Role of RNA backbone groups for ribosomal catalysis

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Summary

The ribosomal peptidyl transferase ribozyme resides in the large ribosomal subunit and catalyzes the two principal chemical reactions of protein synthesis, peptide bond formation and peptidyl-tRNA hydrolysis. With the presentations of atomic structures of the large ribosomal subunit, the questions how an RNA active site can catalyze these chemical reactions gained a new level of molecular significance. The peptidyl transferase center represents the most intense accumulation of universally conserved ribosomal RNA nucleotides in the entire ribosome. Thus, it came as a surprise that recent findings revealed that the nucleobase identities of active site residues are actually not critical for catalysis. Instead RNA backbone groups have been identified as key players in transpeptidation and peptide release. While the ribose 2'-OH of the 23S rRNA residue A2451 plays an important role in peptidyl transfer, its contribution to peptidyl-tRNA hydrolysis is only minor. On the other hand, the ribose 2'-OH of the terminal adenosine of P-site bound tRNA seems to play equally crucial roles in peptide bond formation and tRNA hydrolysis. While it seems that details of ribosome-catalyzed peptidyl-tRNA hydrolysis are just emerging, our molecular insights into transpeptidation are already very advanced. It has been realized that an intricate interaction between the ribose 2'-OH groups of 23S rRNA residue A2451 and tRNA nucleotide A76 is crucial for proton shuttling that is required for efficient amide bond synthesis.

Key words:

ribosomes, rRNA, peptide bond formation, peptidyl-tRNA hydrolysis, translation termination, protein synthesis, ribozyme.

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1. Introduction

Translation of the genetic information encoded within mRNAs into polypeptides represents one of the final steps in gene expression. Pivotal for protein biosynthesis is a multifunctional ribonucleoprotein (RNP) complex, termed the ribosome. In prokaryal organisms, ribosomes sediment at 70S and are composed of two unequal subunits, the small 30S subunit and the large 50S subunit. In bacteria, the ribosome has a molecular weight of 2.6-2.8 MD with about 2/3 of the mass consisting of ribosomal RNA (rRNA) and 1/3 of ribosomal proteins (r-proteins). The 50S subunit is built from two rRNA molecules (the ~2900 nucleotides long 23S rRNA and the 120 residues long 5S rRNA) and about 33 different r-proteins. The 30S subunit on the other hand contains a single rRNA strain (the ~1500 nucleotides long 16S rRNA) and approximately 20 r-proteins.

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 and is located in a deep crevice at the interface side. The two central chemical reactions of protein synthesis are performed by the PTC, namely peptide bond formation and peptidyl-tRNA (pept-tRNA) hydrolysis (for a review see ref. (1)). During the elongation cycle the PTC links amino acids via peptide bonds into polypeptide chains, whereas during the termination phase pept-tRNA is hydrolyzed and the completely synthesized protein released from the ribosome (Fig. 1). The formation of a peptide bond involves aminolysis by the α-amino group of the A-site aminoacyl-tRNA (aa-tRNA) of the ester bond that carries the nascent peptide at the C3' position of the terminal ribose of pept-tRNA. Subsequent to the nucleophilic attack of the α-amino group, a short-lived tetrahedral transition state is formed that breaks down by donating a proton to the leaving oxygen to yield the reaction products deacylated tRNA at the P-site and pept-tRNA (elongated by one amino acid) at the A-site (Fig. 1). This reaction is accurately catalyzed by the ribosome *in vivo* with a remarkable speed of ~ 20 peptide bonds per second but even higher velocities were obtained *in vitro* (2,3). From an energetic point of view formation of a peptide bond does not necessitate additional energy, since almost 8 kcal/mol are 'stored' in the ester bond of aa-tRNA and only ~ 0.5 kcal/mol are needed for amide bond formation (4). Nevertheless, the uncatalyzed reaction (extrapolated from model reactions) occurs very slowly in solution with less than one bond formed per day (5). Thus the ribosome accelerates the rate of peptide bond formation approximately 10^7-fold (6).

The second principle chemical reaction which is promoted by the PTC is pept-tRNA hydrolysis. This reaction is required during translation termination for the release of the fully assembled polypeptide from the ribosome. The termination reaction involves the transfer of the peptidyl moiety of P-site located pept-tRNA to a water molecule (Fig. 1). From a chemical standpoint, pept-tRNA hydrolysis is a more demanding reaction compared to peptide bond synthesis because hydrolysis of the ester bond is driven by a significantly less nucleophilic water oxygen. The cataly-

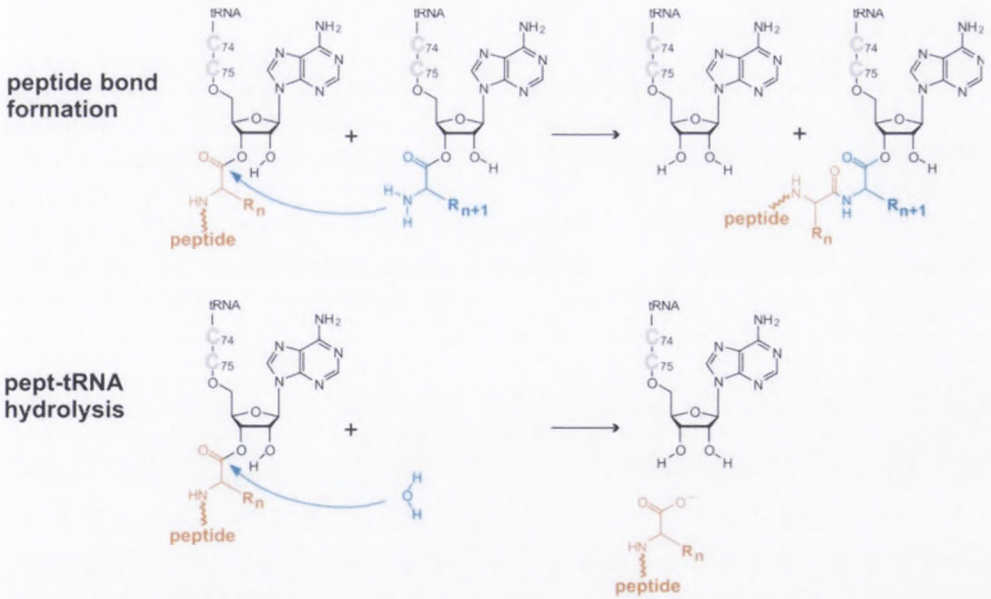

Fig. 1. The two principal chemical reactions of protein synthesis. During peptide bond formation, the α-amino group of aminoacyl-tRNA in the A-site (blue) attacks the ester carbonyl carbon of P-site bound peptidyl-tRNA (orange). A short-lived tetrahedral intermediate is formed (not shown) that decomposes into the reaction products, deacylated tRNA at the P-site and peptidyl-tRNA elongated by one amino acid at the A-site. During translation termination, the ester carbonyl carbon of P-site located peptidyl-tRNA (orange) is nucelophilically attacked by an activated water molecule (blue) which leads to peptidyl-tRNA hydrolysis and polypeptide release. For clarity reasons only the terminal CCA ends of the ribosome-bound tRNAs are shown.

tic rate constant of pept-tRNA hydrolysis has been estimated to be 0.5-1.5 per second and is therefore clearly slower than transpeptidation (7). The switch of the PTC from amino acid polymerization to pept-tRNA hydrolysis is triggered by a protein of the class I release factor family (RF1 or RF2 in bacteria) which binds in response to an A-site displayed mRNA stop codon.

The means by which the PTC catalyzes these fundamental biological reactions has been a subject of intense discussion over the last decades. Initially a ribosomal protein-based scenario was favored, however no r-protein with catalytic properties could be identified (reviewed in (1)). With the presentations of the first high-resolution crystallographic structures of the 50S subunit at the dawn of the new millennium the case was finally sealed (8-10). These structures decisively revealed the PTC as an RNA enzyme and thus place the ribosome as key entry on the list of naturally occurring ribozymes that outlived the transition from the pre-biotic 'RNA World' to contemporary biology.

2. Molecular characteristics of the active site

The active site crater harboring the PTC is built by the universally conserved nucleotides of the central loop of domain V of 23S rRNA (Fig. 2A). The inner core of the PTC is comprised of the universally conserved residues C2063, A2451, U2506, U2585, and A2602 (*E. coli* nomenclature is used here and throughout the review). In all available crystal structures the universally conserved A2451 of 23S rRNA makes the closest approach to the nucleophilic α-amino group of A-site bound aa-tRNA with its nucleobase position N3 as well as its ribose 2'-hydroxyl group (2'-OH) in hy-

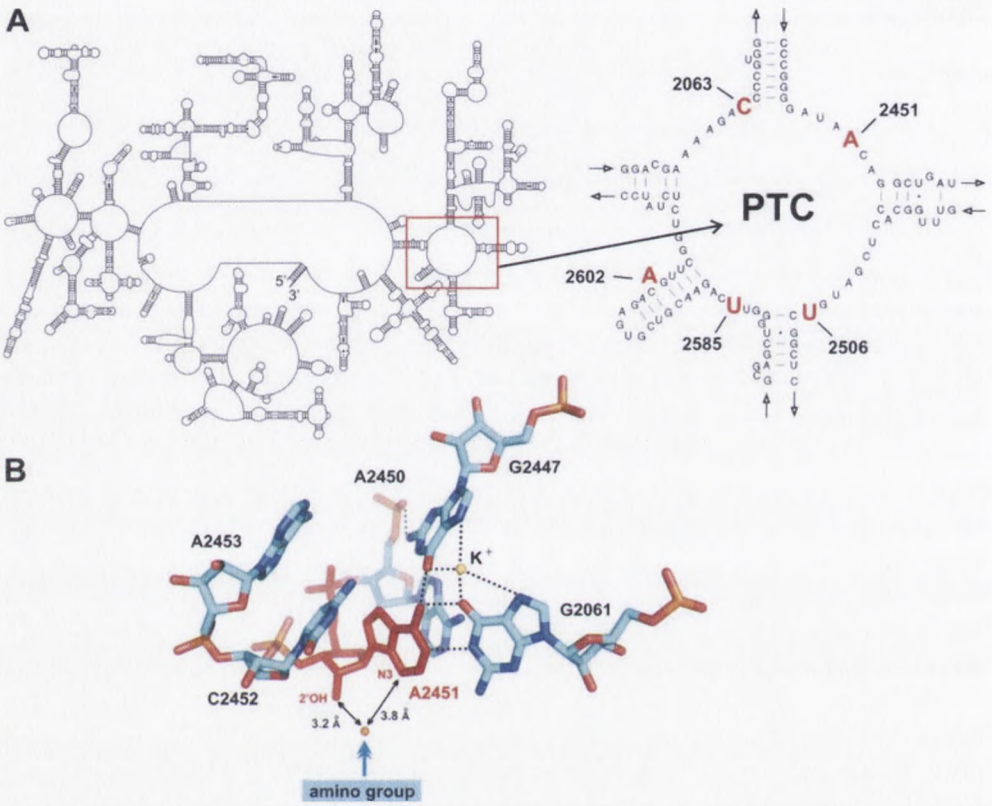

Fig. 2. A2451 is in hydrogen bonding distance to the attacking amine. **A)** Schematic representation of the secondary structure of the 23S rRNA from *Thermus aquaticus* with the PTC located in the central loop of domain V boxed in red (57). The five universally conserved inner core nucleotides that build the active site are bold and highlighted in red. **B)** View of the active site of the PTC structure. A2451 (red) is the only 23S rRNA residue that harbors functional group in hydrogen bonding distance to the attacking amine (orange sphere) of the aminoacyl-tRNA. The distance between the nitrogen atom of the attacking amino group of an aminoacyl-tRNA analog and the N3 or the ribose 2'-OH of A2451 (red) are given in Å and indicated by black arrows. Tertiary interactions that appear to position A2451 in its optimal position are depicted by black dotted lines. The figure was generated from pdb file 1FG0 (10).

drogen bonding distance (Fig. 2B). It is noteworthy that no unambiguous electron density for divalent metal ions near the catalytic center is visible in any of the available crystallographic structures. Thus it is unlikely that the ribosome employs metal ion catalysis for its chemical tasks in contrast to other natural ribozymes (11), such as the group I and group II self-splicing ribozymes, which can be considered metallo-enzymes.

The acceptor arms of tRNA substrates are constrained in the proper orientation in the P- and A-sites of the PTC by forming regular Watson-Crick interactions with conserved nucleotides of 23S rRNA loops. In the P-site, C74 and C75 of the tRNA base-pair to G2252 and G2251 of the 23S rRNA P-loop whereas the CCA end of A-site tRNA is fixed by pairing C75 with G2553 of the 23S rRNA A-loop (10,12-14). The tRNAs ends are further stabilized in both A- and P-sites by A-minor interactions between A76 of tRNA with the 23S rRNA base pairs U2506-G2583 and A2450-C2501, respectively (10,15). Both acceptor ends of the A- and P-tRNAs carrying the peptidyl- or aminoacyl moieties, respectively, approach each other at the bottom of the cone-shaped catalytic cleft directly above the entrance to the nascent peptide exit tunnel.

3. Catalyzing peptide bonds

3.1. From rRNA to tRNA catalysis

These detailed insights into the catalytic heart of the 50S subunit allowed Steitz, Moore and co-workers to postulate a catalytic model for amide bond synthesis on the ribosome (10). In this mechanistic scenario the N3 position of the universally conserved adenine base at position 2451 of 23S rRNA was predicted to function as a key group in a general acid-base mechanism. While this model provided a rational as to why nature has selected an adenine at this PTC position, it immediately raised doubts since some previously published key publications appeared to be in conflict with this model (16). Indeed subsequent biochemical and genetic studies did not support a crucial role of the nucleobase at A2451 for catalysis (17-23). Even though mutations at A2451 are lethal in *E. coli*, the A2451U mutant turned out to viable in *Mycobacterium smegmatis* (24). Furthermore, evidence was presented demonstrating that general acid-base chemistry is unlikely to be used by the PTC to synthesize peptide bonds (2). Cumulatively, the data presented in these studies did not support the model of general acid/base catalysis of peptidyl transfer involving A2451 as the catalytic base.

In search for alternative catalytic mechanisms it was suggested that the PTC might not in fact provide any specific functional group for catalysis, but merely serves as an entropy trap that places and orients the two tRNA substrates optimally for

spontaneous peptide bond formation to occur (6). This scenario was compatible with an earlier proposal called the 'template model' (5). Here, the sole function of the PTC is to precisely organize the universal CCA ends of pept-tRNA and aa-tRNA in a defined stereochemical arrangement. Furthermore, this model provides a suitable interpretation for the observation in *M. smegmatis*, where the A2451U mutation was viable in contrast to *E. coli* (24). It seems that *M. smegmatis* ribosomes in general and the architecture of the PTC in particular, are less sensitive to mutations (25,26). Thus the translational apparatus in the *M. smegmatis* A2451U strain is obviously capable of providing sufficient peptidyl transferase activity by precisely orienting the tRNA substrates (24). In other words, the functional importance of the tRNA reaction substrates was increasingly appreciated and became the new prime focus for mechanistic studies on peptide bond synthesis. Indeed, a critical functional group was identified to reside on one of the substrates, namely the 2'-OH group at the terminal adenosine A76 of P-site located pept-tRNA (27,28). This tRNA 2'-OH was proposed to be essential for transpeptidation in a so called 'substrate-assisted catalysis' (29), thus reducing the role of the PTC during peptide bond formation merely to a passive stage for the main actors, the tRNA reaction substrates. In this model the 2'-OH group of P-site tRNA A76 serves as a 'proton shuttle' in catalysis of peptide bond formation (28,29). The 2'-OH of A76 is in hydrogen bonding distance to the attacking α-amino nucleophile as well as to the 3'-*O* leaving group. The A76 2'-OH was suggested to be part of a 6-membered ring system where it receives a proton from the attacking amine, thus enhancing its nucleophilic character necessary for attack on the carbonyl ester carbon, as well as simultaneously donates a proton to the A76 3'-O leaving group. This elegant model allows proton shuttling without significant charge generation on either the A76 2'-OH or the A76 3'-OH leaving group. Crystallographic (30) and molecular dynamics simulation (31) studies are in agreement with this scenario.

3.2. The comeback of A2451

Does this mean that the PTC does not provide any functional group that directly participates in catalysis? It is still possible that the key functional group on an rRNA residue could not be eliminated or changed in mutational studies. It is a fact that the level of chemical engineering that can be achieved by conventional mutational studies of RNA is fairly limited. Indeed, the putative key functional groups at A2451 that were seen in hydrogen bonding distance to the attacking amine (the nucleobase N3 and the ribose 2'-OH) (Fig. 2B) would retain their chemical characteristics also in the C, G or U mutants. To deepen our understanding of a possible rRNA contribution to ribosomal catalysis, nucleotide-analog interference studies would be required. Thus a novel experimental strategy has been developed that allows functional group replacements on active site 23S rRNA residues. In order to introduce these

non-natural nucleotide analogs into the 23S rRNA a recently established *in vitro* reconstitution technique for 50S subunits was applied (32). The key feature of this approach is the use of circularly permuted 23S rRNA transcripts that place the novel 5' and 3' ends close to the PTC. The new endpoints were designed in such a way to introduce a short sequence gap (between 26-46 nucleotides) within the active site. The missing RNA segment was then provided *in trans* during reconstitution as a chemically synthesized RNA fragment containing the desired nucleotide analog at the 23S rRNA position of interest (Fig. 3). This 'atomic mutagenesis' approach has been applied to all inner core nucleotides of the PTC. We note that these reconstitutions 50S particles do not carry any of the natural posttranscriptional modifications in 23S rRNA. It was previously shown that 50S subunits from thermophilic organisms can be reconstituted with *in vitro* transcribed 23S rRNA and thus do not depend on the natural modifications for functioning (33,34), which is in contrast to reconstituted *E. coli* subunits (35,36).

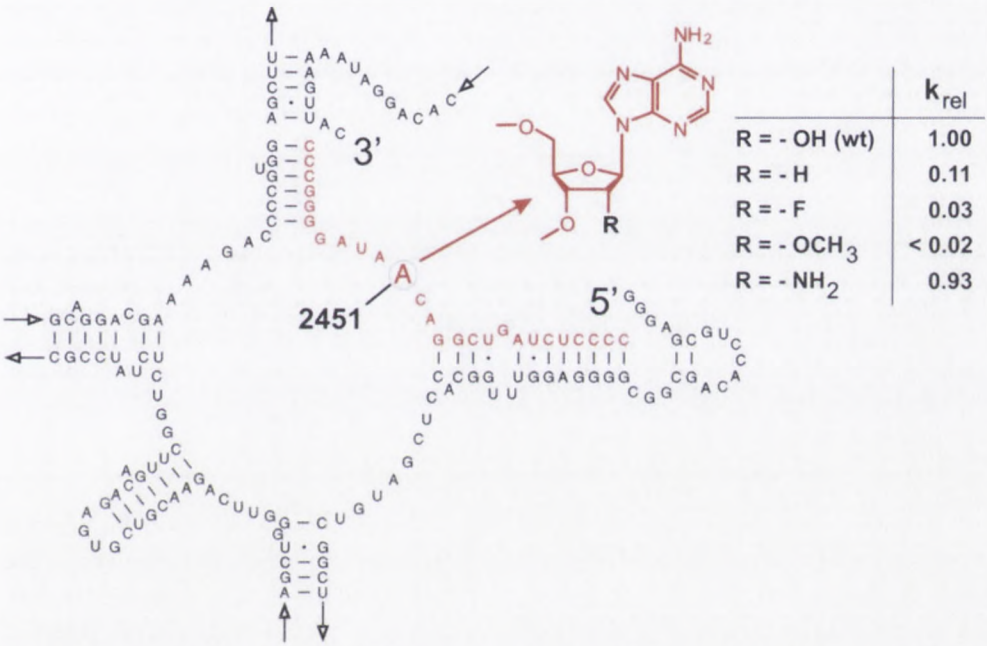

Fig. 3. Atomic mutagenesis at A2451 reveals a functional role of its ribose 2'-OH for peptide bond synthesis. Secondary structure of the PTC of 'gapped-cp-reconstituted 50S subunits' (32) showing the new endpoints of the circularly permuted 23S rRNA at positions 2468 and 2440 (5' and 3', respectively). The chemically synthesized 26-nucleotide RNA, which compensates for the missing rRNA segment, is shown in red. Residue A2451 is highlighted and the different chemical groups that were introduced at the ribose 2' position are depicted next to the chemical structure. The initial peptidyl transferase rates (k_{rel}) of chemically modified ribosomes are shown. The rates were normalized to the rate of gapped-cp-reconstituted ribosomes containing the synthetic wild-type RNA fragment (38).

Applying the 'atomic mutagenesis' technique to all active side residues revealed that all nucleotide modifications were tolerated with a single exception: removal of the ribose 2'-OH group of A2451 severely hampered peptide bond synthesis (32,37). This effect was evident in assays employing minimal (puromycin, CpCp-puromycin) or full length (aa-tRNA) A-site substrates (Tab. 1) (32,37). Recently, an identical effect was observed in *in vitro* translation reactions where the aa-tRNA is bound to the ribosome as ternary complex with EF-Tu and GTP (Erlacher M., Chirkova A., Polacek N., unpublished data), thus demonstrating that the inhibitory effect of the 2'-deoxy modification at A2451 is independent of the nature of delivery of the A-site substrate (Tab. 1). This finding supports a potential involvement of the A2451 2'-OH in the chemistry of the peptidyl transferase reaction.

Table 1

Activity of A2451 modified ribosomes in various peptidyl transferase assays

A2451 modification	Pmn	2 tRNA	poly(Phe)
adenosine (wt)	1.00	1.00	1.00
ribose-abasic	0.53	0.50	0.84
deoxy-abasic	< 0.01	0.05	0.11
2'-deoxy-adenosine	0.11	0.20	0.25
2'-fluoro-adenosine	0.03	n.d.	0.12
2'-O-methyl-adenosine	< 0.01	n.d.	n.d.
2'-amino-adenosine	0.93	n.d.	0.72

The initial peptidyl transferase rates of chemically modified ribosomes carrying modifications at A2451, which were determined from experimental points in the linear range of the respective reactions, are shown. The rates were normalized to the rate of 'gapped-cp-reconstituted' ribosomes containing the synthetic wild-type RNA fragment (wt). Pmn, puromycin reaction; 2 tRNA, dipeptide bond formation using AcPhe-tRNA and Phe-tRNA as P- and A-site substrates, respectively; poly(Phe), poly(U)-directed poly(Phe) synthesis; n.d., not determined.

However, how does A2451 2'-OH of 23S rRNA participate in ribosomal peptide bond formation? Deoxyribose substitution alone provides no information about the precise chemical contribution of a particular 2'-OH. Therefore to investigate the role of the A2451 2'-OH of 23S rRNA during transpeptidation in more detail, selected modifications at the ribose 2' carbon have been introduced that severely influenced the hydrogen bonding potential (Fig. 3). It turned out that efficient peptide bond formation is only possible when the 2' functional group of A2451 is provided with hydrogen donor capability (such as 2'-amino-adenosine) (38). Based on these findings it was proposed that the A2451 2'-OH donates its proton to form a direct hydrogen bond interaction with A76 2'-OH of the pept-tRNA. This proposal is in accordance with crystallographic data (39) and highlights the functional importance of

this particular interaction for the proposed network of the PTC. In other words, by donating its proton to the A76 2'-O of pept-tRNA, A2451 helps to stabilize the 6-membered ring system of the proposed proton shuttle (27,29) in its productive conformation (Fig. 4). Alternatively, since the A2451 2'-OH is also in hydrogen bonding distance to the attacking α-amino group of aa-tRNA, the A2451 2'-OH might also be directly involved within an expanded 8-membered proton shuttle. In this scenario the A2451 2'-OH functions as both – a hydrogen donor (to A76 of pept-tRNA) and acceptor (from the α-amino nucleophile) simultaneously. An additional contribution of this intricate rRNA-tRNA interaction to peptide bond formation might be that migration of the peptidyl-moiety from the productive A76 ribose 3' position to the non-productive 2' position is avoided (38). In summary, these latest mechanistic models of ribosomal peptide bond formation appreciate the concept of 'substrate-assisted catalysis' (29) and combine with it the strict functional requirement of the ribose 2' group at A2451 of 23S rRNA to possess hydrogen donor capability.

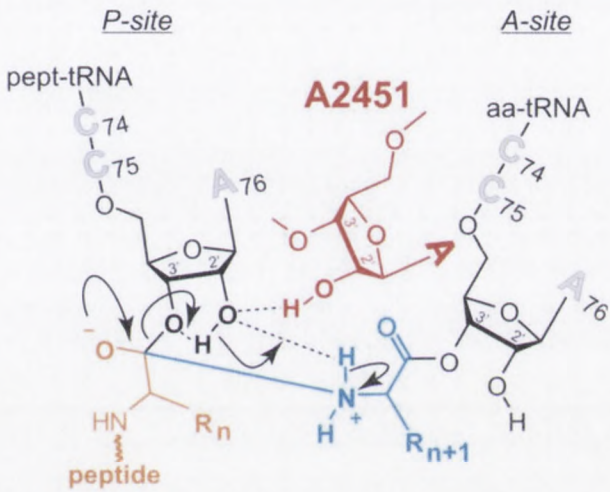

Fig. 4. Model for the mechanism of peptide bond formation proposing a role for 23S rRNA A2451. A2451 of 23S rRNA (red), peptidyl moiety of pept-tRNA in the P-site (orange), and aminoacyl-moiety of A-site bound aa-tRNA (blue); The hydrogen bonding interaction between the A2451 2'-OH and the pept-tRNA A76 2'-O bond assists in P-site tRNA A76 ribose positioning and in suppression of spontaneous intramolecular transesterification. In this model the nucleophilic attack of the α-amino group on the ester carbonyl carbon is accompanied by a concomitant acceptance of a proton from the α-amino group by the A76 2'-O of the pept-tRNA which simultaneously donates its proton to the vicinal 3'-O (38). Black arrows indicate pair-wise electron movement for proton shuttling after the attack of the α-amino nucleophile has established the tetrahedral intermediate. This model represents an extension of the previously proposed 'substrate assisted catalysis' model involving a six-atom 'proton-shuttle' mechanism (27-29).

4. Pept-tRNA hydrolysis: similar but not necessarily the same

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 pept-tRNA hydrolysis. Biochemical and structural data show that the tip of domain III (which harbors the universally conserved GGQ peptide mini-motif at positions 228-230) (40) of the A-site bound RF reaches toward the bottom of the PTC and is in immediate neighborhood of A2602 (41-43). The molecular events that take pace in the PTC upon RF binding, especially the functional group(s) that coordinate and activate the hydrolytic water molecule, remained largely unknown. Models were proposed which suggest that the GGQ motif directly participates in peptidyl-tRNA hydrolysis by coordinating the water molecule (7,40,44-46). However, it has been shown that pept-tRNA hydrolysis can be triggered even in the absence of a class I RF by replacing it with an A-site bound deacylated tRNA (Fig. 5A) (7,47,48), thus hinting at an RNA-catalyzed reaction.

In contrast to peptide bond formation where mutations of all the inner core PTC residues had little or no effect, mutations at position A2602 significantly inhibited the pept-tRNA hydrolysis reaction (23,48). Thus the nucleobase at A2602 became the prime candidate in the PTC to harbor functional groups that directly participate in pept-tRNA hydrolysis (48). The structural flexibility and the central location of A2602 in the PTC are compatible with this proposed role (10,12,49). Surprisingly removal of the entire adenine base at position 2602 (by introducing an abasic nucleotide analog using the atomic mutagenesis approach described above) did not interfere with pept-tRNA hydrolysis activity (50) (Fig. 5B). Only further minimization of the ribose moiety at position 2602 by introducing the C3-linker modification which lacks in addition to the base also the C1', C2', and O4' of the sugar eliminated pept-tRNA hydrolysis (Fig. 5B). This suggests that an intact ribose moiety at the 23S rRNA residue A2602 is crucial for efficient pept-tRNA hydrolysis, while having no apparent functional relevance for transpeptidation. It is noteworthy that removal of the 2'-OH at A2451, which was shown to be so crucial for catalyzing peptide bond formation (Fig. 3 and Tab. 1), had an only very mild effect on the rate of pept-tRNA hydrolysis (50). Even replacing the natural ribose 2'-OH at A2451 by the rather bulky 2'-OCH_3 group, a modification that completely eliminated peptidyl transferase activity (Fig. 3), did not significantly reduce pept-tRNA hydrolysis yields (38).

How can these findings be explained within 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 pept-tRNA (48)? 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') has the chemical potential to hydrogen bond to a water molecule or a hydrated metal ion. Furthermore, the distance from the 2602 ribose to the position where the nucleophilic water molecule is supposed to launch its attack during peptide release,

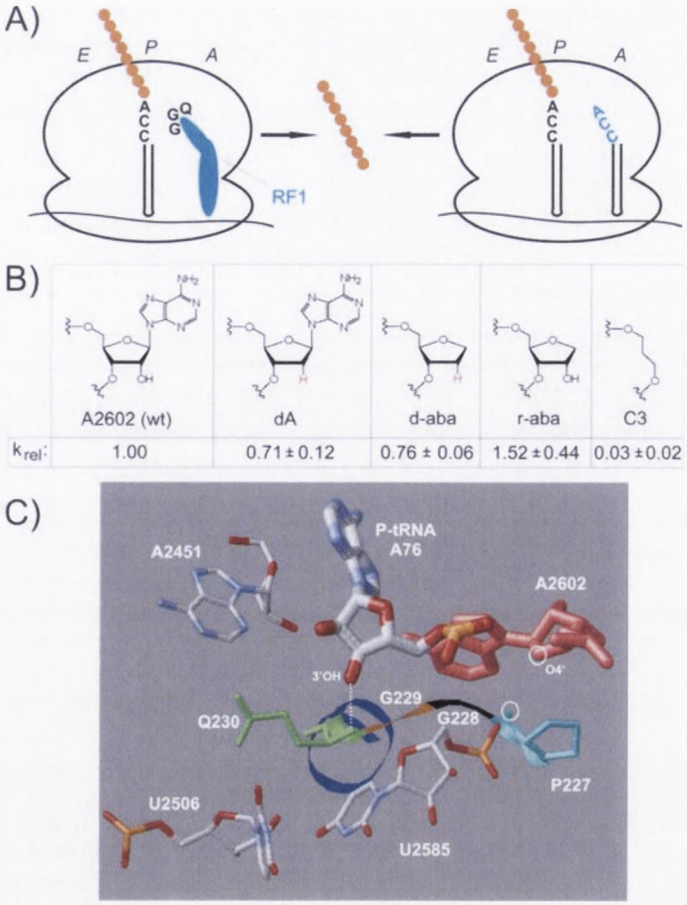

Fig. 5. Ribosome catalyzed peptidyl-tRNA hydrolysis. **(A)** pept-tRNA hydrolysis and release of the peptidyl chain (orange) from P-site bound pept-tRNA is triggered by an A-site bound class I release factor (RF1 or RF2 in bacteria). The 3' CCA tRNA ends and the universally conserved GGQ peptide mini motif of the RF that interact with the PTC are highlighted. *In vitro*, the RF can be functionally replaced by an A-site bound deacylated tRNA in order to initiate pept-hydrolysis (right). **(B)** Modifications at the 23S rRNA residue A2602 and the effect on the RF1-triggered pept-tRNA hydrolysis. Initial rate constants (k_{rel}) of gapped-cp-reconstituted ribosomes carrying the wild-type adenosine (wt) at A2602 was taken as 1.00 and compared to ribosomes containing the depicted nucleotide analogs at this position (50). **(C)** The three-dimensional representation highlights the accommodation of the tip of domain III of RF1 from residue 226 to 236 (depicted as ribbon), including the GGQ motif, into the PTC. For glutamine 230 (green) and proline 227 (cyan) also the side chains are shown. The putative hydrogen bond between the main chain amide of Q230 and the ribose 3'-OH of P-site bound tRNA (43), which formerly carried the nascent peptide, is shown by a white dotted line. Nucleotides A2602 (red) as well as U2585 approach the GGQ loop most intimately, whereas the closest proximity of a 23S rRNA group (distance 3 Å) is seen between the ribose O4' of A2602 and the main chain carbonyl oxygen of proline 227 (encircled). The figure was prepared using the coordinates of pdb 3D5A-D (43).

appears to be too large (11.5 Å) for the direct coordination of the hydrolytic water. Even though we can not completely discard the possibility of direct water coordination by A2602 via structural water molecules forming water 'wires' (51), it seems more likely that A2602 functions as a molecular switch in the ribosome to regulate the specificity of the PTC between amide bond formation, when aa-tRNA is located at the A-site, and pept-tRNA hydrolysis when the RF is bound. It is conceivable that the function of the A2602 switch is to guide or channel the hydrolytic water into the catalytic center for optimal pept-tRNA hydrolysis. Alternatively, or in addition, A2602 might be contributing to pept-tRNA hydrolysis by properly orienting the QQG motif of the class I RF in the PTC.

The question still remains which group actually activates and positions the water molecule in the PTC for optimal nucleophilic attack? The A2451 2'-OH which has been shown to be pivotal for catalyzing peptide bond formation (32,37) does not seem to play an equally important role in pept-tRNA hydrolysis, and thus does not qualify for activating the nucleophile. Groups at other PTC residues are also not critical, leaving the 2'-OH of A76 of P-tRNA, which plays an important role in peptide bond formation (27-29), or a group on the RF as potential candidates for possessing the catalytic moiety for pept-tRNA hydrolysis. In support of this scenario a molecular dynamics simulation was presented that highlights the importance of the P-tRNA A76 2'-OH in activating the water possibly also via the 6-membered proton shuffle (52). Experimental support for this hypothesis was recently presented showing a clear inhibition of RF1-mediated peptide release when this ribose 2'-OH of pept-tRNA was replaced by a 2'-H or a 2'-F substituent (53). Based on molecular dynamics simulations it was suggested that the side chain of the GGQ glutamine of RF1 positions the hydrolytic water molecule while the role of A2602 is to stabilize the GGQ mini peptide motif in its functionally competent conformation (52). This proposed role for A2602 is in full agreement with a recently presented crystallographic structure of a 70S complex mimicking a post-termination state at 3.2 Å resolution (43). Noller and co-workers concluded that a direct contribution of the 23S rRNA residue A2602 to catalysis is unlikely since it was seen to be buried in a cavity of RF1 and blocked from the chemical center. Therefore it seems likely that the observed defects in pept-tRNA hydrolysis by ribosomes carrying mutations or non-natural nucleotides at A2602 are caused by a non-productive binding and/or positioning of the GGQ motif of RF1 for catalysis. In this post-termination complex structure, A2602 is in close proximity (less than 4.5 Å) to several amino acids flanking the GGQ motif from RF1 residues P277 to T235 which adopt a short helical element when bound to the PTC. Interestingly the atom that most intimately approaches this RF1 segment (at residue P227) is the ribose O4' of A2602 (distance: 3.0 Å) (Fig. 5C). This is the very 23S rRNA backbone group that was previously identified by atomic mutagenesis to be strictly needed for effective translation termination (50). The main-chain amide of the GGQ glutamine, and therefore yet another backbone group, was seen in hydrogen bonding distance to the ribose 3'-OH of the terminal A76

of deacylated tRNA bound at the P-site. Thus this glutamine amide group may contribute to the rate enhancement of pept-tRNA hydrolysis by coordinating the leaving group or by stabilizing the oxyanion of the transition state (43).

Peptide bond formation and pept-tRNA hydrolysis do share some important characteristics, but other aspects appear to be distinct (see Tab. 2 for a summary). Based on these similarities it was recently suggested that the PTC catalyzes its two chemical reactions by using a common mechanism (52,53). While this sounds intriguing and also makes sense from an evolutionary perspective, we would like to point out that some experimental data can not be easily explained by this hypothesis. If this theory is correct then one would expect that the same mutations/modifications of active site residues would have comparable effects on either of the two reactions. However, (i) while the A2451 2'-OH was shown to be crucially involved in peptide bond formation, it obviously does not have a comparable functional relevance for the mechanism of pept-tRNA hydrolysis (50). (ii) If the sole role of A2602 is to position the GGQ motif for optimal pept-tRNA hydrolysis it follows that this would also be its purpose in the RF-independent release using deacylated tRNA as A-site substrate. In accordance with this assumption, mutations at 2602 or the entire deletion of this nucleotide essentially kill pept-tRNA hydrolysis using A-site bound deacylated tRNAs (or the CCA end thereof). However, the same mutations have almost no effect on peptide bond formation, even though in the latter reaction a very similar A-site substrate (namely the CCA acceptor end of aa-tRNA) is bound to the PTC A-site (23,48,50). Thus it seems that A2602 as well as A2451 fulfill markedly different tasks in pept-tRNA hydrolysis and peptide bond formation, respectively. Furthermore, before a uniform catalytic mechanism for all PTC-catalyzed reactions will be fully accepted, demonstration of a similar (or identical) activity pattern for both release factors RF1 and RF2 in pept-tRNA hydrolysis in the context of the same active site mutations is a prerequisite. So far, however, only RF1 was employed in almost all PTC mutagenesis studies to date (23,32,48,50,53,54) These open points clearly reflect our still limited molecular understanding of pept-tRNA hydrolysis and emphasize the need for further studies on translation termination to reach comparable mechanistic insights as for peptide bond formation.

Table 2

Comparing molecular requirements for peptide bond formation and pept-tRNA hydrolysis

Contribution of:	Peptide bond formation		Pept-tRNA hydrolysis	
		References		References
1	2	3	4	5
A2451 nucleobase	-	(20,23,32,37)	-	(23,32,50)
A2451 2'-OH	+	(32, 37, 38)	-	(32,38,50)
A2602 nucleobase, 2'-OH	-	(23,48,50)	-	(50)

1	2	3	4	5
A2602 ribose ring	-	(48,50)	+	(50)
Mutations at other PTC residues	-	(20,21,23,48)	- / +	(48,50) / (23)
P-tRNA A76 2'-OH	+	(27-29)	+	(53)

Significant contributions of given residues, groups or principles to either peptide bond formation or pept-tRNA hydrolysis are indicated by '+', if the effects seen were at least 10-fold.

5. Outlook

Do we already know all the mechanistic details of peptide bond synthesis on the ribosome? The answer is most likely no. It is true that after the high resolution structures of 50S subunits (9,10,30) and recently also of 70S particles (14,43), enormous progress has been made in combination with biochemical work (for a review see ref. (55). Although it is evident that we are close to understanding the basic mechanistic principles of ribosome-catalyzed peptide bond synthesis, it is only recently that the role of structured water molecules for ribozyme catalysis has been recognized (51). In the recent 50S structures (30) as well as in molecular dynamics simulations (31,52) ordered water molecules have indeed been spotted in the PTC, hence their contribution to peptide bond synthesis and pept-tRNA hydrolysis still needs to be experimentally deciphered. Furthermore, the case for peptide bond synthesis is not sealed yet since a recent publication questioned the functional significance of the P-tRNA A76 ribose 2'-OH for peptide bond formation (56) indicating that additional mechanistic studies are needed.

Another question that is still largely unanswered is why evolution has selected so many universally conserved nucleotides in the PTC, when standard and atomic mutagenesis studies have shown that the nucleobases are actually not critical for catalysis and that the reactions are mainly driven by RNA backbone groups? Of course it can be argued that these studies were performed exclusively *in vitro* and essentially only single model reactions were investigated. Thus it is possible that under competitive *in vivo* conditions, organisms with mutant PTC residues would be counter-selected. While this is almost certainly true, we would like to point out here that mutations at the key residue A2451 have been introduced into *M. smegmatis* which was viable, indicating that mutations at this pivotal site of the PTC are in principle compatible with cellular life (24). Therefore it is still unclear why evolution did not come up with alternative nucleobase compositions in the PTC for efficient protein synthesis. It is therefore conceivable that the nucleobase identities of the universally conserved active site residues of the ribosome might be crucial for other ribosomal functions distinct from catalysis.

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