REPORT

Peptide release on the ribosome depends critically on the 2' OH of the peptidyl-tRNA substrate

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ABSTRACT

Peptide release on the ribosome is catalyzed by protein release factors (RFs) on recognition of stop codons positioned in the A site of the small ribosomal subunit. Here we show that the 2' OH of the peptidyl-tRNA substrate plays an essential role in catalysis of the peptide release reaction. These observations parallel earlier studies of the mechanism of the peptidyl transfer reaction and argue that related mechanisms are at the heart of catalysis for these reactions.

Keywords: translation termination; release factor; ribosome

INTRODUCTION

Termination of protein synthesis is triggered by the appearance of one of three stop codons (UAA, UGA, and UAG) in the decoding center of the small ribosomal subunit. In bacteria, the stop codons are recognized with overlapping specificity by two conserved release factors (RF1 and RF2) that promote the peptide release reaction. This chemical reaction involves the nucleophilic attack of water on the aminoacyl ester linkage of the peptidyl tRNA, resulting in release of the polypeptide chain and protonation of the tRNA leaving group. This hydrolytic reaction is related to the peptidyl transfer (PT) reaction that results in the extension of the growing polypeptide chain by a single amino acid, where the nucleophile is the primary amine of the aminoacyl tRNA that has been loaded into the A site of the ribosome by the GTPase EF-Tu. These two reactions are diagrammed in parallel in Figure 1.

The peptidyl transfer reaction mechanism has been the focus of a number of studies that emerged following the high-resolution structures of the large ribosomal subunit (for review, see Rodnina et al. 2006). While early models proposed a decisive role for universally conserved rRNA nucleotides in the peptidyl transfer center (PTC) of the large ribosomal subunit, mutational studies indicated that

these residues did not substantially contribute to catalysis (Beringer et al. 2003; Youngman et al. 2004). Subsequent studies established an essential role for the 2' OH of the peptidyl-tRNA substrate (Weinger et al. 2004), which structural studies had identified as ideally poised to play an important role in catalysis (Schmeing et al. 2005). Substitution of the 2' OH of the peptidyl-tRNA substrate with a deoxy (H) or fluoro (F) group diminishes catalysis beyond detectable levels, more than a million-fold slower than the rate of catalysis with normal ribose (OH) tRNA substrates. While the deoxy- and fluoro-substituted tRNA substrates are presumed to be inherently less chemically reactive, the observed million-fold decline in the rate of PT catalysis far surpasses the expected loss in chemical reactivity of these substrates.

While the observation of such losses in catalytic power indicate that the 2' OH of the peptidyl-tRNA substrate plays a substantial role in the PT reaction, it does not reveal how it contributes to catalysis. Multiple models have been considered, including (1) that the 2' OH plays a direct role in general acid-base catalyzed proton transfer, (2) that it binds a metal ion that promotes chemistry through coordinated water species, and (3), the simplest model, that the 2' OH acts to facilitate proton movement in a highly organized active site (discussed in Rodnina et al. 2006). Models based on the involvement of a general acid-base have been discounted based on biochemical experiments (Bieling et al. 2006) whereas involvement of a metal ion has been discounted primarily because of its promiscuous absence from atomic resolution views of the

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.1057908>.

FIGURE 1. Two reactions carried out in the large ribosomal subunit catalytic center. (A) Diagram of the peptidyl transfer reaction with nucleophilic aminoacyl tRNA (A site) reacting with peptidyl tRNA (P site), resulting in deacylated CCA and new peptidyl tRNA elongated by one amino acid. (B) Peptide release reaction with nucleophilic water attacking the peptidyl tRNA (P site) resulting in deacylated CCA and free polypeptide.

active site (for review, see Rodnina et al. 2006). The proton shuttle model, where a proton from the incoming nucleophilic amine on the aminoacyl-tRNA substrate is transferred to the 3' OH of the peptidyl-tRNA leaving group via the 2' OH of the same substrate, reconciles much of the literature surrounding the molecular mechanism of the PT reaction, including site-directed mutagenesis studies (Dorner et al. 2003; Weinger et al. 2004; Okuda et al. 2005), thermodynamic studies (Sievers et al. 2004), and molecular modeling approaches (Das et al. 1999; Trobro and Aqvist 2005).

The termination reaction catalyzed on the ribosome by the class I protein release factors shares certain similarities with the better studied PT reaction but also exhibits clear differences. A priori, there are a number of potential contributors to the chemistry of peptide release (or hydrolysis), including the rRNA in the PT center, the release factor, or the 2' OH of the poised peptidyl-tRNA substrate. Early mutagenesis studies indicated that several of the conserved rRNA nucleotides in the PT center of the ribosome are important for optimal release catalysis (Polacek et al. 2003; Youngman et al. 2004). Mutational analysis of the release factors themselves identified key conserved amino acids that contribute to the release reaction (Frolova et al. 1999; Seit-Nebi et al. 2001; Zavialov et al. 2002; Mora et al. 2003; Shaw and Green 2007). Here we explore the contribution of

the 2' OH of the peptidyl-tRNA substrate to the release reaction using a mutational approach analogous to that previously used for the PT reaction.

RESULTS AND DISCUSSION

Preparation of modified tRNAs and termination complexes

To test the role of the 2' OH of the peptidyl-tRNA substrate in release factor-mediated catalysis on the ribosome, we prepared tRNA^{Lys} lacking the terminal nucleotide A (and its associated ribose) and replaced it with dA76 and fA76 (as well as the control rA76) as previously described (Weinger et al. 2004). Quantitative substitution of the terminal A76 was confirmed by establishing the resistance of the substituted versions (dA76 and fA76) to periodate treatment (data not shown). To evaluate the effects of the substitutions on ribosome-based termination, the substituted tRNA^{Lys} species were incorporated into dipeptidyl-tRNA post-translocation ribosome complexes where the tRNA^{Lys} species occupies the P site and carries the dipeptide moiety f-[³⁵S]-Met-Lys and where the RF1-specific stop codon UAG is positioned in the A site. As previously reported (Weinger et al. 2004), the modified tRNA^{Lys} species are viable A-site tRNA substrates and are efficiently translocated by EF-G following the peptidyl transfer reaction as confirmed by toeprinting experiments (data not shown).

Peptide release with peptidyl tRNA modified at the A76 2' position

The stable dipeptidyl-tRNA ribosome complexes were next reacted with saturating amounts of RF1 and the rate of peptide release evaluated. In these assays, the radioactively labeled dipeptidyl-tRNA species (f-[³⁵S]-Met-Lys-tRNA^{Lys}) is readily resolved from the released dipeptide product (f-[³⁵S]-Met-Lys) using an electrophoretic TLC system. Care was taken in storage and appropriate buffers were selected to minimize background levels of hydrolysis that can occur during the workup or during storage, as this background hydrolysis reaction can easily obscure small amounts of true catalysis that might be observed in this system. Finally, our TLC system was able to distinguish between authentic hydrolysis products and background aminolysis products that can result from small amounts of contaminating nucleophiles in the reactions. To minimize these problems, some of the most obvious nucleophiles were eliminated

from our reaction buffer (HEPES was used instead of Tris and polyamines were excluded).

The rate constant for the natural A76 (rA76) tRNA^{Lys} variant was $\sim 0.1 \text{ sec}^{-1}$, with the reaction proceeding essentially to completion (Fig. 2A,D). Increasing concentrations of RF1 had little effect on the rate constant (data shown for two different concentrations of RF1, 5 μM and 25 μM) indicating that the reaction was saturated (Table 1). The background rate of hydrolysis of this dipeptidyl-tRNA substrate bound to the ribosome in the absence of release factor enzyme was $\sim 1 \times 10^{-4} \text{ sec}^{-1}$ (Fig. 2A,D). The acceleration of the release reaction observed in this system is thus ~ 1000 -fold, consistent with earlier measurements of release activity (Zavialov et al. 2002; Shaw and Green 2007).

We next looked at the rate constants for the RF-mediated release reaction using the dA76 and fA76 substituted tRNA^{Lys} species. The rate of release observed in each case is slightly greater (two- to threefold) than the background rate of hydrolysis observed with these same substrates in the absence of any RF1 (Fig. 2B,C,D). As with the natural (rA76) substrate, there are no substantial rate differences when RF1 concentrations are increased in these reactions (Table 1), indicating that the reactions are saturated for

RF1. The single exponential kinetic reaction endpoints for the rA76 and dA76 substrates of $\sim 70\%$ indicate that a majority of the ribosomal complexes behaved uniformly (Fig. 2B). The fA76 substrate endpoints were somewhat lower at 30%–40%, indicating that a smaller fraction of the population was being evaluated in this experiment (Fig. 2C).

The background rates of hydrolysis for the dA76 substrate were unaffected by increasing concentrations of ribosome (Table 1), consistent with the idea that the measured rate represents hydrolysis occurring on ribosome-bound tRNA species (rather than dipeptidyl-tRNA species that have fallen off the ribosome). Some differences in the background rate of hydrolysis for the fA76 substrate were observed when ribosomes were titrated, suggesting that stable binding might be perturbed for these substrates, though we note that the error for this data set is somewhat larger throughout. The ribosome titration cannot in either case rule out the possibility of an irreversible dissociation, though the documented stability of these complexes during a standard toeprinting protocol ($\sim 30 \text{ min}$) argues against this possibility (Weinger et al. 2004).

These data strongly argue that the 2' OH of the peptidyl-tRNA species is critical for the catalysis of RF-mediated peptide release on the ribosome. The possibility that conformational differences between rA76 and dA76 or fA76 affect the positioning of these substrates for catalysis cannot be ruled out. However, differences in sugar pucker of the A76 ribose seem unlikely to have such drastic effects on chemistry, as it has been noted that energetic barriers between C2'-endo and C3'-endo are small ($<0.5 \text{ kcal mol}^{-1}$) (Hocquet et al. 2000) and fA76 is expected to favor the C3'-endo conformation favored by the wild-type rA76 variant (Uesugi et al. 1979). In addition, structural studies have demonstrated that rA76 and dA76 substrates bind the P site in identical conformations (Schmeing et al. 2005).

Relative reactivities of the peptidyl-tRNA substrates

In both these and earlier experiments evaluating the role of the 2' OH of the peptidyl-tRNA substrate in the related processes of peptide release and peptidyl transfer, there have been concerns about the inherent *chemical* reactivity of the different substrates (rA76, dA76, and fA76). In the absence of detailed information

FIGURE 2. Deoxy- and fluoro-substitutions at the A76 2' position in peptidyl tRNA cause a dramatic decrease in peptide release activity. Representative curves measuring the fraction of dipeptide (fMet-Lys) released over time with modified tRNA substrates; (A) rA76 with (■) or without (□) RF1, (B) dA76 with (▲) or without (△) RF1, and (C) fA76 with (●) or without (○) RF1. (D) Log-scale plot of observed rate constants for peptide release (k_{release} [sec^{-1}]) with various peptidyl-tRNA derivatives; rA76, dA76, and fA76. Data are presented at mean $\pm$ standard error of at least two measurements. All measurements were made with release complex at a concentration of 0.2 μM with or without 5 μM RF1.

TABLE 1. Rate constants for RF1 and release complex titrations

Concentration (μM)		k_{release} (sec^{-1})		
RF1	Complex	rA76	dA76	fA76
5	0.2	0.071 ± 0.004	$(3.1 \pm 0.3) \times 10^{-5}$	$(2.6 \pm 0.4) \times 10^{-5}$
5	0.6	0.088 ± 0.005	$(2.4 \pm 1.0) \times 10^{-5}$	$(16 \pm 8) \times 10^{-5}$
25	0.2	0.115 ± 0.011	$(1.3 \pm 0.02) \times 10^{-5}$	$(1.8 \pm 1.2) \times 10^{-5}$
0	0.2	$(11 \pm 1) \times 10^{-5}$	$(0.95 \pm 0.02) \times 10^{-5}$	$(0.91 \pm 0.17) \times 10^{-5}$
0	0.6	—	$(1.2 \pm 0.4) \times 10^{-5}$	$(3.0 \pm 1.7) \times 10^{-5}$

about these relative reactivities, it is difficult to provide a number that estimates the contribution of the 2' OH moiety to catalysis. We attempted to measure the rate constants for solution hydrolysis of the various substrates using N-acetyl- ^{14}C -Lys-tRNA^{Lys} (rA76, dA76, and fA76) in order to evaluate their chemical reactivity. This experiment was performed by incubating the N-acetyl- ^{14}C -Lys-tRNA^{Lys} substrates (rA76, dA76, or fA76) in reaction buffer C (50 mM HEPES at pH 7.5, 70 mM NH₄Cl, 30 mM KCl, 7 mM MgCl₂, 1 mM DTT) and looking at the rate of hydrolysis of the N-acetyl- ^{14}C -Lys moiety by electrophoretic TLC. These measurements indicated that there were at most minor differences in the inherent reactivity of these substrates in solution (Fig. 3). Similar observations were made by Sievers et al. (2004) in a study characterizing model substrates used in their analysis of the peptidyl transfer reaction. We note that work in the ribozyme field has argued for more significant chemical differences between 2' ribo, deoxy, and fluoro containing phosphodiester substrates (Herschlag et al. 1993). In the absence of more detailed analysis of the nature of the transition state for on-ribosome and off-ribosome hydrolysis reactions for the different substrates, we cannot easily explain their similar chemical reactivities. It seems possible that in solution, as compared to the more hydrophobic active site environment, water can more easily substitute for the missing OH group of the sugar ring and thus effectively

activate the dA76 and fA76 substrates. This inability to define chemical reactivity differences, if any, in these substrates makes it difficult to quantitatively define the contribution of the 2'OH moiety to catalysis in this reaction or, for that matter, in the related PT reaction.

CONCLUSIONS

We show here that the 2' OH of the P-site tRNA substrate plays a critical role in the peptide release reaction catalyzed by class I release factors on the bacterial ribosome. As has been proposed for the related peptidyl transfer reaction, a plausible model for release catalysis involves RF interactions with the ribosome that ultimately involve the 2' OH moiety of the peptidyl tRNA in facilitating proton movement through a highly organized, evolutionarily ancient RNA-rich active site. Despite this substantial similarity in sensitivity of the PT and release reactions to peptidyl-tRNA 2'OH substitutions, other studies have argued that active site nucleotides make distinct contributions to these two reactions (Amort et al. 2007). More detailed studies, eventually guided by RF-ribosome X-ray structures, will help to decipher these various contributions.

MATERIALS AND METHODS

Preparation of modified tRNA substrates

His-tagged CCA-adding enzyme (*Escherichia coli*) and His-tagged lysyl-tRNA synthetase (*E. coli*) were overexpressed and purified as described (Cho et al. 2003; Shimizu et al. 2005). The 3' adenosine (A76) residue of *E. coli* tRNA^{Lys} (Sigma-Aldrich) was removed by one round of periodate-aniline-polynucleotide kinase (PNK) treatment and replaced by incubation with CCA-adding enzyme and adenosine triphosphate, 2'-deoxyadenosine triphosphate or 2'-fluoro,2'-deoxyadenosine triphosphate (TriLink Biotechnologies) essentially as described (Weinger et al. 2004). Quantitative addition of the various adenosine analogs to the truncated tRNAs was confirmed by repeating the periodate-aniline cleavage and looking for a mobility change on a denaturing 10% (w/v) acrylamide gel. Modified tRNA^{Lys} (rA76, dA76, fA76) (13.5 μM) was aminoacylated by incubation at 37°C for 20 min with 27 μM [^{14}C]-lysine (Perkin Elmer Life Sciences), 3 mM ATP, and

FIGURE 3. Deoxy- and fluoro-substitutions at the A76 2' position in peptidyl tRNA have little effect on the rate of spontaneous hydrolysis in solution. Plot of observed rate constants for release of N-acetyl- ^{14}C -Lys from N-acetyl- ^{14}C -Lys-tRNA^{Lys} derivatives in buffered solution. Data are presented at mean $\pm$ standard error of at least two measurements.

14 μ M lysyl-tRNA synthetase in charging buffer (25 mM Tris-Acetate at pH 7.5, 10 mM MgAcetate, 100 mM NH_4Cl , 30 mM KCl, 1 mM DTT).

Preparation of peptide release complexes

E. coli MRE600 (ATCC 29,417) was grown in Luria broth to an A_{600} of 0.6–0.8. Pelleted cells were resuspended in buffer A (20 mM Tris-HCl at pH 7.5, 100 mM NH_4Cl , 10 mM MgCl_2 , 0.5 mM EDTA, and 6 mM β -mercaptoethanol) and lysed using a French press. Clarified lysates were pelleted over sucrose cushions (1.1 M sucrose, 20 mM Tris-HCl at pH 7.5, 500 mM NH_4Cl , 10 mM MgCl_2 , and 0.5 mM EDTA) in a Beckman Ti-45 rotor for 16 h at 37,000 rpm and resuspended in buffer A. Samples were separated by zonal centrifugation over a 10%–40% sucrose gradient in a Ti-15 rotor for 19 h at 28,000 rpm. 70S ribosomes were collected, pelleted, and resuspended to 15 μ M in buffer A before storage at -80°C .

Peptide release activity of modified tRNA species was assayed using dipeptide release complexes. Initiation complexes were formed by loading f- ^{35}S -Met-rRNA^{Met}_f initiator tRNA (2 μ M) into the P site of *E. coli* 70S ribosomes (2 μ M) programmed with mRNA containing a Shine–Dalgarno sequence spaced 8 nt upstream of the coding region AUGAAUAG. The reaction included GTP (2 mM) and initiation factors IF1, IF2, and IF3 and the incubation proceeded at 37°C for 30 min in buffer B (50 mM Tris-HCl at pH 7.5, 70 mM NH_4Cl , 30 mM KCl, 7 mM MgCl_2 , 1 mM DTT). In a separate reaction, EF-Tu was incubated with GTP in buffer B for 15 min at 37°C and subsequently incubated with [^{14}C]-Lys-tRNA^{Lys} (rA76, dA76, or fA76), EF-G, and GTP in buffer B for 30 min to form an elongation mix. Elongation mix was then added to the initiation complex for an additional 5 min at 37°C to form dipeptidyl tRNA post-translocation ribosome complexes with f- ^{35}S -Met-Lys-tRNA^{Lys} in the P site. These complexes were pelleted over sucrose cushions (1.1 M sucrose, 20 mM Tris-HCl at pH 7.5, 500 mM NH_4Cl , 10 mM MgCl_2 , 0.5 mM EDTA) for 2 h at 69,000 rpm and resuspended in reaction buffer C (50 mM HEPES at pH 7.5, 70 mM NH_4Cl , 30 mM KCl, 7 mM MgCl_2 , 1 mM DTT). Dipeptide release complexes were reacted with RF1 (final concentration 5 or 25 μ M) and dipeptide release was observed as a function of time. The reactions were quenched at time points in 25% formic acid and release products were separated from peptide attached to tRNA by TLC electrophoresis. Curves were fit to single exponential equations to obtain a rate constant for each reaction.

N-acetyl-Lys-tRNA^{Lys} reactivity measurements

N-acetylated lysyl-tRNA^{Lys} substrate was generated by incubating lysyl-tRNA^{Lys} variants (15.5 μ M) with 1/100 vol concentrated acetic anhydride (Sigma) in 0.3 M sodium acetate on ice for 1 h and then again with an additional 1/100 vol acetic anhydride for another hour (Haenni and Chapeville 1966). Both the backbone primary amine and the side chain epsilon primary amine moieties are quantitatively modified using this modification procedure. The modified tRNA substrate was recovered by ethanol precipitation and resuspended to a concentration of 3.5 μ M, determined by scintillation counting, in 20 mM potassium acetate (pH 5). Extent of modification was assessed by hydrolysis with KOH and separation by TLC electrophoresis. Differing mobility was observed for the N-acetylated lysine derivative compared to lysine. To compare the relative reactivities of the ribose, deoxy,

and fluoro derivatives of tRNA, hydrolysis of N-acetyl- ^{14}C -Lys-tRNA^{Lys} (rA76, dA76, and fA76) in buffer C was measured over time and rates were obtained using standard methods.

ACKNOWLEDGMENTS

We thank J. Weinger for technical advice and A. Weiner and T. Ueda for providing plasmids for overexpression of CCA-adding enzyme and lysyl-tRNA synthetase. This work was supported with funding from the NIH.

Received March 3, 2008; accepted May 1, 2008.

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RNA 2008 14: 1526-1531

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