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Decoding in the absence of a codon by tmRNA and SmpB in the ribosome

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Abstract

In bacteria, ribosomes stalled at the end of truncated messages are rescued by tmRNA, a bifunctional molecule that acts as both a tRNA and mRNA, and SmpB, a small protein that works in concert with tmRNA. Here we present the crystal structure at 3.2 Å resolution of a tmRNA fragment, SmpB and elongation factor Tu bound to the ribosome. The structure shows how SmpB plays the role of both the anticodon loop of tRNA and portions of mRNA to facilitate decoding in the absence of an mRNA codon in the A site of the ribosome, and explains why the tmRNA-SmpB system does not interfere with normal translation.

Transfer-messenger RNA (tmRNA), also known as 10S RNA or SsrA, is a highly structured RNA that combines properties of tRNA and mRNA in one molecule about 350 nucleotides long (Fig. 1A) (1, 2). The tRNA-like domain (TLD) of tmRNA lacks an anticodon stem loop but contains an acceptor arm (3) that can be aminoacylated at its 3'-end by the same alanyl tRNA synthetase that aminoacylates tRNA^{Ala}. A different region of tmRNA contains a short internal open reading frame (ORF) that acts as an mRNA template. In addition, tmRNA contains several pseudoknots and helices.

Ribosomes that reach the end of prematurely truncated or defective messages are stalled because the absence of a complete codon in the A site prevents either elongation or normal termination. In bacteria, they are rescued by tmRNA in a process called *trans*-translation because it involves continuing translation by changing the mRNA template. In this process, EF-Tu delivers tmRNA to the A site of the stalled ribosome. The nascent polypeptide chain is transferred to the alanine on the TLD. Subsequently, translocation brings the first codon of the ORF into the A site of the ribosome and translation resumes using the ORF as the mRNA (2). The short sequence coded by the ORF thus added to the C-terminus of the partially synthesized protein acts as a degradation tag (4). Thus tmRNA acts both to rescue ribosomes as well as to target incompletely synthesized proteins for degradation.

The binding of tmRNA to stalled ribosomes requires the protein SmpB (5), which can bind to tmRNA simultaneously with EF-Tu (6). Crystal structures of SmpB in complex with the TLD suggest that the protein substitutes for the missing anticodon stem inside the ribosome (7, 8), which was supported by electron microscopy studies at ~15 Å resolution (9, 10). A previous electron microscopy study found two molecules of SmpB with tmRNA in the ribosome, with the carboxy-terminus of one of them near the decoding center of the 30S ribosomal subunit (11). The observed proximity to the decoding center agrees with hydroxyl

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radical and chemical probing experiments that show protection of 16S rRNA bases A1492, A1493 and G530 at the decoding center upon binding of the tmRNA-SmpB complex to the ribosome (*E. coli* numbering used for rRNA throughout) (12, 13). However, mutations at these positions do not reduce SmpB binding to the decoding site (13) or reduce the rate of peptidyl transfer onto tmRNA (14). The mechanism by which tmRNA and SmpB acting in concert can facilitate “decoding” in the absence of codon-anticodon base pairing has remained unclear.

Here we present the crystal structure of the *Thermus thermophilus* ribosome bound to a complex consisting of a fragment of tmRNA (tmRNA Δ^m) along with SmpB and EF-Tu trapped in the GDP form immediately after GTP hydrolysis using the antibiotic kirromycin, as was done previously with the tRNA complex (15). In addition, the ribosome contains deacylated tRNA^{fMet} in the P and E sites of the ribosome, and a truncated mRNA that contains two bases in the A site following the P-site codon. The construct tmRNA Δ^m was designed based on the sequence of the *ssrA* gene from *T. thermophilus* and contained 89 nucleotides (Fig. 1A). It includes the TLD but does not contain the pseudoknots or the ORF of tmRNA. Together with SmpB and EF-Tu, similar constructs of tmRNA were capable of interacting with stalled ribosomes (10), and even of synthesizing polyalanine on 70S ribosomes in the absence of an mRNA template (16).

Crystals were obtained in a new form with only one ribosome in the asymmetric unit. Molecular replacement and initial refinement were carried out using an empty ribosome as starting model (for details see supplementary online materials, SOM). All the components of the Ala-tmRNA Δ^m ·SmpB·EF-Tu·GDP complex were clearly visible in initial unbiased $f_o - f_c$ difference Fourier map (Fig. S1). Ligands were subsequently built and the complete model was refined to 3.1 Å resolution [$I/\sigma(I)$ =2.12 at 3.20 Å] (Fig. 1B and Table S1) resulting in a final $R_{\text{work}} / R_{\text{free}}$ of 23.0% / 27.0%.

The complex of Ala-tmRNA Δ^m with SmpB and EF-Tu·GDP·kirromycin in the A site of the ribosome is shown in Fig. 1C. The model of EF-Tu is complete except for the switch I region (residues 41-66), which was also disordered in a previous crystal structure of EF-Tu in complex with aminoacyl-tRNA (15). The TLD of tmRNA Δ^m was well ordered for residues 1-24 and 314-349. SmpB could be modeled completely, including the entire carboxy-terminus, which is unstructured in solution but critical for tmRNA function (17-19). The overall conformation of the ribosome closely resembles that of the equivalent complex of EF-Tu with acylated tRNA (15). Despite the absence of an anticodon in tmRNA, the 30S subunit is in a “closed” conformation that is normally characteristic of the presence of a cognate codon-anticodon base pairing in the decoding center of the 30S subunit (20, 21). The analysis of this structure now allows detailed insights into how tmRNA and SmpB together recognize stalled ribosomes and facilitate decoding even in the absence of a codon-anticodon interaction.

The complex of SmpB and the TLD resembles a tRNA molecule. Overall, the conformation of the ribosome-bound SmpB-tmRNA Δ^m complex shows the canonical L shape of a tRNA (Fig. 2) and is similar to a closely related isolated crystal structure (rmsd ~1.0 Å) (8). This implies that the complex of SmpB and the TLD undergoes only slight conformational changes during its binding to stalled ribosomes. The only exception is the D loop, whose 5' region moves towards the acceptor stem in the ribosome complex (Fig. S2). This part of the D loop shows weaker electron density and was disordered in one of the isolated crystal structures, indicating that it has structural flexibility (7). Overall the TLD structurally and functionally mimics the acceptor arm and TΨC-arm of tRNA. As suggested by previous isolated structures of the TLD·SmpB complex, the core of SmpB structurally mimics the anticodon stem of tRNA in the ribosome. Helix 2a points out of the ribosome, where its

positioning would allow the pseudoknot modules of tmRNA to form an arc around the beak of the 30S subunit, as previously reported by several electron microscopy studies (22-24). The conformation of the 3'-CCA end of Ala-tmRNA Δ^m , the acceptor arm as well as the T-arm portion that interacts with EF-Tu, closely resemble the pre-accommodation state of aminoacyl-tRNA (Fig. 2). The structure of EF-Tu in the tmRNA complex showed clear extra density for GDP and kirromycin, and is nearly identical to the structure of EF-Tu with cognate aminoacyl-tRNA in the ribosome. As expected, this confirms that the addition of kirromycin has trapped the Ala-tmRNA Δ^m :SmpB particle in the state just after GTP hydrolysis in EF-Tu. Notably SmpB shows no interaction with EF-Tu. The closest distance is about 5 Å between Y68 of SmpB and EF-Tu residue I375 (*T. thermophilus* numbering). Y68 is part of the central loop of SmpB that has high flexibility in solution and likely increases the aminoacylation efficiency of tmRNA (8). Given the EF-Tu and SmpB binding sites on the TLD, the structure does not support the simultaneous binding of two SmpB proteins to the TLD during pre-translocation (11), but rather a model in which tmRNA and SmpB enter the ribosome as a 1:1 complex which structurally mimics a tRNA whose anticodon stem loop has been replaced by SmpB.

The C-terminus of SmpB, which is unstructured in solution, is completely ordered on binding the ribosome (residues 122-144; Fig. 1C). Its interactions with the ribosome are consistent with a recent hydroxyl radical probing study using SmpB mutations of the carboxy-terminal tail (25). The C-terminus first extends from the SmpB core towards the ribosomal decoding base 530 and then continues along the path normally occupied by mRNA downstream of the A-site codon (Fig. 3A and B). This part of the tail from residue 122-132 is well ordered and makes several interactions with 16S rRNA. Residue G122 is highly conserved and its mutation to alanine reduces SsrA-tagging *in vitro* (14), suggesting that conformational flexibility in this position is essential. Several residues further, a well-defined series of interactions starts with Y126 stacking with 16S G530, which is flipped out into the anti conformation. G530 is part of the ribosomal decoding center and a similar conformation is also observed during recognition of cognate codon-anticodon interaction (20). Y126 is a moderately conserved residue (Fig. 3C) and its mutation alone does not inhibit *trans*-translation (25). However, this position of SmpB is highly restricted to aromatic residues, which implies that the stacking onto G530 is important for tmRNA function. Following Y126, two highly conserved basic residues are forming ionic bonds with the negatively charged phosphate backbone. K128 interacts with the phosphate group of G530 and R129 with C532. Together, these residues were previously shown to be required for tagging *in vivo* (14, 19). Starting from residue D132 the sequence then forms a α -helix which extends towards the carboxy-terminus and shows weak interactions with 16S rRNA, e.g. with residues K134 and R138. In *E. coli* the mutation of W147 had a strong effect on tagging efficiency (25), and the corresponding V137 in *T. thermophilus* SmpB together with L141 might be involved in hydrophobic interactions with F28 and V51 on the surface of ribosomal protein S5. Overall the helical structure of this region is highly conserved and essential for SsrA-tagging activity (14, 26). This suggests that these interactions of the carboxy-terminal helix with the ribosome might further stabilize the binding of SmpB to stalled ribosomes. Overall, SmpB appears to recognize stalled ribosomes by making specific interactions with regions of the ribosome that would normally be occupied by mRNA if it were not truncated. After the initial step of *trans*-translation, however, these contacts would need to be disrupted because the ORF of tmRNA would have to replace the C-terminus of SmpB and position itself along the mRNA path before translation can resume.

The structure explains why the tmRNA system does not interfere with active protein synthesis. For crystallization we used an mRNA sequence with two nucleotides in the A site. The second nucleotide is disordered and does not interact with SmpB. This ribosome complex mimics the state of a stalled ribosome after cleavage of mRNA by ribosome-

dependent nucleases like RelE, which cleaves mRNA in the ribosome after the second nucleotide of the A-site codon (27). Indeed, translating ribosomes treated with RelE are efficient targets of tmRNA during adaptation to starvation conditions (28). Recruitment of tmRNA to ribosomes requires truncated mRNAs and it has been shown that complexes with more than 6 nucleotides following the P-site codon are poor substrates for ribosome rescue by tmRNA and SmpB (29). This suggests that tmRNA acts only on ribosomes that have reached the 3'-end of a nonstop message or are stalled during translation with subsequent mRNA degradation (30). A superposition of SmpB with a ribosome structure containing a long mRNA (31) shows that the C-terminal tail of SmpB extends into the space normally occupied by mRNA, leaving space for about 4-5 nucleotides following the P-site codon (Fig. 3A, B). The free 3' end of truncated mRNAs probably has a certain degree of structural flexibility, but significantly longer transcripts would ultimately clash with SmpB, which provides a structural rationale for the observation that the tmRNA·SmpB complex does not compete with translation factors for A-site binding during canonical translation (32).

As in normal decoding with tRNA, the tmRNA·SmpB complex facilitates GTP hydrolysis by EF-Tu by inducing a closed conformation of the 30S subunit. During translation, correct codon-anticodon pairing induces conformational changes in the conserved 16S rRNA nucleotides A1492/A1493 and G530 in the decoding center of the 30S subunit, which lead to a closure of the 30S subunit (15, 20, 21). Together with distortions of A-site tRNA, these changes allow the sarcin-ricin loop of the 50S subunit to stabilize an activated catalytic conformation of EF-Tu (33). In the case of tmRNA there are no codon-anticodon interactions. The tmRNA·SmpB complex shows no major distortions after binding of the ribosome (Fig. S2) and SmpB does not interact with ribosomal protein S12, which has a stabilizing role during canonical decoding. However, two key regions of SmpB are close to the critical 16S rRNA bases involved in decoding (Fig. 3B). As discussed earlier, Y126 near the beginning of the carboxy-terminal tail of SmpB stacks with G530. Additionally, the decoding bases A1492/A1493 are displaced out of the internal loop in helix 44, but in a conformation that is somewhat different from that observed during canonical decoding (Fig. S3). They are close to helix α 1 near the N-terminus of SmpB. This region of SmpB is highly conserved (Fig. 3C). N7 and Y14 appear to be important to stabilize the positioning of the α 1 fold between β 1 and β 2, respectively. The charged residues R8, R9, H12 and D13, whose side chains are only weakly ordered, are within bonding distance of A1492 and A1493. These findings agree with chemical footprinting and hydroxyl radical probing studies, which found a protection of the decoding bases on binding of tmRNA·SmpB to the ribosome (12, 13). On the other hand, mutation of the decoding bases did not affect peptidyl transfer to tmRNA *in vitro* (14). This suggests that regardless of specific interactions with the bases A1492 and A1493, SmpB interacts with the 30S subunit in such a way as to bring the shoulder domain of the 30S subunit (with G530) closer to its 3' major domain (containing A1492 and A1493), resulting in a closed conformation that is similar to that induced by cognate tRNA binding (see Movie S1). This closed conformation is responsible for an increased rate of GTP hydrolysis in EF-Tu and subsequent accommodation of the 3' end of tmRNA into the peptidyl transferase center (34, 35).

The structure raises some interesting questions about what happens during translocation after accommodation of tmRNA and peptidyl transfer. Translocation must bring the ORF of tmRNA into the path normally occupied by mRNA, so that translation can resume on the ORF. However, in the crystal structure this mRNA path is occupied by the C-terminus of SmpB. Therefore, the C-terminus of SmpB has to undergo a major conformational change during translocation. Indeed, in cryo electron microscopy studies of the post-translocated complex of tmRNA, the C-terminus of SmpB has been modeled in a different conformation from that seen here, allowing new interactions to take place between SmpB and the sequence upstream of the resume codon (23, 24).

This structure of Ala-tmRNA^Δ-SmpB-EF-Tu-GDP bound to a stalled ribosome provides insights into the initial steps of *trans*-translation at near atomic-resolution. SmpB, tmRNA and EF-Tu together associate in a 1:1:1 stoichiometry only with ribosomes stalled at or near the 3' end of their transcripts. Interactions of SmpB with the decoding center induce a domain closure of the 30S subunit similar to that seen with cognate tRNA, leading to GTP hydrolysis by EF-Tu. Because the C-terminus of SmpB would overlap with the path of normal mRNA, the structure also shows why the tmRNA-SmpB complex can only act on ribosomes stalled on truncated mRNA that extends only a few nucleotides beyond the P-site codon, and thus would not interfere with normal translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Overview of the structure of Ala-tmRNA Δ^m , SmpB and EF-Tu-GDP bound to the ribosome. **(A)** Secondary structure diagram of tmRNA and sequence of the tmRNA Δ^m fragment used in this study. The tRNA-like domain (TLD) is highlighted in green, the open reading frame (ORF) in magenta and RNA helix 2b in blue. Mutations introduced for improved refolding of the *in vitro* transcribed RNA and the UUCG tetra loop are indicated in magenta, the G-U base pair recognized by alanine tRNA synthetase in yellow. **(B)** Overview of the ribosomal complex with EF-Tu, SmpB and tmRNA Δ^m . **(C)** Detailed view of the ribosomal A site.

Fig. 2. Superposition of $\text{tmRNA}^{\Delta m} \cdot \text{SmpB} \cdot \text{EF-Tu}$ with a complex of A-site tRNA and EF-Tu (*gray*, PDB code 2Y10, ref. 36). In the $\text{tmRNA} \cdot \text{SmpB}$ complex, SmpB substitutes for the anticodon arm of tRNA. The interactions of EF-Tu (red) and the acceptor-stem region of tmRNA (green) are nearly identical to that observed for the tRNA-EF-Tu complex (gray). A close-up view of the 3'-end CCA sequence is shown as inset.

Fig. 3.

Interactions of SmpB with the ribosome. **(A)** The C-terminal tail of SmpB would clash with mRNA downstream of the A-site codon. The mRNA used in this work is colored in magenta and an extension based on the superposition of a longer mRNA (*PDB code 2HGR, ref. 31*) is shown in gray. The mRNA nucleotides are numbered starting with the first nucleotide of the A-site codon. **(B)** SmpB interacts with both the shoulder domain and the 3' major domain of 16S rRNA near the decoding center. Close-up views of the interactions near A1492/A1493 and G530 at the decoding center are shown in insets. **(C)** Sequence alignment for the regions of SmpB interacting with the decoding center with degree of conservation indicated by size. Positively charged residues are highlighted in blue, negative in red and aromatic in yellow. See SOM and Fig. S4 for details.