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Current Opinion in Structural Biology

The ribosome as a versatile catalyst: reactions at the peptidyl transferase center

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In all contemporary organisms, the active site of the ribosome—the peptidyl transferase center—catalyzes two distinct reactions, peptide bond formation between peptidyl-tRNA and aminoacyl-tRNA as well as the hydrolysis of peptidyl-tRNA with the help of a release factor. However, when provided with appropriate substrates, ribosomes can also catalyze a broad range of other chemical reaction, which provides the basis for orthogonal translation and synthesis of alloproteins from unnatural building blocks. Advances in understanding the mechanisms of the two ubiquitous reactions, the peptide bond formation and peptide release, provide insights into the versatility of the active site of the ribosome. Release factors 1 and 2 and elongation factor P are auxiliary factors that augment the intrinsic catalytic activity of the ribosome in special cases.

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Current Opinion in Structural Biology 2013, **23**:595–602

This review comes from a themed issue on **Engineering and design**

Edited by **Florian Hollfelder** and **Stefan Lutz**

For a complete overview see the Issue and the Editorial

Available online 24th May 2013

0959-440X/$ – see front matter,

http://dx.doi.org/10.1016/j.sbi.2013.04.012

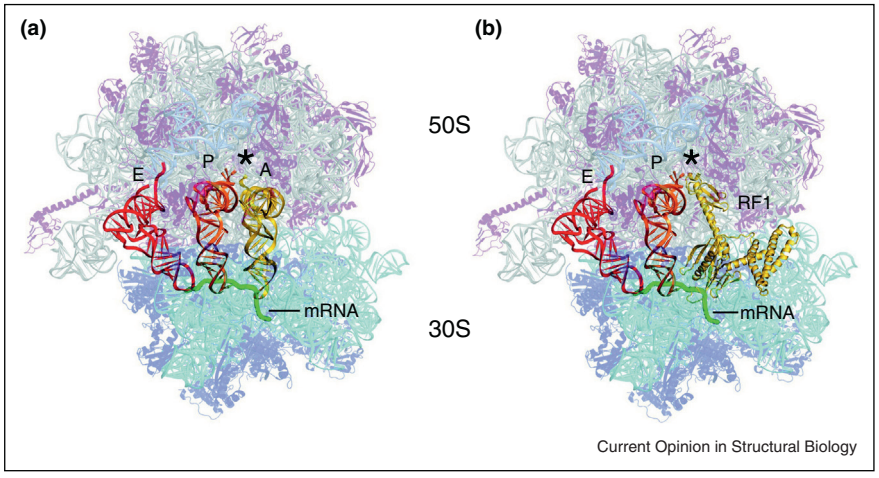
Introduction

In all organisms living today, ribosomes synthesize proteins from L-amino acids using tRNA to recognize the respective mRNA codon. The specificity for the 20 natural L-amino acids is largely determined by aminoacyl-tRNA synthetases, the enzymes that select the matching pair of tRNA and amino acid and produce aminoacyl-tRNA (aa-tRNA). However, recent advances in orthogonal engineering showed a number of ways to overcome the natural limits to substrate specificity: discovery of aminoacylating ribozymes of broad specificity (flexizymes) [1••], *in vitro* evolution of orthogonal aminoacyl-tRNA synthetases [2], and methods for non-enzymatic organic synthesis to create tRNAs charged with non-canonical amino acids [3••] broadened the range of potential substrates of the ribosome. The genetic code can be modified by reassigning the codons or by using quadruplet (four nucleotides) decoding by engineered tRNAs [4••]. The efficiency of orthogonal translation with unnatural substrates is often poor due to numerous cellular control machineries that disfavor the incorporation of unnatural substrates. However, in all cases where the exact step limiting the orthogonal translation was analyzed, the chemistry step itself was not impaired [5,6]. In fact, the repertoire of chemical reactions supported by the ribosome includes formation of esters, thioesters, thioamides, or phosphinoamides, and peptide bond formation with a large variety of unnatural amino acids [3,7] or D-amino acids [8••]. The chemical versatility of the ribosome raises the question of how it can bind these diverse substrates and catalyze different chemical reactions in one active site. This review attempts to provide some answers to these questions, based on recent advances in understanding the mechanisms of the two natural reactions catalyzed at the peptidyl transferase center of the ribosome, peptide bond formation and hydrolysis on peptidyl tRNA (pept-tRNA) (Figure 1).

Peptide bond formation

Peptide bond formation on the ribosome requires that two substrates, pept-tRNA and aa-tRNA, are bound to the P and A sites of the ribosome, respectively. The reaction proceeds through the nucleophilic attack of the α-amino group of aa-tRNA on the carbonyl carbon of the pept-tRNA. The mechanism of the uncatalyzed aminolysis reaction in solution has been studied in great detail. The reaction is expected to proceed through two intermediates, a zwitterionic tetrahedral intermediate (T±), which is deprotonated to form the second intermediate (T−), which then decomposes to form the reaction products [9] (Figure 2). A comprehensive analysis of heavy-atom kinetic isotope effects (KIE) indicated that on the ribosome the formation of the tetrahedral intermediate and proton transfer from the attacking nitrogen take place during the rate-limiting step; the breakdown of the tetrahedral intermediate occurs in a separate rapid step [10••] (Figure 2). These data suggest that the ribosome alters the reaction pathway in such a way that the T± intermediate does not accumulate, in contrast to the uncatalyzed reaction, and predict an early transition state (TS). While those results have been obtained with a relatively slow assay using isolated 50S subunits rather than native ribosomes, we note that the reaction on 50S subunits is slow not because 50S subunits are intrinsically less active than the 70S ribosomes, but simply because they bind the A and P substrates poorly [11•]; at saturation with substrate, similarly high rates of peptide bond formation can be achieved with the 50S subunit as with 70S ribosome, suggesting similar reaction mechanisms. Computational models suggested a late TS [12•] (Figure 3) and argued

Figure 1

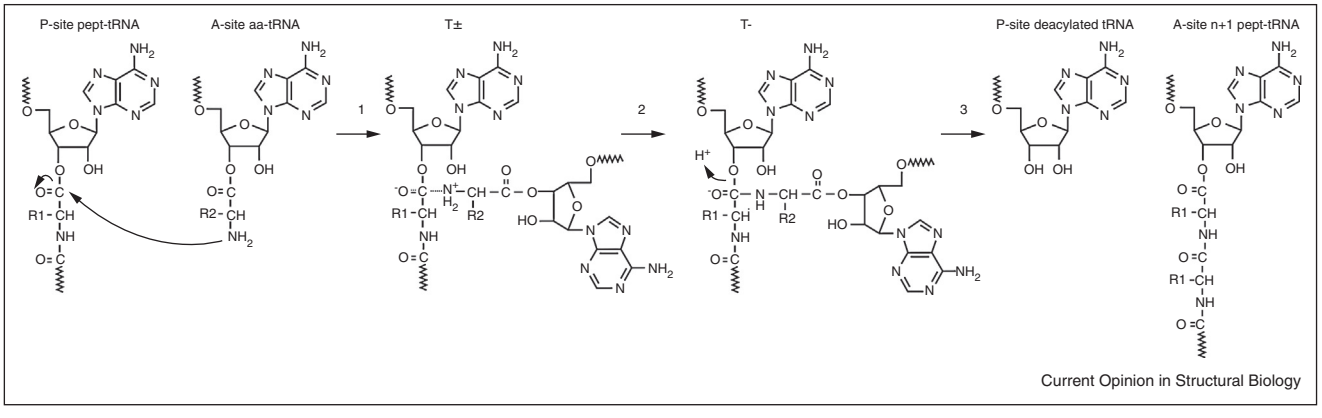


Ribosome complexes during catalysis of peptide bond formation or pept-tRNA hydrolysis. **(a)** During peptide bond formation, aa-tRNA reacts with pept-tRNA. **(b)** Hydrolysis of pept-tRNA is catalyzed by RF1. Positions of tRNAs bound to the E site (red), P site (orange), and A site (yellow), RF1 (yellow) and mRNA (green) bound to the ribosome are indicated. 23S rRNA (gray), 5S rRNA (light blue), 16S rRNA (cyan), 50S proteins (magenta) and 30S proteins (dark blue) are also indicated. The peptidyl transferase center is marked by a star.
Figure modified from Ref. [51••].

that the proposed TS [10••] was likely off the main reaction pathway to products. However, further evidence for an early TS comes from the charge effects of the C-terminal amino acid of pept-tRNA in the P site, which are consistent with a negative charge accumulating in the TS (similar to T−) [13•]. The Brønsted coefficients of the α-amino nucleophile measured with either 50S subunit or 70S ribosome using a series of puromycin derivatives [14•] were close to zero under conditions where the chemistry step was rate limiting. These results indicate that at the TS the nucleophile is uncharged in the ribosome-catalyzed reaction and that the TS involves deprotonation to a degree commensurate with nitrogen-carbon bond formation. Such a transition state is significantly different from that of the uncatalyzed aminolysis reactions in solution.

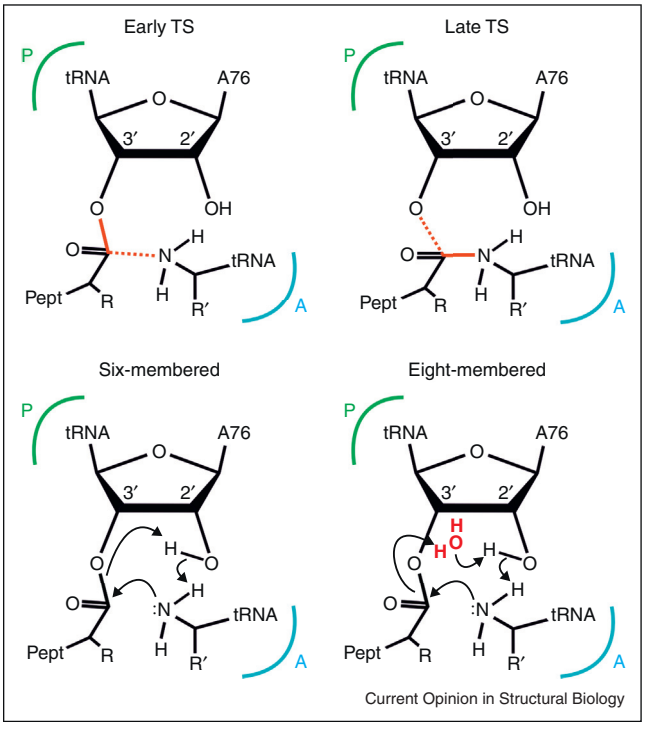
One important question is which groups take part in the TS on the ribosome. Extensive mutational studies of the ribosome's catalytic core and the analysis of pH/rate profiles of peptide bond formation suggested that the ribosome does not provide ionizing groups that contribute to catalysis [15–18]. Crystal structures as well as kinetic and computational work suggested a substrate-assisted

Figure 2



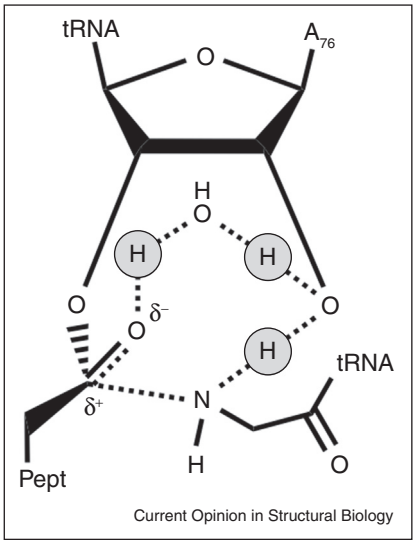
Reaction scheme of peptide bond formation. Step 1, attack of the α-amino group of aa-tRNA on the carbonyl carbon of the pept-tRNA and formation of the zwitterionic tetrahedral intermediate T±. Step 2, deprotonation of the positively charged amino nitrogen resulting in the second intermediate, T−. Step 3, product formation. The mechanism of the uncatalyzed reaction in solution is fully stepwise [9]. On the ribosome, the formation of the tetrahedral intermediate and proton transfer from the nucleophilic nitrogen take place during the rate-limiting step (1 + 2); the breakdown of the tetrahedral intermediate occurs in a separate rapid step (3) [10••].

Figure 3



Schematic overview of different potential transition states and proton shuttle mechanisms of the peptidyl transferase reaction. Adapted from Ref. [12•].

Figure 4



Transition state of peptide bond formation with eight-membered proton shuttle favored by recent KSIE, KIE, and modeling results [10••,14•,24,25•,30••]. Protons involved in the concerted shuttle are encircled.

mechanism of reaction involving the 2′OH group of A76 of the P-site tRNA. The initial proposals envisaged that protons are transferred (shuttled) between the attacking nucleophile, the 2′OH group, and the 3′OH group of A76 [19••,20–22]. However, model studies suggested that the direct proton transfer from the 2′OH to the 3′OH is unfavorable, whereas the carbonyl oxygen of the pept-tRNA was predicted to be a better acceptor [23,24,25•]. The 2′OH of A76 is important, although the magnitude of the rate reduction caused by its replacement of the 2′OH has been recently revised from the initial estimate of 10^6 [26] to >100 [27•]. The exact value remains uncertain, because translation appears to be insensitive to substitution of the 2′OH [28•]. One way to explain this discrepancy is to assume that the effect on the peptidyl transferase is masked by a different, intrinsically slower step which limits the overall rate of translation; alternatively, a water molecule may functionally substitute for the lacking 2′OH at some conditions [29].

A shuttle mechanism may be six-membered, with two protons simultaneously changing their positions in the TS, or eight-membered with three protons 'in flight' in the TS; the latter TS includes a water molecule which is found in the appropriate position in the crystal structures of 50S subunits in the complex with TS analogs [12•,19••]. The analysis of kinetic solvent isotope effects (KSIE) showed that in the rate-limiting TS three protons move in a fully concerted manner [30••]. This not only supports the existence of a concerted proton shuttle, but also favors an eight-membered shuttle with a water molecule taking part in proton transfer. In the rate-limiting TS, the attack of the α-amino group on the ester carbonyl carbon results in an eight-membered transition state in which a proton from the α-amino group is received by the 2′OH group of A76, which at the same time donates its proton to the carbonyl oxygen via an adjacent water molecule (Figure 4) [30••]. Protonation of the 3′OH then would be an independent rapid step [10••], although it is still not clear where the proton for the protonation of the leaving group comes from. The catalytic role of the ribosome is to provide a network of interactions that change the rate-limiting TS and lower the activation entropy [31••,32,33•].

The intrinsic rate of peptide bond formation may depend on the chemical properties of the substrates, the P site-bound pept-tRNA, and the A-site bound aa-tRNA. Depending on the C-terminal amino acid of the pept-tRNA, the rate of reaction with puromycin as A-site substrate varied between 100 s^{-1} and 0.14 s^{-1}, regardless of the tRNA identity, with Pro being by far the slowest [13•]. However, when Phe-$tRNA^{Phe}$ was used as A-site substrate, the rate of peptide bond formation with any pept-tRNA was ~7 s^{-1} (at 20°C in buffer containing 7 mM Mg^{2+}), which corresponds to the rate of accommodation of Phe-$tRNA^{Phe}$ to the A site [13•]. Because accommodation

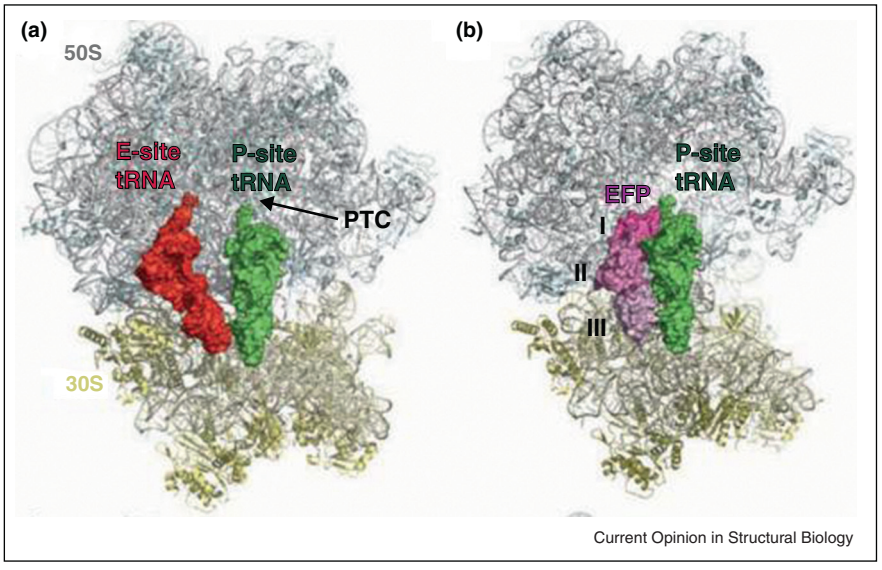
is rate-limiting for peptide bond formation, the reaction rate is uniform for all pept-tRNAs, regardless of the variations of their intrinsic chemical reactivity [34]. On the other hand, the observed 50-fold increase in the reaction rate for pept-tRNA carrying a peptide ending with Pro with aa-tRNA instead of puromycin suggests that full-length aa-tRNA in the A site greatly accelerates peptide bond formation by an as yet unknown mechanism [13•].

The existence of the rate-limiting accommodation step that masks the chemistry of peptide bond formation has been challenged, based on experiments carried out at conditions of very rapid ($200\ s^{-1}$) peptide bond formation, albeit no direct estimation was provided for the rate of accommodation [35]. To test this contention, we measured the rates of Phe-tRNA^{Phe} accommodation and peptide bond formation directly and in parallel at the conditions used in that work [35]. The rates of the two partial reactions turned out to be the same [36••], suggesting that the accommodation is fully rate-limiting for the following peptide bond formation step, at least at those conditions. Notably, aa-tRNA accommodation in the A site is independent of pH [16], whereas peptide bond formation decreases with pH due to inactivation of the nucleophile by protonation. This explains why with some aa-tRNAs, such as Pro-tRNA^{Pro} and Gly-tRNA^{Gly}, which are intrinsically slow in peptide bond formation, show a pH dependence at low pH [35], whereas at high pH, where the nucleophilic nitrogen is deprotonated, accommodation most likely becomes rate-limiting.

EF-P, a specialized translation factor required for rapid synthesis of proteins with consecutive prolines

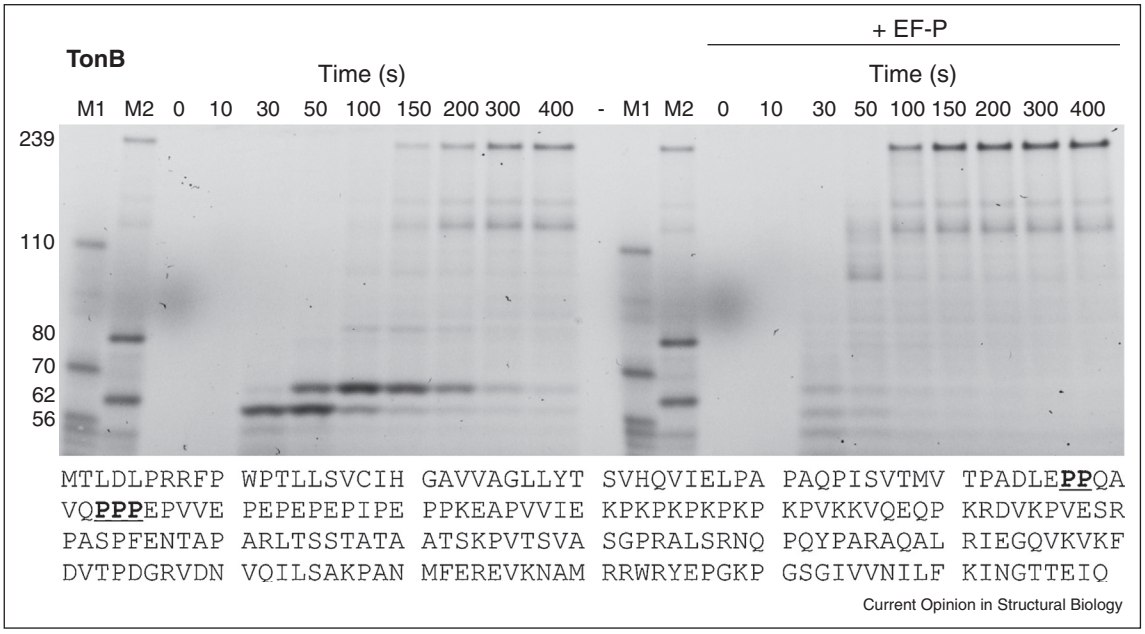
Peptide bond formation with the P-site pept-tRNAs ending with proline or with Pro-tRNA^{Pro} in the A site of the ribosome is surprisingly slow [13•]. In fact, robust stalling on mRNA sequences coding for PP(X) occurs *in vitro* where X stands for Asn, Asp, Glu, Gly, Pro, or Trp [37••]. Toeprinting analysis reveals that upon translation of a PPP sequence, the ribosome stalls with the second Pro codon in the P site [37••]. Two recent papers demonstrate that rapid translation of such sequences requires the auxiliary translation elongation factor P (EF-P) [38••,39••] (Figure 5). EF-P prevents the ribosome from stalling during synthesis of proteins containing consecutive prolines, such as PPG, PPP or longer strings of prolines by facilitating rapid Pro-Pro and Pro-Gly bond formation and by stabilizing pept-tRNA in the catalytic center of the ribosome (Figure 6). Pro is the only imino acid naturally used by the ribosome. Because of the presence of the pyrrolidine ring, the torsion angle for the N–Cα bond may restrict the number of accessible conformations and may impose structural constrains on Pro positioning in the peptidyl transferase center of the ribosome, thereby sterically hindering peptide synthesis. EF-P is post-translationally modified by a hydroxylated β-lysine residue that is attached to a lysine residue [40–43]. The modification enhances the catalytic proficiency of the factor mainly by increasing its affinity to the ribosome [38••]. EF-P works on natural and engineered sequences alike, suggesting that the context of Pro strings is not important. Interestingly, EF-P does not

Figure 5



Structure of EF-P bound to the ribosome. **(a)** E-site (red) and P-site (green) tRNAs bound to the ribosome. **(b)** EF-P and P-site tRNA bound to the ribosome. EF-P is shown in shades of magenta to indicate domains I, II, and III. The 50S subunit is colored gray, the 30S subunit is yellow. Figure reproduced from Ref. [57••] with permission.

Figure 6



EF-P alleviates PPP/PPG-induced stalling in natural proteins. Example of time courses of TonB (239 amino acids) translation in the absence and presence of EF-P. M1, and M2, peptide markers containing TonB sequences of the indicated lengths. The sequence of the protein is shown at the bottom.
Reproduced from Ref. [38••].

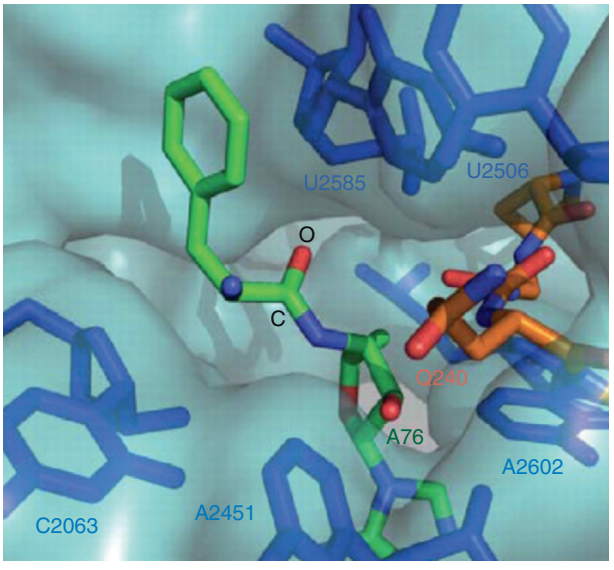
alleviate programed ribosome stalling promoted by specific sequences in nascent peptides [37••]. EF-P is evolutionary conserved; its eukaryotic homolog eIF5A is also post-translationally modified at a position orthologous to that in EF-P. Remarkably, the type of modification of eIF5A (hypusination) is different from that in EF-P [44].

Of more than 4000 annotated proteins in *Escherichia coli*, about 270 contain motifs of three or more consecutive prolines or PPG motifs. Among those, the proteins that belong to the basal transcription-translation machinery are underrepresented, whereas metabolic enzymes, transporters, and regulatory transcription factors are frequent, explaining the pleiotropic phenotypes caused by deletions of the genes coding for EF-P or its modification enzymes, including effects on virulence and bacterial fitness [45•,46]. Given that the synthesis of a peptide bond usually does not require an auxiliary factor, EF-P appears to be a recent evolutionary addition to the repertoire of translation factors, possibly required to properly balance the amounts of regulatory proteins in cells [39••].

Hydrolysis of peptidyl-tRNA

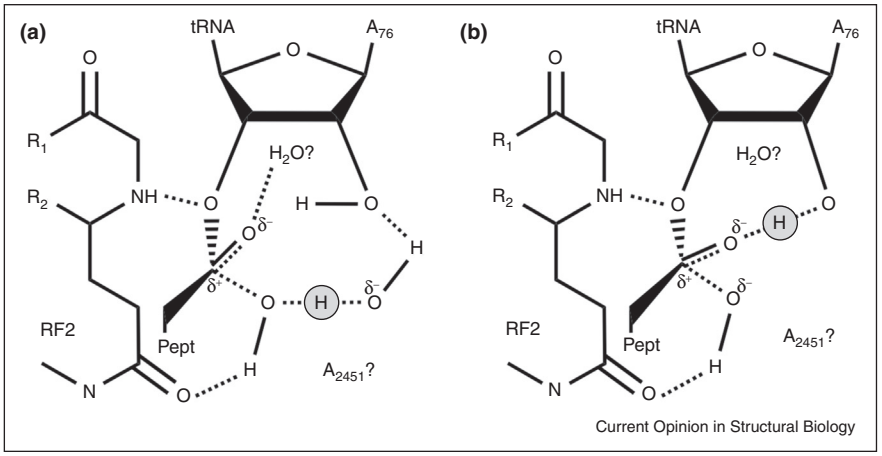
The hydrolysis of pept-tRNA is a second natural reaction catalyzed by the peptidyl transferase center of the ribosome. The reaction occurs during the termination phase of protein synthesis and is facilitated by release factor 1 or

Figure 7



The catalytic pocket of the peptidyl transferase center during translation termination. Upon stop codon recognition by RF1/2, the conserved Q of the GGQ motif is inserted into the catalytic center. Nucleotides of 23S rRNA at the peptidyl transferase center are shown in blue, RF2 is shown in orange, and A76 of the P-site tRNA is shown in green.
Reproduced from Ref. [52••] with permission.

Figure 8



Possible transition states of RF2-catalyzed pept-tRNA hydrolysis on the ribosome (see text). The single low-barrier hydrogen bond that determines the reaction rate is encircled. The possibility of the involvement of another water molecule and/or residue A2451 of 23S rRNA is indicated. Reproduced from Ref. [30••].

2 (RF1/2) in bacteria which recognize stop codons in the decoding site on the ribosome and position the universally conserved GGQ motive into the peptidyl transferase center (for recent review [47]) (Figure 7). The accommodation of RF2 is impaired by the mutations of C2573 and A2572 of 23S rRNA at the putative accommodation gate of the 50S subunit [48]; however, on ribosomes with unmodified 23S rRNA the accommodation of RF2 is rapid and does not limit the chemistry step. Mutations of the conserved Gln in the GGQ motif to most other amino acids have only a modest effect on catalysis [49,50•], except for the Gln to Pro replacement [51••]. Crystal structures indicated that the backbone NH group of Gln230 is positioned within hydrogen bonding distance of the 3′OH of A76 of pept-tRNA, which explains the detrimental effect of Gln to Pro replacement [51••,52••]. Furthermore, Gln controls the specificity of the release reaction for water by excluding nucleophiles larger than water from entering the active site, which also supports the notion that side chain of Gln is positioned in the vicinity of the nucleophile [50•].

The reaction is expected to proceed through a tetrahedral intermediate that breaks down to form the free peptide and deacylated tRNA. Proton inventories indicate that the TS of the RF2-dependent hydrolysis reaction involves only one proton in flight [30••], in agreement with computational models that suggest an early TS and one proton being transferred in the rate-limiting TS [53•]. These findings argue against a concerted proton shuttle in the TS of the hydrolysis reaction, which would require that at least two protons are transferred simultaneously in the TS. The slope of the pH/rate dependence for both ribosome-catalyzed and uncatalyzed hydrolysis reactions turned out to be close to one with a p*K*a > 9 [30••]. For the uncatalyzed reaction, this group must be a water molecule, because removal of the 2′OH, which would be an alternative candidate for an ionizing group with this pKa, does not affect the uncatalyzed reaction [54]. The slope of the pH/rate profile was identical in H_2O and D_2O, suggesting that the proton which is transferred in the TS does not originate from the ionizing group that takes part in the reaction [30••]. These findings are consistent with a reaction mechanism in which the attacking water molecule in the TS donates a proton to a hydroxide ion that facilitates both proton transfer and nucleophilic attack (Figure 8a). Density for a water molecule in the active site was observed in the crystal structure of a complex representing the reactant state [52••]. The effect of replacing the 2′OH group of A76 then may be attributed to a change of the rate-limiting step from an early to a late TS, such that proton transfer to the leaving group becomes rate-limiting. Alternatively, a hydroxide ion could act as attacking group; in this case, the proton in flight can be transferred to, for example, the 2′OH directly or through another water molecule (Figure 8b). Certain amino acid substitutions of the Gln in the GGQ (Ser, Thr, Cys) can rescue the defects associated with loss of the 2′ OH of the P-site substrate, possibly by creating a hydrogen-bond network that provides an alternative stabilization of the TS [55•]. Surprisingly, RF2-catalyzed and RF1-catalyzed reactions show some apparent differences in the properties of the TS (compare Refs. [55•,30••]). The magnitude of the KSIE is much smaller with RF1 (KSIE = 1.4) than with RF2 (KSIE = 4.1), and the slope of the pH/rate profile is substantially smaller than 1 for RF1, compared to almost exactly 1 for RF2. The simplest explanation for these effects is that, unlike RF2, RF1 binding is partially rate-limiting for the chemistry step, at least in the experimental conditions used.

Conclusions

Recent work demonstrates that the ribosome's catalytic site alone or augmented by EF-P or RF1/2 can support rather different reaction mechanisms. That the active site of the ribosome could retain, or gain, this versatility during evolution can be probably explained by the observation that ribosomal residues, and residues of the release factor, do not take part in chemistry, but rather provide a network of electrostatic and hydrogen-bonding interactions that help in orienting the substrates and in stabilizing the respective transition state [12•,31••,33•], more or less independent of the chemical nature of the substrates. Furthermore, the evolutionary pressure favored the optimization of speed and accuracy of the decoding reaction which precedes peptide bond formation. This may explain why the ribosome did not evolve to contain proteins, with their large repertoire of chemical groups suitable for more efficient catalysis, at its catalytic site. In the early days of the RNA world, the primordial ribosome, which most likely comprised only the A and P-site parts of 23S rRNA [56••], might have evolved towards accepting a multitude of chemically versatile substrates, thereby providing potential building blocks of polymers for the evolution of life. The ribosome retained its RNA-based catalytic strategy during the evolution from a prebiotic translational ribozyme into a modern ribosome, which thus appears to be a living fossil of a primitive catalyst of the RNA world.

Acknowledgements

I thank Anja Lehwess-Litzmann for help in preparing the figures. The work was funded by the Max Planck Society and by a grant of the Deutsche Forschungsgemeinschaft.

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