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Small protein B interacts with the large and the small subunits of a stalled ribosome during *trans*-translation

Marc Hallier, Jessie Desreac and Brice Felden*

Université de Rennes I, Upres JE 2311, Inserm ESPRI, Biochimie Pharmaceutique,
2 Avenue du Prof. Léon Bernard, 35043 Rennes, France

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ABSTRACT

During *trans*-translation, stalled bacterial ribosomes are rescued by small protein B (SmpB) and by transfer-messenger RNA (tmRNA). Stalled ribosomes switch translation from the defective messages to a short internal reading frame on tmRNA that tags the nascent peptide chain for degradation and recycles the ribosomes. We present evidences that SmpB binds the large and small ribosomal subunits *in vivo* and *in vitro*. The binding between SmpB and the ribosomal subunits is very tight, with a dissociation constant of 1.7×10^{-10} M, similar to its K_D for the 70S ribosome or for tmRNA. tmRNA displaces SmpB from its 50S binding but not from the 30S. *In vivo*, SmpB is detected on the 50S when *trans*-translation is impaired by lacking tmRNA or a functional SmpB. SmpB contacts the large subunit transiently and early during the *trans*-translational process. The affinity of SmpB for the two ribosomal subunits is modulated by tmRNA in the course of *trans*-translation. It is the first example of two copies of the same protein interacting with two different functional sites of the ribosomes.

INTRODUCTION

Ribosomes that translate truncated mRNAs can stall and generate incomplete polypeptides. In bacteria, these problems are solved by a remarkable translational quality control surveillance system headed by an RNA–protein complex containing transfer-messenger RNA (known as tmRNA, SsrA RNA or 10Sa RNA) and small protein B (SmpB). Owing to a tRNA-like domain and an internal short coding sequence, tmRNA functions as both a tRNA and an mRNA (1). This process, termed *trans*-translation, allows the stuck ribosome to switch mRNA templates, resuming translation on the tmRNA internal reading frame and freeing the ribosome when it decodes tmRNA termination codon. After termination, the polypeptide encoded by the problematic mRNA gains a hydrophobic peptide tag encoded by tmRNA, leading to targeted proteolysis [for recent reviews, see (2,3)].

SmpB is an universal cofactor of tmRNA (4) and adopts a β-barrel fold in solution (5). The protein binds the tRNA part of tmRNA in the elbow region on the D-like loop face (6) and has additional binding sites, including one around the first codon of tmRNA reading frame where translation resumes (7). The C-terminal tail of SmpB emerges from the β-barrel opposite to the face that contacts tmRNA. Although having no defined structure in both free and bound states to tmRNA, it might be ordered when bound to the ribosome. C-terminal tail truncated *Escherichia coli* SmpB, despite being still capable of binding ribosomes and tmRNA (8,9), are inactive for *trans*-translation suggesting that this tail has an essential function.

E.coli SmpB enhances tmRNA aminoacylation (10) and is required for the stable association of tmRNA with ribosomes (4) and remains bound to a 'ribosome–tmRNA' complex isolated from cells with stalled translation at various positions within tmRNA reading frame (11). Therefore, SmpB has essential roles at early and later stages of *trans*-translation. Cryo-EM study of the initial entry of tmRNA into a stalled ribosome indicates that SmpB bridges tmRNA and the 50S subunit (12). We showed previously that SmpB can bind the 70S ribosome in the absence of tmRNA *in vivo,* triggering the subsequent recruitment of tmRNA to process *trans*-translation. Two molecules of SmpB are required for a single ribosome to trigger *trans*-transfer (13). Recent *in vitro* data indicate that SmpB binds 30S and 50S ribosomal subunits (14). Thus, *trans*-translation can be initiated in a different way than originally thought where SmpB in complex with tmRNA and EF-Tu binds to a stalled ribosome (15).

To study the functional role of SmpB proteins during *trans*-translation we looked at the localization of SmpB during the *trans*-translational process *in vivo*. Ribosomes from cells

*To whom correspondence should be addressed. Tel: +332 2323 4851; Fax: +332 2323 4456; Email: bfelden@univ-rennes1.fr

blocked at various stages of the *trans*-translational process were dissociated into ribosomal subunits at low magnesium concentration and the localization of SmpB on the 50S and 30S subunits separated in a sucrose gradient was assessed by western blotting. A similar procedure was used to show the localization of the Hsp15 protein on the 50S ribosomal subunit when translation was blocked by chloramphenicol in *E.coli* (16), the association of the DEVH-box protein Ski2W to the 40S ribosomal subunit (17) and the localization of the GCN2 protein kinase on the 60S ribosomal subunit (18). In this study, we demonstrate that SmpB binds both the small 30S and the large 50S ribosomal subunits *in vivo* and *in vitro* with similar affinities, with a dissociation constant of 1.7×10^{-10} M, similar to its K_D for the 70S ribosome or for tmRNA. Dissociation constants between SmpB and the 70S, SmpB and the two ribosomal subunits, compared with those for tmRNA, were measured by surface plasmon resonance (SPR). Both the SPR and the competition assays *in vitro* indicate that tmRNA can displace SmpB from the 50S subunit, but not from the 30S one. *In vivo*, SmpB is detected on the 50S subunit only when *trans*-translation is impaired, either by omitting tmRNA from the reaction or by using a SmpB mutant that is defective in the first step of *trans*-translation. These results suggest that the binding of SmpB to the large ribosomal subunit is an early and transient step during *trans*-translation. Altogether, our data can be reconciled into a model that illustrates how two molecules of SmpB modulate their affinities towards each of the two ribosomal subunits versus tmRNA to trigger *trans*-translation, allowing the accommodation of tmRNA acceptor branch, *trans*-transfer and translation resumption on tmRNA reading frame.

MATERIALS AND METHODS

Strains and plasmids

E.coli Δ*smpB*, Δ*ssrA* and Δ*smpB*Δ*ssrA* strains have been described previously (13). The promoter of *smpB* gene was amplified by PCR from genomic DNA of *E.coli* using the 5′ primer (TAATATGAATTCTCCTGCTGT) and 3′ primer (CGTCATAAGCTTCGTGAATCA) and then cloned into the ampicillin-resistant pBR322 plasmid at its EcoR1 and HindIII restriction sites to obtain the low-copy pBR322-pS vector. Wild-type *smpB* and an *smpB* gene construct deleted of the sequence coding for the 16 C-terminally amino acids were amplified by PCR and inserted into the HindIII and BamH1 site of the pBR322-pS vector. The truncated *smpB* gene was also cloned into either the pET-21a or pEt-42a to overexpress the Δ16-smpB protein tagged by six histidine to its C or N-terminal portion. His-tagged SmpB proteins were overexpressed and purified as previously described (13).

Complementation and *in vitro trans*-translation assays

Complementation with SmpB and Δ16-smpB was performed by the transformation of the Δ*smpB* strain with either the pBR322-pS-SmpB or the pBR322-pS-Δ16-smpB plasmids. Transformed cells were grown to mid-logarithmic phase at 37°C, diluted at an A600 of 0.01 in an LB broth containing ampicillin and incubated at 45°C. *In vitro* translation of poly(U) template and *in vitro trans*-translation was performed at 37°C in 35 μl with the PURE system described by Shimizu (19) using ribosomes from the Δ*smpB*Δ*ssrA* strain (0.5 μM), His-tagged SmpB proteins (1 μM) and tmRNA aminoacylated with [H^3] alanine (0.5 μM). Each of the final 50 μl reaction mixtures contained 15 pmol ribosomes, 30 pmol of either SmpB wt, delta 16-SmpB or no SmpB and 60 pmol elongation factor EF-Tu. Pure $tRNA^{Phe}$ was adjusted to 4 μM. To stall the ribosomes on a polyphenylalanine polypeptide, a polyUridine RNA (600 pmol) was translated in 47 μl reaction mixture for 30 min at 37°C in the presence of purified PheRS. Separately, 15 pmol tmRNA was aminoacylated by purified aminoacyl–tRNA synthetase in the presence of [H^3] alanine (9.25 kBq) and this mixture was added to the first one to monitor the codon-independent step of *trans*-peptidation.

Determination of tmRNA and SmpB levels in subcellular fractions of *E.coli*

E.coli strains, grown in LB broth, were harvested to mid-logarithmic phase and lysed in buffer A (10 mM HEPES pH 7.5, 100 mM ammonium acetate, 10.5 mM magnesium acetate, 3mM *B*-mercaptoethanol) by freeze/thaw cycles. To block the *in vivo* translation, cells were incubated for 5 min at 37°C with 2 mM chloramphenicol and mixed with an identical volume of ice before centrifugation. The S30 (whole fraction), S100 (soluble material) and P100 (crude ribosome extract) fractions were obtained by differential centrifugations as previously described (13). For each fraction (S30, S100 et P100), an amount of RNAs and proteins corresponding to the same number of *E.coli* cells was analyzed by northern or western blotting. For northern hybridization, RNAs were separated by electrophoresis on a 1.5 % (w/v) agarose gel containing 6.5% (v/v) formaldehyde and transferred in 10× SSC to nylon membrane by the capillary method. Pre-hybridization and hybridization with ^{32}P-labeled DNA oligonucleotides complementary to tmRNA (5′-CGG GTA CGG GTA GGA TCG CAC ACC-3′) or to 16S ribosomal RNA (5′-CCG TCC GCC ACT CGT CAG CAA-3′) were carried out in ExpressHyb according to the protocol (Clontech). SmpB was imunodetected by western blotting using a rabbit polyclonal antibodies directed against His-tagged SmpB protein followed by chemiluminescence detection (Amersham Biosciences).

Sucrose density gradient centrifugation and analysis of 50S and 30S subunits

Sucrose purified ribosomes were dissociated into 50S and 30S subunits by diluting the 70S ribosome with 10-fold volume of Mg^{2+}-free buffer. Ribosomal subunits were separated onto a 10–30% sucrose gradient (32 ml) in 10 mM HEPES pH 7.5, 100 mM sodium chloride, 1 mM magnesium acetate for 15 h at 25 000 r.p.m (Rotor SW32) and 4°C. RNAs were isolated from one-half of each fraction by extraction with phenol and precipitation with ethanol. Proteins were extracted from the other half of samples by TCA precipitation. SmpB and tmRNA were detected by western and northern blotting as described (13).

In vitro binding analysis

70S ribosomes, 50S and 30S ribosomal subunits from Δ*smpB*Δ*ssrA* cells were purified by gradient sucrose centrifugation and incubated 10 min at 4°C with a 10-fold molar excess of His-tagged SmpB protein in buffer E (10 mM Tris–HCl pH

7.5, 100 mM ammonium chloride, 10 mM magnesium acetate and 3 mM 2-mercaptoethanol). The resulting 70S-SmpB, 50S-SmpB and 30S-SmpB complexes were purified further on a 10–30% sucrose gradient in buffer E to eliminate free SmpB proteins. 50S-SmpB subunit (10 pmol) and 30S-SmpB subunit (50 pmol) were incubated for 15 min at 37°C with increasing amount of tmRNA in 400 µl of buffer E and loaded on 10–30% sucrose gradient. Fractions either containing the 50S subunits, the 30S subunit or tmRNA (fractions including tmRNA and soluble proteins) were pooled. 70S-SmpB ribosome was incubated for 10 min at 4°C with a 4-fold molar excess of tmRNA in buffer E. The resulting 70S-SmpB-tmRNA complexes were purified on a 10–30% sucrose gradient in buffer E to eliminate free tmRNA. 70S-SmpB and 70S-SmpB-tmRNA were dissociated into 50S and 30S subunits as described above and separated onto 10–30% sucrose gradient. Proteins were extracted from one-half of samples by TCA precipitation, electrophoresed on 15% acrylamide Tris-glycine gel, transferred to PVDF membranes and SmpB was detected by western blotting. RNA from the other half of pooled fractions was prepared by phenol extraction and ethanol precipitation. TmRNA and 16S ribosomal RNA were detected by northern hybridization as described above.

Surface Plasmon Resonance (SPR) assays

Equilibrium constants of dissociation were measured by SPR using BIAcore X biosensor system (BIAcore). For real-time analyses of molecular interactions between the 70S ribosome or the individual subunits and the SmpB-His protein, anti-histidine antibody was first immobilized on a C1 sensor chip (BIAcore) by covalent linkage between activated carboxyl groups on the chip matrix and lysine residues in the anti-histidine antibody as described by the supplier (BIAapplications Handbook, BIAcore). The chips were then washed 3 times with 20 µl of 50 mM NaOH containing 0.1% SDS at a flow rate of 10 µl/min to stabilize the surface prior to the binding of His-tagged SmpB proteins. Chips were equilibrated in the continuous-flow buffer (10mM HEPES, pH 7.4, 150 mM KCl, 10 mM NH_4Cl, 10 mM MgOAc, 3mM β-mercaptoethanol, 0.05% surfactant P20). Purified His-tagged SmpB proteins were immobilized on one flow cell by injecting an aliquot of 3 nM solution protein in continuous-flow buffer. Various concentrations of 70S ribosome, 50S subunit, 30S subunit or tmRNA diluted in the running buffer were injected at a flow rate of 10 µl/min on 45–55 resonance units (RU) of SmpB proteins until equilibrium was reached. The Sensor Chip was regenerated between each injection by a 60 s pulse of 50 mM NaOH containing 0.1% SDS. Final curves were obtained by subtraction of the signal corresponding to the empty flow cell. For each concentration of 70S ribosome, 50S subunit, 30S subunit or tmRNA, the steady state binding (Req) were measured and used to calculate the equilibrium constants of dissociation following the equation [BIAapplications Handbook, BIAcore, (20)].

$$\frac{\text{Req}}{C} = K_A \times R_{max} - K_A \times \text{Req}$$

where C is the 70S, 50S, 30S or tmRNA concentration, R_{max} is the total surface binding capacity in RU and Req is the steady state binding level in RU obtained when equilibrium was reached for each concentrations of 70S ribosome, 50S subunit, 30S subunit or tmRNA. A plot of Req/C against Req at different concentrations gives a Scatchard plot from which K_D ($1/K_A$) can be calculated.

RESULTS

E.coli SmpB binds the 50S and the 30S ribosomal subunits *in vivo*

In the absence or presence of tmRNA, SmpB binds 70S ribosomes *in vivo* and *in vitro* (13). We have previously shown that SmpB was neither found associated to free 50S or free 30S subunits in wild type or tmRNA-deleted *E.coli* strains (delta ssrA strain) (13). To localize the binding site(s) of SmpB to the subunits of the 70S ribosome, we performed sucrose gradient ultra-centrifugation of purified 70S ribosomes, derived from both wild type and delta ssrA strains. The sucrose gradient experiments were performed at low (1 mM) magnesium salts in order to dissociate the 70S ribosome into its two ribosomal subunits (Figure 1A). Reducing the magnesium concentration dissociates the two ribosomal subunits (21). This procedure was used to purify the individual subunits to solve the structures of the two ribosomal subunits by X-ray crystallography (22,23). The localization of the 30S subunit onto the gradient was confirmed by northern blot hybridization against 16S rRNA (Figure 1B). The presence

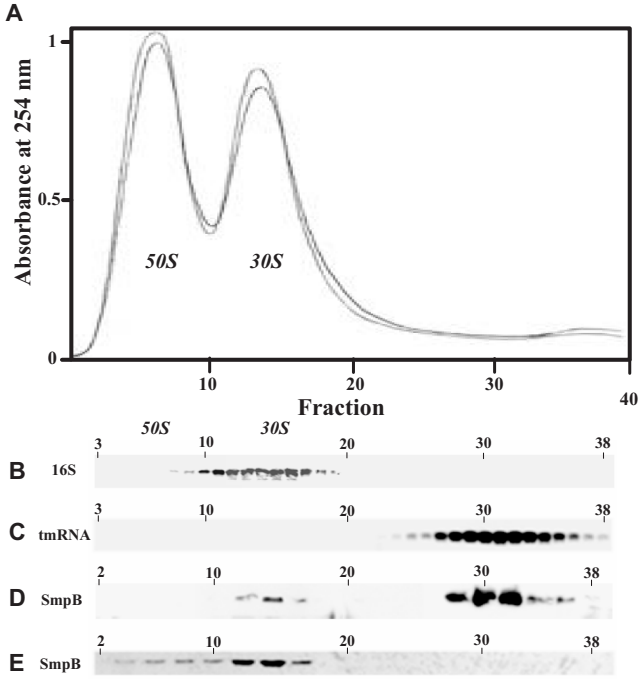
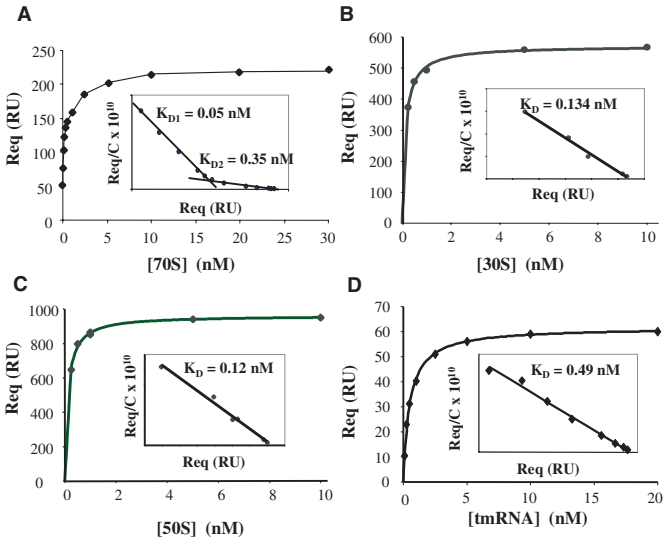


Figure 1. Location of the SmpB protein *in vivo* when the ribosome dissociates into its ribosomal subunits. (**A**) Crude ribosomes from either wild type or delta *ssrA* cells were fractionated by sucrose gradient centrifugation at a low concentration of Mg^{2+} ions. The absorbances of each fraction from wild type or delta *ssrA* cells are shown. The 16S ribosomal RNA (**B**) and tmRNA (**C**) were detected by northern hybridization using complementary ^{32}P-labeled DNA oligonucleotides. The presence of endogenous SmpB in a fraction on two of the sucrose gradient from wild-type (**D**) or delta *ssrA* (**E**) crude ribosomes was detected by western blotting using rabbit polyclonal antibody directed against a histidine-tagged SmpB.

of tmRNA in the different fractions was monitored by northern blotting (Figure 1C). When the 70S ribosomes are dissociated into two subunits, the tmRNA is released from the ribosome to the soluble fraction (Figure 1C). On the other hand, using rabbit polyclonal antibodies directed against His-tagged SmpB, immunoblots show that endogenous SmpB mostly co-sediments with tmRNA, away from the ribosome. The presence of tmRNA and SmpB in the same fractions argues for a release of a tmRNA–SmpB complex during ribosome dissociation. However, a small fraction of the protein remains bound to the 30S subunit (Figure 1D). Interestingly, in the absence of tmRNA (delta ssrA strain), endogenous SmpB is not released from the ribosomal subunits after ribosome splitting, but remains bound predominantly to the 30S subunits (Figure 1E). Furthermore, a minor fraction of the protein is also bound to the 50S subunit. These results demonstrate that SmpB, in the context of 70S ribosome, has affinity to both ribosomal subunits *in vivo*.

E.coli SmpB binds *in vitro* the 30S, the 50S, the 70S and tmRNA with similar affinities.

SmpB binds tmRNA (4), the ribosome (13) and each of the two ribosomal subunits separately (this study). In order to determine the equilibrium dissociation constants (K_D) between SmpB and either tmRNA, the 30S, the 50S or the 70S ribosome, SPR analysis was performed as described in the Materials and Methods section. SmpB proteins were transiently coupled by their C-terminal His-tails to a flow chip preloaded covalently with a commercial anti-histidine antibody. Increasing concentrations of purified small and large ribosomal subunits, purified 70S ribosomes or purified native tmRNA were injected over the sensor chip functionalized with purified SmpB, until steady state binding level is reached. Three independent experiments were performed (Figure 2A–D are representative) and the dissociation constants derived from these experiments are presented in Figure 2E. Scatchard analysis was used to linearize the data from the saturation binding experiments (Figure 2, insets). The dissociation constants between SmpB and each of the purified ribosomal subunits are similar, ~0.17 nM (Figure 2E). For the 70S binding, a curve is observed when the data are visualized on a Scatchard (Figure 2A, inset). Since the 70S ribosome has a 2.5×10^6 Da molecular weight, only one ribosome can bind to one molecule of SmpB immobilized onto the Chip which excludes a cooperative binding in this experiment but argues for a bivalent analyte model with the presence of two binding sites on the ribosome. The dissociation constants between SmpB and these two binding sites on the 70S ribosome are in the same affinity range (0.1–0.33 nM, Figure 2E). Since each ribosomal subunit can bind SmpB with a similar high affinity, the two binding sites identified on the 70S ribosome are consistent with the existence of an independent binding site on each ribosomal subunit. In the context of 70S ribosome, the two binding sites could be more or less accessible to the same extend as in isolated subunits leading to two different K_{Ds}. Alternatively, the binding of SmpB to one site could be stabilized by the overall structure of the 70S ribosome. Moreover, we can not exclude by this experiment a cooperative interaction of SmpB with both ribosome sites which can occur in solution. The K_D between



E

Complexes	K_{Ds} (M)
SmpB-tmRNA	$4.00 \pm 0.85 \times 10^{-10}$
SmpB-30S	$1.67 \pm 0.5 \times 10^{-10}$
SmpB-50S	$1.75 \pm 0.7 \times 10^{-10}$
SmpB-70S	$1.08 \pm 0.51 \times 10^{-10}$ $3.32 \pm 0.88 \times 10^{-10}$

Figure 2. Determination of the dissociation constants between SmpB and either the 70S, the 30S, the 50S or tmRNA by SPR. A plot of the equilibrium binding response of (**A**) 70S, (**B**) 30S, (**C**) 50S and (**D**) tmRNA to SmpB versus concentration were obtained by the injection of increasing amounts of the purified ligands to the flow cell immobilized with SmpB. Req, in RU, was determined when the injection curve showed a plateau and thus equilibrium was reached. Insets: Scatchard analysis linearizing the data from the saturation binding experiments, to determine the K_{Ds}. For the 70S binding, a broken line is observed, favoring two binding sites. The R^2 values for the linear fit were 0.99 and 0.95 for K_{D1} and K_{D2}, respectively. (**E**) The dissociation constants at the equilibrium obtained from three independent experiments are comparable with one another. The standard deviation of each experimental data was indicated.

tmRNA and SmpB measured by SPR is 0.40 nM, consistent to those derived from filter binding assays (0.34 ± 0.18 nM, (8) or from gel mobility-shift assays [1.17 ± 0.18 nM, (9)]. At the equilibrium, the affinities between SmpB and tmRNA, between SmpB and the 70S ribosome or between SmpB and each of the two ribosomal subunits are comparable (Figure 2E). These bindings were specific since the addition of a large excess of tRNA in the continuous flow (440 nM) did not impair the association of SmpB with 70S, 30S, 50S or tmRNA (data not shown). Indeed, SmpB is a RNA binding protein which can bind un-specifically to RNAs and especially to tRNAs (4). At this 440 nM competitor RNA concentration, we found K_ds for the 70S, 50S, 30S and tmRNA to be 7.3, 4.8, 4.4 and 7.5 nM, respectively. Therefore, the affinity of SmpB for the ribosome, ribosomal subunits and tmRNA are specific and similar.

tmRNA can displace SmpB from the 50S subunit but not from the 30S subunit

Purified 30S or 50S subunits were bound to a SmpB-coated chip until saturation of the ligands and the dissociation of each subunit from SmpB was monitored by SPR, after adding an

excess of tmRNA (Figure 3A). tmRNA could only displace the 50S and not the 30S subunit from its binding to SmpB (Figure 3A). To confirm these results, the affinities of SmpB to the 30S and 50S ribosomal subunits were compared with the one between SmpB and tmRNA using competition assays *in vitro*. For this, 30S or 50S ribosomes preloaded with SmpB and purified by a sucrose gradient were titrated with native tmRNA from a 1:1 to 1:10 ratio between the ribosomal subunit-SmpB complexes and the tmRNA concentrations. After incubation, the samples were loaded onto a sucrose gradient. Western blots show that SmpB binds each of the ribosomal subunit *in vitro* in the absence of tmRNA or additional cofactors (Figure 3B and C). Adding increasing amounts of tmRNA to each of the two ribosomal subunits preloaded with SmpB does not lead to ternary complex formation whereas a ternary complex can form between 70S, tmRNA and SmpB [see below and (13)]. At a 1:1 ratio between the 50S subunit and tmRNA, half SmpB stays bound to the 50S while the other half binds tmRNA, as expected from similar K_Ds between the SmpB-50S and the SmpB–tmRNA complexes (Figure 2E). At a 3- to 10-fold molar excess of tmRNA over SmpB, the protein is completely released from the 50S subunit and forms a unique complex with tmRNA in the soluble fraction (Figure 3B). Remarkably, a 10-fold excess of tmRNA over 30S ribosomes failed to pull SmpB from its ribosome-bound state to its tmRNA-bound state (Figure 3C). Thus, although SmpB has similar affinity for tmRNA and for the 30S subunit, tmRNA does not compete for SmpB binding to the 30S subunit suggesting that the conformation of the protein might be modified upon binding the 30S.

To access the affinity of SmpB for tmRNA or ribosomal subunits after *in vitro* 70S dissociation, 70S ribosomes were incubated with a 10-fold molar excess of His-tagged SmpB protein and purified by a sucrose density gradient centrifugation to eliminate the unbound SmpB proteins. The dissociation of the resulting 70S-SmpB complexes at low magnesium concentration reveals the presence of SmpB on the 50S and 30S subunits (Figure 4E) confirming the binding of two SmpB molecules per 70S ribosome (13) one on the 30S subunit and the other on the 50S subunit. Moreover, SmpB still bound to ribosomal subunits at low magnesium concentration indicates that magnesium did not influence the affinity of SmpB for either the 50S or the 30S subunits. The incubation of 70S-SmpB complexes with tmRNA leads to the recruitment of tmRNA without the uncoupling of SmpB from 70S ribosome [Figure 4A and (13)]. Upon dissociation at low-magnesium concentration of purified 70S-SmpB-tmRNA complexes, SmpB was released from 50S subunit in complex with tmRNA (Figure 4C and D) whereas the other SmpB molecule remains bound to the 30S subunit (Figure 4B and D). Thus, tmRNA binds only to 70S-SmpB complexes and then displaces SmpB from the 50S subunit independently of the magnesium concentration (Figures 3A, B and 4D).

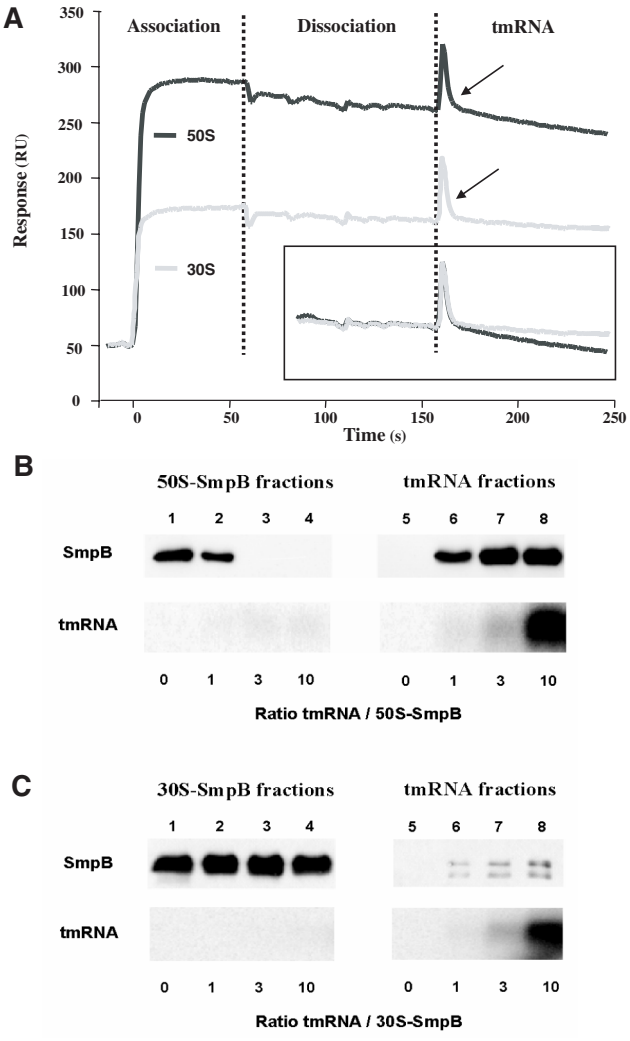


Figure 3. SmpB binds to the isolated 30S and 50S ribosomal subunits *in vitro* and tmRNA can compete the 50S out of the complex with SmpB, but not the 30S. (**A**) Competition between the individual ribosomal subunits and tmRNA for binding SmpB by SPR. The sensorgrams of 30S and 50S binding to SmpB were obtained by the injection of saturating amounts of the purified ligands to the flow cell immobilized with SmpB (association, a 2-fold higher signal for the 50S compared with the 30S reflecting mass differences between both subunits). After 100 s of dissociation, an excess of native tmRNA (400 nM) is added. Boxed inset: superimposition, before adding tmRNA, of the curves corresponding to the dissociation of both 30S and 50S subunits from SmpB; the addition of an excess of tmRNA dissociates the 50S–SmpB complex, but not the 30S–SmpB complex. His-tagged SmpB proteins were added to either the purified (**B**) 50S or the (**C**) 30S ribosomal subunits from delta *smpB-ssrA* cells, and the resulting 30S–SmpB or 50S–SmpB complexes were purified by sucrose gradient centrifugation. The complexes were incubated without (lanes 1 and 5) or with an increasing amount of purified native tmRNA (lanes 2–4 and 6–8) and loaded on a 10–30% (w/v) linear sucrose gradient. For each assay, fractions containing either the ribosomal subunits (lanes 1–4) or soluble molecules including tmRNA (lanes 5–8) were pooled and the amount of SmpB and tmRNA present in the ribosomal and tmRNA soluble fractions were monitored by western blotting (top panels) and by northern hybridization (bottom panels), respectively. The molar ratio between tmRNA and SmpB pre-bound ribosomal subunits in the samples loaded on sucrose gradient is indicated.

A SmpB protein mutant as a tool to investigate *trans*-translation

C-tail truncated SmpB variants are inactive in *trans*-translation but bind tmRNA or the 70S ribosomes and support the association of tmRNA with the stalled ribosomes. Also, the contribution of the tail on *trans*-translation is not due to an effect on SmpB stability (8,9). A recombinant *E.coli* SmpB missing 16 amino acids at its C-terminal tail (delta 16-SmpB) was cloned and sequenced. The phenotype of the SmpB defective strain at 45°C cannot be complemented when

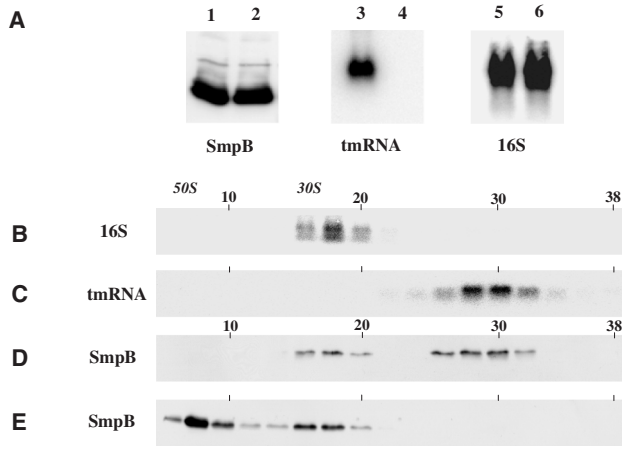


Figure 4. SmpB binds the 30S and the 50S subunits in 70S ribosomes and SmpB is released from the 50S by tmRNA upon 70S dissociation. (**A**) Analysis of SmpB (lanes 1 and 2), tmRNA (lanes 3 and 4) and ribosomal 16S RNA (lanes 5 and 6) levels in purified 70S-SmpB (lanes 2, 4 and 6) or 70S-SmpB-tmRNA (lanes 1, 3 and 5) complexes. Purified 70S-SmpB or 70S-SmpB-tmRNA complexes were fractionated by sucrose gradient centrifugation at a low concentration of Mg^{2+} ions. The 16S ribosomal (**B**) RNA and (**C**) tmRNA were detected by northern hybridization using complementary ^{32}P-labeled DNA oligonucleotides. The presence of SmpB in sucrose fractions from (**D**) 70S-SmpB-tmRNA or (**E**) 70S-SmpB complexes was detected by western blotting using rabbit polyclonal antibody directed against a histidine-tagged SmpB.

expressing delta 16-SmpB from a plasmid whereas a plasmid expressing wild-type SmpB does complement the growth defect (Figure 5A).

In order to access the activity of this truncated SmpB protein into an *in vitro trans*-translation assay, we expressed and purified a delta 16-SmpB protein tagged by six histidines to its C or N-terminal portion. These truncated proteins histidine tagged binds both ribosomal subunits *in vitro*, as wild-type SmpB (data not shown) but are inactive in a 'codon-independent' transfer of a polypeptidyl-tRNA^{Phe} to the alanine from alanyl-tmRNA^{Ala} in the ribosome P-site *in vitro* (Figure 5B). Thus, delta 16-SmpB protein is unable to complement the phenotype of the SmpB defective strain at 45°C because of its inability to perform the first *trans*-peptidation step in the *trans*-translation process.

Inactive C-tail truncated SmpB are not released with tmRNA when the ribosomes are dissociated

What is the location *in vivo* of the SmpB protein defective in *trans*-translation? The inactive C-tail truncated protein, expressed from a plasmid containing its endogenous promoter, is exclusively bound to the 70S ribosomes (P100) and has an expression level slightly lower than endogenous wild-type SmpB (Figure 5C). In the presence of this inactive truncated SmpB protein, tmRNA was found associated with the 70S ribosomes (P100, delta 16-SmpB, Figure 5C). However, in contrast to endogenous SmpB, higher amounts of tmRNA are in the soluble fraction in cells expressing the truncated SmpB (S100, Figure 5C). When the 70S ribosomes from the P100 fraction of the strain that expresses the inactive SmpB variant are dissociated into the 30S and the 50S ribosomal subunits, all the tmRNA molecules are released from the ribosomes without being associated with the mutant SmpB protein (Figure 5G). The inactive protein is bound to the 30S and the 50S ribosomal subunits, in a comparable ratio, between both subunits, with that observed with the strain that does not express tmRNA (see Figure 1E). Despite not working during *trans*-translation, the defective SmpB protein which was able to recruit tmRNA on stalled 70S ribosome still binds the two ribosomal subunits *in vivo* (Figure 5H). The presence of SmpB on the 50S subunit on 70S ribosomes which are all blocked in the early stages of *trans*-translation suggest that SmpB bind early and transiently to the 50S subunit.

The binding of endogenous SmpB to the 50S subunit is an early step *in vivo*

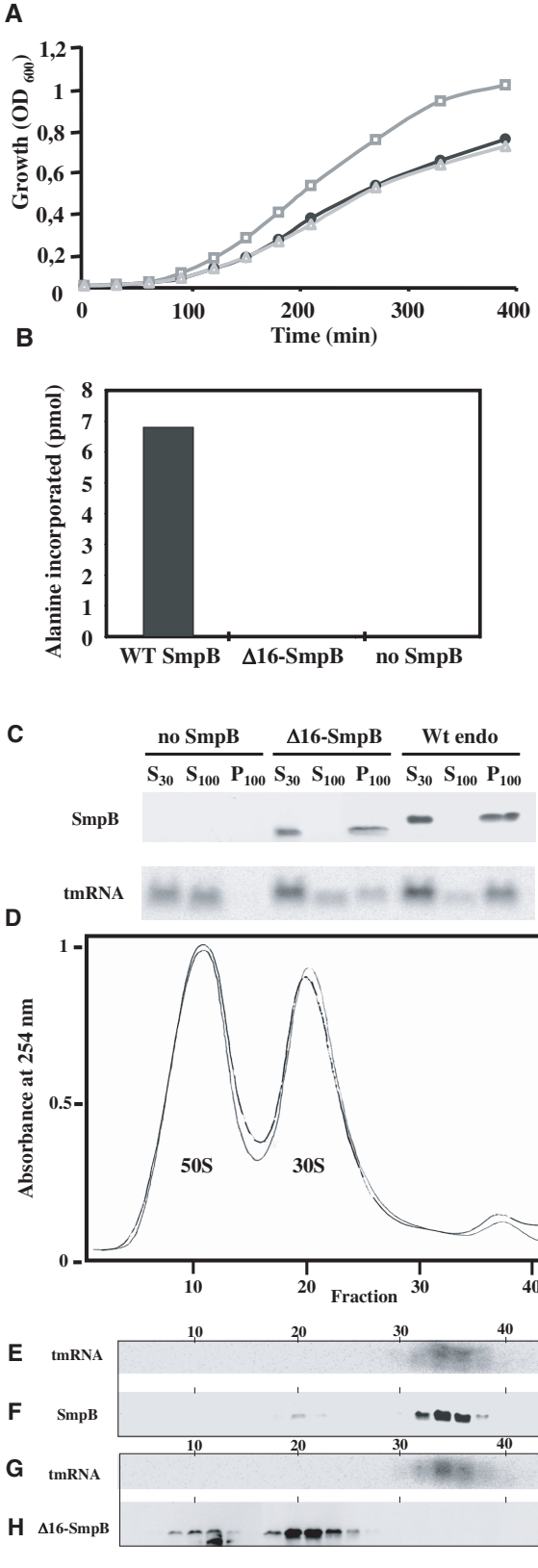
The results presented above suggest that the binding of SmpB to the large ribosomal subunit is an early and a transient step during *trans*-translation. If true, blocking the early steps of *trans*-translation *in vivo* might allow the detection of endogenous SmpB on the 50S subunit. To test that, translation and *trans*-translation are impaired *in vivo* by chloramphenicol that blocks aminoacyl-tRNAs and alanyl-tmRNA^{Ala} interactions with the peptidyltransferase (PT) center (24). Chloramphenicol leads to the enrichment of ribosomes blocked on the first step of *trans*-translation by blocking the late translational step of tmRNA. When these ribosomes blocked at various stages of *trans*-translation are purified and dissociated (Figure 6A), endogenous SmpB, detected by immunoblots, is in complex with the released tmRNA (Figure 6B) and with the 30S subunit (Figure 6C). This SmpB localization is in agreement with the presence of the protein bound to tmRNA and to the 30S subunit on 70S ribosomes engaged on the late step of *trans*-translation in chloramphenicol-untreated cells (Figure 1A). Moreover, a significant amount of SmpB is bound to the 50S subunits (Figure 6C) which represents the SmpB binding during the early step of *trans*-translation. Therefore, impairing translation and *trans*-translation *in vivo* with chloramphenicol allows to visualize the early step of *trans*-translation and transient interaction of SmpB with the large ribosomal subunit.

DISCUSSION

In a previous work, we showed that SmpB is located exclusively on the 70S ribosomes *in vivo* and such complexes can recruit alanyl-tmRNA to trigger *trans*-translation (13). In the present study, we demonstrate that endogenous SmpB associates with both the small and the large ribosomal subunits *in vivo* and *in vitro*. *In vivo*, the association of SmpB with ribosomal subunits occurs only in the context of the 70S ribosome since SmpB was not detected in free 50S and 30S subunits either in the presence or the absence of tmRNA (13). Our *in vivo* data reveal that the dissociation of the 70S ribosomes from wild-type cells leads to the release of all tmRNA from the ribosome, in complex with SmpB. In these ribosomes engaged in *trans*-translation, a small fraction of the SmpB protein remains bound to the 30S subunit after ribosome dissociation. Wild-type SmpB protein is also found associated *in vivo* on the 50S subunit when the 70S ribosomes are purified and dissociated into subunits either from cells lacking tmRNA or from cells treated by chloramphenicol.

The ability of SmpB to interact *in vivo* with the two ribosomal subunits is in agreement with the *in vitro* data demonstrating that SmpB binds to the 30S and the

50S subunits, both prepared to high purity. The binding between SmpB and each of the ribosomal subunits is very tight and specific, with a dissociation constant of $\sim 1.7 \times 10^{-10}$ M for each one, similar to its K_D for the 70S and tmRNA. Although the SmpB protein binds each ribosomal subunit with similar affinity, the domains of the protein in interaction with the 30S and the 50S might be different, in agreement with the existence of two distinct clusters of conserved amino acids at the protein surface (5). SmpB has similar affinity *in vitro* for the two ribosomal subunits, for the 70S ribosome and for tmRNA but are found associated exclusively to the 70S *in vivo* which suggest that SmpB has high affinity for 70S ribosomes stalled on a problematic mRNA and argues for an early binding of the protein onto stalled ribosomes prior to the recruitment of the tmRNA.



SmpB possesses high affinity for the 30S subunit. This is consistent with the structural homology of its tertiary structure (5,25) to that of ribosomal protein S17 from the 30S subunit (5) and especially with that of initiation factor IF1 that transiently binds the A site within the 30S (26). Our biochemical results are in agreement with structural data collected at 11.8 Å resolution in the course of a recent cryo-electron study of stalled ribosomes in complex with SmpB in the absence of tmRNA (R. Gillet, S. Kaur, W. Li, M. Hallier, B. Felden, J. Frank, manuscript submitted). In that study, SmpB was located on the 30S subunit, towards the empty decoding center. Moreover, recent *in vitro* data indicate that SmpB footprints nucleotides that are in the vicinity of the P-site of the small subunit (14).

The binding of SmpB to the 50S subunit is consistent with cryo-EM data obtained with a ribosome from *Thermus thermophilus*, stalled at the end of a short mRNA and reacted with alanyl-tmRNA^{ala}, his-tagged SmpB and EF-Tu-GTP. In this pre-accommodated ribosomal complex, SmpB was initially found to be exclusively located on the 50S subunit (12). A pre-accommodation step reconstructed at a higher resolution, however, reveals the presence of two molecules of SmpB, one interacts with the large subunit while the second is directed towards the decoding site (DS) within the 30S subunit (R. Gillet, S. Kaur, W. Li, M. Hallier, B. Felden, J. Frank, manuscript submitted). The presence of more than one SmpB protein on ribosomal complexes agrees with our previous work showing that two molecules of SmpB are required for *trans*-transfer events *in vitro* (13).

In contrast to SmpB-70S preformed complexes (Figure 4A), neither the SmpB-50S nor the SmpB-30S complexes are

Figure 5. An inactive C-tail truncated SmpB remains bound to the ribosomal subunits but is not associated with tmRNA in the soluble fraction. (**A**) The growth defect of the delta SmpB strain at 45°C is not rescued by a C-tail truncated SmpB. The growth of the delta SmpB strain with either an empty plasmid (closed circle) or a plasmid allowing the expression of the C-tail truncated SmpB (open triangle) and a plasmid containing a wild-type SmpB strain (open square), is compared. (**B**) The truncated protein is inactive in an *in vitro trans*-translation assay showing the incorporation of [H^3] alanine from tmRNA to stalled ribosomal complexes containing poly(U) mRNA. (**C**). Expression levels of tmRNA, truncated and wild type SmpB in the S30, S100 and P100 fractions detected by northern hybridization and western blotting, respectively. (**D**) Fractions from either wild type or cells expressing delta 16-SmpB were fractionated by sucrose gradient centrifugation at a low concentration of Mg^{2+} ions. The absorbance of each fraction from wild type or delta 16-SmpB cells is shown. tmRNA was detected by northern hybridization using complementary ^{32}P-labeled DNA oligonucleotides in either the wild type (**E**) or delta 16-SmpB (**G**) cells. The presence of SmpB (**F**) or of delta 16-SmpB (**H**) in sucrose fractions from wild type or delta SmpB crude ribosomes was detected by western blotting using rabbit polyclonal antibody directed against a histidine-tagged SmpB.

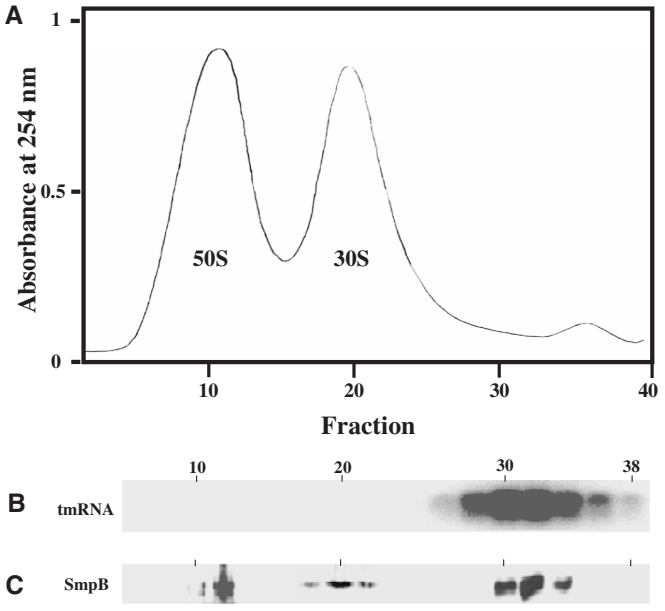


Figure 6. SmpB is detected on the 50S subunit when translation and *trans*-translation are blocked by chloramphenicol *in vivo*. (**A**) Crude ribosomes from wild-type cells which were fractionated by sucrose gradient centrifugation at a low concentration of Mg^{2+} ions. (**B**) tmRNA was detected by northern hybridization using complementary ^{32}P-labeled DNA oligonucleotides. (**C**) The presence of endogenous SmpB was detected by western blotting using rabbit polyclonal antibody directed against a histidine-tagged SmpB.

capable of recruiting tmRNA *in vitro* [Figure 3 and (13)]. When the 70S ribosomes, the 50S or the 30S subunits compete with tmRNA for binding SmpB, tmRNA can only release SmpB from the large ribosomal subunit (Figure 3). Moreover, SmpB is released from the 50S subunits with tmRNA when the 70S ribosomes containing SmpB and tmRNA are dissociated in two subunits. Thus, while SmpB presents a similar affinity for ribosomal subunits and tmRNA, the binding of tmRNA on 70S-SmpB complexes leads to a modulation of SmpB affinity for the two ribosomal subunits versus tmRNA in these ribosomes not engaged in *trans*-translation. It suggests that SmpB is transiently associated to the 50S subunit during *trans*-translation but tightly and stably bound to the 30S subunit. In the presence of tmRNA, SmpB contacts the large subunit by a close interaction with helix 69 in the 23S RNA (12). To avoid steric clashes with the P-site tRNA when the accommodation proceeds, SmpB has to move away from the 50S subunit (6). The transient and early association of SmpB to the 50S subunit agrees with its location on wild-type ribosomes performing *trans*-translation, in which SmpB is only detected on the small subunit (Figure 1D). Moreover, SmpB on the 50S *in vivo* is detected when *trans*-translation is voluntarily impaired by chloramphenicol, which leads to the accumulation of 70S ribosomes blocked at the early stages of *trans*-translation. Since the association between SmpB and ribosomal subunits or tmRNA is independent of the magnesium concentration (Figures 3 and 4), the presence of SmpB on the 50S subunit in chloramphenicol-treated cells is not because of the low level of magnesium but reflects a stage during *trans*-translation.

This notion that SmpB binding to the 50S is a very early and transient step during *trans*-translation is reinforced further by the subunits localization of a mutant SmpB protein. Indeed, when wild type SmpB protein is replaced by C-tail truncated SmpB variant that is inactive in the first step of *trans*-translation, the truncated protein remains bound to both ribosomal subunits *in vivo* upon ribosome dissociation, without being released in complex with tmRNA. Mutant protein binds both ribosomal subunits and recruits tmRNA but is unable to trigger essential conformational rearrangement(s) within the stalled ribosome that are required for its binding and release with tmRNA from the stalled ribosome. This suggests that SmpB has to move away from the 50S subunit during the accommodation of alanyl-tmRNA^{Ala} into the large subunit.

What is the fate of SmpB on the 50S? Complex formation between tmRNA and SmpB was recently monitored by chemical and enzymatic probes combined to SPR measurements, and three independent binding sites of the protein onto tmRNA were identified (7). These data suggest that during *trans*-translation SmpB is able to bind tmRNA at different locations, to induce key structural changes within the tmRNA scaffold required for the sliding of tmRNA through the ribosome to be rescued. The protein could re-associate onto tmRNA structure at a binding site different from the one on the tRNA domain, to participate in resuming translation on tmRNA internal tag reading frame.

We also detect some of the protein bound to the 30S upon subunit dissociation, in agreement with a stably *in vitro* binding of the protein to the 30S, contrasting with its labile binding on the 50S in the presence of tmRNA. tmRNA has a tRNA accepting branch that has to fit within the PT center on the 50S, but lacks an anticodon stem–loop analog that fits into the DS of the 30S during the decoding of aminoacylated tRNAs in canonical translation. Assuming that SmpB binds at or near functional sites within the ribosome, it could rationalize the competition of tmRNA for the binding of SmpB to the large subunit but not on the small subunit. During *trans*-translation, however, when the location of SmpB on the ribosomal subunits from wild-type cells is monitored *in vivo*, the majority of the protein is released with tmRNA with only a minor fraction that remains attached to the 30S. It suggests that during *trans*-translation, the molecules of SmpB bound to the 30S either move onto tmRNA or are released from the ribosome.

Altogether, these data can be reconciled into a working model (Figure 7). During *trans*-translation, SmpB modulates its affinity for the ribosomal subunits versus tmRNA. When a ribosome stalls on a problematic message, SmpB would bind the 70S ribosome in association with tmRNA or prior to recruiting tmRNA. During pre-accommodation, SmpB is still bound to the large and the small ribosomal subunits. The accommodation results in the movement of both the alanylated-tmRNA^{Ala} and the SmpB protein bound to the 50S subunit. SmpB either moves to tmRNA or is released from the 50S subunit, leading to the entrance of the tmRNA acceptor branch within the PT center. Subsequently, there is a codon-independent transfer of the peptidyl residue stalled in the P-site to the alanine from tmRNA. The molecule of SmpB bound to the 30S either binds tmRNA at different site from the tRNA-like domain or is released from the ribosome. Indeed, the molecule of SmpB bound to the small subunit has to move to allow translation resumption on tmRNA reading frame. The molecule that has been recently identified by

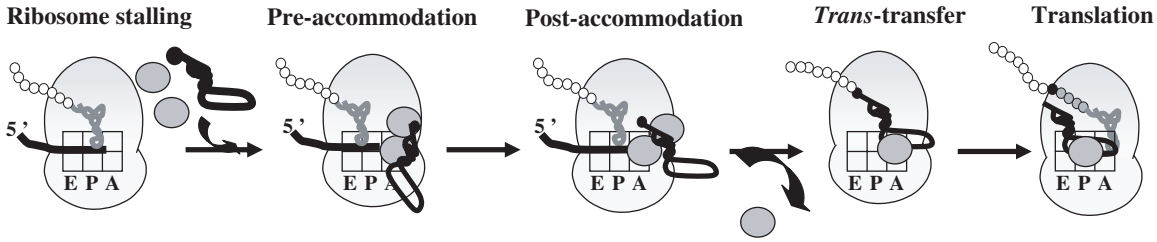


Figure 7. Schematic illustration of the location of the SmpB molecules during *trans*-translation. SmpB proteins bound to both ribosomal subunits and tmRNA when a ribosome is stalled on a problematic mRNA. During the accommodation, SmpB moves from the 50S subunit on the tmRNA to allow the entrance of tmRNA acceptor branch within the PT center. The transfer of the nascent polypeptide chain to the alanine charged on tmRNA requires that SmpB moves away from the 30S subunit. One SmpB is released from the ribosome and the other remains associated with tmRNA during translation of its internal reading frame.

Shpanchenko *et al* (2005) based on monitoring *in vivo* ribosome–tmRNA complexes during *trans*-translation could come from that bound to either the 30S or the 50S (11). This report adds several important insights concerning the location, the functional and structural roles of the molecules of SmpB during bacterial ribosome rescue. Many important questions remain to be elucidated, and among them, when and how the SmpB proteins, alone or in complex with tmRNA, identify the ribosomes that are being stalled on problematic messages.

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