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## Protein synthesis factors (RF1, RF2, RF3, RRF, and tmRNA) and peptidyl-tRNA hydrolase rescue stalled ribosomes at sense codons.

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**Running title:** Factors involved in the rescue of stalled ribosomes

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## **ABSTRACT**

During translation, ribosomes stall on mRNA when the aminoacyl-tRNA to be read is not readily available. The stalled ribosomes are deleterious to the cell and should be rescued to maintain its viability. To investigate the contribution of some of the cellular translation factors on ribosome rescuing, we provoked stalling at AGA codons in mutants which affected the factors, and then analyzed the accumulation of oligopeptidyl- (peptides of up to 6 amino acid residues, oligopep-) tRNA or polypeptidyl- (peptides of more than 300 amino acids in length, polypep-) tRNA associated with ribosomes. Stalling was achieved by starvation for aminoacyl-tRNA<sup>Arg4</sup> upon induced expression of engineered *lacZ* ( $\beta$ -galactosidase Z) reporter gene harboring contiguous

AGA codons close to the initiation codon or at internal codon positions together with minigene AUGAGAUAA accompanied by reduced peptidyl-tRNA hydrolase. Our results showed accumulations of peptidyl-tRNA associated with ribosomes in mutants for release factors (RF1, RF2, and RF3), ribosome recycling factor (RRF), peptidyl-tRNA hydrolase (Pth), and transfer-messenger RNA (tmRNA), implying that each of these factors cooperate in rescuing stalled ribosomes. The role of these factors in ribosome releasing from the stalled complex may vary depending on the length of the peptide in the peptidyl-tRNA. RF3 and RRF rescue stalled ribosomes by “drop off” of peptidyl-tRNA, while RF1, RF2 (in the absence of termination codon), or Pth may rescue by hydrolyzing the associated peptidyl-tRNA. This is followed by the disassembly of the ribosomal complex of tRNA and mRNA by RRF and EF-G (elongation factor G).

## **INTRODUCTION**

A round of protein synthesis ends when the ribosome reaches a stop codon (UAG, UGA, or UAA) in the mRNA. In bacteria, release factors (RF1 or RF2) recognize the stop codons to induce hydrolysis of peptidyl-tRNA (pep-tRNA) at the ribosomal peptidyl transferase center (PTC) and release the complete peptide.<sup>1-6</sup> RF3/GTP accelerates the dissociation of RF1 and RF2 from ribosome<sup>7,8</sup> leaving a post-termination complex (PoTC), which consists of mRNA, tRNA and ribosome. PoTC is disassembled by RRF and EF-G/GTP and its components are used in new rounds of protein synthesis.<sup>9-14</sup>

Factors mentioned above are not limited to disassembly of PoTC but they must play a role also in handling stalled ribosomes at sense codons to ensure high efficiency

of the ribosome usage. There are non-programmed and programmed ribosome stalling. The latter functions as a mechanism of gene regulation.<sup>15-25</sup> Non-programmed ribosomal stalling occurs in two different manners. First, it can occur with ribosome stalled at the end of non-stop mRNA. It has been reported that tmRNA but not RF1, RF3, or RRF are involved in the rescue of stalled ribosomes in this case.<sup>26</sup> Furthermore, YaeJ protein, which carries a GGQ motif (like RFs), induces the hydrolysis of pep-tRNA that resides in a ribosome stalled on a non-stop mRNA.<sup>27,28</sup> In addition, ArfA protein also rescues stalled ribosomes from non-stop mRNAs.<sup>29</sup> Second, the stalling occurs when aminoacyl-tRNA is depleted, leading to empty A-site. The empty A-site has “hungry codon” because it does not have aminoacyl-tRNA corresponding to the triplet. The hungry codon affects cell’s growth.<sup>30</sup> These stalled ribosomes are rescued by a mechanism where mRNA is cleaved by RelE (ribonuclease which cuts mRNA at the A-site) followed by the action of tmRNA. Rel stands for “relax” meaning that the control of RNA synthesis by amino acid is relaxed in the *relE* mutant as opposed to the wild type which “stringently” controls RNA synthesis by amino acid availability. Alanyl-tmRNA enters the A-site and accepts the nascent peptide group of the pep-tRNA in the P-site.<sup>31-36</sup> In addition, RF3 or RRF/EF-G,<sup>37-39</sup> or RRF/EF-G/IF3 “drop off” pep-tRNA<sup>40</sup> and these factors collaborate *in vivo*.<sup>41</sup>

Pep-tRNA released from ribosomes is hydrolyzed by Pth (peptidyl-tRNA hydrolase).<sup>42,43</sup> In Pth reduced cells, expression of minigene coding for two amino acids cause the release of dipep-tRNA from ribosomes decreasing the aminoacyl-tRNA pool.<sup>44,45</sup> Lambda phage codes for short ORF and produces short oligopeptidyl-tRNA, which has to be hydrolyzed by Pth for the growth of the phage. In the *rap* mutant, the

level of Pth is very much reduced and hence the lambda phage cannot grow in this strain. Rap stands for “Restriction of Att<sup>+</sup> Phage”. Att<sup>+</sup> phage means the phage with the gene related to phage attaching to the host. Att<sup>+</sup> contains the DNA region which codes for the minigene. The *rap* mutation restricts the phage growth because with low Pth activity, the host cannot stand the large amount of minigene product (oligopep-tRNA) made by the phage. This mutation has no detectable effect on bacterial growth.<sup>46</sup> In our past publications, non-programmed ribosome stalling was created by the use of *pth(rap)* together with minigene (AUGAGUAA) resulting in the shortage of tRNA<sup>Arg4</sup>.<sup>47</sup>

In this paper, we asked whether or not the factors which mediate translation termination, PoTC disassembly, and hydrolysis of pep-tRNA were involved in rescuing stalled ribosomes caused by the lack of sufficient aminoacyl-tRNA or release factors corresponding to the A-site codon. We either removed or reduced the *in vivo* activity of the factors and estimated the accumulation of pep-tRNA in the cell as an index of ribosome stalling. Our evidence suggests that all six factors (RRF, RF1, RF2, RF3, Pth, and tmRNA) collaborate to rescue stalled ribosomes with hungry codon at the A-site in *E. coli*. We propose that RF1 and RF2 promote *in vivo* hydrolysis of the ester bond of pep-tRNA despite that A-site does not have the termination codon in stalled ribosomes. Furthermore, Pth appears to cleave oligopep-tRNA on the ribosome helping to disassemble the stalled ribosomes with the oligopep-tRNA.

## **MATERIALS AND METHODS**

### **Bacterial Strains, Plasmids, and Growth Conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. P1 phage-mediated transduction was used to transfer genetic markers. The bacterial constructs were selected by resistance to antibiotics or temperature sensitivity. All strains used were derivative of P90C (Table 1). P90C has the following characteristics:  $\Delta(gpt-lac)$ : *gtp* (guanine-xanthine phosphoribosyltransferase) and *lac* genes (*lacA*, *Y*, *Z*, and *I*) located between 5.5 min and 7.8-7.9 min are deleted. Also *proA* and *proB* at 5.6 min (*proA*;  $\gamma$ -glutamyl phosphate reductase and *proB*;  $\gamma$ -glutamyl kinase) are missing. The *lac* deletion was necessary to induce the *lac* gene in the plasmids used in this paper. Each of the strains with one or two plasmids were grown in LB broth (Figs. 1, 2, 3, 6a and 6b) or M9 minimal medium supplemented with 20  $\mu$ g/ml of each amino acid, 20  $\mu$ M biotin and 20  $\mu$ M thiamine (Figs. 4b, 5, and 6c). When required, 100  $\mu$ g/ml ampicillin (Amp), 30  $\mu$ g/ml kanamycin (Kan), 50  $\mu$ g/ml chloramphenicol (Cm) or 12.5  $\mu$ g/ml tetracycline (Tet) were added to the medium. After cultures reached sufficient optical density, *minigene* and/or *lacZ* genes expression were induced with 1 mM isopropylthiogalactoside (IPTG).

### **Extraction and analysis of total oligopep-tRNA<sup>Arg4</sup>**

Oligopep-tRNAs (Figs. 1, 2, 3, 6a, and 6b) were extracted in acidic conditions as described previously<sup>48,49</sup> with some modifications. After induction, 5 ml of LB cultures were immediately mixed with an equal volume of acid phenol. The aqueous phase was recovered by centrifugation at 4°C and extracted with acid phenol (repeated two times). Aqueous phase was treated with EtOH at -20°C. The precipitate present in the RNA



pellet was washed with 70% EtOH, and was treated with diethyl pyrocarbonate (DEPC). Aminoacyl-tRNA in the extract was hydrolyzed with 10 mM CuSO<sub>4</sub>, for 30 min at 37°C. Oligopep-tRNA remains intact under these conditions.<sup>50</sup> The extracted RNA was electrophoresed on urea-acrylamide (6%) acid gels,<sup>51</sup> and blotted onto Hybond N<sup>+</sup> Nylon membranes (Amersham). tRNA<sup>Arg4</sup> was detected by Northern blot with a specific [<sup>32</sup>P]-oligonucleotide probe for tRNA<sup>Arg4</sup> (Table 2).

### **Oligopep-tRNA<sup>Arg4</sup> and mRNA in ribosome fractions**

One hundred ml LB cultures (0.3 OD<sub>600</sub>) (Fig. 2) were induced as described in the legend to Fig.1. Cells were suspended in buffer containing 50 µg/ml Tet,<sup>41,51</sup> disrupted in a French press (450 psi), and centrifuged at 30,000 x g at 4°C for 30 min to obtain S-30. S-30 was incubated in the presence (+) or absence (-) of 2.5 mM puromycin for 10 min at 37°C, and was centrifuged at 100,000 x g at 4°C for 2 hrs to obtain S-100 (S) and ribosomal (R) fractions. S-100 was concentrated by precipitation with EtOH at -20°C. RNA obtained from S-100 and ribosomal pellets by the acid phenol method were treated with CuSO<sub>4</sub> as described in the previous section. Three µl of each fraction were resolved in acidic polyacrylamide gel (Fig. 2a) or in 2% formaldehyde agarose gel<sup>52</sup> (Fig. 2b). Pep-tRNA<sup>Arg4</sup> and *lacZ* AGA3-6 mRNA were probed using specific [<sup>32</sup>P]-oligonucleotides (Table 2).

β-gal-polypep-tRNA<sup>Leu1</sup> detection.

In Fig. 4, 5 ml of cultures were grown at 28 or 32°C (0.5-0.6 OD<sub>600</sub>), induced as described in the figure legend, boiled in the Laemmli buffer,<sup>53</sup> and subjected to 10% SDS-PAGE. The N-terminal His-6 tagged  $\beta$ -gal polypeptides were detected by Western blot using anti-His-6 antibody. In Fig. 5, 100 ml of cultures were induced as in Fig. 4 (32/42), and were processed as described<sup>54</sup> with some modifications. The lysozyme-treated cell pellet was suspended in 450  $\mu$ l of modified lysis buffer (20 mM Hepes-KOH pH 7.8, 50 mM MgCl<sub>2</sub>, 50 mM NH<sub>4</sub>Cl, 1 mM DTT, 200 U DNase I, 100  $\mu$ g/ml Cm), and the cells were disrupted by adding 25  $\mu$ l of 5% Brij58 and sonicated. The lysate was spun down at 30,000 x g for 20 min at 4°C. The extract thus obtained was fractionated by 10-40% SDGC (sucrose density gradient centrifugation) prepared in lysis buffer without DNase I and centrifuged in a Beckman rotor (SW40 Ti rotor) for 3 hrs at 34,000 rpm (145,000 x g). The absorbance of each fraction (500  $\mu$ l, collected from the top of the gradient) was measured at 260 nm. To analyze polypep-tRNA, each fraction (60  $\mu$ l) was suspended in Tris-acetate buffer and subjected to Tris-acetate SDS-PAGE as described<sup>55</sup>. Proteins and RNAs were blotted onto nitrocellulose or nylon membrane in 25 mM Tris-HCl pH 8.3 and 192 mM glycine. The nitrocellulose membrane was subjected to Western blot using anti-His-6 antibody to detect His-tagged  $\beta$ -gal. The nylon membrane was subjected to Northern blot to detect  $\beta$ -gal polypep-tRNA<sup>Leu1</sup> by [<sup>32</sup>P]-oligonucleotide specific probe for tRNA<sup>Leu1</sup> (Table 2).

### **Hydrolysis of polypep-tRNA<sup>Leu1</sup> by Pth**

In Fig. 6c. pHZ352-353-MR expression was induced for 5 min at 32°C to allow sequestering of tRNA<sup>Arg4</sup> by the AGA minigene expression. Cells were then switched to 42°C for 15 min to inactivate temperature-sensitive RF1. These cells (20 ml culture, 0.5 OD<sub>600</sub>) were suspended in 500 µl of 10 mM NaOAc pH 5.0, and sonicated. Debris was removed by centrifugation and the solution was adjusted to pH 7.2. Then, 50 µl of the Pth reaction buffer 10X (100 mM Tris-HCl pH 7.6, 100 mM MgOAc, 200 mM NH<sub>4</sub>Cl, and 60 mM β-ME) and N-terminal-His tagged Pth were added to the extract and incubated at 37°C for 20 min. His-tagged β-gal was detected as described in previous sections.

## RESULTS

### **Reduced Pth, inactive RRF, absence of RF3 or deletion of tmRNA increases ribosome stalling**

To determine the role of RRF, RF3, tmRNA or Pth in the release of pep-tRNA from *in vivo* stalled ribosomes, we examined the accumulation of oligopep-tRNA<sup>Arg4</sup> in strains deficient in these factors that expressed *placZ* AGA3-6 construct<sup>45,56</sup> (Fig. 1a and Table 1). The expression of *lacZ* AGA3-6 mRNA was induced at 42°C and the total RNA was analyzed by Northern blotting (Fig. 1b). In wild type cells, no appreciable oligopep-tRNA<sup>Arg4</sup> was observed (Fig. 1b, lane 1). If one of the factors analyzed were to contribute to the release of oligopep-tRNA<sup>Arg4</sup> from the stalled ribosome, we would expect that in the absence of that factor, the percentages (shown below each lane) of

oligopep-tRNA<sup>Arg4</sup> of total tRNA<sup>Arg4</sup> would increase. In *pth(rap)* strain, a mutant with reduced Pth activity,<sup>57,58</sup> high accumulation of oligopep-tRNA<sup>Arg4</sup> was observed (97.5% of total tRNA<sup>Arg4</sup>) (Fig. 1b, lane 2). In this mutant, the oligopep-tRNA<sup>Arg4</sup> might be ribosome-bound or free. In *frrT*s (the gene coding for factor for recycling of ribosome is mutated to produce temperature sensitive RRF, RRFTs<sup>59</sup>) accumulation of oligopep-tRNA<sup>Arg4</sup> increased at the non-permissive temperature (1.9 vs. 95.5%; Fig. 1b, lanes 1 and 3). *frrT*s is the original LJ14 mutant of *frr* and the colony counts at 32°C was the same as the wild type but it was less than 10<sup>-5</sup> of wild type at 42°C. Bacterial growth rate of this mutant was slightly impaired even at 32°C while it was identical to that of wild type at 28°C (unpublished data). In mutant  $\Delta$ *prfC* (the gene for peptide chain release factor C or RF3 is deleted,  $\Delta$ RF3), oligopep-tRNA<sup>Arg4</sup> accumulation increased compare to wild type cells (58.1% vs. 1.9%; lanes 4 and 1) supporting the idea that these factors help “drop off” of pep-tRNA from ribosomes.<sup>37-39</sup> In the absence of tmRNA ( $\Delta$ *ssrA* strain, the strain missing the gene coding for small stable RNA A), a molecule that rescues stalled ribosomes,<sup>31</sup> oligopep-tRNA<sup>Arg4</sup> increased compared to that in wild type cells (lanes 5 vs. 1) indicating that oligopep-tRNA<sup>Arg4</sup> was on stalled ribosomes. In all mutant strains, the accumulation of oligopep-tRNA<sup>Arg4</sup> caused by the shortage of tRNA<sup>Arg4</sup> was prevented by over-expression of tRNA<sup>Arg4</sup> from the pDC952 construct under the same experimental conditions as those of lanes 1-5 (Fig. 1b, lanes 6-10). This observation that extra tRNA<sup>Arg4</sup> relieves stalled ribosomes under similar conditions has been reported.<sup>60</sup>

## **The oligopep-tRNA<sup>Arg4</sup> detected with reduced factors is bound to the ribosomal P-site and protects the mRNA**

In the preceding section, we observed that the oligopep-tRNA<sup>Arg4</sup> accumulated despite the presence of wild type Pth in some cases (Fig. 1b, lanes 3 to 5). These data imply that the oligopep-tRNA<sup>Arg4</sup> is bound to the ribosome because ribosome protects, to some extent, the bound pep-tRNA<sup>Arg4</sup> from Pth.<sup>42</sup>

To confirm this view, the crude extracts of cells with induced *lacZ* AGA 3-6 mRNA expression were treated, with or without puromycin, an antibiotic which accepts peptidyl group from the oligopep-tRNA<sup>Arg4</sup> bound at the ribosomal P-site. The presence of oligopep-tRNA<sup>Arg4</sup> was analyzed by Northern blot in both supernatant (S) and ribosomal fractions (R) (Fig. 2a).

In strains with *frt*Ts (RRFTs), deletion of the gene for RF3 ( $\Delta$ RF3), or deletion of the gene for tmRNA ( $\Delta$ tmRNA), oligopep-tRNA<sup>Arg4</sup> was detected only in the ribosomal fraction because of the presence of wild type Pth, which hydrolyzes free pep-tRNA very efficiently. Treatment with puromycin resulted in the removal of the oligopep-tRNA<sup>Arg4</sup> from the ribosome in the form of oligopeptidyl puromycin. This shows that it was bound to the P-site in a physiologically active,<sup>61</sup> stalled complex (Fig. 2A, panels 3-6). In the strain with reduced Pth activity (Pth(rap)), oligopep-tRNA<sup>Arg4</sup> was found in both ribosomal and supernatant fractions (panel 3). The oligopep-tRNA<sup>Arg4</sup> found in the supernatant in panel 3 must have been released from ribosomes by RRF and RF3 which are present in Pth(rap) cells. It is important to note that oligopep-tRNA<sup>Arg4</sup> was found on the ribosome when each of four factors including Pth were reduced or deleted.

This implies that, Pth, like other factors, plays an important role in the rescue of the stalled ribosome.

Because the ribosome prevents mRNA hydrolysis by cellular ribonucleases,<sup>62,63</sup> we examined if the ribosomal stalling provoked by depletion of tRNA<sup>Arg4</sup> protected a fragment of *lacZ* AGA3-6 mRNA containing the AGA3-6 sequence. The RNA samples used in Fig. 2a were probed with oligonucleotides specific for *lacZ* AGA3-6 mRNA (Fig. 2b, Table 2). The result showed that the 5'-end fragment of mRNA was detected in ribosomal fractions of the mutant strains *pth*(*rap*), *frt*Ts, or with deletion of the gene for RF3. They all accumulated oligopep-tRNA<sup>Arg4</sup> (compare Fig. 2a and b). The band must be *lacZ* AGA3-6 mRNA because it was not detected in the absence of IPTG (Fig. 2b panel 2). The amount of 5'-end *lacZ* AGA3-6 mRNA accumulated was higher in all mutants than in wild type cell (Fig. 2b compare panels 1 and 3-6).

### **Stalled ribosomes with minigene and Met-Arg-tRNA<sup>Arg4</sup> appear when protein synthesis factors are missing or reduced but no effect of deletion of tmRNA**

The results in Figs. 1 and 2 show that each one of RF3, RRF, Pth and tmRNA removes oligopep-tRNA<sup>Arg4</sup> from stalled ribosomes containing a sense codon in the A-site. We asked whether these factors process the oligopep-tRNA on stalled ribosomes that contain a stop codon in the A-site. To answer this question, a minigene construct carrying the ORF sequence ATGAGAUAA (Fig. 3a) was expressed in mutants of Pth, RF3, RRF or tmRNA. The levels of Met-Arg-tRNA<sup>Arg4</sup> and tRNA<sup>Arg4</sup> were analyzed. The results shown in Fig. 3b revealed that in wild type cells no Met-Arg-tRNA<sup>Arg4</sup> was found

(lane 1). In contrast, with reduced amount of Pth (*pth(rap)*), all of tRNA<sup>Arg4</sup> is in Met-Arg-tRNA<sup>Arg4</sup> (lane 2). This is due to two reasons. First, Met-Arg-tRNA<sup>Arg4</sup> released from the stalled ribosome could not be hydrolyzed.<sup>44</sup> Second, the Pth activity to hydrolyze oligopep-tRNA on the stalled ribosome is compromised leaving bound Met-Arg-tRNA<sup>Arg4</sup> on the ribosome. In other mutants, Met-Arg-tRNA<sup>Arg4</sup> was also detected. Thus, when RRF is inactivated, the accumulated Met-Arg-tRNA<sup>Arg4</sup> was found on the ribosome indicating ribosome stalling (Fig. 3 C) occurred. It should be noted in this case that ribosome-bound deacylated tRNA was found (lane 3). This shows that the PoTC remained due to the inactivation of RRF. Important exception to the effect of various factors relieving stalled ribosomes was the effect of missing tmRNA ( $\Delta$ *ssrA* strain). There was no effect of the loss of tmRNA (Fig. 3b, lane 5). Biological significance of this observation will be dealt with in the discussion section. The data shown in Fig. 3 indicate that minigene can also stall ribosomes suggesting that there is not enough RF1 and 2 in the cell to deal with the large amount of termination complex formed by the minigene. This would cause the empty A-site with the hungry termination codon like the case of depletion of tRNA<sup>Arg4</sup> creating hungry AGA codon.

### **Absence of active RRF, RF3 or tmRNA results in complexes with stalled ribosome harboring long peptide chain-tRNA (polypep-tRNA)**

We asked whether defective factors lead to polypep-tRNA accumulation on stalled ribosomes as was observed with oligopep-tRNA<sup>Arg4</sup>. To answer this question, cells with defective or missing factors were transformed with pHZ352-353-MR (*lacZ* AGA352-353

mRNA)<sup>64</sup> (Fig. 4a and Table 1). Translation of this mRNA to the point where tandem AGAs would result in truncated  $\beta$ -galactosidase. As shown in Fig. 4b, this could be achieved upon tRNA<sup>Arg4</sup> depletion by the simultaneous induction of minigene ATGAGATAA and reduced level of Pth in Pth(rap) cells. In this system, there are only two AGA codons in the  $\beta$ -galactosidase gene. Furthermore, the position of the two tandem codon AGAs is well in the mRNA compared to the system described in Figs. 1 to 3. This makes it more difficult to release truncated  $\beta$ -galactosyl-tRNA from the ribosome. Therefore, it is necessary to express the minigene together with low level of Pth for causing the shortage of tRNA<sup>Arg4</sup>. As shown below, in this particular case, polypep-tRNA<sup>Leu1</sup> is accumulated due to the shortage of tRNA<sup>Arg4</sup> because CUG coding for Leu is located at position 351 (Fig. 4a).

As shown in Fig. 4, the Western blot assay using an anti-His-6 antibody showed the incomplete  $\beta$ -gal-polypeptide (arrow 2) in the double mutants *pth(rap)/ $\Delta$ ssrA*, *pth(rap)/frrTs* (42°C), and *pth(rap)/deletion of the gene for RF3* (Fig. 4b, lanes 4, 6, and 10). The size of the incomplete  $\beta$ -gal-polypeptide is similar to that obtained with a *lacZ* variant carrying a stop codon in position 353 (pHZ-MR construct, Table 1). Either at the permissive temperature of RRFTs (28°C) or by over-expression of wild type RRF (lanes 7 and 8), the stalled ribosomes were rescued. In both cases, intermediate size  $\beta$ -gal bands that correspond to fragments normally produced by high rate translation of *lacZ* were accumulated<sup>65</sup> (arrow 3). Induction of the plasmid expression in single mutants (Pth (*pth(rap)*), tmRNA ( $\Delta$ *ssrA*), RF3 ( $\Delta$ *prfC*) or RRF (*frrTs* at non permissive temperature)) produced full size  $\beta$ -gal protein only (Fig. 4b, arrow 1 in lanes 2, 3, 5, and 9).



The non-acid condition used for SDS-PAGE in Fig. 4 probably hydrolyzed the polypep-tRNA<sup>Leu1</sup> produced during the ribosomal stalling in the middle of *lacZ* mRNA. To avoid this problem, the crude extracts from single mutant *pth(rap)* and double mutants *pth(rap)/frrTs*, and *pth(rap)/prfC* transformed with pHZ352-353-MR were fractionated in SDGC (Fig. 5a) and the incomplete  $\beta$ -gal was detected by Western blot (Fig. 5b) under acidic conditions. In all mutants, full size  $\beta$ -gal (120 kDa, arrow 1) was detected. The ribosomal fraction of the single mutant, *pth(rap)*, should carry various size  $\beta$ -galactosidase which distributed throughout the gel making it undetectable under the experimental conditions (panel Ab). Two incomplete  $\beta$ -gal-polypeptides (arrows 2 and 3) were found in the ribosomal fraction (white arrows) of the double mutants only (panels Bb and Cb). The band labeled with arrow 2 corresponds to incomplete  $\beta$ -gal-polypep-tRNA<sup>leu1</sup>, and the other band (arrow 3) corresponds to the truncated  $\beta$ -gal-polypeptide which lost tRNA<sup>leu1</sup> during the analysis. This was further supported by probing with an oligonucleotide specific for tRNA<sup>Leu1</sup> (panels Bc and Cc). The fast moving band (arrow 4 in Fig. 5c) corresponding to free tRNA<sup>Leu1</sup> was mostly located in the supernatant fractions. These data (Figs 4 and 5) suggest that RRF, RF3, and tmRNA, collaborate in rescuing stalled ribosomes that harbor polypep-tRNA<sup>Leu1</sup> at internal locations of mRNA when sufficient depletion of tRNA<sup>Arg4</sup> was achieved through *pth(rap)* mutation and the presence of minigene.

**RF1 or RF2 relieves ribosomes stalled at AGA codons regardless of the length of pep-tRNA**

It is known that nascent polypeptide goes through the 50S tunnel<sup>66</sup>. This would make it difficult to release polypep-tRNA<sup>Leu1</sup> mentioned in the preceding section from the stalled ribosome by “drop off” of pep-tRNA. It would be easier to rescue the stalled ribosomes if the ester bond of the bound polypep-tRNA<sup>Leu1</sup> is cleaved. In this connection, it has recently been reported that RF2 cleaves pep-tRNA miscoded at the P-site on ribosomes with an empty A-site and sense codon.<sup>67</sup> Their complex is similar to our stalled ribosome in that it has sense codon at the empty A-site while it is different in that the P-site of their ribosome is mismatched. Despite the difference, we wondered if RF1 or RF2 cleaves the ester bond of pep-tRNA bound to the stalled ribosome. This would result in the rescue of stalled ribosomes by the release factors without involving the “drop off” of pep-tRNA from the 50S tunnel.

To examine this possibility, we tested if inactivation of RF1 or RF2 provokes oligopep-tRNA<sup>Arg4</sup> accumulation by expression of *lacZ* AGA3-6 mRNA *in vivo*. The *placZ* AGA3-6 mRNA was expressed in mutant *prfA1* (the gene for peptide chain release factor A is mutated to code for temperature sensitive RF1, RF1Ts) or *prfB2* (the gene for peptide chain release factor B is mutated to code for temperature sensitive RF2, RF2Ts). As shown in Fig. 6a and b, at the non-permissive temperature for mutant RF1 or RF2, the induced expression of *lacZ* AGA3-6 mRNA was accompanied by the accumulation of oligopep-tRNA<sup>Arg4</sup>. This is consistent with the new concept that RFs rescue the stalled ribosome with sense codon at the empty A-site and matched oligopep-tRNA<sup>Arg4</sup> at the P-site. We then examined if RF1 rescues stalled ribosomes harboring a polypep-tRNA<sup>Leu1</sup>. In the experiment shown in Fig. 6c, the plasmid, pHZAGA352-353-MR, was introduced into *pth(rap)/prfA1* double and *prfA1* single

mutants. The accumulation of incomplete  $\beta$ -gal-polypep-tRNA<sup>Leu1</sup> and the polypeptide without tRNA<sup>Leu1</sup> was detected by Western blotting under conditions that preserved the ester bond of  $\beta$ -gal-polypep-tRNA<sup>Leu1</sup>. The results revealed that the  $\beta$ -gal-polypep-tRNA<sup>Leu1</sup> (arrow 2) was present in extracts of the double mutant *pth(rap)/prfA1Ts* (lane 2 in Fig. 6c,) but not in that of the single mutant *prfA1* (lane 1). In these two lanes we observed complete  $\beta$ -gal-polypeptide (arrow 1) representing the case where ribosome did not stall at AGA352-353. The arrow 2 band represents  $\beta$ -gal-polypep-tRNA<sup>Leu1</sup> because it was converted to incomplete  $\beta$ -gal-polypeptide (arrow 3) upon treatment of the extract with Pth (lanes 3 and 4 in Fig. 6c). We conclude that RFs are involved in rescuing stalled ribosomes by hydrolysis of pep-tRNA associated with the stalled ribosome regardless of the peptide size.

## DISCUSSION

### **Various factors are involved in the rescue of stalled ribosomes with hungry codon at the A-site.**

In this paper, we show that factors Pth, RRF, RF1, RF2, RF3, and tmRNA together contribute in the rescue of stalled ribosomes caused by empty A-site. It should be emphasized that the rescue of the ribosomes at the end of termination-less mRNA (for example <sup>26</sup>) is different from the cases discussed in this paper. Stalled ribosomes were created by expressing genes carrying tandem of AGA codons alone or together with expression of the minigene, AUGAGAUAA, under reduced Pth. Stalling was detected by the accumulation of pep-tRNA and mRNA on the ribosome. Since all of the factors collaborate to reduce the stalled ribosomes, missing one factor results in the

accumulation of stalled ribosomes. We postulate three possible pathways for the disposal of the stalled ribosomes (complex A, Fig. 7). Pathway (ii) is the well accepted pathway and pathway (iii) has already been proposed for ribosomes with oligopep-tRNA<sup>37</sup> as well as for those with polypep-tRNA<sup>22,39,41</sup> though *in vitro* experiments did not support the release of polypep-tRNA by RRF and EF-G.<sup>68</sup>

### **RF1, 2 and 3 play a role in the rescue of stalled ribosomes.**

Pathway (i) shows a new concept that RF1, 2 and 3 participate in disassembling the stalled ribosome complex (Fig. 6). The empty A-site gives RF2 the opportunity to bind to the ribosome *even without the termination codon on the A-site* if the P-site is mismatched.<sup>67</sup> The P-site of stalled ribosome is not mismatched but we speculated that RFs may recognize the stalled ribosomes even with matched P-site. This speculation appears to be supported as shown in Fig. 6. In fact, some matched P-site bound pep-tRNA has been reported to be hydrolyzed, though much less extent, by RF2.<sup>67</sup> How RFs recognize the stalled ribosomes or ribosomes with mismatched P-site remains unknown at the present moment. This action of RF1 and 2 is facilitated by RF3 which recycles RF1 or 2. The fact that the loss of RF3 alone can cause ribosome stalling with polypep-tRNA<sup>Leu1</sup> (Figs. 4 and 5) suggests that recycling of RFs<sup>7,8</sup> and binding<sup>69</sup> of RF1 and RF2 stimulated by RF3, may play important roles in the rescue of stalled ribosomes. After the RFs-dependent loss of the peptidyl group that goes through the ribosomal tunnel,<sup>70,71</sup> the rest of the complex will be readily disassembled by RRF and EF-G for recycling of the ribosome, mRNA and tRNA (Fig. 7, complexes B to E).

### **The role of RRF in the rescue of stalled ribosomes with pep-tRNA.**

RRF plays a direct role in pathway (iii) and the accumulation of the stalled ribosome upon inactivation of RRF through this pathway is readily understandable. In addition, we propose that depletion of RRF causes the accumulation of the stalled ribosomes through pathway (i). When RRF is inactivated, its substrate, PoTC with the termination codon at the A-site, accumulates. Since PoTC has affinity for RF1 or 2,<sup>2</sup> the accumulated PoTC can sequester the available RF1 and RF2. Thus, depletion of RRF would have the same effect as inactivating RF1 or RF2.

### **tmRNA is not involved in the rescue of stalled ribosomes at the termination codon.**

Pathway (ii) of Fig. 7 involves tmRNA and should function for the rescue of stalled ribosomes with either oligopep-tRNA<sup>Arg4</sup> or polypep-tRNA<sup>Leu1</sup>. Transfer-mRNA plays a critical role, as has been proposed<sup>72,73</sup> for rescue of stalled ribosomes. The accumulation of pep-tRNA, due to the deletion of the gene for tmRNA, reveals the role of tmRNA in rescuing the stalled ribosomes with oligopep-tRNA<sup>Arg4</sup> (Fig. 1) and polypep-tRNA<sup>leu1</sup> (Fig. 4b). One important exception in the tmRNA rescue activity was observed with ribosomes containing a stop codon in A-site (ATGAGATAA minigene, Fig. 3 lane 5). The lack of tmRNA function in this case is perhaps physiologically important because tmRNA should not interfere with the normal termination process catalyzed by RF1 or RF2. Similar failure of tmRNA to rescue stalled ribosomes with the termination codon at the A-site has been reported.<sup>22</sup>

### **The role of Pth in the rescue of stalled ribosomes.**

In systems used in this paper, we postulate that Pth has two roles. First, Pth helps to replenish tRNA by hydrolyzing free oligopep-tRNA. This is the role we traditionally ascribed to Pth in our past publications dealing with stalled ribosomes. In this connection, we used the AUGAGAUAA minigene expression together with low level of Pth activity (*pth(rap)*) to deplete tRNA<sup>Arg4</sup>.<sup>44</sup> In the present studies, we assign a second direct role to Pth as a factor that rescues stalled ribosomes with oligopep-tRNA as shown in pathway (i). This new concept was created because one of the systems we used did not require the expression of the minigene AUGAGAUAA for induction of stalled ribosomes<sup>56</sup> (Fig. 1). In this system, when the level of any of the factors tested, including Pth, was reduced or deleted, stalling occurred (Fig. 1b). The accumulated oligopep-tRNA<sup>Arg4</sup> was found on the ribosome (Fig. 2). It is possible that in the absence of Pth, a large amount of free oligopep-tRNA<sup>Arg4</sup> may accumulate in the supernatant. Therefore, one can argue that this oligopep-tRNA<sup>Arg4</sup> may bind back to the ribosome. This possibility, however, is unlikely because RRF and EF-G are known to release mRNA actively.<sup>11,74</sup> Since RRF is present in the Pth(rap) strain, there would be no ribosome with mRNA only for the free pep-tRNA<sup>Arg4</sup> binds to. Regarding the Pth activity on the ribosome-bound pep-tRNA, it is known that Pth cleaves about 30% of the ester bonds of ribosome-bound N-acetyl-alanyl-phenylalanyl-tRNA<sup>75</sup> and the ribosome's protection of pep-tRNA from Pth is not 100%.<sup>42</sup> The new role of Pth, hydrolysis of ribosome-bound oligopep-tRNA, does not work for hydrolyzing ribosome-bound incomplete polypep-tRNA. Thus, in the experiments described in Figs. 4 and 5, the *pth(rap)* alone did not cause ribosome stalling at AGAAGA codons corresponding to

the 352<sup>nd</sup> and 353<sup>rd</sup> positions. For stalling before these codons to occur, we needed another mutation such as *frr*(Ts) (RRFTs) (Figs. 4 and 5) or *prfA1*(Ts) (RF1Ts) (Fig. 6c) in addition to *pth*(rap). Clearly, Pth is playing the role of depleting tRNA<sup>Arg4</sup> together with the minigene placed in the plasmid (Fig. 4a). We presume that Pth cannot hydrolyze ribosome-bound polypep-tRNA<sup>leu1</sup> because the nascent polypeptide bound to tRNA<sup>leu1</sup> is fully secured in the 50S tunnel, while the ester-bond of oligopep-tRNA<sup>Arg4</sup> is more exposed to the solvent and may be more accessible to Pth.

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## FIGURE LEGENDS

**Figure 1.** Oligopep-tRNA<sup>Arg4</sup> accumulates upon induction of the *lacZ* AGA3-6 mRNA in strains defective or depletion in one of the following: Pth (*pth*(rap)), RRF (*frr*Ts), RF3 ( $\Delta$ *prfC*), and tmRNA ( $\Delta$ *ssrA*). (a) *placZ* AGA3-6 construct. The boxed sequences represent ORFs. Numbers under boxes show positions of AGAs and termination codon. Italics indicate the *EcoRI* and *HindIII* sites used in the construction. The *placZ* AGA3-6 construct is under the control of P<sub>tac</sub>/O<sub>lac</sub> promoter-operator and T<sub>rmb</sub> is the

transcriptional terminator. The Shine-Dalgarno sequence is underlined. **(b)** Northern blot of tRNA<sup>Arg4</sup> and oligopep-tRNA<sup>Arg4</sup>. Cells harboring the *placZ* AGA3-6 construct (left panel) or cells co-transformed with pDC952 expressing tRNA<sup>Arg4</sup> (right panel) were grown at 32°C to 0.3 OD<sub>600</sub>. Cultures were shifted up to 42°C (non-permissive temperature for *frrTs*) for 5 min. Then, *lacZ* AGA3-6 mRNA expression was induced with 1 mM IPTG for 10 min at 42°C. Oligopep-tRNA<sup>Arg4</sup> and tRNA<sup>Arg4</sup> were isolated and detected by Northern blotting as described in Materials and Methods. Positions of free tRNA<sup>Arg4</sup> and oligopep-tRNA<sup>Arg4</sup> are indicated on the left of the panel. The lane numbers are shown on the gel. The numbers below each lane represent percentage of oligopep-tRNA<sup>Arg4</sup> of total tRNA<sup>Arg4</sup>.

**Figure 2.** Oligopep-tRNA<sup>Arg4</sup> and 5'-end *lacZ* AGA3-6 mRNA fragments are associated with stalled ribosomes in strains defective or depletion in one of the following: Pth (*pth(rap)*), RRF (*frrTs*), RF3 ( $\Delta prfC$ ), and tmRNA ( $\Delta ssrA$ ). Cells transformed with *placZ* AGA3-6 construct were grown and induced as described in the legend to Fig. 1. S-30 was prepared and treated with (+) or without (-) puromycin. S-100 (S) and ribosomal (R) fractions were analyzed for the presence of oligopep-tRNA<sup>Arg4</sup> (a) or *lacZ* AGA3-6 mRNA (b) by Northern blotting using specific [<sup>32</sup>P]-oligonucleotides (Material and Methods, Table 2). The loading controls showing ribosomal RNA after ethidium bromide staining are indicated in the lower panel. Positions of various molecular weight RNAs are indicated on the right side of the panels.



**Figure 3.** Pth, RF3, and RRF but not tmRNA rescue ribosomes stalled at UAA of minigene AUGAGAUAA. (a) pAGA minigene construct placed in various strains indicated above the lanes of Northern blot analysis (b). Plasmid features were as described in Fig. 1a. (b and c) Northern blot analysis showing Met-Arg-tRNA<sup>Arg4</sup>. AGA minigenes were introduced into strains indicated above each lane and induced as described in Fig. 1. (b) Total RNA obtained and treated with CuSO<sub>4</sub> as in Fig. 1, (c) Ribosome and supernatant fractions obtained as indicated in Fig. 2 and these fractions were treated with (+) or without (-) CuSO<sub>4</sub>.

**Figure 4.** Reduction of RRF activity, deleting RF3 or tmRNA promote the stalling of ribosomes at the middle of *lacZ* AGA352-353 mRNA: Accumulation of truncated  $\beta$ -gal-polypeptide. (a) pHZAGA352-353-MR construct. This construct encodes an N-terminal His-6 tagged *lacZ* variant carrying two AGA codons at positions 352 and 353 and a minigene AUGAGAUAA in the same operon. (b) Western blots showing complete and incomplete  $\beta$ -gal-polypeptides. Where indicated as 32/42, induction was performed for 5 min at 32°C to allow sequestering of tRNA<sup>Arg4</sup> by the AGA minigene expression. Cells were then switched to 42°C for 15 min to inactivate temperature-sensitive RRF. All other cells were grown and induced at 28 or 32°C and no temperature shift was performed. Lane 1 shows a truncated  $\beta$ -gal polypeptide generated by expressing a *lacZ* variant carrying a stop codon at position 354 (Table 1). In lane 8, the *frrT*s cells were co-transformed with pPEN907 (plasmid harboring wild type *frr* expressing RRF constitutively, Table 1). Arrow 1, complete  $\beta$ -gal; arrow 2, truncated  $\beta$ -gal; arrow 3,

intermediate size  $\beta$ -gal bands that correspond to fragments normally produced by high rate translation of *lacZ*.

**Figure 5.** The incomplete  $\beta$ -gal-polypeptide is associated with the stalled ribosome as polypep-tRNA<sup>Leu1</sup> in double mutants of *pth(rap)* with temperature sensitive RRF or missing RF3. P90C mutants harboring the plasmid pHZ352-353-MR were induced as described in Fig. 4 (32/42) and subjected to SDGC. (a) Ribosome profiles in SDGC. The numbers above the plots indicate the gradient fractions used for Tris-acetate SDS-PAGE. (b) Ribosome-bound incomplete  $\beta$ -gal-polypeptide (arrows 2 and 3) detected by Western blotting using anti-His-6 antibody. Numbers above each lane of the gel correspond to fraction numbers shown in panel A. (c) Ribosome-bound incomplete  $\beta$ -gal-polypep-tRNA<sup>Leu1</sup> (arrow 2) detected by Northern blot for tRNA<sup>Leu1</sup>. Strains are indicated above panels A, B, and C. For panel Cc, a separate gradient from panels Ca and Cb was run. Therefore, the OD profile of the SDGC (Ca) does not correspond to fraction numbers (indicated above each lane in panel Cc). Molecular weight markers are shown on the right margins in (b) and (c). The white arrows show the ribosomal peak. Arrow 1, complete  $\beta$ -gal; arrow 2, incomplete  $\beta$ -gal-polypep-tRNA<sup>Leu1</sup>; arrow 3, incomplete  $\beta$ -gal polypeptide; arrow 4, free tRNA<sup>Leu1</sup>. Transfer RNA<sup>Leu1</sup> can be seen as the tail toward the ribosomal fractions because fractions were collected from the top and some of tRNA<sup>Leu1</sup> contaminate the ribosomal fractions.

**Figure 6.** Loss of RF1 or RF2 results in accumulation of oligo- and incomplete  $\beta$ -gal-polypep-tRNAs. (a) Northern blot showing oligopep-tRNA<sup>Arg4</sup> and tRNA<sup>Arg4</sup> in US486

(*prfA1Ts*, temperature-sensitive RF1). Cells harboring *placZ* AGA3-6 construct (Fig. 1a) were grown at 32°C, treated with 1mM IPTG for 2 min (lane 2) or not (lane 1). For lanes 3 and 4 they were identically treated as lane 2 except that temperature was raised as indicated followed by the addition of IPTG. (b) As in (a) with YN2971 (*prfB2Ts*, temperature-sensitive RF2). (c) Western blot showing incomplete  $\beta$ -gal-polypeptide upon loss of RF1. Mutants P90C *prfA1Ts* (lane 1) and P90C *pth(rap)/prfA1Ts* (lanes 2-4) transformed with pHZ352-353-MR were grown and the plasmid expression was induced at 32°C and shifted to 42°C the analysis was carried out as in the Materials and Methods. Where indicated, the extract was treated with (lanes 3 and 4) or without (lanes 1 and 2) Pth. Molecular weight markers are shown in the right margin. Arrow 1, complete  $\beta$ -gal; arrow 2, incomplete  $\beta$ -gal-polypep-tRNA<sup>Leu1</sup>; arrow 3, incomplete  $\beta$ -gal-polypeptide.

**Figure 7.** Model for the possible roles of factors in the rescue of stalled ribosomes. (Complex A), stalled ribosomes with pep-tRNA at the P-site, deacylated tRNA at the E-site, and empty A-site. In pathway (i) Pep-tRNA at the P-site is hydrolyzed by the release factors or by Pth. Complex (B), Ribosomes with mRNA and tRNA at the P-site. The structure is similar to the PoTC. This is disassembled to (E), ribosomal subunits, released tRNA and mRNA. Complex (B) to (E) is carried out by the recycling system involving RRF and EF-G. Pathway (ii) should work for all stalled ribosomes except for those with the termination triplet at the A-site. Complex (A) loses the 3' portion of mRNA by the action of RelE (a specific ribonuclease for the A-site bound mRNA) to become complex (C), which does not have the 3' portion of the bound mRNA. Transfer mRNA

binds to this and the ribosome reaches the termination codon of tmRNA followed by the conventional recycling by RRF and EF-G. In pathway (iii), “drop off” of pep-tRNA from the ribosome by RRF, RF3 and EF-G leads to the hypothetical intermediate (D), which is then disassembled by RRF and EF-G. The “drop off” rates of pep-tRNA decrease as the size of peptide chain increases.<sup>76,77</sup> Complex (D) may not exist and the release of deacylated tRNA at the E-site may take place simultaneously with the release of pep-tRNA.

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