tmRNA–SmpB: a journey to the centre of the bacterial ribosome

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Ribosomes mediate protein synthesis by decoding the information carried by messenger RNAs (mRNAs) and catalysing peptide bond formation between amino acids. When bacterial ribosomes stall on incomplete messages, the *trans*-translation quality control mechanism is activated by the transfer-messenger RNA bound to small protein B (tmRNA–SmpB ribonucleoprotein complex). *Trans*-translation liberates the stalled ribosomes and triggers degradation of the incomplete proteins. Here, we present the cryo-electron microscopy structures of tmRNA–SmpB accommodated or translocated into stalled ribosomes. Two atomic models for each state are proposed. This study reveals how tmRNA–SmpB crosses the ribosome and how, as the problematic mRNA is ejected, the tmRNA resume codon is placed onto the ribosomal decoding site by new contacts between SmpB and the nucleotides upstream of the tag-encoding sequence. This provides a structural basis for the transit of the large tmRNA–SmpB complex through the ribosome and for the means by which the tmRNA internal frame is set for translation to resume.

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Introduction

Protein synthesis is an elaborate process that is conducted by ribosomes in all living organisms. By using aminoacyl-transfer RNAs (tRNAs) as substrates, protein synthesis allows the

decoding of messenger RNAs (mRNAs) into a corresponding sequence of amino acids (Schmeing and Ramakrishnan, 2009). When eubacterial ribosomes stall on an mRNA molecule lacking a stop codon, the *trans*-translation quality control mechanism is carried out by the transfer-messenger RNA bound to small protein B (tmRNA–SmpB ribonucleoprotein complex). tmRNA is a chimeric molecule that acts as both a tRNA and an mRNA; depending on the species, tmRNA ranges from 230 to 400 nucleotides in length. The modular architecture of tmRNA includes a tRNA-like domain (TLD), a string of pseudoknots (PKs), a long and disrupted helix H2 connecting the TLD to the PKs, and a short internal open reading frame (ORF) (Figure 1) (Felden *et al.*, 1997). The TLD includes an acceptor stem that is always charged by an alanine, as well as a T arm and a D loop, but no D stem. SmpB is a small basic protein (~18 kDa) that binds both the tmRNA and the ribosome with high affinity (Hallier *et al.*, 2006) and is essential to *trans*-translation (Karzai *et al.*, 1999). With the help of elongation factor EF-Tu and SmpB, tmRNA targets stalled translational complexes and, despite the absence of codon–anticodon interactions, enters the vacant ribosomal A site. The incomplete polypeptide is then transferred to the alanyl-tmRNA, and translation shifts from the problematic mRNA to the tmRNA internal ORF that encodes a peptide tag targeted by several proteases. The stalled ribosomes are recycled because of the presence of the stop codon within the tmRNA, while the incomplete protein and the truncated mRNA are released and degraded specifically (Moore and Sauer, 2007).

Together with a wealth of genetic and biochemical data, the first cryo-electron microscopy (cryo-EM) reconstructions, combined with X-ray and NMR structures, provided insight into the mechanism by which the tmRNA recognizes and binds stalled ribosomes. First, EF-Tu places the TLD into the A site as for canonical tRNAs, except that SmpB overcomes the absence of codon–anticodon interactions (Shimizu and Ueda, 2002; Kurita *et al.*, 2007; Nonin-Lecomte *et al.*, 2009). The PK ring wraps around the beak of the 30S small ribosomal subunit, placing the internal ORF close to the entrance of the mRNA path (Kaur *et al.*, 2006; Gillet *et al.*, 2007). Then, as the accommodation takes place, the TLD swings into the A site (Bugaeva *et al.*, 2008; Cheng *et al.*, 2010; Weis *et al.*, 2010). Although this working model reveals the initial positions of tmRNA–SmpB in and around the stalled ribosome, it also raises questions about the topological rearrangements required to move a complex approximately six times larger than a tRNA from the A to the P site, and to swap the problematic mRNA with the internal ORF of the tmRNA.

Results and discussion

Cryo-EM reconstruction of the accommodated state of tmRNA–SmpB

In the present cryo-EM study, we first revisited the accommodation of tmRNA–SmpB. This investigation resulted in a

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Figure 1 Secondary structure diagram of *Thermus thermophilus* tmRNA. Base pairs are linked by lines, and GU pairs are represented by dots. The nucleotides within the internal ORF are underlined and shown in a larger font, and the resume codon is highlighted in yellow. Domains are shown in the colours used in Figure 3. This figure was adapted from the tmRDB web site (Andersen *et al.*, 2006).

new 3D structure at 13 Å resolution, as well as a much better definition of the densities attributable to both counterparts (Figure 2A and C). A portion of tmRNA-SmpB enters deep into the A site, while the rest of the molecule forms an arc around the beak of the small ribosomal subunit. To facilitate interpretation at the molecular level, we docked atomic models into the electron densities. Using our earlier model (Kaur *et al.*, 2006) as the initial starting point, the tmRNA domains (see Figure 1) were manually docked as rigid bodies into the cryo-EM-density map and, as a final step, linked to each other and optimized in the context of the cryo-EM data (see Materials and methods). According to our data, the TLD contacts to the large subunit resemble an accommodated canonical tRNA: the D loop interacts with helix H38, while the acceptor branch brings the CCA 3' end into the peptidyl transfer centre (PTC) (Figures 3A, 4A, and C; Table I). The lack of a D stem is compensated for by the packing of SmpB into the minor groove of helix H69 of the 23S RNA. This positioning of SmpB realigns helix H2 towards the large subunit, in which it forms extended contacts with protein L11. SmpB interacts with most of the small-subunit regions normally in contact with the anticodon stem loop of the accommodated tRNA, including helix h18 and the top of helix h44 of the 16S RNA and proteins S12/S13 (Figures 3C, 4A, and C; Table I). The 3' end of the internal ORF is still embedded in helix H5 at the hinge between the head and the body of the 30S subunit. The ring of PKs wraps around the beak of the small subunit, with PK1 in front of the beak of 30S and PK2 lying on the surface of S3. However, PK3/PK4 do not contact the ribosome. Compared with the structure of the pre-accommodated state (Valle *et al.*, 2003a; Kaur *et al.*, 2006), accommodation does not trigger large tmRNA movements, but mainly allows the swing of the acceptor branch into the

PTC, allowing transpeptidation with the polypeptide stalled on the P-site tRNA. During this movement, SmpB stays at the same place into the decoding site, but is slightly rotated by ~30° towards the TLD.

The tmRNA ring crosses the ribosome

To understand how such a large RNA molecule navigates through the ribosome, we determined the structure of tmRNA-SmpB translocated into the P site of the ribosome at 13.5 Å resolution (Figure 2B and D). This new cryo-EM structure shows that the overall density of the translocated tmRNA-SmpB complex is as large and structured as in the accommodated step (Figure 2A and C; Supplementary Figure S1). While some portions of the tmRNA may unfold as elongation continues (Wower *et al.*, 2005), the present structure shows that, contrary to current belief, the overall tmRNA structure is conserved during P-site translocation.

TLD-SmpB contacts the ribosome in the same way as a canonical P-site tRNA (Selmer *et al.*, 2006). On the large 50S subunit, SmpB still compensates for the lack of a D stem by contacting H69 (Figures 3B, 4B, and D; Table I), while on the small 30S subunit, it still mimics an anticodon stem loop by interacting with helix h30 of the 16S RNA and protein S13 (Figures 3D, 4B, and D; Table I). Nevertheless, the transit of the large ring to the P site requires a new gap to allow it to progress between the two ribosomal subunits. Twenty-eight per cent of the initial data set for the accommodated state accounts for the ribosome in the ratcheted state, an intermediate known to affect the intersubunit bridge connections on the pretranslocational ribosomes (Frank and Agrawal, 2000). The corresponding map (Figure 5B) shows an intersubunit rotation and a characteristic movement of the L1 stalk towards the E site (Valle *et al.*, 2003b). Following ratcheting, no changes were observed in the tmRNA-SmpB position, except for the acceptor arm of the peptidyl-tmRNA, the weak density of which accounts for its movement into the A/P hybrid position. The deacylated tRNA is recovered in a P/E hybrid state. Further conformational changes include the movement of the tip of helix H38 of the 23S RNA (also known as the A-site finger or ASF, part of bridge B1a) from protein S13 to protein S19 (Reblova *et al.*, 2010). It is known that the ASF is involved in translocation by interacting directly with the A-site tRNA and maintaining the reading frame of canonical mRNAs (Komoda *et al.*, 2006). In the accommodated state, the tip of H38 physically prevents the transit of the tmRNA (Figure 5A). An up-and-down motion of the ASF during ratcheting opens the gate (Figure 5B), allowing helix H2 of the tmRNA to pass to the other side of the bridge (Figure 5C). There, H2 makes additional contacts with the H38 tip of the 23S RNA and with proteins S13/S19 (Figure 4B and D). Interestingly, it was shown recently that mutations in the ASF can inhibit tmRNA tagging in some contexts *in vivo* (Crandall *et al.*, 2010).

Based on our current results, we propose that *trans*-translocation could be facilitated by this disruption, favouring the passage of H2 from the A to the P site. At the same time, PK1 stays at the entrance of the gate, approximately where H2 was positioned in the accommodated state (Figure 5A and C). At this stage, the function of PK1 is mainly structural, as it can be replaced with a variety of hairpin structures (Tanner *et al.*, 2006). As helix H2d connects both PK1 and PK4, the 3' end of PK4 also follows the dynamics of H2, although it does

Figure 2 Cryo-EM map of the accommodated tmRNA-SmpB complex on a stalled ribosome, and a side-by-side comparison with the map of the translocated complex. (A, C) Two views of the *trans*-translational ribosome complex after accommodation. (B, D) Two views of the *trans*-translational ribosome complex after translocation. The density attributable to tmRNA-SmpB is depicted in red, the 50S subunit is in blue, the 30S subunit is in yellow, and the P-site tRNA in (A) and (C) and E-site tRNA in (B) and (D) are shown in purple. Semi-transparent ribosomal subunits in (C) and (D) emphasize the relative position of tmRNA-SmpB and tRNA in the three active sites of the ribosome before and after translocation. Landmarks on the 50S subunit: L7/L12, stalk of proteins L7/L12; L1, stalk of protein L1; and PTC, peptidyl transfer centre. Landmarks on the 30S subunit: b, beak; sh, shoulder; sp, spur; dc, decoding site; and h, head.

not yet make any contacts with the ribosome. In contrast, the 5' region of PK4, together with H5/PK2/PK3, forms a static block packed against the hinge between the head and the body of the small subunit. Accordingly, replacing each of these PKs, except PK1, with a single-stranded RNA does not affect the functions of tmRNA, confirming that PK2, PK3, and PK4 do not interact in a functional manner with the ribosome during the *trans*-translation process (Nameki *et al*, 2000). However, the tension between the large movements of TLD/H2/PK1/PK4 and the immobility of H5/PK2/PK3 stretches the ring of PKs to follow the translocation movement, causing it to become ellipsoidal around the 30S subunit beak and triggering the unfolding of the internal ORF (Figure 3C and D; Supplementary Movie S1).

Contributions of SmpB to decoding

Fitting the atomic model into the cryo-EM-density map confirmed the standard interactions of SmpB with the TLD (Gutmann *et al*, 2003; Bessho *et al*, 2007), but also revealed new contacts between the protein and the nucleotides upstream of the tmRNA tag-encoding sequence (Figures 3D and 6). Indeed, in the crowded environment of the ribosome, placing this region in close contact with SmpB is the only solution to position the resume codon into the decoding site, while maintaining enough space for the next incoming tRNA.

The new frame has to be determined in a unique manner, because the resume codon is not specified by an initiator tRNA or the canonical initiation factors. The five nucleotides just upstream of the tag-encoding sequence are particularly important for resuming translation onto tmRNA internal ORF (Williams *et al*, 1999; Lee *et al*, 2001; Miller *et al*, 2008). Based on several mutations introduced in the upstream of the tag-encoding region on tmRNA (Konno *et al*, 2007) and on a genetic selection for tmRNA activity *in vivo* (Watts *et al*, 2009), it was shown that SmpB and the sequence upstream of the tmRNA template are functionally linked to direct frame selection.

Strikingly, our model links together these two regions: one area comprising the five nucleotides upstream of the resume codon, and a second region formed by the hydrophobic pocket comprising helix α 1, the loop between α 3 and β 8, β 9, and the C-terminal tail of SmpB (Figure 6). Accordingly, this region possesses the highest binding affinity for SmpB (Metzinger *et al*, 2008). This SmpB position is obviously instrumental in specifying the correct resume codon when translation shifts onto the tmRNA.

mRNA swapping

Switching from *trans*-peptidation to *trans*-translation requires a swap between the original truncated mRNA that is stalled in

Figure 3 Models for tmRNA and SmpB in the cryo-EM maps of the accommodated and translocated states. (A, B) Interactions of SmpB and tmRNA with the 50S subunit. (C, D) Interactions of SmpB and tmRNA with the 30S subunit. The densities of tmRNA-SmpB are semi-transparent grey. The orientations with respect to the ribosome are defined by the thumbnails. Landmarks on the 50S subunit: L7/L12, stalk of proteins L7/L12; L1, stalk of protein L1; PTC, peptidyl transfer centre; L11, protein L11; and H69, helix 69 of the 23S rRNA. Landmarks on the 30S subunit: b, beak; sh, shoulder; sp, spur; dc, decoding site; and h44, helix 44 of the 16S rRNA. TLD, blue; H2b-c, red; H2d, grey; PK1, orange; single strand between PK1 and PK2, white (resume codon highlighted in yellow); H5, brown; PK2, green; PK3, pink; and PK4, light blue. SmpB is in purple. This colour scheme is also used in Figure 1. For clarity, the 30S subunit and the tRNA are not shown in (A) and (B), and the 50S subunit and the tRNA are not shown in (C) and (D). Insets: orientation of the tmRNA-SmpB complex (in red) with respect to the ribosome. 30S and 50S subunits are in yellow and blue, respectively.

the mRNA path and the internal tmRNA ORF (Ivanova *et al*, 2005). On the accommodated (Supplementary Figure S2A) and ratcheted (not shown) state maps, the presence of the stalled mRNA is confirmed by the extra density found between the head and the back of the 30S subunit platform, where the Shine-Dalgarno interaction protrudes from the ribosome surface. Accordingly, the translocation of peptidyl-tmRNA to the P site is accompanied by the disappearance of this structural element and a weak E-site tRNA density, indicating a concomitant destabilization of the stalled

mRNA from the ribosome when the tmRNA resume codon is moved into the decoding site (Supplementary Figure S2B). When *trans*-translation triggers stalled polysomes, one can, therefore, presume a sequential delivery of stalled ribosomes one by one at the 3' end of the problematic mRNAs. After each *trans*-translation event, the flawed mRNA will then be subject to competition between the polysome moving forward towards its 3' end, and the 3'-to-5' exoribonuclease R ready to take it for degradation (Richards *et al*, 2006).

Figure 4 Molecular environment of tmRNA-SmpB complex. Cryo-EM-density map of tmRNA-SmpB (in red) with its neighbouring proteins in the accommodated (**A**) and translocated (**B**) states and neighbouring RNA helices of tmRNA-SmpB in the accommodated (**C**) and translocated (**D**) states. For clarity, only the proteins and the ribosomal subunits within 5 Å of the tmRNA-SmpB complex are presented. Landmarks: h18, h30–34, h42, and h44 are 16S rRNA helices and H38, H69–71, H81, H84, and H89–92 are 23S rRNA helices. All atomic coordinates were taken from Selmer *et al* (2006) (PDB code 2J00 and 2J01), except for protein L11, taken from Yusupova *et al* (2006) (PDB code 2HJG), and were rigid body fitted into the cryo-EM maps. Insets: orientation of the tmRNA-SmpB complex (in red) with respect to the ribosome. 30S and 50S subunits are in yellow and blue, respectively. The tRNA present in the P site during the accommodation and in the E site after the translocation is in purple.

Table 1 Molecular environment of the A- and P-site tmRNA on both ribosomal subunits

	A-site tmRNA		P-site tmRNA	
	30S	50S	30S	50S
TLD		H38/H43/H71/H89/H90/H92 L16/L25	S13	H38/H69/H71/H80/H81/H84/H90/ H92/H93 L5/L16
SmpB	h18/h30/h44	H69	h30 S13	H69
H2b–d	h32 S19	L11	h42 S13/S19	H38 L5/L11/L31
PK1			h30/h31/h32 S13/S19	
MLD	S3		h18/h34/h44 S12	
H5	S3/S4/S5		S3/S4/S5	
PK2	S3		S3	
PK3			S10	
PK4				

H, large subunit helix; h, small subunit helix; S, small subunit protein; L, large subunit protein.

Figure 5 A-site finger motion as the tmRNA-SmpB complex translocates to the P site. (A) Cryo-EM map of the 70S ribosome with tmRNA-SmpB in the accommodated state. (B) Cryo-EM map of the 70S ribosome in the ratcheted state prior to the translocation of tmRNA-SmpB. Note the motion of the H38 tip, which permits the transit of tmRNA helix H2 (white arrow). (C) Cryo-EM map of the 70S ribosome with tmRNA-SmpB in a translocated state. Helix H2 of the tmRNA moves to the other side of bridge B1a, while PK1 remains at the entrance of the gate. Landmarks are as follows: H38, helix 38 of the 23S rRNA; CP, central protuberance; and b, beak. PK1, PK4, and H2b-c refer to tmRNA domains described in Figure 1.

Navigating through the ribosome

To summarize, the present cryo-EM structures address a number of questions regarding the process of translocation of tmRNA-SmpB. After transpeptidation, the complex moves to the P site as a canonical tRNA, except that codon:anticodon pairing is supported by SmpB. Owing to its large size and complicated topology, the gap in bridge B1a triggered by the ratcheting of the ribosome is necessary for the transit of tmRNA. Helix H2 crosses the bridge while PK1 stays on the other side. PK4 follows this dynamic, but almost all of the rest of the molecule stays in place around the beak of the small subunit. The truncated mRNA is immediately released, while simultaneous binding of SmpB to the TLD and to the upstream region of the tagging sequence sets the resume codon into the decoding site. At this step, the internal ORF is

only partially unfolded, as its internal helix H5 is still highly structured. This model lends support to a mechanism in which the next transition of tmRNA-SmpB, from the P site to the E site, would follow the same dynamics. In line with the first translocation, the movement of the TLD to the E site is likely to drag PK1 over bridge B1a. Accordingly, an isolated tmRNA-ribosomal complex blocked with tmRNA in the E site was recently analysed using chemical probing. The model resulting from this study suggests a positioning of PK1 and H2 near the E site (Bugaeva *et al*, 2009). The dynamics of the other modules are more elusive and mainly depend on when helix H5 will unwind to be correctly translated into the mRNA path. Cryo-EM work is now underway in our laboratory to follow the fate of tmRNA-SmpB during the next steps of *trans*-translation, inside and outside of the ribosome.

Figure 6 Docking of SmpB into the cryo-EM density of the translocated complex (close-up view). Interactions of SmpB with tmRNA indicate two different binding sites. While the standard binding site links part of the OB (oligonucleotide/oligosaccharide binding) fold of SmpB with the TLD, the second binding site links the hydrophobic pocket at the bottom of the protein and the C-terminal tail of SmpB to the upstream region of the tmRNA resume codon. The resume codon is in yellow; helices h30 and h34 of the 16S rRNA are in pink; the TLD is in grey; and SmpB is in purple. Helices $\alpha 1$ and $\alpha 3$, strands $\beta 8$ and $\beta 9$, and the C-terminal helix are indicated. Locations of SmpB mutations known to affect frame choice are highlighted in orange (Watts *et al*, 2009).

Materials and methods

Preparation of ribosomal complexes

One of the main hurdles in preparing stalled ribosomal complexes is the overall heterogeneity found in the samples (Kaur *et al*, 2006; Bugaeva *et al*, 2009; Cheng *et al*, 2010; Weis *et al*, 2010). To overcome that problem, the ribosomal complexes were prepared *in vitro* by using ribosomes, SmpB, EF-G, and Ala-tmRNA from *Thermus thermophilus*; these molecules are more suitable for structural studies than the analogous components from *Escherichia coli*. All complexes were prepared in the absence of EF-Tu·GTP that traps some ribosomes in a pre-accommodated state, thus increasing sample heterogeneity (Weis *et al*, 2010). Indeed, the *trans*-transfer reaction can proceed without EF-Tu·GTP, albeit at a much lower efficiency (Shimizu and Ueda, 2002; Hallier *et al*, 2004). Salt-washed *T. thermophilus* 70S ribosomes free of S1 were prepared as previously described (Clemons *et al*, 2001). His-tagged AlaRS from *E. coli* and *T. thermophilus* EF-G and His-tagged SmpB were overexpressed in *E. coli* using the T7 expression system, then isolated by Ni^{2+} precharged HisTrap chelating column, according to the manufacturer's procedures (GE Healthcare, Orsay, France). The *T. thermophilus* tmRNA gene was directly cloned into a pUC19 plasmid, downstream of a T7 RNA polymerase promoter. Plasmids were linearized by BsmB1 digestion, and the corresponding RNAs were synthesized overnight using the T7 MEGAscript kit, according to the manufacturer's protocol (Ambion, Courtabœuf, France). Purification was performed by denaturing gel electrophoresis, followed by passive elution overnight. The tmRNA was heated at 80°C for 2 min in folding buffer (10 mM Hepes-KOH (pH 7.5), 20 mM NH_4Cl , 5 mM MgCl_2 , 0.1 mM EDTA), then cooled for 30 min at room temperature prior to aminoacylation. Aminoacylation was performed by incubation at 37°C for 30 min in 50 mM Hepes-KOH (pH 7.5), 60 mM NH_4Cl , 7 mM MgCl_2 , 0.1 mM EDTA, 2 mM ATP, 30 μM alanine, 2 μM SmpB, 1 μM tmRNA, and 1 μM AlaRS. The

sample was four-fold concentrated on a 5-kDa cutoff Microcon centrifugal unit (Millipore). Stalled ribosomes were obtained by incubating 1 μM 70S ribosomes with a short mRNA containing a Shine-Dalgarno sequence and a P-site codon: (i) 2 μM of an mRNA consisting of the GGCAAGGAGGUAAAAAUG sequence (bold: SD sequence; underlined: P-site codon) and 2 μM *E. coli* deacylated tRNA^{Met} for the accommodated complex or (ii) 2 μM of an mRNA sequence consisting of the AGGAGGUGAGGUUUU sequence (bold: SD sequence; underlined: P-site codon), and 2 μM *E. coli* acylated Phe-tRNA^{Phe} for the translocated complex, for 30 min at 37°C in 5 mM Hepes-KOH (pH 7.5), 10 mM NH_4Cl , 10 mM MgOAc , 50 mM KCl, 0.1 mM EDTA, and 6 mM β -mercaptoethanol. The final complexes were obtained by mixing 1 μM of stalled ribosomes with 2 μM SmpB for 5 min at 37°C, before adding the concentrated solution of Ala-tmRNA-SmpB complex (4:1 tmRNA:stalled ribosome) in the absence (accommodated complex) or in the presence (translocated complex) of 10 μM EF-G and 2 mM GTP for 15 min at 42°C.

Cryo-EM and image processing

The accommodated and translocated state complexes were diluted to a final concentration of 80 nM. Suspensions were applied to Quantifoil holey-carbon grids (Quantifoil, Jena, Germany) previously glow discharged. Excess solution was blotted, and the grids were flash frozen in liquid ethane, using the Vitrobot automat (FEI). This process resulted in ribosomal complexes embedded in a thin film of vitrified ice. Electron micrographs were recorded under low-dose conditions at liquid nitrogen temperature with a JEOL JEM-2200FS operated at 200 kV. Images were collected at $\times 45\,700$ magnification with a defocus range of 0.9–2.2 μm . In order to obtain the maximal number of complex ribosomal particles, good micrographs were digitized on a Nikon Coolscan 9000 ED with a step size of 7.5 μm . Approximately 138 000 and 270 000 particles for the accommodated and translocated complexes, respectively, were selected semi-automatically from raw micrographs using the e2boxer subroutine of EMAN2 (Tang *et al*, 2007) and were coarsened by a factor of 2, resulting in a pixel size corresponding to 3.28 Å. Contrast-transfer function parameters were determined using ctffind3 (Mindell and Grigorieff, 2003) and corrected by phase flipping. Images were processed using the IMAGIC-V software package (van Heel *et al*, 1996). The final 'accommodated' and 'translocated' maps were computed from $\sim 49\,000$ and $\sim 70\,000$ particles, respectively, after eliminating particles with heterogeneous composition and conformation by a supervised classification method (see Supplementary data). The final resolutions were 13 and 13.5 Å, based on the Fourier shell correlation 0.5 bit criterion (Supplementary Figure S3) (van Heel *et al*, 2000), and the maps were filtered accordingly. The visualization and the fitting of the cryo-EM densities with the atomic models were performed using the Chimera (Pettersen *et al*, 2004) and VMD (Humphrey *et al*, 1996).

Atomic model of the accommodated tmRNA-SmpB complex

Using our earlier model (Kaur *et al*, 2006) as our initial starting point, we separated the tmRNA-SmpB into small fragments corresponding to the PK and helices, which we subsequently fitted into the density corresponding to the tmRNA-SmpB complex. Owing to its characteristic shape, we started with the TLD-SmpB complex, using the 2CZJ crystal structure (Bessho *et al*, 2007) for coordinates. This complex was docked as a rigid body into the cryo-EM-density map using CHIMERA (Pettersen *et al*, 2004). We then simultaneously placed helices H2b and H2c into the dense region following the TLD. We manually positioned PK1 and H5 helix on both sides of the poorly defined domain corresponding to the upstream sequence tag. In order to allow the correct fitting of PK2 and PK3 into the density map, we split them into their respective helical regions. As for helices H2b, H2c, H5, H6a, H6b, H7, H8, and H9, PK2 and PK3 were manually fitted in the denser region of the cryo-EM map. We docked PK4 as a rigid body into its corresponding density map. We then placed the H2d helix in the remaining density between PK1, PK4, and H2c, carefully positioning this last structural element to connect it to the neighbouring structural fragments. To finish, all the single strands (including the long one connecting PK1 to H5) were manually positioned in the density map to connect the different structural domains.

In order to adjust the position of flexible elements, the whole structure was minimized *in vacuo* for 35 000 steps using NAMD

(Phillips *et al*, 2005) and the CHARMM27 force field (Mackerell *et al*, 1998, 2004; Foloppe and Mackerell, 2000; MacKerell and Banavali, 2000), while maintaining the protein and the helical region of the RNA. The model was then optimized by molecular dynamic flexible fitting (MDFF) (Trabuco *et al*, 2008, 2009), a method consisting of the application of forces proportional to the gradient of the EM-density map in molecular dynamic simulations to drive the atomic structure into the high-density regions. The MDFF simulations were performed *in vacuo* using NAMD (Phillips *et al*, 2005) and the CHARMM27 force field (Mackerell *et al*, 1998, 2004; Foloppe and Mackerell, 2000; MacKerell and Banavali, 2000). A multiple time-step integration scheme was used to calculate bonded interactions every femtosecond (fs), and nonbonded interactions every 2 fs. A cutoff distance of 10 Å was used for the nonbonded interactions. A dielectric constant of 80 was applied to adjust electrostatic interactions, and the temperature was maintained at 300 K using a Langevin thermostat with a damping coefficient of 5 ps⁻¹. To avoid the 'overfitting' artefact (Tama *et al*, 2004), supplementary restraints had to be applied to both the protein and RNA (Trabuco *et al*, 2008, 2009). To preserve the secondary structure of the protein, the ψ and ϕ dihedral angles of the amino-acid residues in helices or β -sheets were harmonically restrained using a force constant of 200 kcal mol⁻¹ rad⁻². Identically, the helical part of the RNA was preserved using two 200 kcal mol⁻¹ Å⁻² restraints to maintain the distance between paired bases, and a set of 200 kcal mol⁻¹ rad⁻² restraints to maintain the dihedral angles. The dihedral angle of the last four bases of the tmRNA were also restrained to maintain this fragment in a conformation similar to that of the corresponding base in the tRNA, using the 2J00 (Selmer *et al*, 2006) crystal structures as our reference. We decided to use a three-step protocol to optimize our model. First, we performed an additional 65 000 steps of minimization on the tmRNA-SmpB complex, using a 0.3-kcal mol⁻¹ scaling factor and a threshold of 0.01 to adjust the strength of the influence of the cryo-EM map on the molecular system. We then slowly heated our system to 300 K over the course of 20 000 simulation steps. In the last step, we performed 1 ns of molecular dynamic simulation, using the same scaling factor and force constant as in the previous MDFF steps. The convergence of the MDFF simulations was assessed during the course of the simulation by monitoring the cross-correlation coefficient between the atomic model and the original cryo-EM-density map. The convergence of the MDFF was usually found after 600 ps (data not shown).

From the accommodated model to the translocated tmRNA-SmpB

We started from the model of the accommodated state in order to create an atomic model of the translocated tmRNA. By comparing the two cryo-EM maps, we noticed that the density region corresponding to the H5 helix and PK2/PK3 did not differ between the two states. These three structural domains did not move significantly during translocation; therefore, we reused the coordinates of the accommodated model for the translocated tmRNA-SmpB. For the remaining sections, we used rigid-body docking to

position the TLD-SmpB complex, the H2b, H2c, and H2d helices, and PK1 and PK4 in their respective densities. We then connected the various regions by placing the appropriate single strands in the density map. We took special care of the resume codon that we positioned into the decoding site. We also added a fragment of the 30S subunit, including the h30-34 helices, in order to properly position the long single strand connecting PK1 to H5, leaving enough space for an incoming tRNA. The coordinates of this fragment were once again taken from crystal structure 2J00 (Selmer *et al*, 2006). NMR studies have shown that the C-terminal 20-30 residues are disordered in solution (Dong *et al*, 2002; Someya *et al*, 2003), and the crystal structure of these residues has not yet been shown (Gutmann *et al*, 2003; Bessho *et al*, 2007). Nevertheless, as previously predicted, we modeled the C-terminal tail of the SmpB in an α helix to account for the extra density found in the P site of the 30S (Jacob *et al*, 2005). We decided not to restrain this region during the optimization process, in order to let it relax and adjust freely.

The same minimization and MDFF protocols were used to optimize this second model. The only difference resides in the threshold of 0.03, which was used this time to adjust the strength of the influence of the cryo-EM map on the molecular system. Both the resume codon and the h30-34 helices were held fixed during the minimization and the MDFF simulations.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: RG, DT, PB and BF designed research; FW and RG performed biochemical work; FW, PB, and J-PR performed cryo-EM; FW and PB performed image processing; EG performed the atomic models; all authors contributed to the interpretation of the data, and RG, FW, and EG wrote the paper with important input from others. Coordinates of the cryo-EM maps and atomic models of the accommodated and translocated states have been deposited in the EMDB (accession codes EMD-5188 and EMD-5189, respectively) and in the PDB (accession codes 3IYQ and 3IYR, respectively).

Conflict of interest

The authors declare that they have no conflict of interest.

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