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An intact ribose moiety at A2602 of 23S rRNA is key to trigger peptidyl-tRNA hydrolysis during translation termination

Melanie Amort[1], Brigitte Wotzel[1], Kamilla Bakowska-Zywicka[1,2], Matthias D. Erlacher[1], Ronald Micura[3] and Norbert Polacek[1,*]

[1]Innsbruck Biocenter, Medical University Innsbruck, Division of Genomics and RNomics, Fritz-Pregl-Strasse 3, 6020 Innsbruck, Austria [2]Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland and [3]CMBI, Leopold-Franzens-University Innsbruck, Institute of Organic Chemistry, Innrain 52a, 6020 Innsbruck, Austria

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ABSTRACT

Peptide bond formation and peptidyl-tRNA hydrolysis are the two elementary chemical reactions of protein synthesis catalyzed by the ribosomal peptidyl transferase ribozyme. Due to the combined effort of structural and biochemical studies, details of the peptidyl transfer reaction have become increasingly clearer. However, significantly less is known about the molecular events that lead to peptidyl-tRNA hydrolysis at the termination phase of translation. Here we have applied a recently introduced experimental system, which allows the ribosomal peptidyl transferase center (PTC) to be chemically engineered by the introduction of non-natural nucleoside analogs. By this approach single functional group modifications are incorporated, thus allowing their functional contributions in the PTC to be unravelled with improved precision. We show that an intact ribose sugar at the 23S rRNA residue A2602 is crucial for efficient peptidyl-tRNA hydrolysis, while having no apparent functional relevance for transpeptidation. Despite the fact that all investigated active site residues are universally conserved, the removal of the complete nucleobase or the ribose 2′-hydroxyl at A2602, U2585, U2506, A2451 or C2063 has no or only marginal inhibitory effects on the overall rate of peptidyl-tRNA hydrolysis. These findings underscore the exceptional functional importance of the ribose moiety at A2602 for triggering peptide release.

INTRODUCTION

The ribosomal peptidyl transferase center (PTC) is the catalytic heart of the ribosome and plays a fundamental role in protein synthesis. It is a part of the large ribosomal subunit (50S in eubacterial ribosomes), a complex dynamic ribo-nucleoprotein ensemble with a molecular weight of ~1.8 MD. The crystallographic structures compellingly confirmed that peptidyl transferase is an RNA enzyme [reviewed in (1)]. This places the ribosome as the key entry on the list of naturally occurring ribozymes that outlived the transition from the pre-biotic RNA World to contemporary biology. The PTC is characterized by the most pronounced accumulation of universally conserved rRNA nucleosides in the entire ribosome. The prime functions of the PTC are (i) transpeptidation to covalently link amino acids via peptide bonds into polypeptides during the elongation phase of protein synthesis and (ii) peptidyl-tRNA hydrolysis, which is required for termination of translation and release of the fully assembled polypeptide from the ribosome. The termination reaction likely involves the transfer of the peptidyl moiety of P-site located peptidyl-tRNA to a water molecule (2). In the course of the reaction, the nucleophilic attack of an activated water molecule in the PTC acceptor site onto the carbonyl carbon of the peptidyl-tRNA ester leads to formation of a tetrahedral intermediate. A proton is subsequently transferred to the 2′(3′)-hydroxyl of the 3′-terminal adenosine of peptidyl-tRNA breaking the ester bond between the peptide and tRNA. From a chemical point of view, peptidyl-tRNA hydrolysis is a more challenging reaction than transpeptidation because hydrolysis of the ester bond is driven by a significantly less

*To whom correspondence should be addressed. Tel: +43 (0)512 9003 70251; Fax: +43 (0)512 9003 73100; Email: norbert.polacek@i-med.ac.at

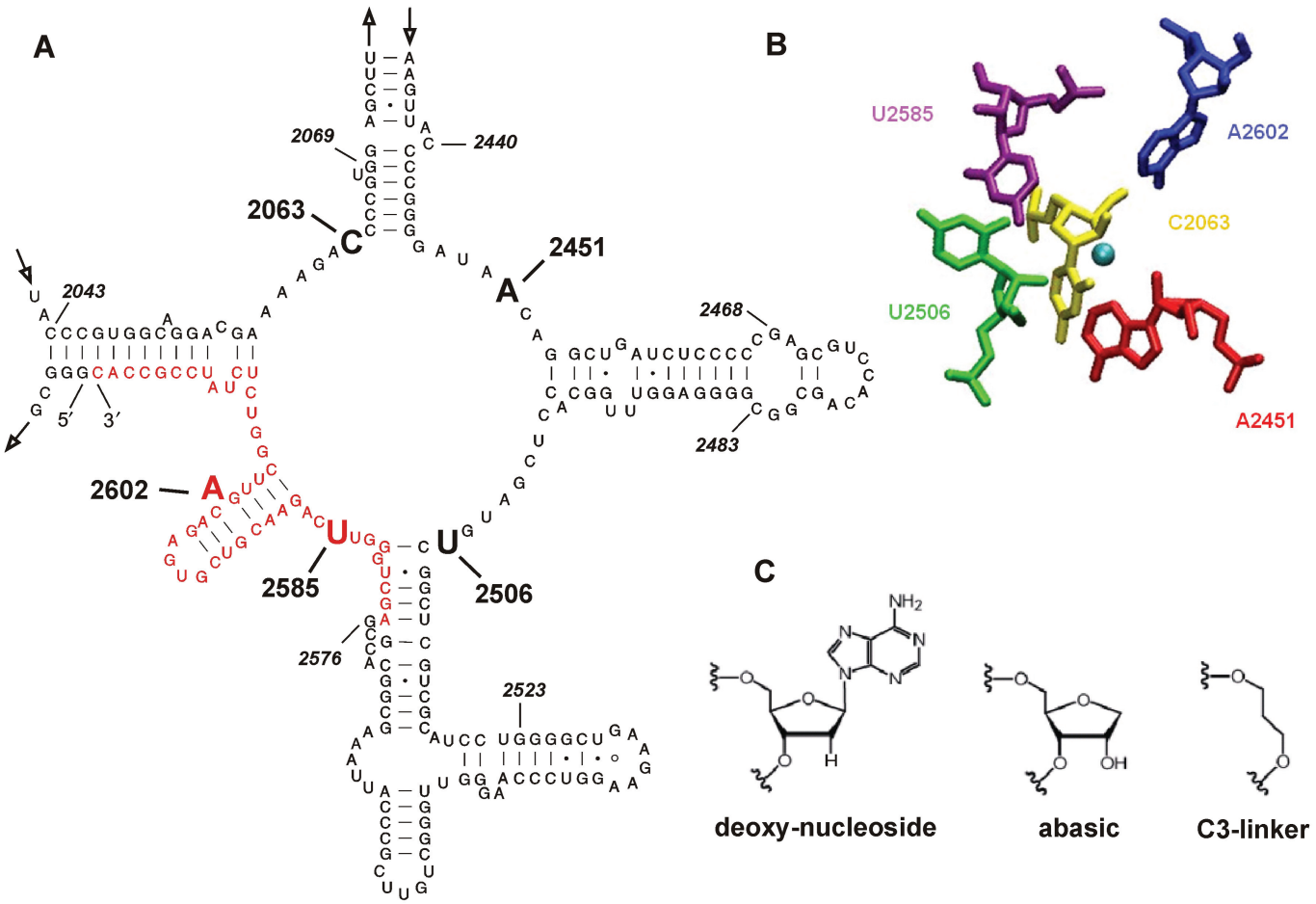


Figure 1. Structural aspects of the PTC of gapped-cp-reconstituted 50S subunits. **(A)** Secondary structure of the central loop of domain V of 23S rRNA of *T. aquaticus* showing the new endpoints of the cp-23S rRNA at positions 2623 and 2622 (5′ and 3′, respectively) which was constructed to investigate the effects of nucleoside modifications at positions A2602, U2585 and U2584. The chemically synthesized 45-nucleotides RNA, which was ligated to the 3′-end of the cp-23S rRNA transcript at position 2576, is shown in red. Additional active site residues that have been replaced by the modified nucleoside analogs depicted in (C) using the gapped-cp-reconstitution approach are in bold. The endpoints of the corresponding cp-23S rRNA constructs are italic. Cp2523-2483, cp2468-2440 and cp2069-2043 were constructed to study U2506, A2451 and C2063, respectively, whereas the first nucleotide always indicates the new 5′-end and the second residue the 3′-end of the cp-23S rRNA transcript. **(B)** View of the three-dimensional architecture of the PTC structure. The green sphere marks the location of the nitrogen atom of the α-amino nucleophile of an aminoacyl-tRNA mimicry. The figure was generated from pdb file 1FG0 (6). **(C)** The chemical structure of the three nucleoside analogs that have been introduced at all inner shell residues shown in (B) employing the gapped-cp-reconstitution.

nucleophilic water oxygen compared to the strongly nucleophilic α-amino group of aminoacyl-tRNA during peptide bond formation. It is therefore expected that the PTC-promoted reaction of peptide release should involve specific coordination and activation of the water molecule. The catalytic rate constant of peptide release has been estimated in an *in vitro* translation assay to be 0.5–1.5/s (3), and is therefore clearly slower than transpeptidation (15–300/s) (4,5). The change of the mode of action of the PTC from amino acid polymerization to peptidyl-tRNA hydrolysis is triggered by class I release factors (RF1 or RF2 in bacteria) which bind in response to an mRNA stop codon displayed in the decoding A-site of the small ribosomal subunit.

The peptidyl transferase cavity is densely packed and decorated with nucleotides of the central loop of domain V of 23S rRNA (Figure 1). The residues exposed on the surface of the catalytic center (A2602, U2585, U2506, A2451 and C2063; *Escherichia coli* nomenclature is used here and throughout the manuscript) are universally conserved and are referred to as 'inner shell' PTC nucleotides. In the non-translating large ribosomal subunit, the peptidyl transferase cavity is hollow except for the 23S rRNA bases of nucleosides A2602 and U2585, which bulge into its core. The orientation of these two universally conserved residues depends on the functional state of the ribosome and the nature of the bound substrate (6–11) suggesting functional relevance of these nucleosides. Biochemical, cryo-electron microscopic and crystallographic data show that the tip of domain III (which harbors the universally conserved GGQ peptide mini-motif) of the A-site-bound RF reaches toward the bottom of the funnel-shaped active site crater of the PTC and is in immediate neighbourhood of A2602 and U2585 (12–17). What happens in the PTC in response to the RF binding, what the role of the GGQ motif is and which

functional groups are involved in the coordination and activation of the water molecule remain unknown. Models were proposed which suggest that the GGQ motif directly participates in peptidyl-tRNA hydrolysis by coordinating the water molecule (3,12,18–20). However, mutational studies do not seem to support a direct participation of the GGQ sequence of the RF in catalyzing peptide release (3,21,22). The fact that peptide release can be efficiently triggered even in the absence of the RF by A-site-bound deacylated tRNA (3,23,24), strongly suggests that peptidyl-tRNA hydrolysis is a PTC-catalyzed reaction.

Mutational studies employing *in vitro* reconstituted *Thermus aquaticus* 50S subunits showed that A2602 of 23S rRNA may be one of the critical components of the reaction for peptide release (24). Whereas mutations of the other inner shell PTC residues C2063, A2451 and U2585 had only moderate effects on either of the reactions, substitution of A2602 with C or its deletion dramatically reduced the ribosome's ability to promote peptidyl-tRNA hydrolysis but had little effect on transpeptidation (24). Furthermore, in experiments with affinity-tagged *in vivo* derived *E. coli* ribosomes, again mutations at A2602 had the most significant impact although also mutations at U2585 reduced, albeit to a lesser degree, the rate of peptide release (40-fold compared with 350-fold for the A2602C mutation) (25). This indicates a possible additional contribution of U2585 for the peptide release mechanism. Based on the results of mutational studies, a model was proposed in which the class I RF triggers peptide release by reorienting A2602 in the PTC so that it can coordinate and possibly activate a water molecule for the attack onto the carbonyl carbon atom of the ester bond of the peptidyl-tRNA (24). The structural flexibility and the central location of A2602 in the PTC are compatible with this proposed role (6–9). The repositioning of A2602 for peptide release can potentially be coordinated with the movement of U2585, the second most flexible nucleotide in the PTC (11,26).

An important aspect that has not yet been directly addressed experimentally is the nature of the chemical group in the PTC that activates the water molecule or possibly stabilizes the oxyanion at the transition state. To address this issue we have employed a recently developed experimental system, the gapped-cp-reconstitution of 50S subunits, that allows non-natural RNA nucleoside analogs to be site-specifically placed into the 23S rRNA of the PTC (27,28). This experimental strategy expands the potential of standard mutagenesis approaches since it is possible to investigate PTC-catalyzed reactions on the functional group, and even at the single atom level of active site residues. The centerpiece of the gapped-cp-reconstitution is the use of circularly permuted 23S rRNA (cp-23S rRNA) that carries a short internal sequence deletion and a chemically synthesized RNA fragment (containing the desired nucleoside modification) that fills the 23S rRNA gap, for *in vitro* assembly of functional 50S particles. This system has already been successfully applied to demonstrate the importance of the A2451 2′-hydroxyl group as functionally critical component for efficient peptide bond formation (27,28). The A2451 ribose 2′-hydroxyl interacts with another important 2′-hydroxyl located at A76 of P-site-bound peptidyl-tRNA (29–31) via hydrogen-bonding. This interaction is considered to be pivotal for a pre-organized electrostatic hydrogen bond network that promotes transpeptidation (32,33), likely primarily via positional (34,35) or entropic forces (36,37). Here, we show that the A2451 2′-hydroxyl does not play an equally fundamental role in peptide release, whereas an intact ribose moiety at the universally conserved A2602 is absolutely required to trigger RF1-mediated peptidyl-tRNA hydrolysis.

MATERIALS AND METHODS

Gapped-cp-reconstitution

Cp-23S rRNA constructs for investigating A2451, U2506, U2584, U2585 and A2602 in the gapped-cp-reconstitution were generated as described previously (27,28). To investigate the contribution of C2063, a cp-23S rRNA was constructed in analogy to the published procedure (28). To generate the DNA template for cp-23S rRNA transcription, the forward and reverse PCR primer pair TAATACGACTCACTATA**G$_{(2069)}$**GAGCTTTACTGCA GCCTG and **T$_{(2043)}$**AGGCCGCATCTTCACGG was used on the plasmid pCPTaq23S carrying tandemly repeated 23S rRNA genes from *T. aquaticus*. The T7 promoter sequences in the forward primer is underlined and the positions defining to the new 5′ and 3′ ends of the cp-23S rRNA are bold and numbered according to *E. coli* nomenclature. Cp-23S rRNA constructs allowing to study A2451 (cp2468-2440; whereas the first number always indicates the 5′-end and the second number the 3′-end of the cp-23S rRNA, respectively), U2506 (cp2523-2483) or C2063 (cp2069-2043) were assembled, and subsequently reassociated with native *T. aquaticus* 30S subunits as described previously (28). For the gapped-cp-reconstitution of 10 pmol cp2069-2043 RNA, 20 pmol of the synthetic RNA oligonucleotide (CCCGUGGCAGGAC GAAAAGACCCCGU) was used to fill the sequence gap during *in vitro* reconstitution which was otherwise performed as described (28). Prior to *in vitro* assembly of cp2623-2576, which allows to study U2584, U2585 and A2602, the chemically synthesized RNA fragment was ligated to the 3′-end of the cp-23S rRNA transcript. Therefore, 60 pmol of cp2623-2576 were mixed with 90 pmol of the 5′-phosphorylated synthetic RNA oligonucleotide AGCUGGGUUCAGAACGUCGUGAGACA GUUCGGUCUCUAUCCGCCAC and 60 pmol of the DNA splinter oligo TCACGACGTTCTGAACCCA GCTCGCGTGCCGCTTTAATGGGCGTTCTGCCC in 16.6 µl water. The mixture was incubated for 3 min at 90°C and subsequently placed on ice for 15 min. 2.4 µl of 10× ligation buffer (400 mM Tris/HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, 2.4 µl of PEG 4000 (Fermentas), 40 u of RNase inhibitor (Fermentas) and 60 u of T4 DNA Ligase (Fermentas) were added and the reaction incubated at 30°C for 4 h. Subsequently, the DNA splinter was digested by the addition of 1 u DNase I (Fermentas) at 37°C for 30 min. The RNA was phenol/chloroform extracted, precipitated, dissolved in water and subsequently used in the gapped-cp-reconstitution.

None-modified RNA oligonucleotides and those carrying a single deoxyribose residue or the C3-linker modification were purchased from *Dharmacon*, while synthetic RNAs that contained the abasic site analog were synthesized by solid-phase synthesis as described (27).

Primer extension analysis for determining ligation efficiencies

The splinter ligation efficiencies between the cp2623-2576 transcript and all used RNA oligonucleotides was always checked via a modified primer extension reaction (38). The 5′ P^{32} radio-labelled DNA primer CGACGTTCTGAACCCAG was annealed to the synthetic RNA oligo two nucleotides downstream of the ligation site and extended by 2 u AMV reverse transcriptase (Promega) as described (39) with the difference that cDNA synthesis was performed in the presence of 66.7 μM ddGTP, 833 μM dATP, 833 μM dCTP and 833 μM dTTP. Under these conditions the reverse transcriptase stops cDNA synthesis at the first encountered C nucleotide which results in a product of 'primer plus 4' nucleotides in the case of a ligated cp-rRNA species, whereas unligated rRNAs produce a 'primer plus 2' product. The cDNA products were resolved on a 15% denaturing polyacrylamide gel and the ligation efficiency quantified by comparing the intensities of the two RT products using the molecular dynamics Storm phosphorimager.

tRNA

Formyl-[^{3}H]Met-tRNA^{fMet} (30 000 cpm/pmol) was prepared and purified by a reversed phase C4 HPLC column as described previously (28).

RF1-mediated peptidyl-tRNA hydrolysis

70S ribosomes (containing 'gapped-cp-reconstituted' 50S subunits assembled from 10 pmol cp-23S rRNA and 4 pmol native 30S subunits from *T. aquaticus)* were programmed with 250 pmol synthetic mRNA UUCAUGUAA (Dharmacon Research, Inc.) and incubated with 0.3 pmol formyl-[^{3}H]Met-tRNA^{fMet} (30 000 cpm/pmol) for 15 min at 30°C in 15.9 μl of the reconstitution buffer containing 20 mM Tris/HCl pH 7.4, 20 mM $MgCl_2$, 400 mM NH_4Cl, 5 mM β-mercaptoethanol and 0.2 mM EDTA (40). The peptidyl-tRNA hydrolysis reaction was initiated by the addition of 15 pmol *T. thermophilus* RF1 (41) and performed at 25°C in a final volume of 18.9 μl. The reaction was stopped and quantified by liquid scintillation counting as described (24). Under these single turnover conditions (28), *in vitro* reconstituted particles containing the full-length wild-type 23S rRNA transcript hydrolyzed the peptidyl-tRNA analog quantitatively, whereas in ribosomes containing gapped-cp-reconstituted 50S subunits the fraction of P-site substrate that was converted into product was in average between 20% (cp2623-2576) and 50% (cp2069-2043).

RF-independent peptidyl-tRNA hydrolysis

The release factor-independent peptidyl-tRNA hydrolysis assay using 70S ribosomes (containing 'gapped-cp-reconstituted' 50S subunits assembled from 10 pmol cp-23S rRNA and 4 pmol native 30S subunits from *E. coli)* was performed as described (24) with the following modifications: 70S were programmed with 30 pmol of a 98 nt long mRNA *in vitro* transcript (42) placing a unique Met codon in the P-site, incubated with 0.3 pmol formyl-[^{3}H]Met-tRNA^{fMet} (30 000 cpm/pmol) and the reaction was initiated by the addition of 780 pmol of the synthetic CCA tri-nucleotide (Dharmacon Research, Inc.) and 30% acetone. The final reaction volume was 36 μl.

Peptide bond formation

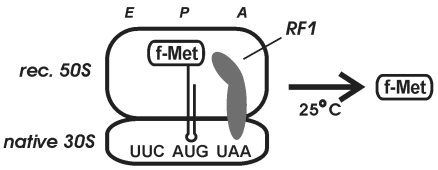
To measure transpeptidation activities of the gapped-cp-reconstituted 50S subunits carrying nucleoside analogs in the PTC, the puromycin reaction was employed. Therefore, 10 pmol reconstituted 70S were programmed with 60 pmol synthetic mRNA containing a unique AUG codon and incubated with 0.3 pmol formyl-[^{3}H]Met-tRNA^{fMet} (30 000 cpm/pmol) in order to fill the P-site. Puromycin was added to a final concentration of 2 mM, the peptidyl transferase reaction was performed, and the reaction product identified as described (28).

RESULTS

Experimental system for studying peptide release

The gapped-cp-reconstitution system offers the advantage of performing nucleoside analog interference studies to unravel crucial 23S rRNA groups for PTC functions (27,28). Here we applied this experimental tool to characterize the functionally pivotal groups on active site PTC residues for RF1-mediated peptide release. Previous standard mutagenesis studies highlighted the potential involvement of A2602 (and to a lesser extent U2585) in the catalysis of peptidyl-tRNA hydrolysis (24,25). We therefore constructed a cp-23S rRNA that harbors its novel 5′-and 3′-ends at positions 2623 and 2576 (cp2623-2576), respectively, which places a gap of 45 nt inside the PTC encompassing both prime candidates A2602 and U2585 (Figure 1A and B). While this 23S rRNA variant has been shown to possess clear peptidyl transferase activity when the missing rRNA fragment was added *in trans* during the *in vitro* assembly (27), no RF1-mediated peptide release could be measured (Figure 2). Obviously the precise positioning of helix 93 (harboring residue A2602) which packs against helix 74 of 23S rRNA via a GNRA tetraloop/helix interaction (43), cannot optimally be achieved under these reconstitution conditions. In order to improve the catalytic performance of this gapped-cp-23S rRNA, the synthetic 45-mer RNA fragment was ligated to the 3′-end of the cp-23S rRNA transcript via the 'splinter ligation' approach (44). To test whether this ligated cp-23S rRNA construct would be active in peptidyl-tRNA hydrolysis, we first generated a cp-23S rRNA placing its 5′- and 3′-ends at positions 2623 and 2622, respectively, by transcribing the corresponding PCR template (28). This cp-23S rRNA mimics a 100% successful ligation event of the 45-mer RNA oligo to cp2623-2576 (Figure 1A). Under the applied conditions, P-site-bound f-[^{3}H]Met-tRNA^{fMet} could be efficiently and almost quantitatively hydrolyzed (Figure 2B).

A



B

construct	RF1	f[^{3}H]Met released (cpm)	fraction f[^{3}H]Met-tRNA hydrolyzed
reaction buffer	—	217 ± 18	0.02
30S	+	411 ± 26	0.04
rec.50S_(cp2623-2576)	+	445 ± 11	0.05
rec.50S_(cp2623-2576) + wt-oligo	—	328 ± 113	0.04
rec.50S_(cp2623-2576) + wt-oligo	+	2,142 ± 26	0.24
rec.50S_(cp2623-2622_wt)	+	8,097 ± 460	0.90
rec.50S_(cp2623-2622_Δ2602)	+	483 ± 63	0.05

C

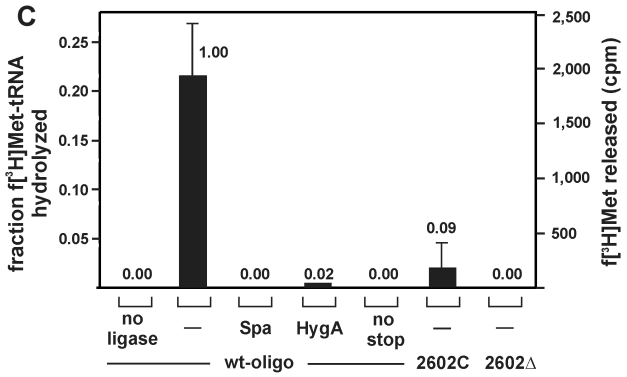


Figure 2. RF1-mediated peptide release activities of gapped-cp-reconstituted 50S subunits. **(A)** To measure peptidyl-tRNA hydrolysis activity, gapped-cp-reconstituted 50S (rec. 50S) were reassociated with native 30S subunits and programmed with an mRNA analog placing the stop codon UAA into the A-site. The peptidyl-tRNA analog f-Met-tRNA was bound to the P-site and the reaction initiated by the addition of RF1 from *T. thermophilus*. **(B)** The amount of hydrolyzed f-[^{3}H]Met-tRNA in the absence of ribosomal particles (reaction buffer), in the presence of the small ribosomal subunit (30S) or in the presence of 70S ribosomes containing native 30S and gapped-cp-reconstituted 50S after 30 min of incubation is shown. Gapped-cp-reconstituted 50S particles assembled from cp2623-2576 were tested in the absence and presence of the ligated 45-mer RNA fragment containing the wild-type sequence (+ wt oligo). 50S assembled form cp2623-2622 were assayed in the context of the wild-type sequence (cp2623-2622_wt) and in the context of the A2602 deletion mutant (cp2623-2622_Δ2602). RF1 was absent (−) or present (+) at a concentration of 0.8 μM. The mean and SD of released f-[^{3}H]Met from two representative experiments are shown (cpm) as well as the fraction of the 0.3 pmol input peptidyl-tRNA that was hydrolyzed are indicated. **(C)** Peptidyl-tRNA hydrolysis product yields of reconstituted 50S subunits that have been assembled from cp2623-2576 to which the compensating synthetic wild-type 45-mer RNA (wt-oligo) or the 2602C or 2602Δ variants have been ligated. The yield of f-[^{3}H]Met released by particles carrying the wt-oligo at the end of the reaction curve after 30 min of incubation has been taken as 1.00. The relative activities of gapped-cp-reconstituted 50S subunits carrying the ligated wt-oligo in the presence of antibiotics (sparsomycin: Spa; hygromycin A: HygA), or in the absence of an A-site-bound stop codon (no stop), as well as the activities of the two 2602 mutants are shown above the respective bars. The RF1-mediated hydrolysis strictly depends on the covalent connection of the synthetic RNA fragment to the cp-23S rRNA, since no product formation was observed in control experiments where the T4-DNA ligase has been omitted (no ligase). Values shown represent the mean and the SD of at least two independent experiments.

In accordance with our previous findings (24), deletion of the entire nucleotide A2602 also in this cp-23S rRNA context, completely abolished the hydrolytic activity of the PTC (Figure 2B). Thus, it appears that the ligation approach could potentially solve the functional problems of gapped-cp-reconstituted 50S containing the cp2623-2576 construct in peptidyl-tRNA hydrolysis.

Ligation efficiencies for the synthetic 45-mer RNA oligonucleotide (either containing the wild-type sequence or specific nucleoside modifications) were determined via a modified primer extension approach (see Materials and Methods section) to be in the range from 20% to 55% (data not shown). Importantly, while ligation efficiencies varied between different experiments, very similar efficiencies were obtained within one set of ligations performed in parallel (SDs < 23%). Therefore, we always compared the peptide release activities of gapped-cp-reconstituted 50S carrying different modifications that originated from the same set of parallel ligation experiments. This minimizes the possibility that potential differences in the peptide release activities between differently modified gapped-cp-reconstituted 50S result from varied ligation yields. Reconstituted 50S particles containing cp-23S rRNA with the ligated wild-type RNA fragment showed clear RF1-dependent peptide release activity (Figure 2B). Importantly, control experiments revealed peptidyl-tRNA hydrolysis to be ribosome-dependent, RF-dependent and strictly required an A-site-bound stop codon on the 30S subunit (Figure 2B and C). Furthermore, the peptidyl-tRNA hydrolysis activity was sensitive to known inhibitors of translation termination, such as sparsomycin and hygromycin A (Figure 2C). These controls strongly indicate that an authentic peptide release reaction is monitored in our experimental system. With the ligated wild-type sequence the gapped-cp-reconstituted 50S particles catalyzed peptidyl-tRNA hydrolysis with a rate of 0.1/min, which is about 300-fold slower than reported termination rates in an optimized translation system employing native *E. coli* ribosomes and factors (3). Despite these intrinsic limitations of the gapped-cp-reconstitution approach (27,28), our experimental system was able to assess severe reductions in RF1-triggered peptide release such as those observed with the 2602 deletion mutant (2602Δ) or the 2602C base mutation (Figure 2B and Table 1). Previously, very similar results were obtained with these two mutants in the context of *in vitro* assembled full-length 23S rRNA transcripts with native 5′- and 3′-ends (24) as well as with *in vivo* derived 2602 mutant ribosomes (25). Therefore, the experimental system appears to report reliable PTC activities, and is therefore amenable for nucleoside modification interference studies.

Nucleoside modifications at A2602 and U2585 and the effects on peptide release

We first introduced the abasic site analog at A2602 (Figure 1C), the prime candidate in the PTC residue for peptide release. Unexpectedly, this modification did not interfere with the hydrolytic activity of the PTC. In fact, a slight but reproducible stimulation of peptide release

Table 1. Peptide release activities of chemically modified ribosomes

23S rRNA position	Modification	k_{rel}[a]	Rel. decrease	Rel. increase
wt	None	1.00	(1.0)	(1.0)
A2602	C	0.03 ± 0.04	33.0	–
	Δ	<0.02	>50.0	–
	dA	0.71 ± 0.12	1.4	–
	abasic	1.52 ± 0.44	–	1.5
	d-abasic	0.76 ± 0.06	1.3	–
	C3-Linker	0.03 ± 0.02	33.0	–
U2585	dU	1.77 ± 0.10	–	1.8
	abasic	2.57 ± 0.41	–	2.6
	C3-Linker	1.27 ± 0.19	–	1.3
U2584	dU	0.65 ± 0.18	1.5	–
U2506	dU	1.16 ± 0.17	–	1.2
	abasic	1.14 ± 0.18	–	1.1
	C3-Linker	0.61 ± 0.11	1.6	–
A2451	dA	0.80 ± 0.21	1.2	–
	abasic	0.56 ± 0.25	1.7	–
	C3-Linker	0.31 ± 0.02	3.2	–
C2063	dC	0.94 ± 0.03	1.1	–
	abasic	0.73 ± 0.17	1.4	–
	C3-Linker	0.20 ± 0.08	5.0	–

[a]Initial peptide release rate (k_{rel}) of ribosomes carrying the wild-type (wt) synthetic RNA-oligomer was taken as 1.00 and compared to ribosomes containing modified nucleoside analogs at positions A2602, U2585, U2584, U2506, A2451 and C2063 or the 2602C as well as 2602Δ mutation. k_{rel} were determined from time points in the linear range of two to five independent time course experiments. The activity of the 2602Δ mutation was below the detection limit of our experimental system ($k_{rel} < 0.02$). Tested nucleoside analogs (see also Figure 1C): 2′-deoxyribonucleosides (dN), the ribose-abasic site analog (abasic), or the C3-linker modification (C3-linker). At position 2602 also the deoxyribose-abasic site analog (d-abasic) has been tested.

was observed (Figure 3A and Table 1). It appears that a PTC that does not harbour any base at position 2602 is actually more capable of releasing the peptide from the peptidyl-tRNA than a ribosome with the C mutation (Table 1). This suggests that the 2602C base mutant is trapped in an unproductive conformation and explains why this mutant ribosome never produces the same product yield compared to wild-type ribosomes after 30 min of incubation [Figure 2B and (24)]. These data also exclude the possibility that the crucial functional group of A2602 resides on the nucleobase. Removal of the 2′-hydroxyl group either in the context of the abasic site analog (by introducing the deoxyribose-abasic modification) or in the context of the complete nucleoside (by introducing deoxy-adenosine) also did not show significant reductions (Figure 3A and Table 1). However, introducing the C3-linker modification, which lacks in addition to the entire nucleobase also the C1′, C2′ and the O4′ of the ribose sugar (Figure 1C), almost completely deprives the PTC of any catalytic power to hydrolyze the P-site-located peptidyl-tRNA. Only marginal amounts of released f-[^{3}H]Met above the background could be measured after a prolonged incubation time (Figure 3A). Compared to the gapped-cp-reconstituted 50S subunits carrying the ligated wild-type RNA fragment, the rate of peptidyl-tRNA hydrolysis with the C3-linker variant decreased by at least 33-fold (Table 1). Increasing the RF1 concentration 7-fold (from 0.8 to 5.6 μM) did not rescue any release activity of the 2602 C3-linker modified PTC, thus strongly indicating RF binding deficiencies not to be the reason for the diminished termination activity (data not shown). To more directly clarify this, we applied a peptidyl-tRNA hydrolysis assay that is independent of ribosome-bound release factors. In this set up, the hydrolytic power of the PTC is activated by A-site-bound deacylated tRNA or the 3′ terminal CCA end thereof (23). We employed this RF-independent assay with the CCA tri-nucleotide to test the peptide release activities of all 50S subunits carrying modifications at position 2602. Also under these conditions the C3-linker modification at 2602 almost completely inhibited peptidyl-tRNA hydrolysis, while all other modifications showed no or only minor inhibitory effects (Figure 4). This is independent evidence that the severely reduced activities of 50S subunits carrying the C3-linker at 2602 are resulting from a catalytic deficiency of the PTC to hydrolyze peptidyl-tRNA and is independent of the bound A-site substrate.

Like A2602, also U2585 has been shown to be a structurally flexible residue in the catalytic cavity of the PTC in various 50S subunit crystal structures (6,7,9,11,26,45). Furthermore, mutations at this base also showed reductions in peptide release activities, albeit to a weaker extent compared to A2602 (24,25). Here we introduced the abasic, the deoxy-uridine and the C3-linker modification at U2585 and tested the chemically engineered subunits in the termination assay. It turned out that none of the U2585 modifications interfered with RF1-medited peptide release. In fact all of them showed slightly enhanced (~ 2-fold) peptide release rates (Table 1). Based on crystallographic studies it has been suggested that the base of U2585 protects the ester bond of P-site-bound peptidyl-tRNA from premature hydrolysis during the elongation phase of protein synthesis (9). We therefore tested if the accelerated peptide release rates of the abasic 2585 subunit are the result of an RF-independent peptidyl-tRNA hydrolysis. To this end, the 2585-abasic particle as well as gapped-cp-reconstituted 50S carrying the wild-type sequence were incubated in the absence of RF1. Removal of the nucleobase at U2585 indeed slightly increased the premature background hydrolysis of peptidyl-tRNA. Compared to the wild-type control the background increased ~2-fold in the 2585 abasic PTC (300 versus 600 cpm) after an incubation of 30 min. However, this amount of released f-[^{3}H]Met does not completely account for the observed release stimulation of the abasic 2585 50S subunits, since it only raises the measured product yield by 15% (data not shown).

Peptide release of 50S subunits carrying modifications at other active site residues

To investigate whether any of the other active site residues carry functional groups essential for peptide release, we introduced deoxy-ribose nucleosides, the abasic analog as well as the C3-linker modification at U2506, A2451 and C2063 (Figure 1). It is of note that all of the respective cp-23S rRNA constructs were active in RF1-mediated

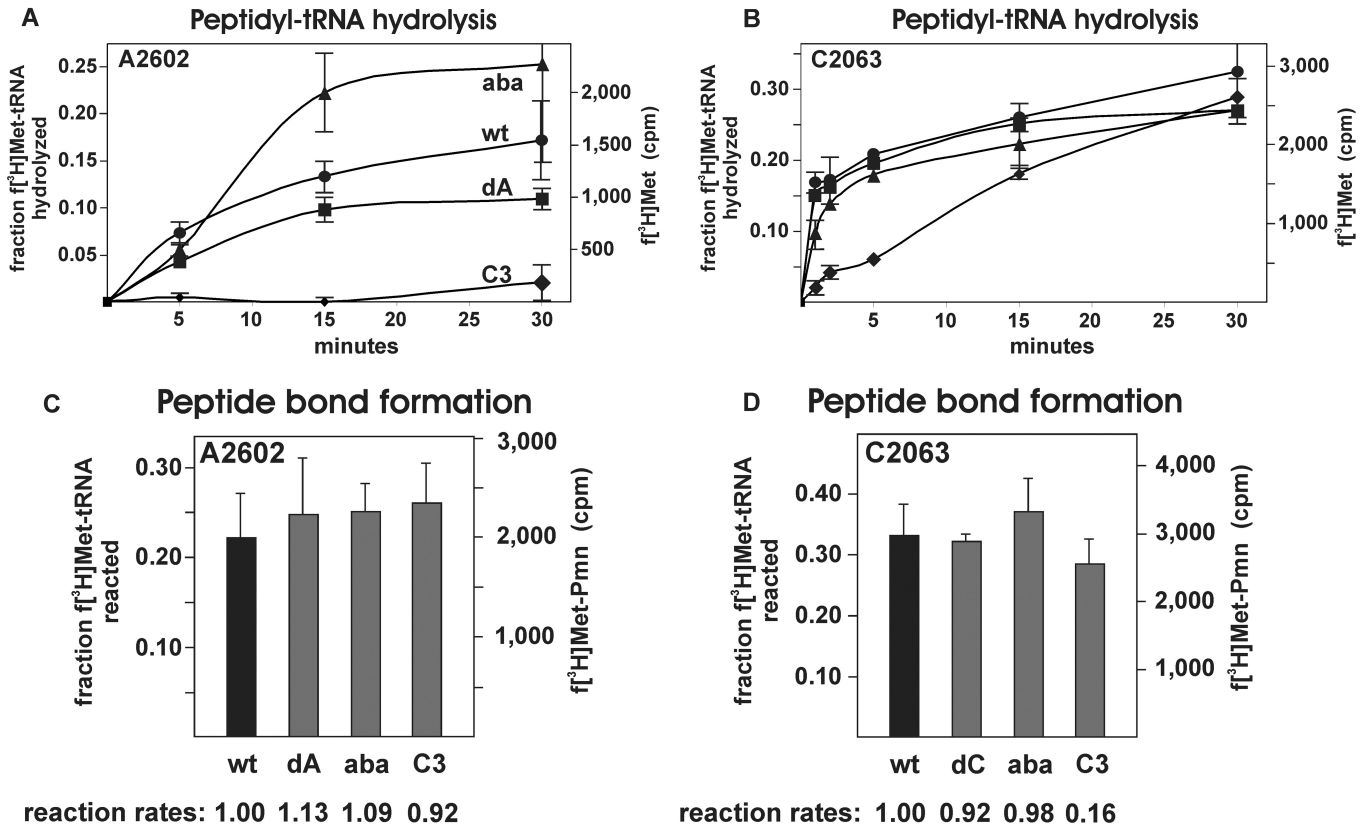


Figure 3. The catalytic activities of the PTC carrying nucleoside modifications at position A2602 or C2063. Time course of peptidyl-tRNA hydrolysis promoted by ribosomes containing gapped-cp-reconstituted 50S carrying either the wild-type sequence (wt; circles), the 2′-deoxyribonucleoside (dN; squares), the abasic site analog (aba; triangles), or the C3-linker modification (C3; diamonds) at 23S rRNA positions A2602 **(A)** or C2063 **(B)**. The curves represent the mean and the SD of five independent time course experiments. The same particles were also used in peptidyl transferase reactions employing puromycin as an A-site substrate. The amount of product formation at the reaction plateau (incubation time 120 min) formed by ribosomes carrying modifications at A2602 **(C)** or C2063 **(D)** are shown (gray bars) and compared to the yields produced by the respective gapped-cp-reconstituted wild-type ribosomes (black bars). The initial peptidyl transferase rates, which were determined from experimental points in the linear range of the reactions within the first 15 min of incubation, are shown below the graphs. The rates were normalized to the rate of gapped-cp-reconstituted ribosomes containing the synthetic wild-type RNA fragments. In all cases (A–D) the background values (amount of product formation in reactions containing 50S subunits that have been reconstituted in the absence of the synthetic RNA fragments) were subtracted from all experimental data points and the fraction of the 0.3 pmol input f-[^{3}H]Met-tRNA that reacted are indicated.

peptide release even when the synthetic RNA oligomer, that was required to fill the introduced gap, was added *in trans* during reconstitution, and therefore not ligated to the 3′-end of the cp-23S rRNA transcript. Also the reaction rates increased compared to the cp2623-2576 construct used to study A2602 and U2585 and were in the range from 0.2/min (cp2468-2440) to 1.0/min (cp2523-2483). This likely indicates that none of these regions of the PTC is equally important to promote peptidyl-tRNA hydrolysis compared to the A2602 region. In agreement, none of the modifications introduced at U2506, A2451, or C2063 had equally severe effects on termination compared to the 2602 C3-linker (Figure 3B and Table 1). With the exception of the A2451 C3-linker modification (see subsequently), all chemically modified 50S particles reached essentially the same product yield compared to the wild-type control after 30 min of incubation and the peptide release rates were only slightly affected (Figure 3B and Table 1). The strongest effects in these experiments were seen with the PTC containing the C3-linker at position 2063 whose release rate was inhibited 5-fold (Figure 3B). However, similar rate reductions were also measured in the puromycin reaction with this modified ribosome (6-fold), thus indicating a more general, probably conformational, defect of the PTC carrying this 2063 modification for 50S-catalyzed reactions (Figure 3D). We would like to point out here that removing the 2′-hydroxyl group at A2451, which was shown before to be crucial for peptide bond formation (27,28), reduced RF1-mediate peptide release only marginally by 1.2-fold (Table 1). This indicates the catalytic mechanism for peptide bond formation to be distinct from that of peptide release, as suggested earlier (24).

Modified ribosomes carrying the 2602 C3-linker are active in peptide bond formation

It is possible that any changes in the peptide release activities of chemically modified 50S particles, especially the 2602 C3-linker variant, are the result of varying reconstitution efficiencies. To investigate this possibility we employed the modified 50S particles in a peptidyl

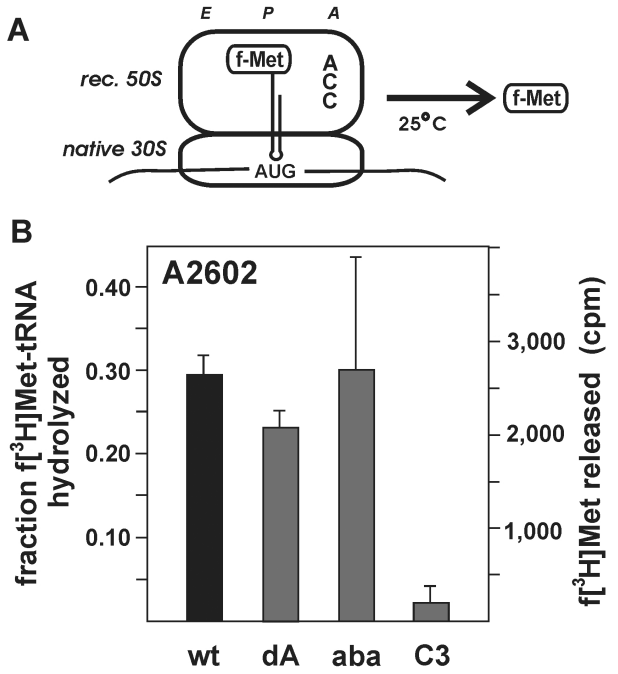


Figure 4. RF-independent peptidyl-tRNA hydrolysis activity of ribosomes carrying modifications at 23S rRNA position 2602. **(A)** In the RF-independent assay the hydrolytic activity of ribosomes containing gapped-cp-reconstituted 50S was initiated by binding of the CCA tri-nucleotide to the 50S A-site. **(B)** The product yields of hydrolyzed f-[^{3}H]Met-tRNA using ribosomes carrying the wild-type sequence (wt), the 2′-deoxy-adenosine (dA), the abasic site analog (aba), or the C3-linker modification (C3) at 23S rRNA positions A2602 after 30 min of incubation are shown. The values represent the mean and SD of three independent experiments. The amount of hydrolyzed peptidyl-tRNA in reactions containing 50S subunits that have been reconstituted in the absence of the synthetic RNA fragment encompassing position A2602 was subtracted from all data points.

transferase reaction using puromycin as A-site substrate. It turned out that all the 2602 modified ribosomes, including the C3-linker variant, produced the same amount of reaction product in the peptidyl transferase assay compared to the wild-type particles (Figure 3C). We have established previously that both peptide release and peptide bond formation are single turnover reactions under the applied conditions (28). Thus, these data indicate that comparable fractions of catalytically competent 50S particles were *in vitro* assembled with all 2602 variants, and therefore exclude the possibility of gross reconstitution deficiencies of the C3-linker containing cp-23S rRNA. Similarly, ribosomes harboring modifications at position C2063, a residue not tested previously in the gapped-cp-reconstitution system, reached wild-type levels of product yields in the puromycin reaction (Figure 3D). The effects of placing deoxy-nucleoside as well as abasic or C3-linker modifications at positions U2585 and U2506 have been shown previously not to affect the peptidyl transfer rates (27). The only modified 50S particle whose reconstitution efficiency could not be assessed by employing the peptidyl transferase reaction was the 2451 C3-linker variant. This modification deprives A2451 of the key functional ribose 2′-hydroxyl, and therefore renders the PTC inactive for transpeptidation (28). Nevertheless, since the product yield of the 2451 C3-linker variant in the peptide release assay was only reduced by ~2-fold (data not shown) suggest that a catalytically competent 50S can even be formed in the presence of this rather rigorous nucleoside analog introduced at this central PTC residue.

DISCUSSION

Based on the results of previous mutagenesis studies an unequivocal functional role for the universally conserved inner shell nucleobases for PTC-catalyzed reactions could not be assigned [reviewed in (1)]. Here we present evidence that none of the 23S rRNA nucleobases at A2602, U2585, U2506, A2451 or C2063 harbor fundamentally crucial functional groups for peptidyl-tRNA hydrolysis since high peptide release activities remained when abasic site analogs were introduced at those sites (Table 1). The severe reductions seen before in ribosomes carrying base changes at A2602 (24,25) or U2585 (25) are therefore likely the result of a conformationally distorted PTC that trapped the active site in a non-productive state for RF1-mediated peptidyl-tRNA hydrolysis. In support of this conclusion, we found strongly stimulated peptidyl-tRNA hydrolysis rates when the mutant base was entirely removed from the 2602C (Figure 2B and Table 1) or the 2585C (data not shown) mutant 50S particle. Likely these active site residues require a certain amount of structural flexibility for full peptide release activity which is obviously not necessarily the case when an incorrect base is attached to the ribose. However, full termination activities can be re-gained when the mutant bases are removed at 2602 or 2585 by introducing abasic site analogs thus hinting at an important contribution of the ribose moiety for translation termination. Introducing deoxy-ribose nucleosides at A2602, U2584, U2585, U2506, A2451 or C2063 did not interfere significantly with peptide release, excluding the possibility that any of these 2′-hydroxyl groups are crucial for peptidyl-tRNA hydrolysis (Table 1). However, further minimization of the ribose moieties of active site residues by removing in addition to the base also the C1′, C2′ and O4′ of the sugar by introducing the C3-linker (Figure 1C) clearly emphasizes the importance of a single ribose, namely at position 2602 (Figure 3A and Table 1). Compared to the abasic 2602 ribosome, the C3-linker containing PTC showed at least 50-fold slower peptidyl-tRNA hydrolysis rates. Peptidyl-tRNA-binding deficiencies to the P-site in the 2602 C3-linker ribosome are unlikely since essentially wild-type levels of peptidyl transfer activities remained (Figure 3C) and none of the interactions of P-tRNA with the 23S rRNA that have been identified recently to be important for efficient peptide release (46), have been manipulated. Applying an RF-independent peptidyl-tRNA hydrolysis assay which was activated by the A-site-bound 3′-terminal CCA end of deacylated tRNA, again revealed a significantly decreased activity when the C3-linker modification was introduced at position 2602 (Figure 4). This strongly suggests that the reason for the

diminished peptidyl-tRNA hydrolysis activities of the modified PTC was not due to A-site substrate-binding deficiencies. Since the CCA tri-nucleotide required in the RF-independent assay interacts solely with the 50S part of the ribosomal A-site (47), we can further conclude that no function of the 30S decoding center is causally linked to the observed compromised peptide release activity of the PTC with the C3-linker modification at position 2602. Thus, even though the P-site (peptidyl-tRNA) and A-site (RF1 or CCA) substrates are most likely present, the PTC carrying the C3-linker at 2602 was essentially unable to hydrolyze the peptidyl-tRNA, indicating that the nucleophile (the water molecule) is absent or strongly misplaced. The same C3-linker modification at all the other investigated PTC residues had no (2585) or relatively minor (2506, 2451 and 2063) inhibitory effects (Table 1). This highlights the distinct functional role of the A2602 ribose ring for peptidyl-tRNA hydrolysis.

How can these findings be explained in the context of the proposed model of translation termination, in which A2602 has been suggested to coordinate and possibly activate the water molecule for the nucleophilic attack on the ester bond of peptidyl-tRNA (24)? With the exception of the O4′ position, which possesses lone-pair electrons, none of the crucial positions of the 2602 ribose (the C1′ and C2′) identified here has any functional groups to hydrogen bond to a water molecule or a hydrated metal ion. Furthermore, the distance of the 2602 ribose to the position where the attacking α-amino of aminoacyl-tRNA has been located in the crystal structure, and from where the nucleophilic water molecule is also supposed to launch its attack during peptide release, appears to be too large for the direct coordination of the hydrolytic water. The distance of the A2602 O4′ to the nitrogen atom of the α-amino group of puromycin is 11.5 Å (45). Even though we cannot completely discard the possibility for an important direct water coordination by the ribose O4′ during the peptide release reaction, it seems more likely that A2602 functions as a molecular switch in the ribosome that regulates the specificity of the PTC from amide bond formation, in the case where aminoacyl-tRNA is located at the A-site, to peptidyl-tRNA hydrolysis when the RF is bound. For this switching function the nucleobase at 2602 is not actually required, however, an intact ribose moiety appears to be strictly necessary. For this crucial task during translation termination, however, it is not important whether the sugar at position 2602 is a ribose or a deoxyribose, since the PTC carrying the deoxyribose-abasic analog showed wild-type-like peptidyl-tRNA hydrolysis activities (Table 1). This modified model of peptide release is compatible with the structurally mobile nature of A2602 which involves movements of both the ribose and the base (6,7,9,10,26) as well as with the recent structure of RF1-bound 70S particles, showing close proximity of the universally conserved GGQ mini-motif of the RF to A2602 (17). However, which group actually activates or positions the water molecule for optimal attack? The A2451 2′-hydroxyl which has been shown to be pivotal for catalyzing peptide bond formation (27,28) does not seem to play an equally important role in peptide release, and thus does not qualify for activating the nucleophile. Groups at other PTC residues are also not critical (Table 1), leaving the 2′-hydroxyl of A76 of P-tRNA, which plays an important role in peptide bond formation (29–31), or a group on the RF as potential candidates for possessing the catalytic group for peptidyl-tRNA hydrolysis. Since all previous mutational studies suggest that peptide release is an RNA-promoted reaction rather than directly catalyzed by the protein RF [reviewed in (1)], we favor the A76 2′-hydroxyl of P-site-bound peptidyl-tRNA. In fact, a clear inhibition of RF1-mediated peptide release has been observed when this 2′-hydroxyl was removed from peptidyl-tRNA (R. Green, personal communication). In this scenario, A2602 functions as a molecular trigger that is pulled in response to the A-site-bound RF to allow the nucleophilic water to access the active site. In our view, A2602 however does not simply function as a passive lid that prevents premature water access to the peptidyl-tRNA ester bond, since removal of the entire nucleotide in the 2602Δ mutant did not increase the background hydrolysis of P-site-bound fMet-tRNA in the absence of RF1 (data not shown). It is possible that the ribose at A2602 specifically channels or guides the hydrolytic water molecule into the PTC which is then activated and optimally positioned for the attack by a non-ribosomal group, most plausibly by the A76 2′-hydroxyl of peptidyl-tRNA. It is of note that as for transpeptidation where the 2′-hydroxyl at A2451 is crucial (27,28), the ribosome again provides a 23S rRNA backbone group, the ribose moiety at A2602, rather than any group on a nucleobase to promote the second chemical reaction of the PTC, namely peptidyl-tRNA hydrolysis.

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