Molecular determinants of release factor 2 for ArfA-mediated ribosome rescue

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Translation termination in bacteria requires that the stop codon be recognized by release factor RF1 or RF2, leading to hydrolysis of the ester bond between the peptide and tRNA on the ribosome. As a consequence, normal termination cannot proceed if the translated mRNA lacks a stop codon. In Escherichia coli, the ribosome rescue factor ArfA releases the nascent polypeptide from the stalled ribosome with the help of RF2 in a stop codon-independent manner. Interestingly, the reaction does not proceed if RF1 is instead provided, even though the structures of RF1 and RF2 are very similar. Here, we identified the regions of RF2 required for the ArfA-dependent ribosome rescue system. Introduction of hydrophobic residues from RF2 found at the interface between RF2 and ArfA into RF1 allowed RF1 to associate with the ArfA-ribosome complex to a certain extent but failed to promote peptidyl-tRNA hydrolysis, whereas WT RF1 did not associate with the complex. We also identified the key residues required for the process after ribosome binding. Our findings provide a basis for understanding how the ArfA-ribosome complex is specifically recognized by RF2 and how RF2 undergoes a conformational change upon binding to the ArfA-ribosome complex.

At the end of protein synthesis, the ribosome encounters one of three stop codons (UAA, UAG, or UGA) on an mRNA (1–5). In bacteria, the class I release factors, RF1 and RF2, recognize the stop codon in the A-site and promote hydrolysis of peptidyl-tRNA on the ribosome. Whereas UAA codons are recognized by both RF1 and RF2, RF1 is specific for UAG and RF2 for UGA (6). Although release factors have slight differences in codon specificity, they share a similar overall structure. Each release factor is composed of four domains, with domains II and IV forming a stable compact structure to which domains I and III are connected. Genetic and biochemical studies revealed that a conserved tripeptide motif in domain II, Pro-Xaa-Thr (PXT) in RF1 or Ser-Pro-Phe (SPF) in RF2, is essential for stop codon recognition (7). Both release factors have a universally conserved Gly-Gly-Gln (GGQ) tripeptide motif in domain III that interacts with the peptidyl transferase center of the ribosome to promote peptidyl-tRNA hydrolysis (8–10). The termination process requires coordination between stop codon recognition on the 30S subunit and peptidyl-tRNA hydrolysis on the 50S subunit.

Accurate recognition of the stop codon is important for the cell because premature termination on sense codons may lead to accumulation of truncated proteins. During translation elongation, high-fidelity tRNA discrimination is achieved by two consecutive selection steps, initial selection and proofreading. Whereas the former step occurs without energy consumption, the latter step includes GTP hydrolysis by EF-Tu to prevent incorporation of an incorrect amino acid into the nascent polypeptide (11). On the other hand, recognition of the stop codon by RF1 or RF2 is achieved by a one-step process without energy consumption (12). RF3, the only GTPase in the termination process, induces rotation of the small subunit to facilitate dissociation of class 1 RF from the ribosome after hydrolysis of peptidyl-tRNA but is not involved in stop codon recognition (13–15). According to the “tripetide anticodon” model, the conserved tripeptide, PXT in RF1 or SPF in RF2, determines stop codon specificity. Structural and computational studies revealed that the stop codon is recognized by a complex network between RF, tRNA, and water molecules rather than simply by the tripeptide motif (16–21).

When the ribosome is trapped by truncated mRNA lacking a stop codon, the ribosome stalls at the 3’ end of mRNA, as neither RF1 nor RF2 functions. In bacteria, tmRNA/SmpB relieves the stalled ribosome and adds a degradation tag to the nascent polypeptide through a process called trans-translation (22). In trans-translation, tmRNA receives the nascent polypeptide from peptidyl-tRNA and provides an internal stop codon to terminate the protein synthesis. Trans-translation is a sophisticated system combining ribosome rescue system with nascent polypeptide quality control mechanism. Although tmRNA is universally conserved in bacteria, it is dispensable for the viability of some species, including Escherichia coli, suggesting the presence of other ribosome rescue systems (23).

Recently, it was reported that alternative ribosome rescue factors, ArfA and ArfB (YaeJ), are involved in peptidyl-tRNA hydrolysis of stalled ribosomes (24–26). Unlike ArfB, ArfA itself cannot promote peptide release due to the absence of the catalytic GGQ motif, and instead it requires RF2, but not RF1, for ribosome rescue (27, 28). In our previous work, we found that ArfA binds to the 70S ribosome but not to RF2 and that RF2 binds to the ArfA-70S ribosome complex, indicating that ArfA binds to the 70S ribosome prior to RF2 binding rather than in complex with each other (29). Recent cryo-EM structures revealed that the N-terminal region of ArfA is sandwiched between the decoding center of the 30S subunit and the

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switch loop of RF2, whereas its C-terminal region extends toward the mRNA entry channel on the small subunit (30–34). However, it remains unclear how RF2 but not RF1 recognizes the ArfA-ribosome complex in the absence of a stop codon in the A-site.

In this study, we identified the residues in RF2 required for ribosome binding and peptidyl-tRNA hydrolisis (PTH) activity. Our results suggest that RF2 recognizes the ArfA-ribosome complex through hydrophobic interactions and β-sheet formation between RF2 and ArfA and undergoes productive conformational change to allow hydrolisis of peptidyl-tRNA in the P-site.

Results

Swapping residues between RF1 and RF2 by site-directed mutagenesis

Although RF1 and RF2 share 36% amino acid sequence identity in E. coli (Fig. 1A) and similar function in translation termination, only RF2 plays a distinct role in the ArfA-mediated ribosome rescue system. To elucidate the specificity determinant of RF2, we designed several kinds of swapping mutants and tested their PTH activities in vitro. Given that the binding site of RF2 on the ribosome is in close proximity to that of ArfA, we focused on the residues at the interface between RF2 and ArfA. Recent cryo-EM structures of ArfA-RF2-stalled ribosome (nonstop complex) have revealed that ArfA forms the interface with the switch loop (residues 319–321, E. coli RF2 numbering) and β-sheet (residues 213–224) of RF2 via hydrophobic interactions (31). A structural comparison of RF2 between the nonstop complex and canonical termination complex showed the different conformation of switch loop (Fig. 1B) (35). In canonical termination, the switch loop extends to the decoding center, allowing the interaction of RF2 (Trp-319) with 16S rRNA (A1492). In contrast, in the nonstop complex, the switch loop interacts with ArfA rather than 16S rRNA due to occupation of the decoding center by ArfA. Trp-319 in the switch loop of RF2 forms a hydrophobic pocket with Phe-217 of β5 and the central portion of ArfA (Fig. 1, C and D). Introduction of mutations in the hydrophobic residues of RF2 or ArfA decreases PTH activity in vitro (31). Therefore, we hypothesized that RF2 preference in ArfA-mediated ribosome rescue is derived from these hydrophobic interactions between RF2 and ArfA.

Effects of mutations on PTH activity and ribosome binding

To verify this hypothesis, we designed swapping mutants and assessed their in vitro PTH activities as we have reported previously (29, 31). Stalled ribosomes programmed with an mRNA encompassing the Shine–Dalgarno sequence to the P-site AUG codon and N-formyl-[3H]Met-tRNA in the P-site were prepared, and they were reacted with the ArfA and RF mutants. Using this system, we confirmed that RF2 WT has ArfA-dependent PTH activity, whereas RF1 does not (Fig. 2A). Then we swapped the residues of the switch loop and/or β5 of RF1 for the corresponding residues in RF2. We found that all three variants—“RF1 switch loop” in which the switch loop of RF1 was replaced by the corresponding residues of RF2, “RF1 A200F” in which Ala-200 in β5 of RF1 was replaced by the corresponding residue Phe-217 in RF2, and “RF1 switch loop/A200F”—failed to promote ArfA-dependent PTH activity (Fig. 2A), whereas they had stop codon-dependent PTH activity comparable with WT RF1 (Fig. 2B). In contrast, introduction of the switch loop and/or β5 of RF1 into RF2 (RF2 switch loop, RF2 F217A, and RF2 switch loop/F217A) had effects on neither ArfA—nor stop codon—dependent PTH activity (Fig. 2, A and B).

To test the effects of these mutations on ribosome binding, we performed filter-binding assay. As reported previously, the ribosome-bound RF was analyzed by centrifugal ultrafiltration followed by SDS-PAGE (29). Interestingly, RF1 switch loop, RF1 A200F, and RF1 switch loop/A200F bound to the ArfA-ribosome complex to a certain extent, whereas WT RF1 did not (Fig. 2C). To compare the affinities of RF mutants, ArfA-ribosome complex was incubated with various concentrations of RF (Fig. 2D). The $K_d$ value of RF2 WT was found to be 105 nM. This is in line with the cellular concentration of RF2 (>1 μM) (36). We observed the difference in ribosome binding between WT RF1 and RF1 switch loop/A200F, although we could not determine accurate $K_d$ values due to its low affinity. Given that RF1 switch loop/A200F required a >10-fold higher concentration of RF for complex formation than RF2 WT, the $K_d$ value of RF1 switch loop/A200F should be higher than 1 μM (Fig. 2E). On the other hand, RF1 switch loop/A200F exhibited stop codon—dependent PTH activity and ribosome binding comparable with WT RF1 and RF2 (Fig. 2, B and C), suggesting that the switch loop and β5 have little effect on canonical termination. Taken together, these residues of RF2 contribute to ribosome binding to a certain extent, but they are not sufficient for PTH activity in ArfA-mediated rescue.

Domain-swapping experiments

To explore the regions of RF2 required for ribosome rescue, we performed domain-swapping experiments. As RF1 consists of four domains, 1–106 (domain I), 107–208 (domain II), 209–300 (domain III), and 301–360 (domain IV) (37), we designed all possible combinations of domain-swapping RF1 (Fig. 3A). Note that all mutants have switch loop and A200F mutations that are derived from the RF2 sequence. Mutants with combinations of “domain I of RF1” and “domain II of RF2” were not available due to lack of expression of proteins, in agreement with a previous study (28). The mutant with replacement of residues 1–106 (domain I) of RF1 with the corresponding residues of RF2 is called “RF1 1–106”. Interestingly, RF1 1–300 (domain I/II/III) and RF1 1–208, 301–360 (domain I/II/IV) exhibited ArfA-dependent PTH activities (Fig. 3B) as well as stop codon—dependent PTH activities (Fig. 3C) comparable with RF2 WT. We found that RF1 1–208 (domain I/II) had a moderate ArfA-dependent PTH activity, whereas its stop codon—dependent activity was comparable with WT RF1. Conversely, all mutants with RF1 sequences of either domain I or II exhibited defects in ArfA-dependent PTH activity. Taken together, these results indicated that both residues 1–106 (domain I) and 107–208 (domain II) of RF2 are essential for ArfA-dependent PTH activity and that 209–300 (domain III) or 301–360 (domain IV) is supplemental.
To specify the regions in domains I and II of RF2 required for ribosome rescue, we swapped a segment(s), which is a smaller structural unit than the domain, using RF1: 1–208 and 301–360 variant (domain I/II/IV). As domain I of RF1 is composed of three α-helices, we divided domain I into three segments, 1–32, 33–72, and 73–106, and designed hybrid RF containing all six possible combination sets of segments (Fig. 4A). We found that all mutants possessing segment 73–106 derived from RF2 exhibited both ArfA-dependent (Fig. 4B) and stop codon–dependent PTH activity (Fig. 4C). On the other hand, mutants with 73–106 derived from RF1 were not available due to low protein expression level. These results raise the possibility that domain II may require the α-helix (α3) of domain I for stability of RF2.

Unlike domain I, domain II is more complex, as it contains many inter- and intradomain interactions. Domains II and IV form a compact structure with four α-helices and an eight-stranded β-sheet. These interactions make it difficult to replace domain II of RF1 with that of RF2. Therefore, we examined which region of domain II in RF2 is dispensable for PTH activity. Domain II in RF2 can be divided into six segments (125–139, 140–160, 161–178, 179–196, 197–213, and 214–225, RF2).

Figure 1. Sequence alignment of RF1 and RF2 and their structural comparison. A, alignments are colored as follows: blue, positively charged amino acids (Lys and Arg); red, negatively charged amino acids (Asp and Glu); green, hydrophobic amino acids (Ala, Phe, Ile, Leu, Met, and Val). Conserved residues between RF1 and RF2 are marked by vertical arrows. The positions of the tripeptide anticodon (PAT in RF1 and SPF in RF2), GGQ motif, Ala-200/Phe-217, and switch loop are marked with asterisks. B, structural comparison of RF2 in the canonical termination complex (red; Protein Data Bank entry 6OG7) and nonstop complex (green; Protein Data Bank entry SHSU). The switch loop of RF2 in the nonstop complex is colored yellow. C, structure of ArfA and RF2 in nonstop complex. ArfA and RF2 are colored purple and green, respectively. D, close-up view of the interface between ArfA and RF2. The residues of Phe-217 in domain II and the switch loop in domain IV of RF2 are shown in stick representation and colored blue.
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numbering), and these segments were replaced by the corresponding residues in RF1. When residues 140–160 of RF2 were replaced by the corresponding residues of RF1, we could not obtain soluble protein. We found that swapping 179–196 or 197–213 reduced ArfA-mediated PTH activities as well as stop codon–dependent PTH activities (Fig. 5, A and B). Given that residues 140–160 forming a long α-helix (α5) are surrounded by an eight-stranded β-sheet including residues 179–196 and 197–213, the helix may be stabilized by intradomain interactions. We observed no defects in PTH activity of RF2 214–225, although residues 214–225 (β5) form an antiparallel β-sheet with β4 of RF2 and β1 of ArfA.

Requirement of the residues in domain II of RF2

To identify the residues in these three segments (140–160, 179–196, and 197–213) required for PTH activity that are not conserved between RF1 and RF2, we introduced mutations and measured the initial velocity of PTH activity. We found that RF2 142–147 (α5), 179–181 (β3), V198G (β4), and R213I (β5) mutations affected both ArfA-dependent (Fig. 6A) and stop codon–dependent PTH activities (Fig. 6B). The reduced PTH activity of RF2 V198G is consistent with cryo-EM structures showing that the side chain of Val-198 forms hydrophobic interactions with those of Phe-217 (β5) and Trp-319 (switch loop) of RF2 and those of Ile-16, Leu-20, and Phe-25 of ArfA. When we introduced valine into the corresponding residue (Gly-181) in RF1 to obtain RF1 G181V/A200F/switch loop mutants, soluble protein was not available. We found that R213I affected PTH activity, in agreement with a previous biochemical study (38). The initial velocity of ArfA-dependent PTH activity plotted against RF2 concentrations generated hyperbolic curves that fit the Michaelis–Menten equation (Fig. 6C). The $K_m$ values of RF2 142–147 and RF2 179–181 were $6.1 \pm 0.9 \times 10^{-6}$ and $9.8 \pm 3 \times 10^{-6}$ s$^{-1}$, respectively, whereas that of RF2 WT was $0.49 \pm 0.07 \times 10^{-6}$ s$^{-1}$ (Table 1). The $k_{cat}$ values of these mutants were $0.056 \pm 0.003$ and $0.050 \pm 0.01$ s$^{-1}$, respectively, comparable with that of RF2 WT (0.037 ± 0.005 s$^{-1}$). According to the cryo-EM structures, the α5 helix, including residues 142–147, is surrounded by the β-sheet. The β3-strand (including residues 179–181) is sandwiched between β1 and β2. Notably, the side chain of Trp-144 can form hydrogen bonds with the backbone carbonyl oxygen atom in His-199 (β4). Therefore, partial replacement of residues 142–147 or 179–181 may affect the β-sheet or surrounding structure, resulting in reduction of ribosome binding activity. On the other hand, RF2 V198G and R213I showed 7- and 3-fold higher $K_m$ (3.4 ± 1 × 10$^{-6}$ and 1.5 ± 0.5 × 10$^{-6}$ M, respectively) than RF2 WT.

Role of conserved histidine residues in RF2

According to molecular dynamics simulation, the distance between SPF and GGQ motif is affected by the charge state of conserved histidine residues (39). There are five conserved histidine residues in RF2, two in domain II and three in domain III, and we tested the roles of these histidine residues by replacing each with alanine. We found that H199A, H253A, H265A, and H281A exhibited significant reductions in PTH activity (Fig. 6, A and B). We also determined the Michaelis–Menten parameters for RF variants (Fig. 6C). The $K_m$ values of H199A and H265A were $5.4 \pm 0.8 \times 10^{-6}$ and $2.8 \pm 0.4 \times 10^{-6}$ M, respectively. The $k_{cat}$ values of these mutants were comparable with WT. The higher $K_m$ and similar $k_{cat}$ values of H199A and H265A indicate significant defects in ribosome binding (Table 1). On the other hand, H253A and H281A showed lower $k_{cat}$ values ($0.0031 \pm 0.00007$ and $0.0078 \pm 0.008$ s$^{-1}$, respectively) than RF2 WT (0.037 ± 0.005 s$^{-1}$), although the $K_m$ values of these mutants were comparable with RF2 WT. The fact that H253A and H281A affect PTH activity but not ribosome binding suggests that these histidine residues play a role in the step after ribosome binding.

Discussion

To elucidate the specificity determinant of RF2 in ArfA-mediated ribosome rescue, we designed RF1 variants in which residues were swapped with those of RF2 and tested their peptide release and ribosome binding activities in vitro. Our previous cryo-EM analysis showed that RF2 establishes a large interaction interface with the N-terminal region of ArfA mainly via hydrophobic interactions (31). Unlike RF2, RF1 seems to be incapable of such hydrophobic interactions with ArfA. Biochemical studies showed that mutations in ArfA or RF2 at the interface decreased in vitro peptide release activity (31, 33). These results prompted us to examine whether these interactions serve as a major determinant of RF2 in the ArfA-mediated ribosome rescue system. Contrary to our expectations, introduction of hydrophobic residues at the switch loop and/or β5 of RF2 into RF1 failed to promote PTH activity, although the swapped mutant supported ribosome binding to a certain extent. These data indicated that a different region(s) of RF2 is required for efficient PTH activity.

Domain-swapping experiments revealed that domain II of RF2 is a prerequisite for ArfA-mediated ribosome rescue (Fig. 3A). Additional mutation studies identified the residues of domain II required for ribosome binding and PTH activity. Some of the effects of mutations are consistent with the cryo-EM structures. Based on our results, we propose a model in which ArfA functions as a scaffold protein forming a multiple interaction network.
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Figure 3. Activity of in vitro peptidyl-tRNA hydrolysis for domain-swapping RF mutant. A, structure of E. coli RF1. Domain I is shown in red, domain II is in yellow, and domain IV is in blue. B and C, ArfA-dependent (B) and UAA stop codon–dependent (C) PTH activities. Open and closed boxes represent RF1 and RF2 segments, respectively. NA, not available due to low expression of soluble protein. Means with S.D. (error bars) of at least three independent experiments are shown.

Figure 4. Activity of in vitro peptidyl-tRNA hydrolysis for hybrid RF containing a different part(s) of domain I of RF2. A, structure of E. coli RF1. Residues 1–32 are shown in red, residues 33–72 are in yellow, and residues 73–106 are in green. All constructs consist of domains II and IV of RF2 and domain III of RF1. B and C, ArfA-dependent (B) and UAA stop codon–dependent (C) PTH activities. Open and closed boxes represent RF1 and RF2 segments, respectively. NA, not available due to low expression of soluble protein. Means with S.D. (error bars) of at least three independent experiments are shown.

to recruit RF2 to the stalled ribosome (Fig. 7A). RF2 recognizes ArfA by two types of interaction (i.e. hydrophobic interactions and main-chain hydrogen bonding to form β-sheet). As each β-strand is stabilized by intra- and interdomain interactions (Fig. 7B), partial introduction of RF2 residues into RF1 may be insufficient.
According to earlier structural studies, the structure of free (ribosome-unbound) RF2, which adopts a closed conformation, is different from that of ribosome-bound RF2, which has an open conformation (8, 9, 16, 17, 19, 20, 37, 40). In the closed conformation, the distance between SPF and GGQ motifs is only 23 Å, much shorter than 73 Å, which would be necessary to span the distance between the decoding center and the peptidyl transferase center. Kinetics studies indicated that RF undergoes a conformational change from the closed to open conformation upon stop codon recognition (41, 42). Recently, X-ray crystallography and time-resolved cryo-EM captured the preaccommodation complex with the closed form of RF at a very early step of the termination process (43, 44). Similar conformational transition was observed in the ArfA-RF2–mediated ribosome rescue system (30, 32). However, the molecular mechanism by which ribosome binding of RF induces a conformational change of RF remains unclear. Our results suggest that the conserved histidine residues (His-253 and His-281) of RF2 play a role in the step after ribosome binding in ArfA-mediated ribosome rescue. Given that the charge state of the side chain of histidine is changed by environmental conditions, the histidine protonation may induce a conformational transition of RF2 from a closed to open conformation that allows proper positioning of the catalytic GGQ motif in the peptidyl transferase center. This is in agreement with a previous molecular dynamics simulation, in which protonation of histidine residues was shown to affect the RF2 conformation (39). Taking this into consideration, it makes sense that a closed form of RF2 was observed in ArfA A18T (loss-of-function) nonstop complex with the GGQ loop of RF2 (residues 248–257, including His-253) disordered (30). When the closed form of RF2 binds to the ArfA-ribosome complex, proper positioning of the histidine residues in the ribosome surrounded by the overall negatively charged RNA backbone induces histidine protonation of RF2, enabling dynamic conformational change. A similar compact conformation of RF2 was visualized in a nonstop complex using Thermus thermophilus RF2 instead of E. coli RF2. However, the histidine residues at 253 and 281 are not conserved in T. thermophilus RF2 (31), suggesting that the driving force of the conformational change may be different. In E. coli, these two histidine residues are conserved in RF1 and RF2, suggesting that canonical termination and ArfA-mediated termination have a common mechanism of the conformational change of RF. A recent cryo-EM study revealed an intermediate structure, in which the catalytic GGQ loop is rearranged into the extended β-hairpin structure to plug the peptide tunnel after hydrolysis of peptidyl-tRNA (35), suggesting that local rearrangement of catalytic domain III promotes efficient release of the polypeptide.

Figure 5. Activity of in vitro peptidyl-tRNA hydrolysis for RF2 mutant containing different part(s) of domain II of RF1. A and B, ArfA-dependent (A) and UAA stop codon–dependent (B) PTH activities. Open and closed boxes represent RF1 and RF2 segments, respectively. NA, not available due to low expression of soluble protein. Means with S.D. (error bars) of at least three independent experiments are shown.
Recently, an alternative ribosome rescue factor, named arfT, was found in the human pathogen Francisella tularensis as the gene showing synthetic lethality with ssrA coding for tmRNA (45). Interestingly, F. tularensis ArfT can promote hydrolysis of peptidyl-tRNA in combination with either F. tularensis RF1 or RF2. Whereas the primary sequences of RF are conserved across species, those of ribosome rescue factors vary between species. Presumably, the sequences of ribosome rescue factors have been optimized and coevolved with the evolutionarily ancient release factors to deal with the stalled ribosome in each species. The observations that ArfA activates E. coli RF2 but not Thermus thermophilus RF2 and that ArfT activates F. tularensis RF1/2 but not E. coli RF1/2 indicate that interactions between rescue factors and release factors are not interchangeable across species. This is consistent with another recently discovered ribosome rescue factor, BrfA, found in Gram-positive Bacillus subtilis, which has a different amino acid sequence from E. coli or F. tularensis and activates B. subtilis RF2 but not E. coli RF2 (46).

In summary, our biochemical study provided a basis for understanding how RF2, but not RF1, recognizes the ArfA-ribosome complex and triggers its conformational change to achieve stop codon–independent translation termination.

Table 1

| RF2 Mutant | $K_m$ (M) | $k_{cat}$ (s$^{-1}$) |
|------------|------------|----------------------|
| RF2 WT     | $10^{-6}$  | 0.037 ± 0.005        |
| RF2 142–147| 0.49 ± 0.07| 0.056 ± 0.003        |
| RF2 179–181| 9.8 ± 3    | 0.049 ± 0.01         |
| RF2 V198G  | 3.4 ± 1    | 2.030 ± 0.004        |
| RF2 R213I  | 1.5 ± 0.5  | 0.024 ± 0.004        |
| RF2 H199A  | 5.4 ± 0.8  | 0.012 ± 0.0001       |
| RF2 H214A  | 0.79 ± 0.2 | 0.032 ± 0.003        |
| RF2 H253A  | 0.36 ± 0.07| 0.0031 ± 0.00007     |
| RF2 H265A  | 2.8 ± 0.4  | 0.019 ± 0.005        |
| RF2 H281A  | 0.32 ± 0.02| 0.0078 ± 0.0008      |

Figure 6. Initial velocities of in vitro peptidyl-tRNA hydrolysis for hybrid RF containing a different part(s) of domain II of RF2. A and B, ArfA-dependent (A) and UAA stop codon–dependent (B) PTH activities. Open and closed boxes represent RF1 and RF2 segments, respectively. Means with S.D. (error bars) of three independent experiments are shown. C, data were fitted to the Michaelis–Menten equation.
**Experimental procedures**

**Preparation of RF mutants, ribosomes, and tRNA**

Mutations were introduced into a plasmid for overproduction of His-tagged RF1 or RF2 from *E. coli*. RF variants were prepared as described previously (29). 70S ribosomes were prepared from *E. coli* A19 as described (29). tRNA<sub>fMet</sub> was purchased from Sigma–Aldrich. N-Formyl-[<sup>3</sup>H]Met-tRNA<sub>fMet</sub> was prepared as described (29), except for the following modification: tRNA<sub>fMet</sub> was charged with [<sup>3</sup>H]Met and [<sup>14</sup>C]Met (molar ratio 1:19).

**Peptidyl-tRNA hydrolysis assay**

The stalled ribosome was formed by incubating 10 pmol of ribosomes, 50 pmol of mRNA (AAGGAGUAAAAUG), and 10 pmol of N-formyl-[<sup>3</sup>H]Met-tRNA<sub>fMet</sub> in 20 μl of buffer A at 37 °C for 10 min. Buffer A contained 80 mM Tris-HCl (pH 7.8), 7 mM MgCl₂, 150 mM NH₄Cl, 2.5 mM DTT, and 2 mM spermidine. After incubation, the stalled ribosome mixture was incubated with 5 μl of 20 pmol of ArfA and 100 pmol of RF (RF1 or RF2) in buffer A. At specified time points, reaction was quenched in 150 μl of 0.1 M HCl. Hydrolyzed N-formyl-[<sup>3</sup>H]Met was extracted with 300 μl of ethyl acetate, and 200 μl of the ethyl acetate layer was spotted onto glass fiber filters (Advantec). Radioactivity on the filter was measured using a liquid scintillation counter. Data were fit to the Michaelis–Menten equation in GraphPad Prism5.

**Pretermination complex was formed by incubating 10 pmol of ribosomes, 50 pmol of mRNA (AAGGAGUAAAAU-GUAUACAAAAAAAGAAAAAA), 10 pmol of N-formyl-[<sup>3</sup>H]Met-tRNA<sub>fMet</sub>, 20 pmol of IF1, 20 pmol of IF2, 20 pmol of IF3, and 0.2 mM GTP in 20 μl of buffer A at 37 °C for 10 min. After incubation, pretermination complex was incubated with 5 μl of 100 pmol of RF (RF1 or RF2) and 0.2 mM GTP in buffer A. Hydrolyzed N-formyl-[<sup>3</sup>H]Met was quantified in the same way as described for the ArfA-mediated peptidyl-tRNA hydrolysis assay.

**Ultrafiltration assay**

A mixture of 20 pmol of 70S ribosome, 100 pmol of mRNA and 100 pmol of tRNA<sub>fMet</sub> was incubated in 40 μl of buffer A at
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37 °C for 10 min. After incubation, 20 pmol of ArfA and 100 pmol of RF (RF1 or RF2) in 10 µl of buffer A were mixed with the ribosome mixture and incubated at 37 °C for 10 min. Unbound ArfA and RF were removed by centrifugal ultrafiltration using Amicon Ultra (100 kDa). The ribosome fraction was subjected to SDS-PAGE and visualized by Oriole Fluorescent Gel Stain (Bio-Rad). Data were fit to an equation in GraphPad Prism5 to determine the $K_d$ values.

Data availability
All data are included in the article.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: RF, release factor; tmRNA, transfer-messenger RNA; PTH, peptidyl-tRNA hydrolysis.

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