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Ribosomal protein S1 induces a conformational change of tmRNA; more than one protein S1 per molecule of tmRNA

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Abstract

tmRNA (10Sa RNA, *ssrA*) acts to rescue stalled bacterial ribosomes while encoding a peptide tag added trans-translationally to the nascent peptide, targeting it for proteolysis. Ribosomal protein S1 is required for tmRNA binding to isolated and poly U-programmed ribosomes. Mobility assays on native gels indicate that the binding curves of both recombinant and purified proteins S1 from *E. coli* is biphasic with apparent binding constants of ~90 and ~300 nM, respectively, suggesting that more than one protein interacts with tmRNA. Structural probing of native tmRNA in the presence and absence of the purified protein suggest that when S1 binds, tmRNA undergoes a significant conformational change. In the presence of the protein, nucleotides from tmRNA with enhanced (H2, H3, PK1, PK2, PK4, in and around the first triplet to be translated), or decreased (H5 and PK2), reactivity towards a probe specific for RNA single-strands are scattered throughout the molecule, with the exception of the tRNA-like domain that may be dispensable for the interaction. Converging experimental evidence suggests that ribosomal protein S1 binds to pseudoknot PK2. Previous structural studies of tmRNA in solution have revealed several discrepancies between the probing data and the phylogeny, and most of these are reconciled when analyzing tmRNA structure in complex with the protein(s). Ribosomal protein(s) S1 is proposed to set tmRNA in the mRNA mode, relieving strains that may develop when translating a looped mRNA. © 2002 Société française de biochimie et biologie moléculaire/Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Bacterial ribosome; Trans-translation; Transfer-messenger RNA; Ribosomal protein S1; Structural probing

1. Introduction

Transfer-messenger RNA (tmRNA), also known as SsrA or 10Sa RNA, is a bacterial ribonucleic acid that recycles 70S ribosomes stalled on problematic messenger RNAs (mRNAs) and also contributes to the degradation of incompletely synthesized peptides [1,2]. tmRNA acts initially as a transfer RNA (tRNA), being aminoacylated at its 3' end by alanyl-tRNA synthetase, to add alanine to a stalled polypeptide chain. Translation then resumes not on the mRNA on which the ribosomes were stalled but at an internal position in tmRNA. Termination soon occurs, tmRNA recruiting the appropriate termination factors allowing the release of the tagged protein that is subsequently recognized and degraded

by specific proteases. As a consequence, the stalled ribosome can be recycled.

tmRNA interacts specifically with several macromolecules including SmpB (small protein B) [3], EF-Tu [4], phosphoribosyl pyrophosphate synthase [5], RNase R and YfbG [6], and with the tRNA that decodes the resume codon [7]. Moreover, ribosomal protein S1 is required for tmRNA binding to isolated and poly U-programmed ribosomes [8]. Protein S1 is the largest ribosomal protein, 68 kDA, present in the small subunit of the *E. coli* 70S ribosome. Association of S1 to the ribosome is weak and reversible. In the work reported here, we present experimental evidence that purified and recombinant ribosomal protein S1 binds native tmRNA. The binding curve is biphasic, suggesting that several molecules of protein S1 bind tmRNA. When the protein(s) bind tmRNA, the RNA undergoes a conformational change involving several domains, but not the tRNA-like portion. These results are discussed with the other

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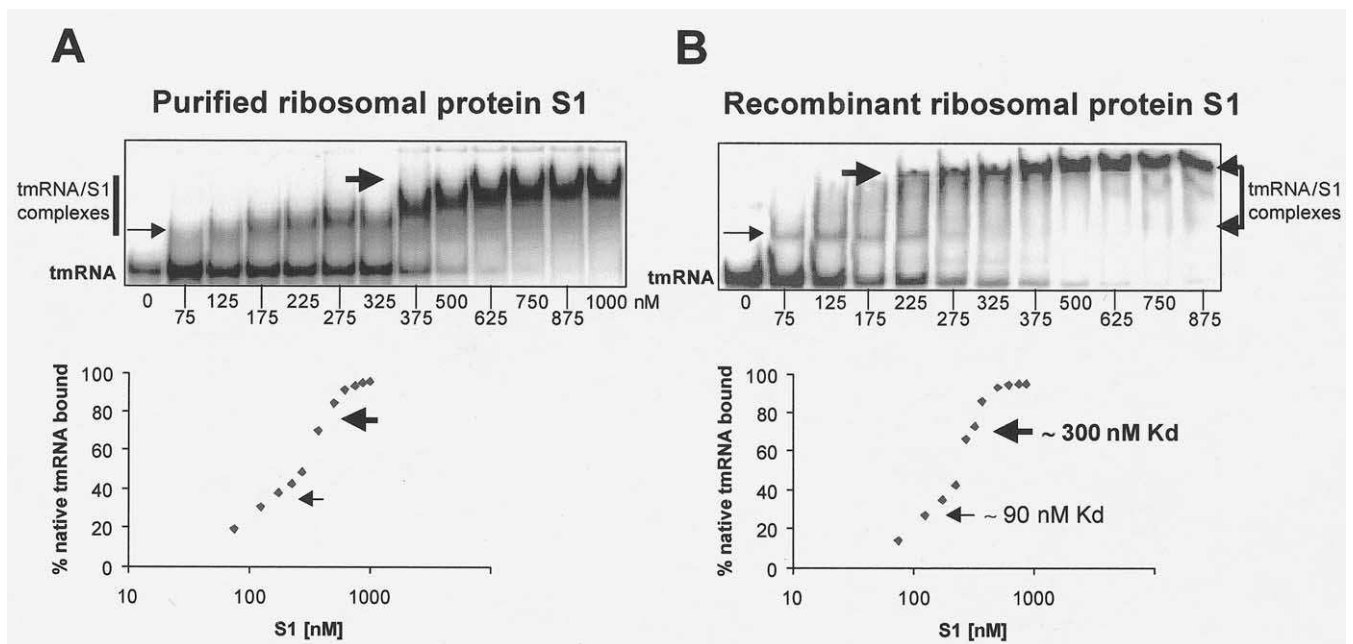


Fig. 1. Native gel retardation assays between purified-labeled tmRNA and purified (A) or recombinant (B) ribosomal protein S1 from *E. coli*, with a graphical representation of the experimental data analyzed on a phosphorimager. Notice that the binding curves is biphasic (arrows), suggesting that several molecules of protein S1 interact with tmRNA.

available experimental evidence, including the identification of several UV-induced cross-links between His-tagged S1 and a 4-thioU-substituted tmRNA transcript [8].

2. Materials and methods

2.1. Expression and purification of tmRNA and ribosomal protein S1

A derivative of plasmid pGEMEX-2 with the *E. coli* *ssrA* gene expressed from T7 promoter is transformed into *E. coli* JM109 (DE3) that contains the T7 RNA polymerase gene driven from a *lac* promoter. In vivo produced tmRNA is purified as described [9]. Expression and purification of the His-tagged *E. coli* ribosomal protein S1 is performed as described [8].

2.2. RNA labeling

Labeling at the 5'-end of the RNAs is performed with [γ - 32 P]-ATP and phage T4 polynucleotide kinase after dephosphorylation with alkaline phosphatase [10]. Labeling at the 3'-end is done by ligation of [γ - 32 P]-pCp using T4 RNA ligase. After labeling, tmRNA is gel purified, eluted passively, and ethanol precipitated.

2.3. Gel retardation assays

RNAs are denatured 2 min at 80 °C in RNase-free water and then slowly cooled down to room temperature for 20 min. Standard gel retardation assays contain 0.2 pico-

mole (100 000 cpm) of labeled tmRNA with the appropriate concentrations (see Fig. 1) of either purified or recombinant ribosomal protein S1 in a binding buffer (10 mM Tris-HCl, pH 7.4, 10 mM Mg acetate, 200 mM NH₄Cl, 0.1 mM EDTA, 7 mM β -mercapto ethanol), to a final volume of 20 μ l. A 45 min incubation at 4 °C is performed in the presence of 20 units of RNase, followed by adding 10 μ l of 30% glycerol and subjected directly to electrophoresis in a 5% (19/1 acryl/bisacrylamide) non-denaturing PAGE in 90 mM Tris-HCl (pH 8.3), 90 mM boric acid, 2.5 mM EDTA, at 4 °C (a 4 h migration at 150 V). The gel is then fixed, dried and the data are analyzed on a phosphorimager.

2.4. Structural mapping procedures

Enzymatic digestions or chemical modifications are performed on both 3'- and 5'-labeled tmRNA (300 000 cpm/reaction). Labeled RNA is heated to 80 °C for 2 min and slowly cooled down to room temperature. The labeled RNA is incubated with or without 500 nM of purified ribosomal protein S1 in 50 mM Hepes pH 7.5, 20 μ M Mg acetate, 1 mM Zinc acetate for 45 min at 4 °C in a final volume of 20 μ l. The reaction mixture is then supplemented with 1 μ g of total rRNA. Digestions of the RNA are performed with 100 units of nuclease S1 for 7 min at 37 °C, followed by a phenol extraction and ethanol precipitation. The RNA pellets are washed twice with ethanol 75%, dried and the radioactivity counted. Identical amounts of radioactivity are loaded per lanes. Cleavage or modification sites are detected by gel electrophoresis by direct identification with the statistical cleavage patterns of the RNA itself. The denaturing PAGE is fixed, dried and the

data are analyzed on a phosphorimager. The quantitation of each fragment is scored manually.

3. Results

3.1. Apparent association constants of purified and recombinant S1 with native tmRNA

To assess the optimal concentration of protein S1 required for the enzymatic footprint assays, interactions between native tmRNA and either purified or recombinant ribosomal protein S1 were analyzed by gel mobility shift assays (Fig. 1). The overall apparent association constant (K_a) was derived by determining the concentration of the protein that led to equal amounts of free and bound RNAs. Recombinant and purified protein S1 both bind native tmRNA with an overall apparent association constant of $3.25 \times 10^7 \text{ M}^{-1}$, demonstrating that the his-tag of recombinant S1 has no deleterious effects in binding tmRNA. With recombinant S1, two different complexes are observed: a lower one with [S1] from 75 to 225 nM with an apparent binding constant of $\sim 90 \text{ nM}$, and a higher one starting from a concentration of S1 of 225 nM and higher, with an apparent binding constant of $\sim 300 \text{ nM}$ (Fig. 1B). The separation of these two complexes is less obvious with the purified protein (Fig. 1A), but notice that the major band has a slower migration with increased protein concentration. Also, the binding curves seem to be biphasic (arrows in Fig. 1), giving further experimental support to the fact that several molecules of protein S1 interact with tmRNA.

3.2. Monitoring the interaction between native tmRNA and purified ribosomal protein S1

We employed footprint assays to monitor the interaction between tmRNA and purified ribosomal protein S1, an approach that is instrumental in establishing the contacts between RNAs and proteins (for a recent work, see [11]). In this study, purified tmRNA from *E. coli* cells was end-labeled, and chemicals and enzymes probed its solution conformation in the absence or presence of purified ribosomal protein S1. Ribonuclease V1 cleaves double-stranded RNA or stacked nucleotides, while nuclease S1, lead and imidazole cleave single-stranded RNA. The reactivity towards these probes was monitored for each nucleotide of the 363 nt-long RNA. Ten independent experiments were performed (Fig. 2 is representative). Among them, nuclease S1 has revealed dramatic differences in the reactivity of selected nucleotides from tmRNA (Fig. 2), but not the other three probes (not shown). These data are summarized in Fig. 3 on a secondary structure model of *E. coli* tmRNA [12,13].

In the presence of the protein, the ribose-phosphate chain of tmRNA is cleaved at positions G23, G24, C331 and G332 (Fig. 2, red); interestingly, these four positions are close in tmRNA secondary structure (H5, Fig. 3). Twelve nucle-

otides from tmRNA (G54, G61–G62, A83–A84, A92, C189, G267, G277–G278, A301 and A305) are not (or weakly) cut by nuclease S1 in the absence of ribosomal protein S1, but are susceptible to the nuclease in the presence of the protein (Fig. 2). Conversely, 11 nucleotides from tmRNA (A33–A34, A38–G43, C157–U158 and C199) are cleaved by nuclease S1 in the absence of ribosomal protein S1, but become protected (or the cleavage is significantly reduced) in the presence of the protein (Fig. 2). The reactivity of domains H1, H4, H6 and PK3 towards structural probes is not affected by the presence of ribosomal protein S1 (Figs. 2 and 3).

4. Discussion

We report that both purified and recombinant protein S1 bind purified tmRNA with an overall $\sim 3.25 \times 10^7 \text{ M}^{-1}$ apparent association constant (K_a). It was reported that an identical recombinant protein binds synthetic tmRNA with a $\sim 1 \times 10^8 \text{ M}^{-1} K_a$ [8]. Both studies have been performed in comparable experimental conditions. The only difference between synthetic and native tmRNA is the presence of two post-transcriptional modifications in the tRNA-like domain of native tmRNA; since its tRNA-like domain is not involved in binding with S1 ([8] and this report), this is the rationale for observing similar K_a for both RNAs. Interestingly, our data suggest that several molecules of S1 interact with tmRNA. Upon binding of a ligand, in our case a protein, to an RNA, the reactivity of a subset of nucleotides towards structural probes can be either increased, decreased or unaffected. If increased, it originates from a conformational change of the RNA induced by the protein; if decreased, it could originate either from a direct contact of the protein with the RNA (footprint) or from a conformational change of the RNA, when the protein binds. As mentioned earlier, only nuclease S1 has revealed significant changes in the cleavage pattern upon protein S1 binding. For the other probes (lead, imidazole and RNase V1) to work, concentrations of protein S1 higher than 500 nM might have been required to detect reactivity changes in tmRNA structure. At a concentration of 500 nM of protein S1, more than one protein might be in complex with tmRNA, as already discussed previously. A more systematic footprint analysis of tmRNA as a function of protein S1 concentration, including additional probes that are less sensitive to steric hindrance and to the RNA structure, will be necessary to delineate the complete set of binding site(s) of protein(s) S1 to tmRNA.

Phylogenetically supported helix H5, that connects the two ends of tmRNA, and pseudoknot PK2, are both unstable in solution (Fig. 4, left), as evidenced by structural probing of naked *E. coli* tmRNA [12]; when ribosomal protein S1 binds tmRNA, the lower portion of H5 is either stabilized or protected against single-stranded cuts, whereas its upper part becomes exposed to spontaneous cleavages (Figs. 2–4).

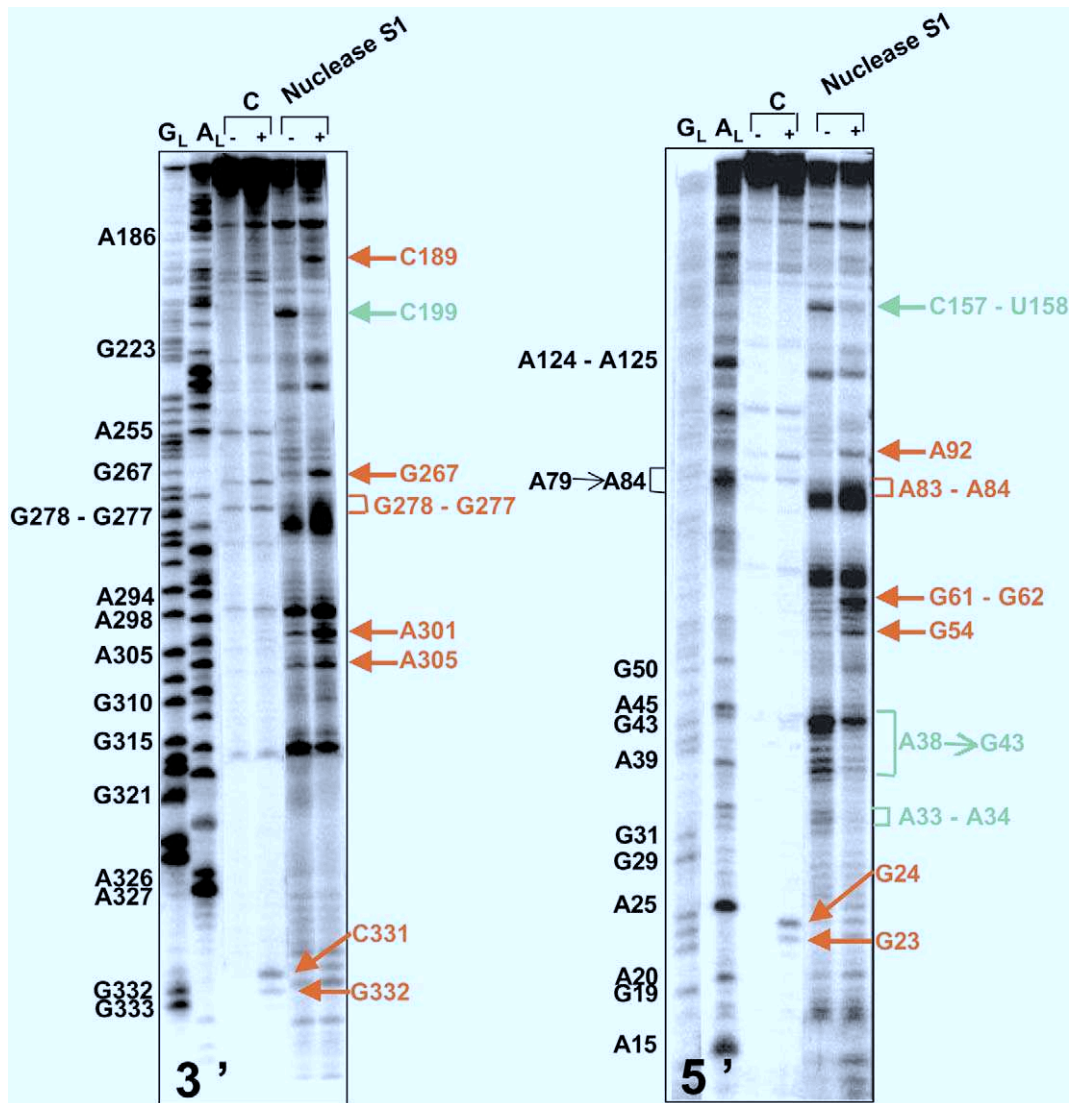


Fig. 2. Autoradiograms of cleavage products of 3'- (left) and 5'- (right) labeled RNA by nucleases S1, in the presence (+) or absence (-) of 500 nM of purified ribosomal protein S1. Structural probing is collected on the 5' and 3' halves of a purified *E. coli* tmRNA. Lanes C, incubation controls; Lanes GL, RNase T1 hydrolysis ladder; Lanes AL, RNase U2 hydrolysis ladder; Sequence is indexed on the left sides. The red and green nucleotides are the positions with enhanced or decreased reactivity in the presence of the protein, respectively.

In naked tmRNA, pseudoknot PK2 is cleaved by lead and by nuclease S1 at positions C157 and U158 [12], whereas these two nucleotides are protected against single-stranded cuts in the presence of ribosomal protein S1 (Figs. 2 and 3). In naked tmRNA, C189, from the strand crossing the shallow groove of PK2, is cleaved by ribonuclease V1 (cleaves double-stranded RNA or stacked nucleotides) whereas C189 is subjected to single-stranded cuts in the presence of S1 (Figs. 2 and 3). These experimental evidences suggest that phylogenetically supported PK2 is not folded, or only transiently, as a pseudoknot in naked tmRNA, but becomes folded as such in the presence of the ribosomal protein. Strong protections have been identified at positions C157, U158 and C199, and a UV-induced crosslink was detected at position U198 (Fig. 3, blue) between His-tagged S1 and a 4-thioU-substituted tmRNA

transcript off the ribosome [8]. Taken together, these data suggest that ribosomal protein S1 binds pseudoknot PK2.

Nucleotides from both stems of pseudoknot PK4 (G267, G277, G278), one nucleotide from helix H2 (A305) and one nucleotide between H2 and PK4 (A301), are subjected to single-stranded cuts only in the presence of the protein, suggesting that H2 and PK4 are destabilized when S1 binds tmRNA (Fig. 4, right). Moreover, nucleotides G54, G61 and G62 from PK1 are subjected to single-stranded cuts only in the presence of the protein, suggesting that the conformation of PK1 is modified when S1 binds. Based on a mutational analysis, PK1 was shown to be essential for protein tagging [14]. Since protein S1 modifies its conformation, it could be one reasonable explanation for its functional importance. Nucleotides in and around the resume codon, A83, A84 and A92 (the resume codon is G90C91A92) become more

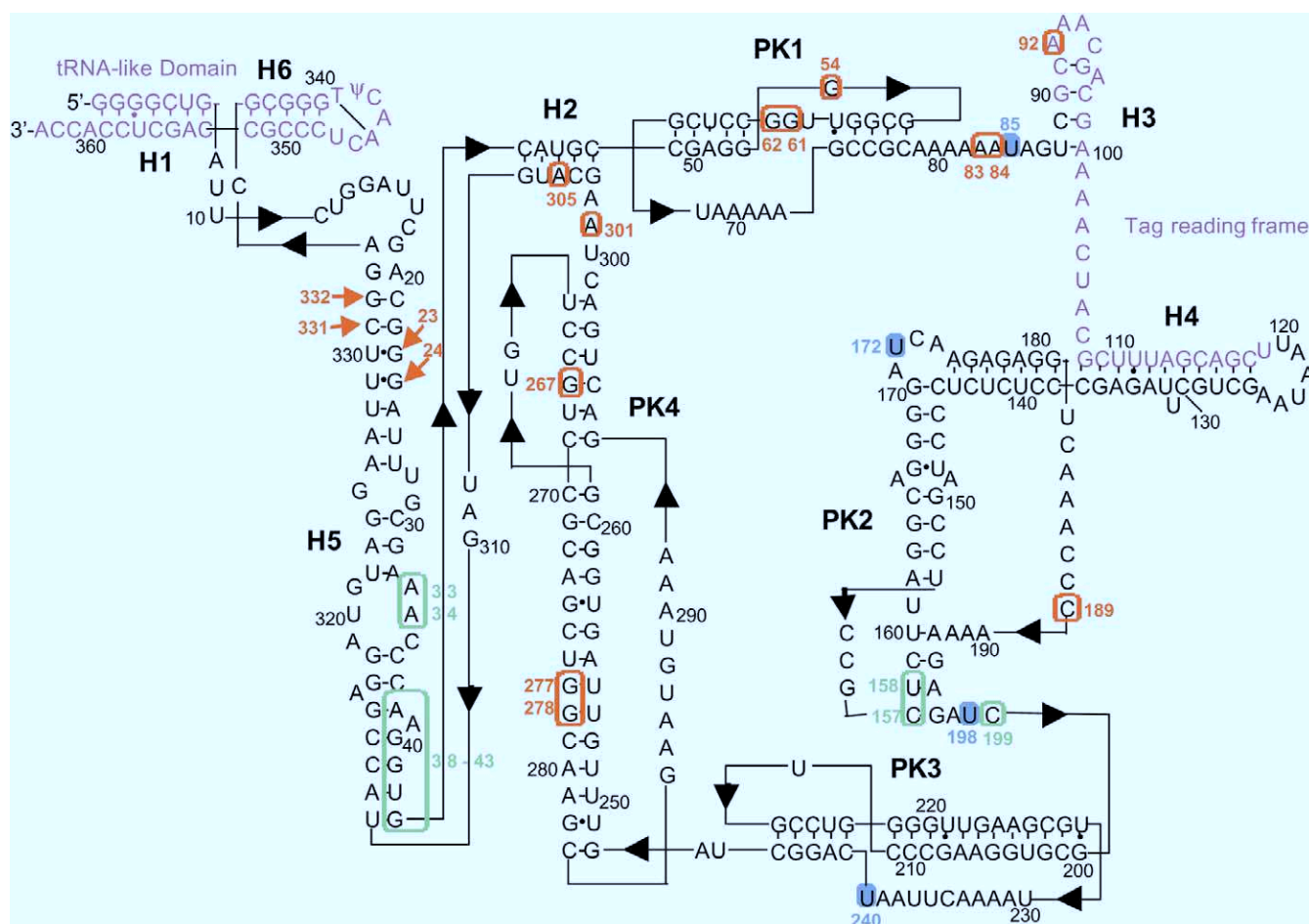


Fig. 3. Results of the footprints between native tmRNA and purified ribosomal protein S1, depicted onto *E. coli* tmRNA secondary structure [12,13]. Nucleotides that are protected against nuclease S1 cuts upon binding of the protein are circled in green; those that become accessible to S1 cuts when the protein binds are circled in red; the red arrows show spontaneous degradation sites when the protein binds. For comparison, the location of the nucleotides of 4-thioU-substituted tmRNA transcript cross-linked to protein S1 in the absence of the ribosome is indicated (in blue). The two functional parts of tmRNA, the tRNA-like domain and the tag reading frame, are in purple.

accessible when S1 binds. Earlier data [8] have identified a UV-induced cross-link at position U85 (Fig. 3, blue), between synthetic tmRNA and his-tagged S1, suggesting that this residue is close to the protein; Interestingly, in vivo randomization-selection experiment performed around the resume codon suggest that local upstream elements, including positions 83, 84 and 85, provide the strongest determinants to determine the resume codon in tmRNA [15]. Overall, these data suggest that S1 might participate in determining the internal reading frame of tmRNA.

Nucleotides U172 (from PK2) and U240 (from PK3) are cross-linked with S1 off the ribosome [8], but no evidences were gathered about these two regions that are not or poorly accessible towards nuclease S1. Additional structural perturbations of tmRNA, when S1 binds, might await further structural mapping to be delineated, including other probes such as Iron-EDTA (riboses) or ethyl nitroso urea (phosphates). Ribosomal protein S1 affects the conformation of most, if not all, pseudoknots of tmRNA; using selection/amplification methods (SELEX), pseudoknot-containing high affinity RNA ligands were generated against

purified S1 protein [16]. Moreover, the loops of the selected pseudoknots were highly conserved, implicating them in binding. In tmRNA, pseudoknot PK2 is a strong candidate for the binding of ribosomal protein S1. In the pseudoknot motif that was selected by SELEX, loop 1 contains always three nucleotides, ACA, ACG or ACC [16]; loop 1 from PK2 contains also three nucleotides, CCG. The length and sequence of loop 1 among the known tmRNA sequences from eubacteria, however, are not conserved.

tmRNA structure has a tRNA-like domain (H1–H6) connected by a long paired region (H5 and H2) to a looped domain composed of a tag reading frame inserted into a string of pseudoknots (PK1–PK4). In naked tmRNA, the conformation of H5 and PK2 is poorly defined in solution [12], whereas phylogenetically supported [12,17]. When proteins S1 bind tmRNA, H5 and PK2 are stabilized further, to the expense of other parts of the molecule, including H2 and PK4. Interestingly, the destabilized elements cluster together at the junction between the looped mRNA and the tRNA-like domain (Fig. 4, right). Opening of H2 and PK4 probably gives more conformational freedom to the looped

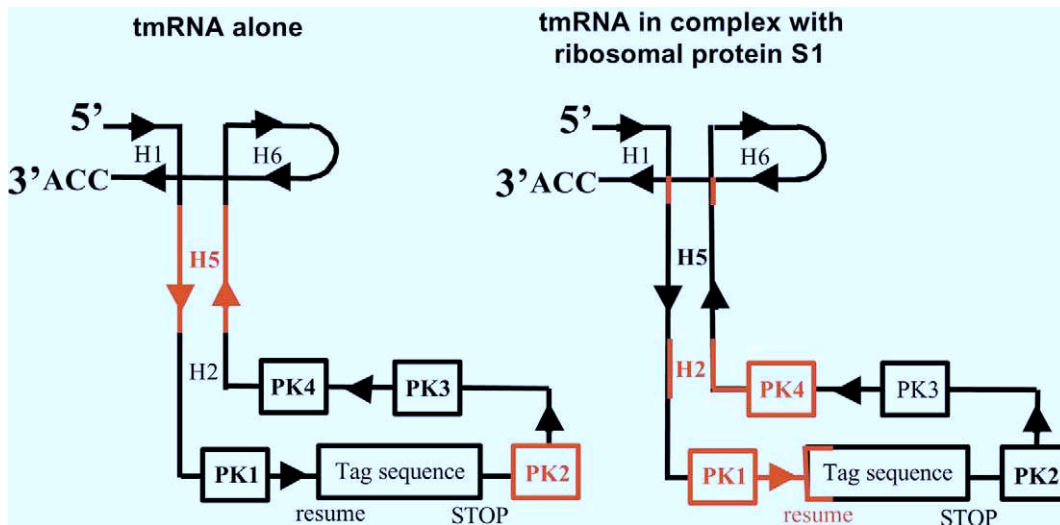


Fig. 4. Schematic representation of tmRNA structure illustrating the conformational changes induced by the binding of *E. coli* ribosomal protein S1. Some phylogenetically supported structural domains are destabilized when the RNA is naked (left, in red) and others when the protein binds (right, in red). For naked tmRNA, discrepancies between the probing data and phylogeny were reported for helix H5 and pseudoknot PK2 [12]. When S1 binds tmRNA, PK2 and parts of H5 are stabilized; conversely, nucleotides from H2, PK1, the resume codon, between PK1 and the tag, and from PK4 are more susceptible to single-stranded specific cuts.

mRNA domain of tmRNA. Ribosomal protein S1 may facilitate the access of the resume codon to the translational apparatus, during ribosome re-registration onto the internal open reading frame of tmRNA. S1 may also act during tag translation, relieving strain that could develop in the looped mRNA domain. Stabilizing the conformation of the downstream pseudoknot (PK2) might prevent putative translational readthrough at the tmRNA-encoded termination codon tmRNA has no obvious SD sequence analogs; for mRNAs lacking an upstream Shine-Dalgarno (SD) sequence, ribosomal protein S1 is required during translation initiation [18]. Based on cryoelectron microscopy, S1 was visualized within the *E. coli* 30S ribosomal subunit, and the protein is proposed to interact with 11 nucleotides of the mRNA, immediately upstream of the SD sequence [19]. Our data shows protections by the S1 proteins in helix H5 and pseudoknot PK2, suggesting that the interaction between tmRNA and proteins S1 is quite different than that with canonical messenger RNAs. Interestingly, our results suggest that several molecules of ribosomal protein S1 bind tmRNA. S1 is weakly attached to the ribosome and can be found free in solution. One hypothesis is that soluble S1 binds tmRNA first outside the ribosome, inducing a conformational change of tmRNA that favors its recruitment to a stalled ribosome; subsequently, another molecule of S1 that is attached to the stalled ribosome may also participate in the recruitment of tmRNA.

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