechanism through rapid kinetic experiments (Fig. 2). Importantly, following binding of tRNA, TruB induces the reversible disruption of tertiary interactions in the elbow region such that tRNAs undergo multiple opening and refolding events before becoming eventually pseudouridylated and released. The ability of TruB to bind and fold tRNA is critical for bacterial fitness as evident in coculture competition assays (Fig. 3), which indicates that this property of TruB has been selected for during evolution. Notably, for all tested TruB variants, the ability to fold tRNA in vitro (Fig. 1) correlates with the ability to rescue the fitness disadvantage of the *truB* KO strain (Fig. 3). Last, we clarified the function of the PUA domain of TruB as contributing to the initial binding of tRNA and to the catalysis of pseudouridine formation, likely by correctly positioning the tRNA (Fig. S7). Moreover, the PUA domain is not involved in restructuring the elbow region of tRNA, is not required for tRNA folding in vitro and is dispensable for bacterial fitness (Fig. 3).

In general, RNA chaperones are proteins that assist RNA in folding. To further discuss TruB's function as a tRNA chaperone, we use a theoretical definition for RNA chaperones that was proposed by Reneé Schroeder and coworkers, but which may not be rigorously met by all proteins with some form of RNA chaperone activity: "A protein that binds transiently and nonspecifically to RNA and resolves kinetically trapped, misfolded conformers. RNA chaperone activity entails the disruption of RNA-RNA interactions and the loosening of RNA structures. The interaction with the protein is needed for the unfolding of the RNA but not to maintain its structure. The protein does not require ATP binding or hydrolysis for its activity." (1).

First, does TruB "bind transiently and nonspecifically to RNA"? As a multiple turnover enzyme, TruB clearly binds tRNA transiently (14, 18, 26). TruB does not interact entirely nonspecifically with RNA as it does not bind a single-stranded or a structured RNA (Fig. S8). TruB does, however, modify all elongator tRNAs in bacteria in the TΨC arm and is therefore not specific to a single tRNA (10, 26). Together, these properties designate TruB as a tRNA chaperone rather than a general RNA chaperone. The tRNA specificity of TruB is similar to protein chaperones that are often specific to a certain type of substrates rather than folding all proteins (27).

Second, RNA chaperones are supposed to "resolve kinetically trapped, misfolded conformers." We show that TruB increases the fraction of folded and aminoacylation-competent tRNAs. In this experiment, tRNAs were first unfolded and allowed to fold at 0 °C in the presence or absence of TruB. Under these low temperature conditions, tRNA is likely to adopt an at least partially unfolded or mis-folded conformation and therefore correct tRNA folding is rate-limiting for aminoacylation. Clearly, TruB is able to accelerate the rate of folding and to increase the fraction of folded tRNA rendering it active for aminoacylation. We have not fully characterized the tRNA conformation in this assay before interaction with TruB and cannot state whether it is kinetically trapped, but this seems likely. Moreover, we have shown that TruB interacts similarly with a potentially mis- or unfolded tRNA G18A as with WT tRNA demonstrating its capability to act on misfolded tRNAs (Fig. 2*C*).

Third, we experimentally assessed TruB's activity in "the disruption of RNA-RNA interactions and the loosening of RNA structures." The crystal structures of TruB bound to the TΨC arm reveal that TruB disrupts interactions between the TΨC and the D arm of tRNA to gain access to the target uridine 55 (Fig. 4) (16, 17). Using tRNA with a 2AP base analog at position 57 allows us to observe the flipping of bases directly (Fig. 2). This conformational change in tRNA loosens the tRNA structure in the elbow region, which contains numerous tertiary RNA–RNA interactions. Importantly, this base-flipping and the associated conformational change can occur multiple times while tRNA is bound to TruB and before pseudouridylation occurs (Fig. 4). Thus, TruB resembles a protein chaperone that can repeatedly unfold its substrate, ensuring multiple chances to fold correctly (28). Indeed, pseudouridine formation may act as a timer and may have evolved to be slow to facilitate this repeated conformational change (18).

Fourth, the statement that "the interaction with the protein is needed for the unfolding of the RNA but not to maintain its structure. The protein does not require ATP binding or hydrolysis for its activity" is obvious for TruB. tRNA independently interacts with many other proteins and the ribosome in the cell while maintaining its structure, and all TruB assays are conducted in the absence of ATP. In conclusion, our experimental evidence clearly demonstrates that TruB acts as a tRNA-specific chaperone.

Why is tRNA folding by TruB critical for cellular fitness? Under optimal growth conditions, TruB is dispensable, suggesting that tRNAs can fold independently (10). However, in nature, bacteria typically grow under stress and in competition with other organisms. Under these conditions, tRNA binding by TruB, but not pseudouridine formation, would be important for cellular fitness. The importance of only tRNA binding is evident in comparing TruB K64E and TruB ΔPUA, which form similar levels of pseudouridine in tRNA (Fig. 3*D*). Only the fitness of the strain expressing TruB K64E is impaired, indicating that pseudouridylation is not the determining factor for cellular fitness (Fig. 3*C*). Rather our in vitro data (Fig. 3*A*) suggest that only a few tRNAs will bind to TruB K64E and will be pseudouridylated, whereas a significant fraction of tRNAs will not bind and will not benefit from the tRNA chaperone function of TruB. In contrast, a large proportion of tRNAs will interact with TruB ΔPUA (Fig. 3*B*) and become correctly folded, although only a small portion of these will be pseudouridylated due to the reduction of the catalytic rate (Fig. 3*A*). This suggestion implies that there are kinetically trapped and misfolded tRNAs in the cell that benefit from TruB's tRNA chaperone activity.

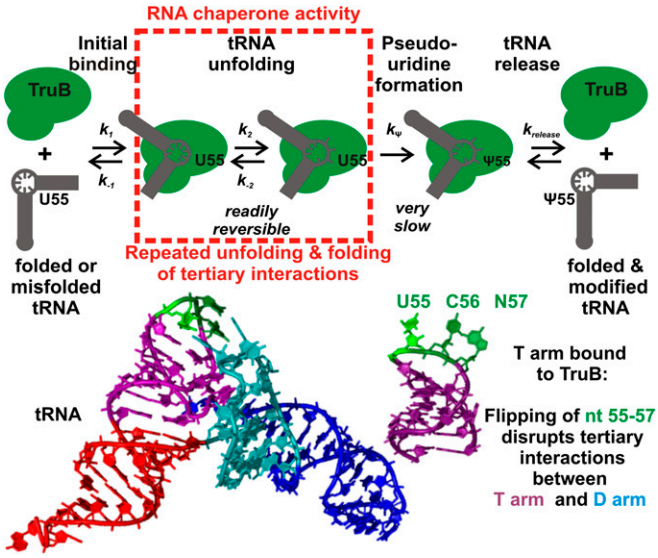

Fig. 4. Mechanism of TruB acting as a tRNA chaperone while introducing pseudouridine 55. Rapid tRNA binding is followed by local tRNA unfolding in the elbow region that allows TruB to gain access to the modification site. By flipping out nucleotides 55–57 in the T arm when binding to TruB (PDB ID code 1K8W), the tertiary interactions between T and D arm in tRNA (PDB ID code 4TRA) are disrupted (*Bottom*), and the tRNA is opened such that TruB gains access to U55. Because the reversion of the tRNA rearrangement (k_{-2}) is faster than catalysis (k_{Ψ}) (Table 1), TruB allows the tRNA to repeatedly open and refold before becoming pseudouridylated. This repeated folding-unfolding transition in the elbow region of tRNA constitutes the tRNA chaperone activity of the pseudouridine synthase TruB.

Indeed, there are several lines of evidence suggesting the presence of misfolded tRNAs and other potential tRNA chaperones in the cell. For instance, mutations in mitochondrial tRNAs are implicated in human diseases (29), and such a mutation has been reported to result in tRNA misfolding (30). Similarly, mutations in cytoplasmic $tRNA^{His}$ lead to tRNA misfolding and in turn reduced processing of precursor tRNAs (31). In eukaryotes, the La protein functions as a tRNA chaperone that is important for pre-tRNA folding (32). Moreover, the La protein functions redundantly with catalytically active Pus4, the yeast homolog of TruB, indicating that these proteins together contribute to tRNA folding and stability (33). The importance of Pus4 for tRNA stability and/or folding is also supported by a report of a genetic interaction between a $tRNA^{Ser}$ mutant and Pus4 (34). Last, several biochemical studies have addressed the folding of WT tRNAs revealing intermediate structures and possible formation of misfolded structures (23, 35, 36). In summary, misfolded tRNAs are present in cells, and TruB can thus enhance cellular fitness by accelerating tRNA folding and/or increasing the fraction of correctly folded tRNA.

It is astonishing that the PUA domain of TruB is not required for its tRNA chaperone function and consequently for bacterial fitness (Fig. 3). Aligning 100 bacterial TruB sequences revealed that this domain is generally conserved, but absent in TruB proteins of *Chlamydia* species, which have a significantly reduced genome size compared with *E. coli*. Hence, *Chlamydia* may have lost the less-important PUA domain as TruB's catalytic domain alone can still support cellular fitness. Our biochemical studies demonstrate that TruB's PUA domain binds RNA (Fig. 3*B*), as expected for this domain (25). Superimposing the TruB-T arm structure (16, 17) with a full-length tRNA structure suggests a large binding interface between the PUA domain and the acceptor arm of tRNA, which is supported by our results with tRNA labeled at the 3′ end (Fig. S6). This interaction of TruB's PUA domain with the 3′CCA end of tRNA is similar to the PUA domain in the TruB homolog Cbf5, which is in direct contact with the related 3′ACA motif of H/ACA guide RNA (37, 38). Our data suggest that this interaction contributes to tRNA binding but is more important for catalysis. A plausible explanation is that the tRNA is positioned slightly differently on TruB ΔPUA because a significant portion of the interaction surface is lost.

Our findings show that the pseudouridine synthase TruB acts as a tRNA chaperone. Could this be a common phenomenon for RNA modification enzymes? The idea of a dual function for RNA modification enzymes is not new (3). Similar to Ofengand's fitness studies with for TruB (10), the yeast tRNA methyltransferase Trm2, which forms T54 adjacent to pseudouridine 55, has been shown by genetic interaction to stabilize tRNA independent of its catalytic activity and could thus also act as a tRNA chaperone (34). Also, the archaeosine tRNA-guanine transglycosylase (ArcTGT) induces an alternative λ conformation in tRNA to access its target nucleotide G15, thereby drastically refolding tRNA (39). Therefore, this enzyme could also possess tRNA chaperone function. Interestingly, it has been speculated that ArcTGT can bind simultaneously with TruB to tRNA in the λ conformation, which could possibly suggest that RNA modification enzymes work together as chaperones. Despite these supporting studies, to the best of our knowledge, direct mechanistic evidence for an RNA modification enzyme acting as an RNA chaperone has been lacking. Many RNA modification enzymes have been proposed or demonstrated to use a base-flipping mechanism to gain access to the target nucleotide for modification (40–42), but base-flipping alone may not represent an RNA chaperone function. Rather, we hypothesize that RNA rearrangements, such as base-flipping, that are coupled to tertiary interactions such as in the elbow region of tRNA, could be the hallmark of the RNA chaperone activity by RNA modification enzymes.

A fine-tuned relationship between RNA folding and modification or binding by proteins may have arisen during the transition from an RNA world to a ribonucleoprotein world and could thus be an ancient and general mechanism. Proteins may enhance RNA structure and function not just by permanently associating with RNAs to form ribonucleoprotein complexes, but also by acting as RNA chaperones, which transiently interact with RNA and induce unfolding of incorrect RNA structures. Therefore, the finding that TruB acts as a tRNA chaperone simply by binding and rearranging the tRNA elbow region requires us to change how we think about RNA-protein interactions in general. It is possible that some of the more than thousand known RNA-binding proteins also have a second function in acting as RNA chaperones with significant consequences for RNA biology.

In conclusion, we provide direct evidence that an RNA modification enzyme also acts as an RNA chaperone in vitro and in vivo. This discovery alters our understanding of RNA maturation as modification and folding can no longer be considered as separate processes. Instead, it is likely that more examples of enzymes with dual modification and chaperone activities will be identified. Apparently, evolution has selected an efficient mechanism that integrates seemingly different events: RNA modification to expand the repertoire of chemically distinct ribonucleotides and RNA folding to adopt the biologically active conformation.

Materials and Methods

For details of all materials and methods, see *SI Materials and Methods*. In brief, QuikChange site-directed mutagenesis (Table S3), protein expression, purification (Fig. S9), tRNA in vitro transcription and purification, filter binding assays, tritium release assays, generation of 2-aminopurine–labeled tRNA, fluorescence titrations, and stopped-flow experiments were performed as described previously (18, 24). tRNA folding was monitored by measuring tRNA aminoacylation as reported by the Perona group (23). Coculture competition assays were conducted as described by the Ofengand and Stansfield groups (10, 21). tRNAs were isolated from *E. coli* by phenol/chloroform extraction, precipitated, reacted with 1-cyclohexyl-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate (CMCT) for 1 h at 37 °C, reprecipitated, and subjected to alkaline lysis in 50 mM Na_2CO_3 solution, pH 10.3, incubated at 50 °C for 2 h. Reverse transcription was carried out with avian myeloblastosis virus (AMV) reverse transcriptase, and samples were separated on a 15% sequencing urea-PAGE.

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