SmpB Triggers GTP Hydrolysis of Elongation Factor Tu on Ribosomes by Compensating for the Lack of Codon-Anticodon Interaction during Trans-translation Initiation*

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Bacterial tmRNA rescues ribosomes that stall because of defective mRNAs via the trans-translation process. Although entry of the charged transfer messenger RNA (tmRNA) into the ribosome proceeded in the absence of elongation factor (EF-Tu) and in the presence of EF-Tu and the antibiotic kirromycin, evidence was found for the involvement of EF-Tu in trans-translation initiation. The polyalanine synthesis system attained by using a tmRNA variant consisting of only the tRNA-like domain revealed that it was completely dependent on the presence of SmpB and greatly enhanced by EF-Tu and EF-G. Actually, ribosome-dependent GTPase activity of EF-Tu was stimulated by the addition of SmpB and tmRNA but independently of template mRNA, demonstrating that SmpB compensates for the lack of codon-anticodon interaction during the first step of the trans-translation initiation. Based on these results, we suggest that SmpB structurally mimics the anticodon arm of tRNA and elicits GTP hydrolysis of EF-Tu upon tmRNA accommodation in the A site of the ribosome.

Bacterial cells employ a trans-translation system mediated by tmRNA (also known as 105a RNA or SsrA RNA) to rescue stalled ribosomes that are translating incomplete mRNAs that lack termination codons (reviewed in Refs. 1 and 2). In the first step of the trans-translation process, tmRNA enters the A site of the stalled ribosome in an alanine-charged form and functions as tRNA without deciphering the codon, followed by the addition of an alanine residue to the nascent polypeptide. Subsequently, tmRNA serves as a substitute for mRNA using a short open reading frame (ORF) with a termination codon within tmRNA.

By switching the message from that encoded in an incomplete mRNA to that encoded within tmRNA, protein synthesis is completed, and the protein is tagged with a specific C-terminal sequence that marks it for degradation (3). The stalled ribosomes are released to participate in new translation reactions. The addition of the tmRNA-encoded tag peptide causes the degradation of newly synthesized incomplete protein that may be unnecessary or harmful to the cell, because of its inability to fold correctly. In this way, bacterial cells maintain the quality of translation and newly synthesized proteins.

It is known that SmpB (Small protein B) binds to tmRNA to participate in trans-translation. SmpB interacts with the tRNA-like domain (TLD) of tmRNA (4–7) and the ribosome (8, 9), enhances the alanyl-acceptance efficiency of tRNA (4, 6, 10), and facilitates tmRNA binding to the ribosome (6, 11) and proper entry of the alanyl-tmRNA into the A site of the ribosome for the subsequent peptidyl-transferring reaction (10). A recent report that C-terminally truncated or -mutated SmpB facilitates tmRNA binding to the ribosome, but cannot attach an alanine residue to nascent peptide, indicates that SmpB is also involved in the peptidyl transfer reaction at the A site of the ribosome in addition to conveying tmRNA onto the ribosome (12).

It is known that EF-Tu binds to an aminoacyl moiety of alanyl-tmRNA (13–15) simultaneously with SmpB (4) and EF-Tu is thought to be important for the initial entry of the alanyl-tmRNA into the A site of the ribosome, in a manner similar to the entry of common aminoacyl-tRNA. Indeed, it has been demonstrated that EF-Tu enhances tmRNA entry into the ribosome (8). The binding of EF-Tu to the alanyl-tmRNA, as well as the binding of SmpB, on the ribosome has been shown with a cryoelectron microscopy density map (16). In contrast, the dispensability of EF-Tu for the initial step of trans-translation also has been described (8). It has not been shown whether the hydrolysis of GTP occurs in the EF-Tu-dependent tmRNA entry into the ribosome.

In our previous report (10), we analyzed the role of SmpB in the trans-translation reaction by using a reconstituted in vitro system with purified components of the translation process (PURESYSTEM) (17). Here, by using this system, we have carried out further investigations of trans-translation initiation, including the functional analysis of SmpB and EF-Tu. The involvement of EF-Tu in trans-translation initiation is evaluated by using purified stalled ribosomes and revealed that alanyl-tmRNA entry into the A site of the ribosome can proceed independently of EF-Tu.

However, protection analysis reveals that EF-Tu, as well as SmpB, binds to the TLD of tmRNA with high affinity, although excess amounts of SmpB decrease this affinity by competing for the same binding site on the TLD. Surprisingly, template-independent polyalanine synthesis on the ribosome is observed using SmpB and a tmRNA variant that possesses only the TLD, and the rate of polyalanine synthesis is enhanced by the addition of EF-Tu or by the addition of EF-G but inhibited by the addition of kirromycin and template RNA. Furthermore, the complex consisting of SmpB and tmRNA or SmpB and TLD directly stimulates the ribosome-dependent GTPase activity of EF-Tu, suggesting SmpB conveys a signal equivalent to the codon-anticodon pairing. These results, combined with the previous structural studies, suggest that SmpB mimics an anticodon arm of the canonical tRNA and compen-
Function of SmpB on the Ribosome

sates for the lack of the codon-anticodon pairing in the trans-translation initiation.

MATERIALS AND METHODS

Preparation of Protein Factors—The components used in the PURESYSTEM were purified as described in a previous report (17). SmpB was purified as described previously (10); however, the digestion with thrombin and subsequent Mono-S purification steps were abbreviated. The plasmid encoding mutant SmpB that lacks the C-terminal seven amino acid residues was generated using a QuickChange site-directed mutagenesis kit (Stratagene). This mutant SmpB was purified in the same manner as wild-type SmpB. The concentration of the purified protein was determined using a Bio-Rad protein assay kit with bovine serum albumin as the standard.

Preparation of RNA Samples—E. coli tRNA<sup>Pho</sup> was prepared as described (18). Escherichia coli tmRNA, tRNA<sup>Ala</sup>, and the tmRNA mutant, TLDwAL, were runoff-transcribed using T7 RNA polymerase. DNA templates for in vitro transcription were prepared as follows. DNA fragments encoding the respective RNA sequences were amplified from genomic DNA (tmRNA) or without template DNA (tRNA<sup>Ala</sup>, TLDwAL) and cloned into pUC18. With the resulting plasmids as templates, DNA fragments containing a T7 promoter directly upstream of the RNAs were amplified by PCR using the appropriate primers. The in vitro transcription products were purified by 5 (tmRNA) or 12% (tRNA<sup>Pho</sup>, tRNA<sup>Ala</sup>, and TLDwAL) denaturing PAGE.

Aminoacylation—Reaction conditions and methods for the aminoacylation were as described previously (10). <sup>3</sup>H-Labeled alanyl-tRNA, alanyl-TLDwAL, and alanyl-tRNA<sup>Pho</sup> for protection analysis were prepared as follows. The reaction mixtures (100 μl) contained 5 mM magnesium acetate, 20 mM potassium phosphate, pH 7.3, 95 mM potassium glutamate, 5 mM potassium carbonate, 0.5 mM magnesium chloride, 1 mM spermidine, 8 mM putrescine, 1 mM ATP, 1 mM each of ATP and GTP, 1.85 kBq of [14C]alanine, 5 mM phosphoenolpyruvate, 0.2 unit of pyruvate kinase, 1 mM DTT, 1 mM each of ATP and GTP, and 70 Units of alanyl-tRNA synthetase. The reaction mixtures were spotted on Whatman 3MM filter paper and boiled in 10% trichloroacetic acid at 90 °C for 30 min to deacylate the alanyl-tRNA. The radioactivity in the 10% trichloroacetic acid-insoluble fraction was measured with a liquid scintillation counter.

Translation Experiments—The template plasmid for the truncated DHFR was prepared as described previously (10). The reaction conditions for the PURESYSTEM were as described previously (17), with a slight modification. Synthesized proteins containing [14C]alanine were separated by 12% SDS-PAGE and visualized with a BAS-5000 bio-imaging analyzer (FujiFilm). Radioactive alanine incorporation into the synthesized protein on the ribosome was measured as follows. The reaction mixtures (50 μl), with poly(U) as a template (Fig. 6, A and B), contained 5 mM magnesium acetate, 20 mM potassium phosphate, pH 7.3, 95 mM potassium glutamate, 5 mM ammonium chloride, 0.5 mM calcium chloride, 1 mM spermidine, 8 mM putrescine, 1 mM DTT, 1 mM each ATP and GTP, 50 μM phenylalanine, 9.25 kBq of [14C]alanine, 12 pmol of ribosome, 1 μg of EF-G, 2 μg of EF-Tu, 1 μg of EF-Ts, 10 μg of AlaRS, 10 μg of PheRS, and 1.1 μM SmpB. The reaction mixtures (50 μl) without template (Fig. 6, C–E) contained 7 mM magnesium acetate, 5 mM potassium phosphate, pH 7.3, 95 mM potassium glutamate, 5 mM ammonium chloride, 0.5 mM calcium chloride, 1 mM spermidine, 8 mM putrescine, 1 mM DTT, 1 mM each ATP and GTP, 5 mM phosphoenolpyruvate, 2.3 units/ml pyruvate kinase (Sigma), 40 μM cold alanine, 185 kBq of [3H]alanine, 12 pmol of ribosome, 1 μg of EF-G, 1 μg of EF-Ts, and 10 μg of AlaRS. The reactions were carried out at 37 °C for the specific times. Aliquots (8 μl) were withdrawn, spotted on Whatman 3MM filter paper, and boiled in 10% trichloroacetic acid at 90 °C for 30 min to deacylate the aminoacyl-RNAs. The radioactivity in the 10% trichloroacetic acid-insoluble fraction was measured with a liquid scintillation counter. For the Tricine-SDS-PAGE analysis, aliquots (5 μl) were withdrawn and mixed with SDS-PAGE loading buffer at specified times. Synthesized proteins containing [14C]alanine were separated by Tricine-SDS-PAGE (19) and visualized with a BAS-5000 bio-imaging analyzer (FujiFilm).

Preparation of the Pre-trans-translation Complex—The pre-trans-translation complex was prepared as follows. A template for the truncated DHFR without a stop codon was translated in the PURESYSTEM (100 μl) containing 100 pmol of ribosome. After a 1-h incubation at 37 °C, reaction mixtures were loaded on 300 μl of a 20% sucrose buffer (10 mM Hepes, pH 7.6, 60 mM NaCl, 5 mM Mg(OAc)<sub>2</sub>, 20% sucrose, 7 mM 2-mercaptoethanol), followed by ultracentrifugation at 66,000 rpm for 3 h using a TLA 100.2 rotor (Beckman Coulter). The supernatant was discarded, and the pellet was dissolved in 20 μl of ribosome buffer (20 mM Hepes-KOH, pH 7.6, 30 mM KCl, 6 mM Mg(OAc)<sub>2</sub>, 7 mM 2-mercaptoethanol). The sample was used immediately in the trans-translation experiment.

Trans-translation Experiment Using the Pre-trans-translation Complex—The reaction mixtures (10 μl) used for the trans-translation experiment contained 9 mM magnesium acetate, 5 mM potassium phosphate, pH 7.3, 95 mM potassium glutamate, 5 mM ammonium chloride, 0.5 mM calcium chloride, 1 mM spermidine, 8 mM putrescine, 1 mM DTT, 1 mM each of ATP and GTP, 1.85 kBq of [14C]alanine, 5 mM phosphoenolpyruvate, 0.2 unit of pyruvate kinase, 1 mM DTT, and 70 units of alanyl-tRNA synthetase. After a preincubation at 37 °C for 10 min to allow pyruvate kinase to put the EF-Tu in the GTP form, the reaction was started by the addition of 10 pmol of tmRNA and 2 μl of pre-trans-translation complex (10 pmol of ribosome). After a 30-min incubation at 37 °C, the reaction mixtures were spotted on Whatman 3MM filter paper and boiled in 10% trichloroacetic acid at 90 °C for 30 min to deacylate the alanyl-tmRNA. The radioactivity in the 10% trichloroacetic acid-insoluble fraction was measured with a liquid scintillation counter.

Protection Analysis—Protection analysis was performed according to Hanada et al. (20). The reaction mixtures (100 μl) contained 50 mM Hepes-KOH, pH 7.6, 7 mM magnesium chloride, 60 mM ammonium chloride, 1 mM DTT, 200 μM GTP, 2.25 mM phosphoenolpyruvate, 2.3 units/ml pyruvate kinase, 60 μg/ml bovine serum albumin, and the specified concentrations of EF-Tu and SmpB. After a 15-min incubation at 37 °C for the conversion of EF-Tu/GDP to EF-Tu/GTP, the reaction mixtures were placed on ice. After the reaction mixtures were precultured at 30 °C, [3H]-alanylated RNA (0.07 nmol of alanyl-tRNA, 0.08 nmol of alanyl-TLDwAL, or 0.13 nmol of alanyl-tRNA<sup>Ala</sup>) was added. After 0, 10, 20, 30, and 40 min of incubation, 15 μl were withdrawn from each sample and spotted on Whatman 3MM filter paper. The radioactivity of the 5% trichloroacetic acid-insoluble fractions was measured with a liquid scintillation counter. The natural logarithmic ratios of the remaining [3H] counts at the specified times over the one at 0 min were plotted, and the reciprocal slopes of the linear fits were calculated as the rate constants of alanylated RNA decay.

GTPase Assay—Ribosome-dependent GTPase activity of EF-Tu was measured spectrophotometrically with a GTP-regenerating system as described (21). The reaction mixtures (100 μl) contained 7 mM magnesium acetate, 5 mM potassium phosphate, pH 7.3, 95 mM potassium glutamate, 5 mM ammonium chloride, 0.5 mM calcium chloride, 1 mM spermidine, 8 mM putrescine, 1 mM DTT, 1 mM each ATP and GTP, 5 mM phosphoenolpyruvate, 2.3 units/ml pyruvate kinase (Sigma), 40 μM cold alanine, 185 kBq of [3H]alanine, 12 pmol of ribosome, 1 μg of EF-G, 1 μg of EF-Ts, and 10 μg of AlaRS. The reactions were carried out at 37 °C for the specified times. Aliquots (8 μl) were withdrawn, spotted on Whatman 3MM filter paper, and boiled in 10% trichloroacetic acid at 90 °C for 30 min to deacylate the aminoacyl-RNAs. The radioactivity in the 10% trichloroacetic acid-insoluble fraction was measured with a liquid scintillation counter.
EF-Tu Is Dispensable for tmRNA Entry into the Ribosome—To observe the effect of EF-Tu on tmRNA entry into the ribosome, a pre-trans-translation complex was prepared, corresponding to a ribosome stalled by a truncated mRNA. An E. coli dhfr gene that had been digested in the middle of the ORF was translated using the PURESYSTEM. The stalled ribosome was pelleted for the trans-translation experiment. 8, EF-Tu is necessary for tag peptide synthesis but not for the first alanine attachment from the charged tmRNA. Truncated DHFR with the tag peptide was synthesized in the presence of 4 μM EF-Tu, whereas truncated DHFR with an alanine residue, but not the tag peptide, was synthesized in the absence of EF-Tu or in the presence of 4 μM EF-Tu and 50 μM kirromycin. 0.112 A₄₅₀ units of tRNA mixtures (Roche Applied Science), 10–70 units of aminoacyl-tRNAsynthetase mixtures required for tag peptide synthesis, 0.1 mM each of 5 amino acids encoded in the tag peptide, 1.85 kBq of [14C]alanine, 0.2 μg of EF-G, 0.2 μg of EF-Ts, 0.1 μg of RF1, 0.1 μg of RF3, and 0.1 μg of ribosome recycling factor were additionally added in the 10-μl trans-translation reaction mixtures (10 μl). Products containing [14C]alanine were separated by 12% SDS-PAGE and visualized.

RESULTS

EF-Tu Is Dispensable for tmRNA Entry into the Ribosome—To observe the effect of EF-Tu on tmRNA entry into the ribosome, a pre-trans-translation complex was prepared, corresponding to a ribosome stalled by a truncated mRNA. An E. coli dhfr gene that had been digested in the middle of the ORF was translated using the PURESYSTEM. The stalled ribosome-mRNA-polypeptide complex was carefully isolated through sucrose density ultracentrifugation to eliminate soluble factors, especially EF-Tu (Fig. 1A).

By using this complex, the tagging reaction mediated by tmRNA and SmpB in the presence and absence of EF-Tu was monitored by the incorporation of the [14C]alanine into the nascent polypeptide, as determined by SDS-PAGE. As shown in Fig. 1B, the complete tagging reaction proceeded in the presence of EF-Tu, whereas the omission of EF-Tu resulted in the synthesis of shorter polypeptide but labeled with [14C]alanine corresponding to the alanylated truncated DHFR. In the presence of EF-Tu, the addition of kirromycin, an antibiotic that specifically inhibits the conformational change of EF-Tu on the ribosome, thereby hindering the proper positioning of the aminoacyl-tRNA on the ribosome and the subsequent peptidyl transfer reaction (22), also resulted in the synthesis of [14C]alanine-labeled truncated DHFR. The fact that the depletion of EF-Tu and the addition of kirromycin both failed to carry out the complete tagging reaction indicated that it progressed in an EF-Tu-dependent manner and that the pre-trans-translation complex preparation contained no EF-Tu. However, these results also demonstrated that [14C]alanine could be incorporated into the protein even in the absence of EF-Tu, indicating that alanyl-tmRNA was able to enter the A site of the ribosome without EF-Tu.

In order to define the minimal components necessary for alanyl-tmRNA entry into the ribosome and peptidyl transfer to the alanyl-tmRNA, the incorporation of the alanine residue into the nascent polypeptide on the pre-trans-translation complex was examined in the absence of tmRNA, in the presence and absence of EF-Tu, and in the presence of EF-Tu and kirromycin (Fig. 2A). As expected, similar levels of alanine were incorporated into the nascent polypeptide in the presence or absence of EF-Tu and in the presence of EF-Tu and kirromycin. SDS-PAGE anal-
ysis showed that the product corresponded to the alanylated truncated DHFR (data not shown). Fig. 2B shows that use of the GDP-form of EF-Tu in place of the GTP-form did not affect the incorporation of alanine into the nascent polypeptide. These observations could not be attributed to nonspecific binding to the ribosome, because normal tRNAAla could not bring about the incorporation of alanine (data not shown). These data demonstrate that EF-Tu was dispensable for the alanylation of the polypeptide.

EF-Tu Strongly Binds to Alanyl-tmRNA—The finding that the trans-translation initiation was independent of EF-Tu led to a reinvestigation of the interactions among alanyl-tmRNA, EF-Tu, and SmpB. Previous studies on the interaction of EF-Tu and alanyl-tmRNA were conducted in the absence of SmpB (13–15). Although the interaction of EF-Tu and alanyl-tmRNA was demonstrated in the presence of SmpB (4), the inter-relationships among the three molecules were not elucidated clearly. The affinity of EF-Tu for alanyl-tmRNA has been reported as both $K_d$ $\approx 110$ nM (13) and $K_d$ $\approx 50$ nM (15). Therefore, the affinity of EF-Tu for alanyl-tmRNA was examined in the absence and presence of SmpB. Because both SmpB and EF-Tu had been shown to protect against RNase A cleavage (4), the protection of alanyl-tmRNA against nonenzymatic deacylation in a neutral buffer was examined.

As shown in Fig. 3A, the addition of increasing concentrations of EF-Tu had a drastic effect on the protection of alanyl-tmRNA. Almost all the alanyl-tmRNA was protected in the presence of 0.8 $\mu$M EF-Tu. Half-maximal protection was observed in the range of 0.05–0.1 $\mu$M EF-Tu, in agreement with the results of Stepanov and Nyborg (15) but not with those of Barends et al. (13).

Surprisingly, the addition of increasing amounts of SmpB to reaction mixtures containing 0.8 $\mu$M EF-Tu, a concentration that conferred nearly complete protection of the aminoacyl moiety of alanyl-tmRNA, decreased the protection of the alanyl-tmRNA. Equimolar amounts of SmpB to the amounts of EF-Tu almost completely eliminated the protection (Fig. 3B). Because SmpB did not protect the aminoacyl moiety of alanyl-tmRNA in the previous study (4), these data indicated that the binding site of SmpB competed with its counterpart in EF-Tu. This result is also inconsistent with previous results indicating that SmpB had no effect on the protection by EF-Tu (4). However, those experiments used 40 $\mu$M EF-Tu in the reaction mixtures, a concentration at which competition by SmpB probably was not detectable.

**Positive and Negative Cooperativity of SmpB and EF-Tu toward the Alanylated TLD of tmRNA**—The tertiary interactions between EF-Tu and aminoacyl-tRNA have been well investigated. The interacting region is concentrated in the acceptor arm of tRNA (23). In contrast, structural analysis of SmpB and the TLD of tmRNA revealed that SmpB bound to the upper side of the anticodon stem region (5), which is opposite the EF-Tu interacting region. Our results indicated that the tmRNA-binding site of SmpB competed with the EF-Tu binding region, which is inconsistent with the structural results.

Therefore, to evaluate EF-Tu binding to the alanylated TLD of tmRNA, a tmRNA variant consisting of only the TLD of tmRNA was prepared. The truncated tmRNA with an anticodon corresponding to Ala (GCA or GCG) is shown in Fig. 4A. The efficient charging of the resultant RNA, TLDwAL, with alanine by AlaRS was strongly dependent on SmpB (Fig. 4B). This result indicated that the complex composed of SmpB and TLDwAL, but not the RNA alone, was the AlaRS substrate.

EF-Tu binding to the alanyl-TLDwAL was also analyzed. Most surprisingly, EF-Tu did not protect alanyl-TLDwAL in the range of 0.05–
0.8 μM, indicating that there was no interaction between EF-Tu and alanyl-TLDwAL (data not shown). However, the addition of 0.2–0.8 μM SmpB to the reaction mixtures containing 0.8 μM EF-Tu protected alanyl-TLDwAL, demonstrating a stimulating effect by SmpB on the binding of EF-Tu to the alanyl-TLD (Fig. 5A). Because SmpB enhanced EF-Tu binding to the alanyl-tmRNA, the protection of alanyl-TLDwAL by EF-Tu in the presence of 0.4 μM SmpB was reevaluated (Fig. 5B). Half-maximal protection occurred in the presence of 0.05–0.1 μM EF-Tu, similar to the results obtained with intact tmRNA, and 0.8 μM EF-Tu was sufficient to protect almost all of the alanyl-TLDwAL, indicating that the EF-Tu concentration was saturable in the experiment in Fig. 5A. Fig. 5A also indicated that, as with intact tmRNA, high concentrations of SmpB dissociated EF-Tu from alanyl-TLD, demonstrating that two molecules of SmpB could bind to the TLD of tmRNA. The tighter binding of SmpB that stimulated EF-Tu binding to the aminoacyl moiety of the alanyl-TLD may have been the canonical binding to the upper side of the anticodon stem, as visualized in the crystal structure of the complex consisting of the TLD and SmpB (5). The looser binding that competed with EF-Tu for the TLD may have been the alternative binding to the acceptor arm region. The same experiment was carried out using alanylated tRNA<sub>Ala</sub>. Fig. 5C shows that this alternative binding to the acceptor arm region may be general to the TLD structure.

These results suggested that at least two SmpB molecules could bind to the TLD of tmRNA, and the competitive binding of SmpB and EF-Tu to the intact alanyl-tmRNA was due not to the canonical binding but to alternative binding to the TLD. To test this hypothesis, the effect of SmpB on the protection of alanyl-tmRNA was reevaluated using higher concentrations of alanyl-tmRNA and EF-Tu. As shown in Fig. 3C, in the presence of 4 μM EF-Tu, a semi-saturable concentration for the protection of 20 nM alanyl-tmRNA, a small amount of SmpB (~0.5 μM) actually promoted EF-Tu binding to the alanyl-tmRNA, although the effect was less than for the alanyl-TLD (Fig. 5A). Equimolar amounts of SmpB and EF-Tu resulted in the complete dissociation of EF-Tu from the alanyl-tmRNA, in agreement with the previous result (Fig. 3A). These observations showed that the canonical binding SmpB, in which the K<sub>d</sub> value was reported to be ~1 nM for intact tmRNA (12), could occur and stimulate EF-Tu binding of both the alanyl-tmRNA and the alanyl-TLD. However, this canonical binding of SmpB had very little effect on the binding of EF-Tu to the intact tmRNA, because EF-Tu itself bound well to the intact alanyl-tmRNA in the absence of SmpB (Fig. 3A).
Function of SmpB on the Ribosome

EF-Tu Promotes the Accommodation of the TLD of tmRNA in the A Site of the Ribosome—The question of whether the mutant TLDwAL could enter the A site of the ribosome in a similar way as the intact tmRNA was examined. The TLDwAL was added to the poly(U)-dependent polyalanine synthesis system consisting of poly(U), tRNA^{ Ala }, PheRS, and three elongation factors. SmpB and AlaRS for the aminocacylation of TLDwAL were also added to the system.

As shown in Fig. 6A, alanine residues were incorporated into the polypeptide with the addition of TLDwAL, demonstrating that alanyl-TLDwAL attached alanine to the polyphenylalanine on the stalled ribosome that was translating poly(U), in a similar way as the trans-translation reaction. The effect of SmpB on the ribosome binding and subsequent accommodation in the A site was tested in this system using pre-charged alanyl-TLDwAL to eliminate the SmpB effect on the amination of TLDwAL. The result showed that alanine incorporation correlated with the concentration of SmpB, indicating the indispensability of SmpB for alanyl-TLD entry into the ribosome, as well as for the intact tmRNA (data not shown). These results strongly suggested that the TLD of tmRNA could enter the A site of the ribosome in the same manner as the intact tmRNA and that one of the major roles of SmpB is the proper entry of the TLD of tmRNA into the A site of the ribosome.

A slight incorporation of alanine into the polypeptide was observed even in the absence of tRNA^{ Phe } (Fig. 6A). Such incorporation was also observed in the absence of phenylalanine, tRNA^{ Ala }, and PheRS (Fig. 6B), demonstrating that the observed alanine incorporation was not into polyphenylalanine synthesized with residual tRNA^{ Ala }. Because there were no other amino acids or tRNA-like molecules in the reaction, the incorporation of alanine showed that polyalanine was synthesized on the ribosome by the actions of TLDwAL and SmpB. Moreover, the omission of poly(U) from the reaction mixture increased the incorporation of alanine residues (Fig. 6B), demonstrating that the polyalanine synthesis by TLDwAL and SmpB was independent of template mRNA and that the mRNA binding to the ribosome inhibited polyalanine synthesis.

[^14]C-Alanine-labeled product was also separated by Tricine-SDS-PAGE (19) to analyze the length of the product (Fig. 6C). The data showed that the product was not detected in the separating gel (20% acrylamide gel), but the large parts of the product were stacked at the top of the spacer gel (10% acrylamide gel) in relation to the incubation time, suggesting that the high degree of polymerization of[^14]C-alanine proceeded in the reaction mixture. It also should be noted that such polyalanine synthesis was not observed by using normal tRNA^{ Ala } (Fig. 6E), demonstrating that this template-independent...
In the polyalanine synthesis, TLDwAL might execute the multiround A site entry and subsequently translocation to the P site of the ribosome with the help of SmpB. This assumption was tested by performing the reaction in the presence and absence of EF-G (Fig. 6D, open circles and open triangles, respectively). The results indicated that EF-G-catalyzed TLDwAL translocation was necessary for polyalanine synthesis. Hence, the rate of polyalanine synthesis should reflect the rate of the alanyl-TLDwAL entry as far as this step is rate-limiting. Therefore, polyalanine synthesis was measured in the presence of different concentrations of EF-Tu and SmpB. As shown in Fig. 6D, although a small amount of the polyalanine was synthesized in the absence of EF-Tu, the addition of EF-Tu enhanced the rate of the polyalanine synthesis in the presence of SmpB, indicating that EF-Tu enhanced alanyl-TLDwAL entry into the ribosome. Furthermore, the addition of kirromycin inhibited the reaction (Fig. 6D), suggesting that GTP hydrolysis by EF-Tu is necessary for the TLDwAL entry into the A site of the ribosome. It was also found that excess SmpB inhibited the polyalanine synthesis (Fig. 6D). By binding to the acceptor arm region of TLDwAL, as was observed in the protection analysis (Figs. 3 and 5), SmpB may block the binding of AlaRS or EF-Tu and decrease the efficiency of alanylation and TLDwAL entry into the ribosome, thus decreasing the rate of the polyalanine synthesis. However, proper concentrations of SmpB and EF-Tu increased the efficiency of polyalanine synthesis, demonstrating that SmpB and EF-Tu play a cooperative role in the A site entry of the TLD of tmRNA.

**GTP Hydrolysis Actually Occurs in the Step of tmRNA or TLD Entry by the Concerted Action of EF-Tu and SmpB But Independently of Template mRNA**—The effect of SmpB on the ribosome-dependent GTPase activity of EF-Tu in the presence of TLDwAL was monitored spectrophotometrically by applying an ATP-regenerating system that utilizes pyruvate kinase and lactate dehydrogenase as enzymes and phosphoenolpyruvate, ADP, and NADH as substrates (21). This system allows the detection of ATP hydrolysis as a decrease of absorbance at 340 nm that represents an oxidation of NADH. Because GDP is also a substrate for pyruvate kinase, the hydrolysis of GTP can be detected in this system. The dependence of EF-Tu was monitored by integrating ribosome, TLDwAL, AlaRS, SmpB, EF-Tu, and the GTP-regenerating system (Fig. 7A). In this reaction mixture, polyalanine synthesis did not take place because of the absence of EF-G. It also should be noted that the hydrolysis of ATP by an aminoacylation reaction could be negligible because the reaction alters ATP to AMP, which was not the substrate for the pyruvate kinase. As shown in the figure, in the presence of EF-Tu, the rate of the GTP hydrolysis increased by the addition of the ribosome, although almost no such increase was observed in the absence of EF-Tu, demonstrating that all GTPase activities arose from the concerted action of EF-Tu and the ribosome. Surprisingly, in the presence of EF-Tu, GTP hydrolysis proceeded for ~2 min until NADH was fully oxidized, and the hydrolysis rate was calculated as ~20 μM/min. These values indicated that at least ~40 μM of GTP was hydrolyzed, which was extremely higher than the concentration of the ribosome (0.5 μM) or TLDwAL (0.5 μM). Thus, this result suggested multiple accommodation of alanyl-TLDwAL in the A site of the ribosome in the reaction mixture. Because there was no peptidyl-tRNA at the P site of the ribosome, accommodated alanyl-TLDwAL may be dissociated fast from the A site of the ribosome and be recycled by the lack of subsequent acceptance of the nascent peptide.

By using this system, participation of SmpB in the GTP hydrolysis was also examined (Fig. 7B). The data showed that GTP hydrolysis by the addition of the ribosome was stimulated only in the presence of both SmpB and EF-Tu, demonstrating that SmpB is indispensable for the ribosome-dependent GTPase activity of EF-Tu. Intact tmRNA and tRNA^Ala^ were also examined (Fig. 7B). In the case of tmRNA, although the rate of the GTP hydrolysis is lower than in the case of TLDwAL, stimulation of the hydrolysis rate was observed only in the presence of both EF-Tu and SmpB. On the other hand, in the case of tRNA^Ala^ or in the absence of tRNA-like molecules, no such increase was observed even in the presence of both factors. According to all the results shown here, it was demonstrated that SmpB played a functional role of transferring the GTPase signal to EF-Tu in the tmRNA or TLDwAL accommodation step that lacked the canonical codon-anticodon interaction.

**DISCUSSION**

**SmpB Function on the Acceptor Arm Region of tmRNA**—There are significant differences between a canonical aminoacyl-tRNA entry and an alanyl-tmRNA entry into the A site of the ribosome. tmRNA contains a TLD and other regions, including four pseudoknots and an ORF. Thus, it is about 5-fold larger than canonical tRNA, and its entry into the ribosome requires several additional conditions. These include the involvement of SmpB (11), the presence of the pseudoknot structure (24), and the absence of mRNA upstream of the A site codon (25–27).

The requirement of SmpB for tmRNA entry has been well studied. Although it has been proposed that the role of SmpB is to enhance tmRNA alanylation and properly position alanyl-tmRNA on the ribosome, the detailed function of this protein remains ambiguous.

By adding an anticodon loop structure equivalent to that of canonical tRNA, we have constructed a mutant tmRNA, TLDwAL, whose structure has only the TLD (Fig. 4A). SmpB has the same effects on this mutant as on the intact tmRNA. SmpB stimulates both aminoacylation (Fig. 4B) and A site entry (Fig. 6D), even though the mutant has no PK1
Function of SmpB on the Ribosome

structure, which has been reported to be a sub-essential element for the aminoacylation and subsequent A site entry of intact tmRNA (24).

Although the qualitative effects of SmpB on the mutant are same as those on intact tmRNA, the quantitative effects of SmpB are somewhat different. The mutant is dependent on SmpB for the aminoacylation (Fig. 4B), whereas intact tmRNA can be aminoacylated even in the absence of SmpB (10). SmpB is also required for the protection of the aminoacyl moiety of alanyl-TLDwAL by EF-Tu (Fig. 5, A and B), whereas the intact tmRNA does not require SmpB for the protection (Fig. 3A).

These quantitative differences can be explained by the short, weak helix of tmRNA that is equivalent to the anticodon stem region of canonical tRNAs. Because this helix is smallest in the short form mutants and contains two GU wobble base pairs, the region may not form a rigid helix and, as a result, the 3’ end of the D loop and 5’ end of the T stem may be opened. This may reduce the interaction of the D and T loops, which has been reported to be important for trans-translation by tmRNA (28, 29). Accordingly, the structure of this region, which is equivalent to the hinge region of canonical tRNA, may be disordered. The strong effect of SmpB on AlaRS and EF-Tu binding to the mutant may be due to the interaction of SmpB and the upper side of the weak helix, as visualized in the crystal structure (5). This interaction may substitute for the weak helix, regenerating the interaction of the D and T loops.

Thus, one role of SmpB binding to the TLD may be to support the interaction of the D and T loops to form a highly structured acceptor arm region, which is essential for aminoacylation by AlaRS, protection by EF-Tu, and possibly the fitting to the A site of the ribosome. Intact tmRNA does not necessarily require this support because the anticodon stem region is tighter than in the mutant construct. Although intact tmRNA is also strongly dependent on SmpB for entry into the ribosome, the reason for this dependence may be somewhat different. The unstructured C-tail region of SmpB, which x-ray analysis has shown to be irrelevant for the interaction between tmRNA and SmpB, may facilitate the proper entry of the TLD into the ribosome, as discussed below.

tmRNA Entry into the A Site of the Ribosome—It has been reported previously that when a canonical aminoacyl-tRNA-EF-Tu-GTP complex binds to the A site of the ribosome, codon-anticodon interaction on the ribosome triggers GTP hydrolysis, leading to the conformational change of EF-Tu and the dissociation of EF-Tu/GDP (22). Further investigation using a cryoelectron microscopy density map has shown that the signaling that leads to the EF-Tu conformational change corresponds to the kinked structure of the aminoacyl-tRNA formed by the canonical codon-anticodon interaction (30–32).

However, the molecular mechanism of tmRNA entry into the ribosome appears to be somewhat different from the canonical aminoacyl-tRNA entry because of two features of tmRNA as follows: the lack of an anticodon and an extended L-shaped conformation as compared with the canonical tRNA. The lack of codon-anticodon interaction may prevent the formation of the supporting point of the kinked structure of the tmRNA, which has been observed in the pre-accommodation state of the complex consisting of the aminoacyl-tRNA, EF-Tu, and the ribosome (32). The extended L-shaped conformation of tmRNA, resulting in a significant structural difference between the TLD of tmRNA and the canonical tRNA, as visualized by x-ray analysis (5), has also obscured the tmRNA entry mechanism.

However, here we identify functional similarities between these two molecules. Template-independent polyalanine synthesis proceeds via the actions of TLDwAL, SmpB, elongation factors, and the ribosome (Fig. 6B), indicating that the TLD of tmRNA is capable of acting on the ribosome in the same manner as canonical tRNAs, via entry into the A site and translocation to the P site of the ribosome. The rate of the polyalanine synthesis is increased by the addition of EF-Tu but is inhibited by the addition of kioromycin that specifically inhibits the conformational change of EF-Tu on the ribosome (Fig. 6D and E), suggesting that the binding of EF-Tu to alanyl-TLDwAL complexed with SmpB (Fig. 5, A and B) enhances the alanyl-TLDwAL entry into the A site of the ribosome through the hydrolysis of GTP, as with canonical tRNAs. In fact, EF-Tu-dependent GTP hydrolysis occurs on the ribosome in the presence of both SmpB and TLDwAL, although in the absence of canonical codon-anticodon interaction (Fig. 7).

These functional similarities between canonical tRNAs and the TLD of tmRNA, which also strongly suggest structural similarities, can be explained by hypothesizing that the complex composed of SmpB and the TLD of tmRNA concurrently passes through the ribosome. Fig. 8 shows the structural comparison of E. coli tRNA and the complex comprised of SmpB and the TLD of tmRNA as indicated previously (5). In this image, the L-shaped conformation of the TLD of tmRNA forms an 120° angle, and SmpB and the acceptor arm region form a 90° angle, similar to the L-shaped conformation of canonical tRNAs, and places the C-tail of SmpB in proximity to the decoding center of the 30 S ribosomal subunit. The enhancement of alanyl-TLDwAL entry by EF-Tu suggests that the C-tail of SmpB, by interacting with the decoding region of the small ribosomal subunit, forms the supporting point of the kinked structure of the complex comprised of SmpB and the TLD of tmRNA. EF-G-dependent translocation of TLDwAL from the A site to the P site of the ribosome (Fig. 6C), a step in which the anticodon arm structure is essential for canonical tRNAs (33), can also be explained by assuming that SmpB mimics the anticodon arm structure of canonical tRNAs. Previous work has shown that mutations in the conserved residues of the unstructured C-tail region of SmpB (Asp-137, Lys-138, Arg-139, Ile-154, and Met-155 in E. coli) prevent the attachment of the...
first alanine residue to the polypeptide, indicating that these mutants are incapable of facilitating the alanyl-tmRNA entry into the A site of the ribosome (12). Certainly, we also find that the use of the mutant SmpB that lacks C-terminal seven amino acid residues in our polyalanine synthesis system results inefficient synthesis of polyalanine (Fig. 6D). In accord with the above hypothesis, it can be argued that these C-terminal residues are involved in the interaction with the decoding region of the 30 S subunit, thereby allowing the supporting point of the kinked structure of the complex comprised of SmpB and the TLD of tmRNA to be formed.

This model is different from that proposed by Gutmann et al. (5), in which the fitting is performed by synchronizing the T loop region. In our model the fitting is performed by synchronizing the T stem region. Because the entire structure of the complex was not visible in the previous work (5), better defined three-dimensional structural images will be necessary to elucidate the correct positioning of SmpB. However, our biochemical analyses suggest that SmpB may play a role by interacting with the 30 S ribosome and mimicking the anticodon arm region of the canonical tRNA.

**Schematic Model for the Mechanism of Trans-translation Initiation**—SmpB dissociates EF-Tu from alanyl-tmRNA, alanyl-TLDwAL, and alanyl-TRNA<sub>36A</sub> (Figs. 3 and 5), indicating that two molecules of SmpB bind to the TLD of tmRNA. This alternative interaction of SmpB with the TLD of tmRNA has also been observed previously (4, 7, 34). However, we find that EF-Tu promotes the entry of alanyl-TLDwAL into the ribosome. Therefore, this interaction of SmpB may be nonfunctional or harmful to tmRNA function. Because TLDwAL is small, there may be no space for SmpB binding other than the canonical and alternative bindings described above. Thus, only one molecule of SmpB may function in TLDwAL-derived polyalanine synthesis. Two main functions of SmpB, enhancement of tmRNA alanylation and an obligatory effect on tmRNA entry into the A site of the ribosome, can be explained by the canonical binding of SmpB, as shown here. Therefore, we propose that intact tmRNA-derived trans-translation is also facilitated by a single molecule of SmpB.

In terms of the requirement of EF-Tu for the A site entry of alanyl-tmRNA, we find it proceeds in the absence of EF-Tu (Figs. 1 and 2), which is consistent with the previous result (8). The finding that the alanyl-tmRNA entry proceeds even in the presence of kikromycin and in the absence of GTP (Figs. 1 and 2) strongly suggests that alanyl-tmRNA can accommodate the A site of the ribosome without the help of EF-Tu. However, this EF-Tu independent A site entry would not be physiologically relevant because there are certain amounts of EF-Tu in cells. As far as alanyl-tmRNA interacting with EF-Tu in the same manner as canonical tRNAs, it cannot be conceived that the alanyl-tmRNA entry proceeds independently of EF-Tu. Because intact tmRNA interacts with tmRNA (Fig. 3) and enhances the rate of alanyl-tmRNA entry into the ribosome (8), efficient trans-translation may require EF-Tu in a physiological condition. This speculation is consistent with our finding that EF-Tu also enhances the rate of alanyl-TLDwAL entry into the ribosome in the polyalanine synthesis system (Fig. 6D), and that both intact tmRNA and TLDwAL stimulate the ribosome-dependent GTPase activity of EF-Tu in the presence of SmpB (Fig. 7B).

Fig. 9 shows a proposed schematic model of the trans-translation initiation, based on our results and previous findings. In this model, the canonical binding of SmpB to the upper side of the anticodon stem region supports interaction of the D and T loops to form a highly structured acceptor arm region. This enhances alanylation, EF-Tu interaction, and probably the fitting to the A site of the ribosome. This interaction may be highly specific for tmRNA, and no other molecule may interfere. After the alanylation of tmRNA, alanyl-tmRNA complexed with EF-Tu may interact with EF-Tu and bind rapidly to the stalled ribosome. At this stage, the basic C-tail of SmpB may bind to the 30 S subunit and be immobilized, in the same way as the codon-anticodon interaction between tRNA and mRNA. This interaction may stimulate the hydrolysis of GTP by EF-Tu and promote the accommodation of alanyl-tmRNA in the A site of the ribosome. After the tmRNA-SmpB complex enters the A site, it may be translocated to the P site of the ribosome by EF-G, followed by the dissociation of mRNA (35) and the proper positioning of the “resume codon” (36) to the A site of the ribosome.

It has been shown previously in *in vivo* and *in vitro* experiments that the tagging reaction by tmRNA does not take place on a ribosome that has mRNA downstream of the A site codon (25–27), which is also replicated in our reconstituted cell-free translation system. These results are in agreement with our polyalanine synthesis experiment, in which polyalanine synthesis is inhibited by the presence of poly(U) (Fig. 6B). In
Function of SmpB on the Ribosome

this case, because of the depletion of tRNA^Phe, poly(U) may occupy the A site of the ribosome and block the entry of TLDwAL and SmpB complex, which results in the inhibitory effect on the polyalanine synthesis. This result also suggests the interaction of the C-tail of SmpB with the 30 S subunit and supports the hypothesis that SmpB compensates for the misplaced anticodon arm-like region of tmRNA. The presence of mRNA downstream of the A site codon may protect the SmpB binding region of the 30 S subunit and, consequently, block the binding of the tmRNA-SmpB complex to the ribosome. Thus, the C-tail of SmpB also may function as the discriminating sensor of a translating or stalled ribosome, so as not to disturb the canonical translation.

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