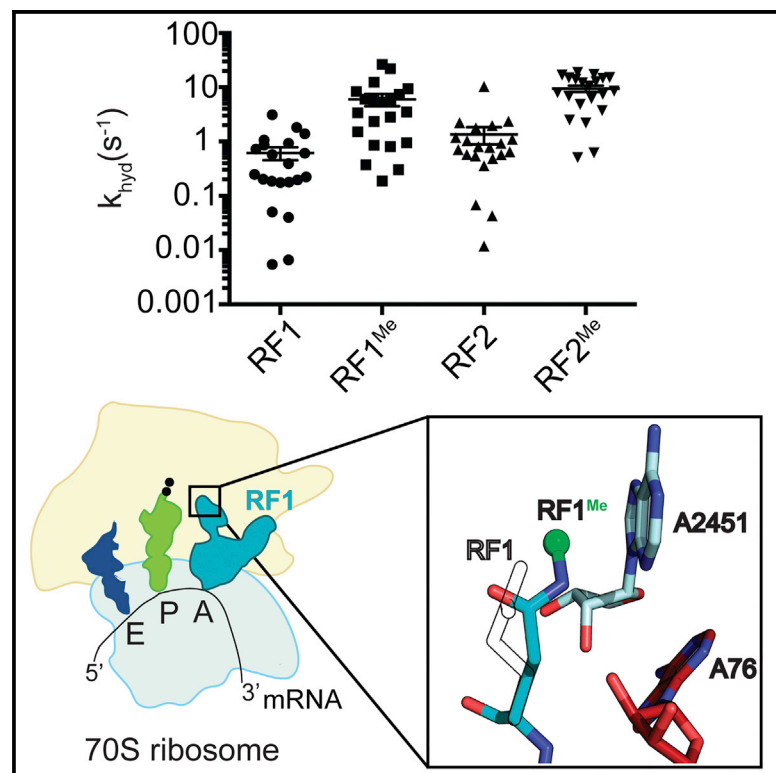


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Uniformity of Peptide Release Is Maintained by Methylation of Release Factors

Graphical Abstract



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In Brief

Methylation of release factors is a universal process found in all organisms. Pierson et al. show that the modification substantially increases rates of peptide release on a subset of amino acids that are otherwise slow. Structures of the methylated factors show repositioning of the active-site glutamine in the peptidyl-transferase center.

Highlights

- Efficiency of peptide release is dependent on the C-terminal amino acid identity
- Methylation of release factor has disparate effect, depending on the substrate
- Structures show methylation to reposition the glutamine in the active site

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Uniformity of Peptide Release Is Maintained by Methylation of Release Factors

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SUMMARY

Termination of protein synthesis on the ribosome is catalyzed by release factors (RFs), which share a conserved glycine-glycine-glutamine (GGQ) motif. The glutamine residue is methylated *in vivo*, but a mechanistic understanding of its contribution to hydrolysis is lacking. Here, we show that the modification, apart from increasing the overall rate of termination on all dipeptides, substantially increases the rate of peptide release on a subset of amino acids. In the presence of unmethylated RFs, we measure rates of hydrolysis that are exceptionally slow on proline and glycine residues and approximately two orders of magnitude faster in the presence of the methylated factors. Structures of 70S ribosomes bound to methylated RF1 and RF2 reveal that the glutamine side-chain methylation packs against 23S rRNA nucleotide 2451, stabilizing the GGQ motif and placing the side-chain amide of the glutamine toward tRNA. These data provide a framework for understanding how release factor modifications impact termination.

INTRODUCTION

Protein synthesis is an intricate process that involves many coordinated events on the ribosome, ensuring that the genetic code is decoded accurately and efficiently. Termination of protein synthesis occurs when one of three nearly universally conserved stop codons (UAA, UGA, and UAG) enters the A site of the ribosome. Stop codons are recognized by protein factors called release factors (RFs), which mediate the hydrolytic reaction in the peptidyl transferase center (PTC) of the ribosome. Unlike eukaryotes and archaea, which have one factor (eRF1 and aRF1, respectively) that recognizes all stop codons (Konecki et al., 1977), bacteria have two very similar factors with overlapping

specificities (Scolnick et al., 1968). RF1 recognizes UAG and UAA, whereas RF2 recognizes UGA and UAA.

Despite the conserved nature of the translation machinery in all domains of life, bacterial and eukaryotic RFs are unrelated and instead appear to have evolved independently (Frolova et al., 1994). The factors, however, share a universally conserved sequence motif of Gly-Gly-Gln (GGQ) (Frolova et al., 1999). This motif is critical for the hydrolytic reaction (Frolova et al., 1999), and early low-resolution structures of termination complexes revealed that the motif engages the PTC of the ribosome (Petry et al., 2005; Rawat et al., 2003). Mutation of either glycine to any other amino acid (aa) completely inhibits activity, whereas certain substitutions at the glutamine residue are only partially defective for promoting catalysis (Frolova et al., 1999; Shaw and Green, 2007). The ribosome also plays a critical role during the reaction. This is best highlighted by early observations showing that binding of a cognate deacylated tRNA to the A site accelerates the hydrolysis of the peptidyl-tRNA (Vogel et al., 1969). Interestingly, in the presence of a deacylated tRNA, the specificity for water as a nucleophile is compromised akin to what has been observed for the RF glutamine mutants (Shaw and Green, 2007). These findings suggest that RFs contribute largely to catalysis through inducing conformational rearrangements in the PTC that expose the ester carbon for the nucleophilic attack. Consistent with this proposal, mutations of the so-called inner shell nucleotides of the PTC as well as the 2'-OH of the terminal adenosine of the peptidyl tRNA are detrimental for RF-mediated release, but not for peptidyl transfer (Pavlov et al., 2009; Polacek et al., 2003; Youngman et al., 2004; Zaher et al., 2011).

Crystal structures of post-termination complexes revealed important clues about the mechanism of the reaction. The glycine residues in the GGQ motif adopt a conformation in the PTC that is incompatible with any other amino acid rationalizing earlier mutational studies (Jin et al., 2010; Korostelev et al., 2008; Korostelev et al., 2010; Laurberg et al., 2008; Weixlbaumer et al., 2008; Zhou et al., 2012). In contrast, the role of the glutamine in catalysis has been a source of considerable debate. One group argued that the main-chain amide of the glutamine is involved in coordinating a nucleophilic water molecule (Laurberg et al.,



2008). Another group argued for both the main-chain amide and the side-chain carbonyl oxygen playing a critical role in coordinating the hydrolytic water (Weixlbaumer et al., 2008). The conserved nature of the residue adds support to the side chain of glutamine contributing in some way to the mechanism.

It is worth noting that, although the 3.0–3.5 Å 70S-RF structures are at resolutions typically sufficient to observe interpretable electron density of protein side chains, there is little to no electron density surrounding the unmethylated glutamine residue in all structures but the pre-termination 70S-RF2 complex and the recent post-termination 70S-*E. coli* RF1 (Figure S1; Jin et al., 2010; Korostelev et al., 2008; Korostelev et al., 2010; Lauberg et al., 2008; Weixlbaumer et al., 2008; Zhou et al., 2012). Therefore, it is still ambiguous whether the glutamine side-chain carbonyl or amide is directed toward the 3'-OH of the substrate tRNA A76, where it could potentially coordinate the catalytic water molecule (Shaw and Green, 2007). Recent kinetic analyses examining the pK_a of the termination reaction suggested instead that a hydroxide ion could participate in the reaction, with the glutamine side-chain amide group donating a proton to the hydroxide to stabilize the reaction (Indrisiunaite et al., 2015).

The glutamine residue of the GGQ motif in bacterial and eukaryotic RFs is N⁵ methylated. In *E. coli*, RFs are methylated by prnC (Heurgué-Hamard et al., 2002). The methylation is required for optimal growth in certain minimal media (Mora et al., 2007), and in vitro, the methylation contributes modestly to the rate of release by RF2 (Dinçbas-Renqvist et al., 2000). Therefore, the coordination of water in the active site of the ribosome could be further stabilized by the methylation. Indeed, molecular dynamics studies suggest that the methylation removes the side-chain amide of the glutamine from the active site and helps in orienting the carbonyl oxygen toward the active site to coordinate the catalytic water (Trobro and Aqvist, 2007, 2009). However, beyond these models, a mechanistic understanding for the role of the modification during peptide release is missing.

Here, we explore the effects of methylation on the efficiency of peptide release using a well-defined bacterial reconstituted translation system. We measured the rates of peptide release on all 20 common amino acids with unmethylated and methylated RFs. Whereas the modification was found to increase the rate of release on all amino acids, this enhancement varies significantly, depending on the identity of the amino acid. We report four structures of unmethylated and methylated *E. coli* RF1 or RF2 in complex with the *Thermus thermophilus* (*Tth*) ribosome. In all structures, the placement of the glutamine backbone amide is within hydrogen bonding distance to the 3'-OH of the terminal A76 of the P-site tRNA. However, in the 70S-RF1^{Me} post-termination structure, the side-chain amide packs against A2451 of 23S rRNA and is proximal to the tRNA, both suggesting an unexpected role of the side-chain amide in stabilizing the GGQ motif.

RESULTS

Experimental Approach

To explore the full contribution of the methylation of RFs on peptide release, we took advantage of our in vitro bacterial reconstituted system to generate dipeptidyl-tRNA ribosomal nascent chains (DRNCs) programmed with a UAA stop codon in the A

site (Figure S2A). Twenty initiation complexes (ICs) were generated by incubating 70S ribosomes with 20 mRNAs that had the following coding sequence (AUG NNN UAA) in the presence of f[³⁵S]-Met-tRNA^{fMet}; initiation factors 1, 2, and 3; and GTP. We note that the NNN sequence coded for the most-abundant *E. coli* codon for each amino acid. DRNCs were generated by incubating the initiation complexes with their corresponding ternary complexes composed of the appropriate aa-tRNA, elongation factor Tu (EF-Tu), and GTP in the presence of elongation factor G (EF-G). Following peptide-bond formation and EF-G-catalyzed translocation, the complexes were purified over sucrose cushions to remove unincorporated factors. We assessed the yield of the peptidyl-transfer reaction using an electrophoretic TLC system to follow the release reaction (Youngman et al., 2004; Figure S2B).

Uncatalyzed Hydrolysis of Dipeptidyl tRNAs On and Off the Ribosome

Previous experiments showed that the efficiency of nonsense suppression in *E. coli* is dependent on the amino acid prior to the stop codon (ultimate amino acid; Björnsson et al., 1996), suggesting that RF-mediated release is influenced by the identity of the C-terminal amino acid. Before exploring this hypothesis in our in vitro system, we sought to determine the rates of nonenzymatic release on all 20 dipeptidyl-tRNAs to assess whether any differences observed are due to inherent chemical differences among amino acids. Nonenzymatic hydrolysis and aminolysis off the ribosome were initiated by diluting the complexes in the presence of EDTA, which facilitates splitting of the ribosomal subunits and the dissociation of the dipeptidyl-tRNA. To increase the rate of release, the pH of the dilution buffer was increased to 8.0 using Tris. Fortuitously, in these assays, Tris serves as an analog for peptide bond formation, because similar to the α -amine group of aa-tRNA, its amine group attacks the peptidyl-tRNA bond to form an amide bond (Figures 1A and 1B). The rates we observed for hydrolysis and aminolysis (Figures 1C and 1D) were generally similar to one another for the same dipeptides, with one exception. Aminolysis rates were higher than hydrolysis rates for the fMet-Trp dipeptide. Apart from fMet-Ile, fMet-Lys, fMet-Pro, fMet-Arg, fMet-Thr, and fMet-Val, which displayed overall reduced rates, the rates of hydrolysis/aminolysis were overall similar among the 20 dipeptides. The reduced rate for fMet-Pro can be easily rationalized given the unique structure of proline. Similarly, it appears that having a positive side chain stabilizes the ester bond of the dipeptidyl tRNA for lysine and arginine. The remaining fMet-Ile, fMet-Thr, and fMet-Val all share the common feature of having a branched β -carbon, but beyond that, no discernible similarities are obvious.

The environment in the PTC of the ribosome is very different from that in solution and is likely to affect the accessibility/orientation of the attacking water. We measured the nonenzymatic hydrolysis rates on the ribosome in the absence of release factor. Hydrolysis rates on the ribosome were significantly slower—2- to 30-fold but on average 10-fold—than those determined off the ribosome (Figure 1E). Similar to the solution rates of hydrolysis, fMet-Pro exhibited the slowest rates on the ribosome. Apart from this similarity, we observe no correlation between the nonenzymatic solution rates to rates on the ribosome

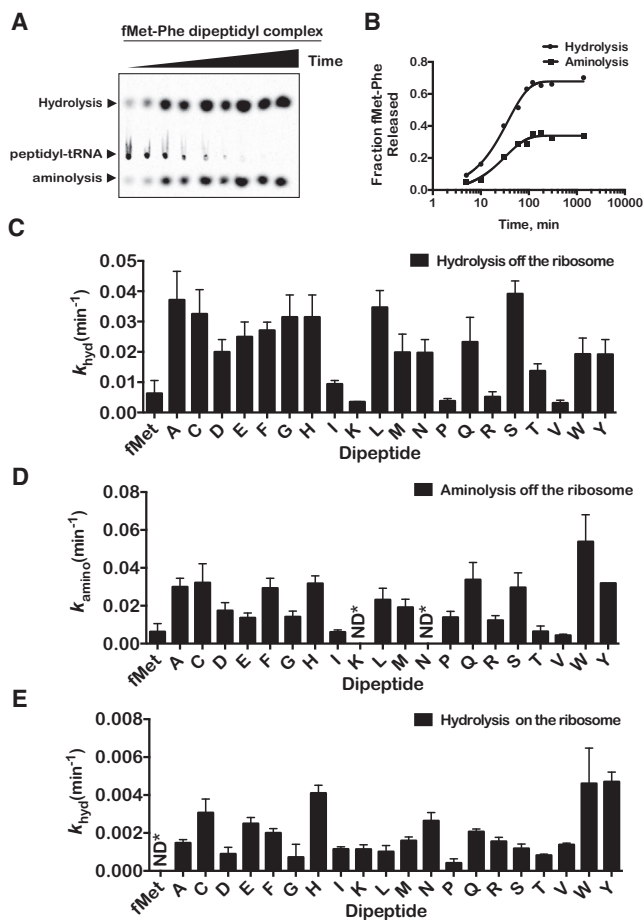


Figure 1. Nonenzymatic Dipeptide Hydrolysis and Aminolysis

(A) Representative electrophoretic TLC of a time course of nonenzymatic hydrolysis and aminolysis from free fMet-Phe peptidyl tRNA. (B) Quantification of data in (A). Fractional radioactivity corresponding to the released product was plotted against time and fit to one-phase exponential association equation. (C) Dipeptide release rates calculated for hydrolysis of free peptidyl-tRNA. (D) Dipeptide release rates calculated for aminolysis of free peptidyl-tRNA. Note the general similarity between hydrolysis and aminolysis rates. (E) Dipeptide release rates calculated for nonenzymatic hydrolysis of ribosomal peptidyl-tRNA. The error bars represent the SE obtained from the nonlinear regression. Most of the reactions were done in duplicates, and the deviation was less than 20% between the observed rates.

(Figure S3A), suggesting that the environment in the PTC has an effect on the rate of hydrolysis. However, we note that the nonenzymatic rates on the ribosome, except for proline, do not vary significantly relative to what we observe for those measured off the ribosome (<4-fold; Figures 1D and 1E). Thus, it appears that the environment in the PTC provides some uniformity to the termination process.

GGQ N⁵-Methylation Significantly Increases the Rate of Peptide Release on Proline- and Glycine-Terminating Peptides

Having established that the nonenzymatic rates of release do not vary significantly among the 20 amino acids, we set out to mea-

sure the RF-mediated rates of peptide release. Our experiments were motivated by previous *in vivo* studies, which suggested that peptide release is particularly inefficient on peptides terminating with proline and glycine residues (Björnsson et al., 1996). In agreement with these studies, we measured greatly reduced rates of RF1-mediated peptide release for fMet-Pro and fMet-Gly of 0.0050 s⁻¹ and 0.013 s⁻¹ (Figure 2A), respectively. Similarly for the RF2 reaction, we measured slow rates of 0.020 s⁻¹ and 0.035 s⁻¹ (Figure 2B). These rates are almost two orders of magnitude slower than those previously reported rates for fMet-Ala (Freistroffer et al., 2000; Figures 2C and 2D). Furthermore, similar to what has been observed for other amino acids (Freistroffer et al., 2000), the addition of RF3 has no effect on RF1- and RF2-mediated peptide release on proline and glycine residues (Figure S4; data not shown). As a result, our measurements suggest that peptide release is likely to be a major bottleneck for gene expression on proline- and glycine-terminating peptides. In direct contrast to this notion, an *E. coli* proteome-wide analysis shows that these residues are not underrepresented at the C terminus (Figure S4C). Therefore, we wondered whether the above rates we measured *in vitro* do not truly represent the *in vivo* efficiency of peptide release and that some other factor might be contributing to peptide release.

Recently, it has been shown that, during peptide-bond formation, the elongation factor P (EF-P) increases the rate of peptide-bond formation on ribosomes stalled on dipropyl motifs (Doerfel et al., 2013; Ude et al., 2013). As a result, we sought to determine whether the factor could also increase the rate of release of peptides having C-terminal proline and glycine residues. Lys³⁴ of *E. coli* EF-P is modified post-translationally through the addition of a hydroxylated lysine residue by three enzymes (Peil et al., 2012). We generated modified EF-P by coexpressing the modifying enzymes (Doerfel et al., 2013; Figure S5A), which resulted in high levels of modified protein as judged by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure S5B). We further examined the function of the EF-P variants—unmodified, lysylated, and hydroxylated—by assessing their effect on the yield of polypeptide peptides (Figure S5C). Having established that our modified EF-P is active, we next compared the release rates of fMet-Pro and fMet-Gly dipeptides from DRNC in the presence and absence of EF-P. Incubation with EF-P had little to no effect on the release rate of fMet-Pro and fMet-Gly, regardless of the RF added (Figures 3 and S5D). These observations suggest that, in contrast to peptide-bond formation, EF-P does not significantly promote peptide release on proline and glycine residues.

As discussed earlier, in *E. coli*, the glutamine residue of the GGQ motif is N⁵ methylated by prmC (Heurgué-Hamard et al., 2002). Our experiments, so far, have been conducted with over-expressed release factors (Figure S5E), which exhaust prmC and as a result are unmethylated (Figure S5F). To address the effect of methylation on peptide release of fMet-Pro and fMet-Gly, we generated methylated RFs by coexpressing the methyltransferase prmC alongside the His-tagged RF. Using this strategy, we obtained fully methylated RF1 (99.8%) and 75% methylated RF2 as assessed by LC-MS/MS (Figures S5F and S5G).

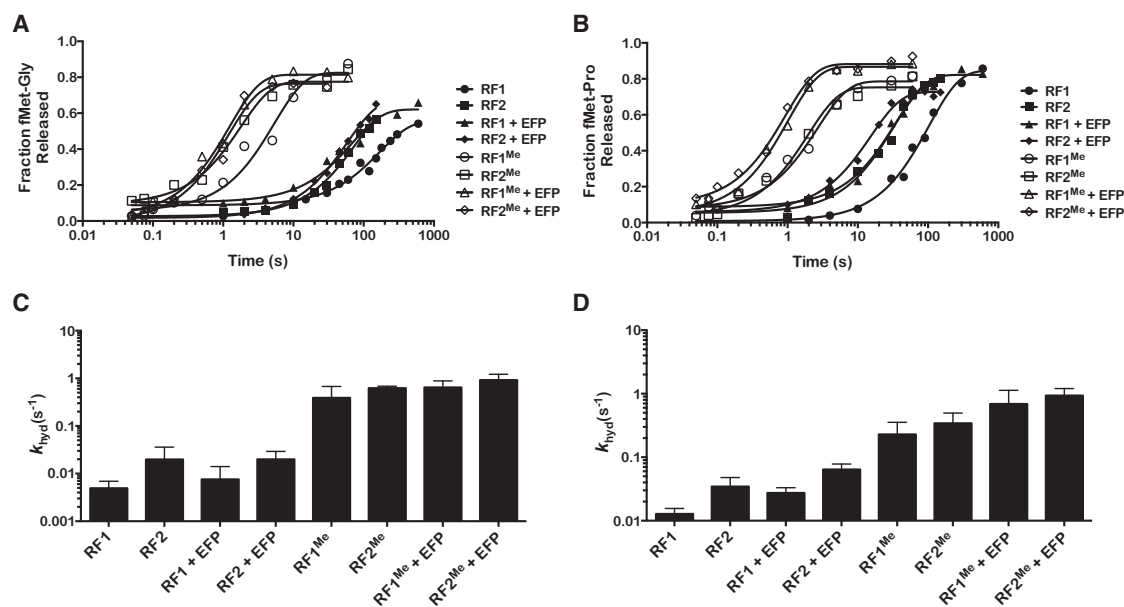


Figure 2. RF-Mediated Peptide Release of fMet-Glycyl and fMet-Prolyl Dipeptidyl tRNAs

(A and B) Representative time courses of (A) fMet-Gly and (B) fMet-Pro release in the presence of the indicated factors. For both dipeptides, only the addition of methylated RF1 or RF2 results in a significant increase in the rate of peptide release.

(C and D) fMet-Gly (C) and fMet-Pro (D) dipeptide release rates under the indicated conditions. Error bars represent SE of measurement calculated from at least three replicates.

Although we expected the methylation to stimulate peptide release (Dinçbas-Renqvist et al., 2000), we were surprised by its extent of stimulation on particular dipeptides. Methylation of RF1 increased the rate of peptide release by ~70-fold and 30-fold for glycine and proline, respectively (Figure 2). Likewise, for RF2, methylation increased the rate of peptide release by 30- and 10-fold for glycine and proline, respectively (Figure 2). Similar to the unmethylated factors, the addition of RF3 had minimal effects on RF2^{Me}-mediated release (Figure S4B). In contrast, the addition of EF-P to the methylated factors further increased the rate of release by 2- to 5-fold, bringing the rates of peptide release to ~1 s⁻¹ (Figures 2 and S5D). Collectively, these findings suggest that methylation is critical for peptide release on a subset of amino acids.

Effects of GGQ N⁵-Methylation on Peptide Release on All Amino Acids

Our data on peptide release with glycine- and proline-terminating peptides suggest that methylation of release factor may play a larger role in catalysis than previously appreciated (Dinçbas-Renqvist et al., 2000) and is likely to be specific to certain amino acids. To test this, we measured the k_{hyd} values for the remaining 18 amino acids by incubating our DRNCs with saturating concentrations of RF1 and RF2. In agreement with previous in vivo studies (Björnsson et al., 1996), release rates were significantly influenced by the identity of the terminal amino acid. The rates vary by more than 500-fold for RF1-mediated and by more than 800-fold for RF2-mediated release (Figures 3A and 3B). We note that, although rates of peptide release by both factors on glycine and proline were the obvious outliers,

rates on other amino acids were also relatively slow. This variation in the rates of peptide release is in direct contrast to what we observe for the nonenzymatic reactions, for which we measure a maximum of 10-fold difference in the rate of hydrolysis (Figure 1E). These data suggest that the release factor significantly reshapes the environment in the PTC and perhaps alters the reaction pathway. In agreement with this proposal, rates for RF1-mediated release do not correlate well to those of nonenzymatic release ($R = 0.332$; Figure S3B). RF2-mediated release correlates a little better to nonenzymatic release ($R = 0.562$; Figure S3C). This is consistent with recent data from our laboratory showing that, whereas RF1 and RF2 share many similarities, under certain conditions, the factors interact differently with the ribosome (Petropoulos et al., 2014). Nevertheless, statistically significant correlation ($R = 0.544$; $p = 0.01$) was observed between RF1 and RF2 k_{hyd} values (Figure S3D). In summary, our observations suggest that, in the absence of methylation, peptide release varies significantly and could potentially affect gene expression.

This disparity in the rates of peptide release appears to be alleviated by the methylation of the factors. GGQ N⁵ methylation increased release rates for both factors (Figures 3A and 3B). Similar to our earlier analysis, we observe little to no correlation between the nonenzymatic rates and those of RF^{Me}-mediated release (Figures S3E and S3F). We do observe, however, significant correlation between rates of release for RF1^{Me} and RF2^{Me} ($R = 0.651$; $p = 0.0014$; Figure S3G). Perhaps more interesting was the observation of a correlation between the effect of RF methylation on dipeptide release and the release rate observed for the unmethylated RF (Figure 3D). DRNCs that exhibited

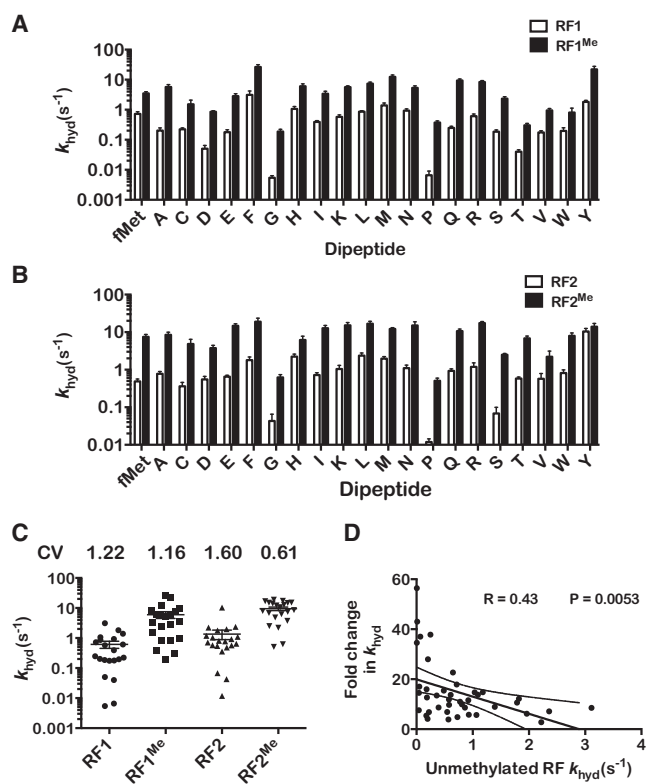


Figure 3. Effect of RF Methylation on Dipeptide Release Rate
 (A and B) Comparison of dipeptide release rates for (A) RF1 and RF1^{Me} and (B) RF2 and RF2^{Me}. Note the near universal rate increase for methylated RFs. The error bars represent the SE obtained from the nonlinear regression. For most dipeptides, reactions were carried out at least in duplicates and the observed variation was less than 20% between the rates.
 (C) Variation in dipeptide release rates for each release factor. Each data point corresponds to the release rate of a different dipeptidyl ribosome complex. Numbers above plot indicate the coefficient of variation (CV) for the release rates for each RF.
 (D) Negative correlation between release rate for unmethylated RF and increase in release rate due to GGQ N⁵ methylation, consistent with the notion that methylation has a more significant effect on slow release reactions.

slow dipeptide release rates for RF1 and RF2 generally exhibited disproportionately high release rates for RF1^{Me} and RF2^{Me}, whereas those that exhibited high release rates for RF1 and RF2 exhibited more-modest increases for RF1^{Me} and RF2^{Me}. For instance, methylation of RF2 increased the rate of fMet-Ser release by nearly 40-fold (0.07 s⁻¹ to 2.5 s⁻¹) but had little effect on the rate of fMet-Tyr release (10 s⁻¹ to 14 s⁻¹). In addition, dipeptide release rates varied less between DRNCs for RF1^{Me}- and RF2^{Me}-mediated release than for RF1 and RF2 (Figure 3C). The coefficient of variation (CV) for dipeptide hydrolysis rates was 1.215 for RF1, 1.164 for RF1^{Me}, 1.599 for RF2, and 0.613 for RF2^{Me}. This is also visible in the condensed vertical spread of data points for RF1^{Me} and RF2^{Me}. These observations suggest that the GGQ N⁵ methylation plays an important role in tuning the environment of the PTC during peptide release to ensure that different peptidyl-tRNA substrates are released with somewhat similar efficiency.

Structural Insights into Catalysis by RF1^{Me} and RF2^{Me}

To understand the structural basis of the methylated glutamine, we next determined four structures of *E. coli* unmethylated and methylated RF1 and RF2 bound to the *Tth* 70S ribosome. In all previous structures of *Tth* RFs bound to the 70S, domain three closely packs against 23S rRNA nucleotides of the PTC (Figure 4A; Jin et al., 2010; Korostelev et al., 2008; Santos et al., 2013; Weixlbaumer et al., 2008). The universally conserved GGQ motif is located at the tip of domain three, and the glutamine (Q235 in RF1; Q252 in RF2) backbone amide is located in an equivalent position as the 2' end of the A-site tRNA as previously reported (Figures 4B and S1; Jin et al., 2010; Korostelev et al., 2008; Santos et al., 2013; Weixlbaumer et al., 2008). The structures of the 70S-RF1^{Me} with and without the Q235 methylation reveal that the overall location of the backbone of the GGQ motif is unaffected by the modification. Surprisingly, in the 70S-RF1^{Me} complex, the side-chain amide of glutamine in RF1^{Me} is positioned facing A76 of the tRNA and packs against 23S rRNA A2451 and C2452 (Figure 4B). This positioning is clear based on our electron density maps and leaves positive difference density if rotated 180° with the side-chain carbonyl facing the tRNA instead (Figure S1). In the RF2 structure, the methylated Q252 positioning is more ambiguous, likely due to the lower resolution (3.4 Å; Figure S1). In contrast to the side-chain carbonyl, the side-chain amide has historically been proposed to play little to no role in catalysis (Weixlbaumer et al., 2008). Recent studies have suggested that the packing of the methyl group against A2451 of the 23S provides support for the glutamine side-chain amide group to participate in hydrogen bonding with a hydroxide ion (Indrisiunaite et al., 2015). However, future studies assessing the role of the methylated glutamine in pre-termination states and with different substrates will be required to reconcile the mechanism of termination.

DISCUSSION

During termination of protein synthesis, release factors encounter a diverse set of peptidyl-tRNA substrates. As expected, for the nonenzymatic reaction, this diversity of the leaving group was found to impart a significant effect on the rate of hydrolysis (Figure 1) that is likely the result of chemical differences among the 20 amino acids. However, on the ribosome, this variation in the rates of hydrolysis is not simply the result of intrinsic chemical differences among the leaving groups, as we observe no correlation between ribosome-independent and dependent reactions (Figure S3A). Instead, it appears that the side chain of the C-terminal amino acid, through interactions with the active site of the ribosome, affects the environment of the PTC and hence possibly the reaction pathway.

Release factors play an active role during the course of the hydrolysis reaction and accelerate the rate of the reaction by a factor of ~10⁵ (Figures 2 and 3). It is therefore reasonable to assume that variations in the efficiency of peptide release that we observe for the uncatalyzed reactions are irrelevant during cellular protein synthesis. Release factors, in principle, could bring about uniformity to the reaction by reshaping the environment in the PTC, which they are known to do (Jin et al., 2010; Korostelev et al., 2008; Korostelev et al., 2010; Laurberg et al.,

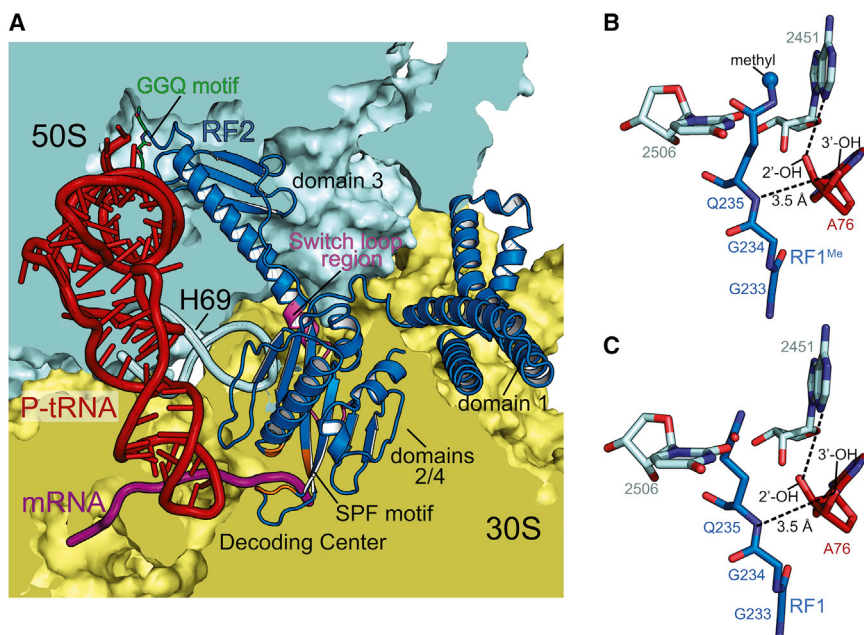


Figure 4. 70S-RF^{Me} Interactions

(A) Overview of *E. coli* RF2^{Me} (blue) interactions with the *Thermus thermophilus* 30S (yellow) and 50S (blue) subunits programmed with P-site tRNA^{Met} (red) and mRNA (magenta).

(B) Interactions between RF1^{Me} GGQ motif (blue), the peptidyl transferase center 23S rRNA residues 2506 and 2452 (cyan), and the terminal A76 of P-site tRNA^{Met} (red). The backbone amide of Q235 is within hydrogen bond distance to the 3'-OH of A76. The side-chain methyl group (blue sphere) packs against the nucleobase of 2451, placing its side chain positioned toward A76.

(C) Interactions between RF1 GGQ motif. The backbone amide is still within hydrogen bond distance to the 3'-OH of A76, but the glutamine side chain no longer interacts with 2451. The color scheme is the same as (B).

2008; Weixlbaumer et al., 2008; Zhou et al., 2012), and/or by altering the rate-limiting step of the reaction. As a result, we were surprised by the observations that, in the presence of RF1 and RF2, the rates are much more dramatically different relative to the uncatalyzed reaction. For fMet-Pro and fMet-Gly dipeptidyl tRNAs, the rates of release were so greatly reduced (0.005–0.01 s⁻¹) relative to other dipeptidyl tRNAs that, if the rates we measure reflect the *in vivo* values, ribosomes terminating on these amino acids are likely to stall. Interestingly, Hayes and colleagues have previously documented that transcripts having proline codons before stop codons induce ssrA tagging in *E. coli*, indicating that termination following proline residues is inefficient and causes ribosomal stalling (Hayes et al., 2002). In *E. coli*, 113 and 279 genes terminate with proline and glycine, respectively, and they are in fact not underrepresented, taking into account the overall amino acid frequencies (Figure S4C); unless there are mechanisms *in vivo* that improve termination efficiency on these amino acids, cellular fitness is likely to be impeded given the large fraction of ribosomes that would be unavailable for translation. This termination inefficiency appears to be partly alleviated by adjusting the sequence context around the C-terminal amino acid. In particular, the penultimate amino acid appears to play a critical role during the hydrolysis reaction as it affects the efficiency of stop-codon readthrough (Mottagui-Tabar et al., 1994). In agreement with these findings, proteome-wide analysis of *E. coli* shows that the same subset of amino acids are either forbidden or significantly underrepresented at the penultimate position for genes terminating with proline (Figure S4D). We note, however, all of our *in vitro* analysis conducted here was done with Met as the penultimate amino acid, which is not significantly underrepresented at this position for genes terminating with proline. Hence, it is highly unlikely that the termination inefficiencies that we document for certain peptidyl-tRNAs are the result of

poor sequence context. Instead, we argue that, in addition to the sequence context around the stop codon, the methylation status of release factor plays a critical role in ensuring termination pro-

ceeds uniformly, irrespective of the identity of the C-terminal amino acid.

What is clear from our analysis and a previous *in vivo* survey of nonsense suppression (Björnsson et al., 1996) is that the identity of the C-terminal amino acid is important for efficiency of the hydrolysis reaction. Our data suggest that the side chain of the ultimate amino acid is likely to influence the orientation of the catalytic water. Our structures of RF1^{Me} and RF2^{Me} bound to 70S reveals that the backbone placement of the GGQ motif is unaffected by the methylation and preserves the previously noted key interaction of the main-chain amide interaction of the glutamine with the 3'-OH of A76 (Figures 4B and 4C). Previous models for how the methylation contributes to release argued that only the main-chain amide is responsible for coordinating the water whereas a second model argued that the side-chain carbonyl is also involved in coordinating the water (Korostelev et al., 2008; Korostelev et al., 2010; Laurberg et al., 2008; Santos et al., 2013). A recent alternative hypothesis is that methylation could position the side-chain amide toward the 3'-OH of A76, where it forms a hydrogen bond with a hydroxide ion (Indrisiunaite et al., 2015). Our structures show that the methyl-glutamine of the GGQ motif maintains the main-chain amide interaction with the P-site tRNA. Unexpectedly, the methylation causes the side-chain carbonyl to face away from the active site and, instead, positions the side-chain amide toward the P-site tRNA and A2451 of the 23S (Figure 4B). Clearly presenting the side-chain amide adjacent to the P-site tRNA must be beneficial to the hydrolysis reaction, and two possibilities exist. One is that the methylation rigidifies the important glutamine to provide a tight pocket for the catalytic water, regardless of the ultimate amino acid. The other possibility is that the methylation stabilizes the glutamine in the PTC to allow for the side-chain amide to interact with a hydroxide ion in the reaction. Future studies aimed at the determination of pre-termination state structures with

different substrates will be required to reconcile this important mechanistic question.

EXPERIMENTAL PROCEDURES

Detailed methods are provided in the [Supplemental Experimental Procedures](#).

Release Assays

Ribosome complexes were prepared as previously described (Simms et al., 2014). Rates of peptide release were determined on a Quench-Flow instrument (RQF-3 Rapid Quench-Flow; KinTek) and reactions resolved with electrophoresis on cellulose TLC plates (Youngman et al., 2004). Observed k_{hyd} values were calculated by fitting a first-order rate equation to the curve using GraphPad Prism software.

Nonenzymatic Hydrolysis and Aminolysis

Nonenzymatic hydrolysis on and off the ribosome were performed as previously described (Zaher et al., 2011).

Structures of 70S and Release Factor Complexes

T70 70S ribosomes were purified and complexed with mRNA, tRNA, and RFs as described (Selmer et al., 2006). Crystals were screened for diffraction at the SER-CAT 22-ID beamline, and X-ray data were collected at the NE-CAT 24-IDC beamline, both at the APS, Argonne National Laboratory. The data were integrated and scaled using XDS (Kabsch, 2010), refined with PHENIX (Adams et al., 2010), and modeled using Coot (Emsley et al., 2010; Table S1).

ACCESSION NUMBERS

The accession numbers for the coordinates and structure factors reported in this paper are PDB: 5CZP, PDB: 5DFE, PDB: 5J30, and PDB: 5J3C.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.08.085>.

AUTHOR CONTRIBUTIONS

W.E.P. designed and performed the peptide release assays. E.D.H. designed and carried out the X-ray crystallography assays. H.E.K. performed the nonenzymatic release assays. C.L.S. carried out the *in vivo* readthrough assays. All authors interpreted the results. C.M.D. and H.S.Z. designed the experiments and wrote the manuscript.

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