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Point of View

Ribosomal catalysis

The evolution of mechanistic concepts for peptide bond formation and peptidyl-tRNA hydrolysis

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Abbreviations: RNP, ribonucleoprotein; rRNA, ribosomal RNA; r-protein, ribosomal protein; PTC, peptidyl transferase center; pept-tRNA, peptidyl-tRNA; aa-tRNA, aminoacyl-tRNA; RF, release factor

Key words: ribosomes, rRNA, peptide bond formation, peptidyl-tRNA hydrolysis, translation termination, protein synthesis, ribozyme

Over time the mechanistic concepts to describe the two principal chemical reactions that are catalyzed by the ribosome, peptide bond formation and peptidyl-tRNA hydrolysis, have undergone dramatic changes. While the initial models were based on a ribosomal protein-based mechanism, evidence for a direct functional contribution of ribosomal RNA for catalysis has accumulated over the past years. The presentation of high resolution crystallographic structures of the large ribosomal subunit at the beginning of the new millennium dramatically increased our molecular insight into the organization of the active center and finally placed the ribosome amongst the list of RNA enzymes. Combined with elaborate biochemical and biophysical approaches the translation field has made significant progress in understanding mechanistic details of ribosomal catalysis. While it seems that the mechanism of ribosome-catalyzed peptidyl-tRNA hydrolysis is just emerging, the knowledge on transpeptidation is already very advanced. It has been realized that intricate interactions between ribosomal RNA and the transfer RNA substrate are crucial for proton shuttling that is required for efficient amide bond formation.

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 build 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.

Functions of the ribosome in translation are complex and involve different types of activities critical for decoding of the genetic information, covalent linkage of amino acids via amide bonds to form polypeptide chains, as well as the release and the proper targeting of the synthesized protein. During the course of the elongation cycle the ribosome has to interact dynamically with various RNA (e.g., mRNA), RNP (e.g., aminoacyl-tRNA-EF-Tu) and protein (e.g., EF-G, RF) ligands. 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 locate in a deep cleft at the interface side of the large subunit (Fig. 1). Since ribosomes are so fundamental to life, and represent the main target for clinically relevant antibiotics, comprehending how they work is at the heart of molecular understanding of biology.

The two central chemical reactions of protein synthesis are performed by the PTC, namely peptide bond formation and peptidyl-tRNA (pept-tRNA) hydrolysis (reviewed in 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. 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 reaction intermediate 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 an amazing 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]

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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 catalytic 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 mRNA stop codon displayed in the A-site.

The means by which the PTC catalyzes these fundamental biological reactions has been a subject of intense discussion over the last decades. With the presentations of the first high-resolution crystallographic structures of the 50S subunit at the dawn of the new millennium,[8-10] the controversial discussions about mechanistic details of ribosomal catalysis intensified and, unfortunately, sometimes left pure scientific grounds. Emotions have largely calmed down in the meantime, which makes a more impassive retrospective look at the evolving concepts of ribosomal catalysis timely.

Forging Peptide Bonds on the Ribosome

In the beginning of protein synthesis research it was believed that peptide bonds are most likely formed by inversed proteolytic mechanisms catalyzed by specialized protein enzymes. Nevertheless, already in 1939 a connection between protein synthesis and RNA was made based on a correlation between high concentrations of RNA with fast dividing cells.[11] A few years later small cytoplasmic particles were discovered that were termed 'microsomes' and were shown to consist of about 50% lipids, 35% proteins and 15% nucleic acids.[12] It was a decade later when an experimental connection between microsomes and protein synthesis could be established.[13] With the availability of radioactive tracers in the form of labeled amino acids, research on protein synthesis received a huge boost. Now it was possible to monitor protein synthesis with the help of elaborate centrifugation protocols and cell free translation assays (reviewed in ref. 14). It was around this time that the first models of protein synthesis emerged. An important observation was made showing that the soluble fraction of a 105,000 x g supernatant activated amino acids in an ATP-dependent manner and attached them to ribonucleic acids called soluble RNA (sRNA),[15] that were later renamed to tRNA. These sRNAs were obviously the adaptor molecules needed for templated protein synthesis that Francis Crick had already postulated in 1955.[16] Subsequently, an intriguing hypothesis was introduced that charged sRNAs directly bind to the microsomal RNA and use it as template for protein synthesis.[17] Consequently, every protein would need its own microsome to be synthesized, a hypothesis that could not be substantiated. The term *ribosomes* was finally coined by Dick Roberts for "ribonucleoprotein particles in the size range 20 to 100S".[18]

After realizing that the ribosome is the enzyme that synthesizes peptide bonds, efforts to experimentally identify the crucial r-protein that provides catalytic power to the ribosome began—a rather futile task as we now realize (reviewed in ref. 19). At that time, only protein enzymes were known in nature, thus it was reasonable to assume that the ribosome also uses functional amino acid group(s) of r-proteins for catalysis. However, not a single r-protein nor a mixture of proteins could be identified with catalytic activity. The identification of the first RNA enzymes in the early 80's radically changed the field of molecular biology and made rRNA-mediated catalysis on the ribosome fashionable.[20] In the light of the accumulating evidence for a functional and possibly catalytic role of 23S rRNA (reviewed in ref. 21) the rather prophetic hypothesis of Francis Crick, namely that the primordial ribosome was an all-RNA composed molecular machine,[22] appeared more than just a ribocentric fantasy of a celebrated molecular biologist. The case was finally sealed in the years 2000 and 2001 when the first crystal structures at atomic resolution of the large 50S ribosomal subunit were presented.[8-10] These structures conclusively revealed the PTC to be devoid of any r-proteins, thus confirming the peptidyl transferase as an RNA enzyme. These findings finally ended the long-term controversy as to whether or not rRNA or r-proteins constituted the catalytic domain and moved the field forward enabling more detailed mechanistic questions to be addressed.

A Molecular Glimpse of the Active Site

The high resolution structural data gave unprecedented insight into molecular aspects of translation and pinpointed the PTC at the bottom of a large cleft on the interface side of the large ribosomal subunit underneath the central protuberance[8,9] (Fig. 1). The active site crater is densely packed and decorated with universally conserved nucleotides of the central loop of domain V of 23S rRNA. Protein side chains from r-proteins are absent in the immediate vicinity of the chemical center. 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 manuscript). In the non-translating 50S subunit, the cavity is empty except for the bases of nucleotides A2602 and U2585, which bulge into its center. The orientation of these two residues depends on the functional state of the ribosome and the nature of the bound substrate (reviewed in ref. 1). In all available crystal structures with bound reaction substrates, 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 hydrogen bonding distance. 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,[19] such as the group I and group II self-splicing ribozymes, which can be considered metallo-enzymes.

The acceptor arms of tRNA substrates are locked 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.[10,23-25] The tRNAs ends are further stabilized in both A- and P-sites by A-minor interactions between A76 of tRNA with the

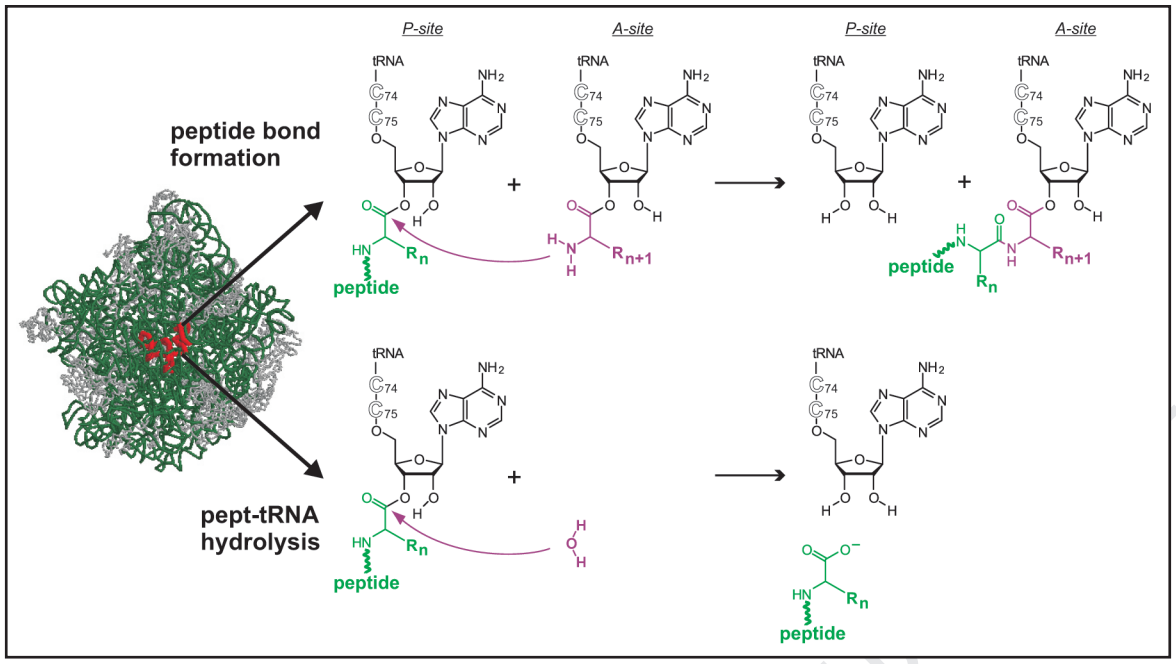

Figure 1. The two principal chemical reactions of protein synthesis. The ribosomal PTC is composed of highly conserved nucleotides of domain V (red) of 23S rRNA (dark green) and is located at the interface side of the 50S subunit. Ribosomal proteins are shown as grey ribbons. The figure was generated using the pdb file 1FFK.[10] During peptide bond formation, the α-amino group of aminoacyl-tRNA in the A-site (purple) attacks the ester carbonyl carbon of P-site bound peptidyl-tRNA (green). A short-lived tetrahedral transition state 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 (green) is nucelophilically attacked by an activated water molecule (purple) which leads to peptidyl-tRNA hydrolysis and polypeptide release.

23S rRNA base pairs U2506-G2583 and A2450-C2501, respectively.[10,26] Both acceptor ends of the A- and P-tRNAs carrying the peptidyl- or aminoacyl moieties, respectively, meet at the bottom of the funnel-shaped catalytic cleft directly above the entrance to the nascent peptide exit tunnel.

RNA-Catalyzed Peptide Bond Formation

From rRNA to tRNA catalysis. These detailed insights into the catalytic center of the 50S subunit allowed Steitz, Moore and coworkers to postulate a very comprehensive 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. During the course of the reaction it was proposed that the N3 receives a proton from the attacking α-amino group of aa-tRNA, stabilizes the tetrahedral intermediate and finally donates its proton to the P-tRNA 3'-O leaving group. While this elegant model provided a rational as to why nature has selected an adenine at this position of the PTC in all ribosomes on this planet, it immediately raised doubts since some previously published key findings appeared to be in conflict with this model.[27] Indeed subsequent biochemical and genetic studies, did not support a crucial role of the nucleobase at A2451 for catalysis.[28-34] Even though mutations at A2451 are lethal in *E. coli*, the A2451U mutant turned out to viable in *Mycobacterium smegmatis*.[35] These important findings demonstrated that the base identity at position 2451 is not absolutely critical for transpeptidation and shows that in principle life is possible without this universally conserved adenine nucleobase. 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.

Subsequently it appeared that the 'momentum' in the ribosome community changed again since 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. 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.[36,37] This tRNA 2'-OH was proposed to be essential for transpeptidation in a so called 'substrate-assisted catalysis',[38] 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.[37,38] 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 (Fig. 2). This elegant model allows proton shuttling

without significant charge generation on either the A76 2'-OH or the A76 3'-OH leaving group. Crystallographic[39] and molecular dynamics simulation[40] studies are in agreement with this scenario and additional interactions with other PTC residues and coordinated water molecules have been suggested to stabilize this 6-membered ring conformation (Fig. 2). It has been suggested that the PTC works by providing a pre-organized electrostatic network that reduces the free energy of forming the transition state (reviewed in ref. 41).

The return of A2451. Which of these predicted network interactions at the PTC are critical for peptide bond formation? It is obvious that not all of the hydrogen bonds that generate the predicted electrostatic network are possible or at least can be formed simultaneously (Fig. 2). To address this question we have developed a novel experimental strategy that allows functional group replacements to be done on active site 23S rRNA residues. In order to introduce these non-natural nucleotide analogs into the 23S rRNA we applied a recently established in vitro reconstitution technique for 50S subunits.[42] 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. We applied this 'atomic mutagenesis' approach to all inner core nucleotides of the PTC and modified single functional groups (or even single atoms) of the nucleobases, removed the ribose 2'-OH, eliminated the entire nucleobase or the entire base as well as most of the ribose sugar (Fig. 3). It turned out that all the nucleotide modifications were tolerated with a single exception, namely removal of the ribose 2'-OH group of A2451 severely hampered peptide bond synthesis.[42,43] We could demonstrate this using minimal (puromycin, CpCp-puromycin) or full length (aa-tRNA) A-site substrates.[42,43] Recently, we observed an identical effect in model in vitro translation reactions where the aa-tRNA is delivered to the ribosome as ternary complex with EF-Tu and GTP (Erlacher M, Chirkova A and 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. This finding further supports a potential involvement of the A2451 2'-OH in the chemistry of the peptidyl transferase reaction.

How 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 detail, we have recently introduced selected modifications at the ribose 2' carbon that severely influence the hydrogen bonding potential (Fig. 3). We could show 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).[58] We propose 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[44] and highlights the functional importance of this particular interaction for the proposed network of the PTC (Fig. 2). In other words, by donating its proton to the A76 2'-O of pept-tRNA, A2451 helps to stabilize the 6-membered

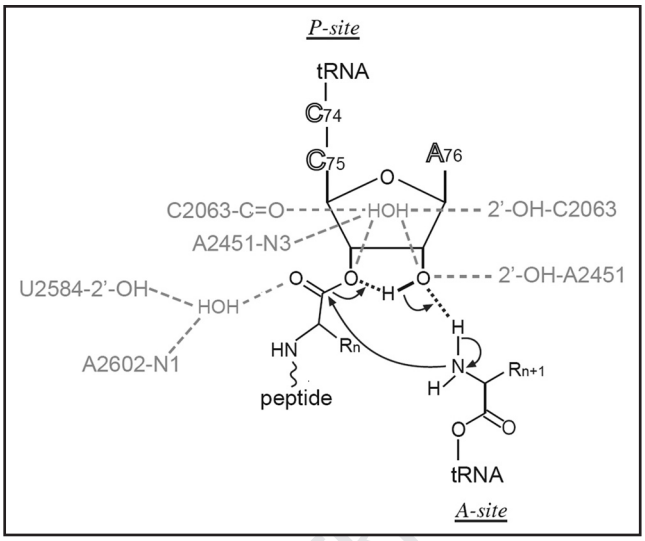

Figure 2. Substrate assisted catalysis of peptide bond formation by a proton shuttle mechanism. The CCA terminal sequence of P-site pept-tRNA and the α-amino nucleophile of A-site tRNA are shown in black while rRNA residues and structural water molecules are in grey. In this model the nucleophilic attack 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. Additional interactions with PTC residues and water molecules that have been proposed to stabilize this 6-membered transition state (reviewed in ref. 41) are depicted by grey dotted lines.

ring system of the proposed proton shuttle in its productive conformation (Fig. 4A). 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 (Fig. 4B). 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 crucial 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. In summary, these latest mechanistic models of ribosomal peptide bond formation appreciate the concept of 'substrate-assisted catalysis'[38] and combine with it the strict functional requirement of the ribose 2' group at A2451 of 23S rRNA to possess hydrogen donor capability.

Pept-tRNA Hydrolysis: Similar but not 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 during the termination phase of translation. Biochemical and structural data show that the tip of domain III (which harbors the universally conserved GGQ peptide mini-motif)[45] of the A-site bound RF reaches toward the bottom of the PTC and is in immediate neighborhood of A2602 and U2585 of 23S rRNA.[46,47] The molecular events that take place 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

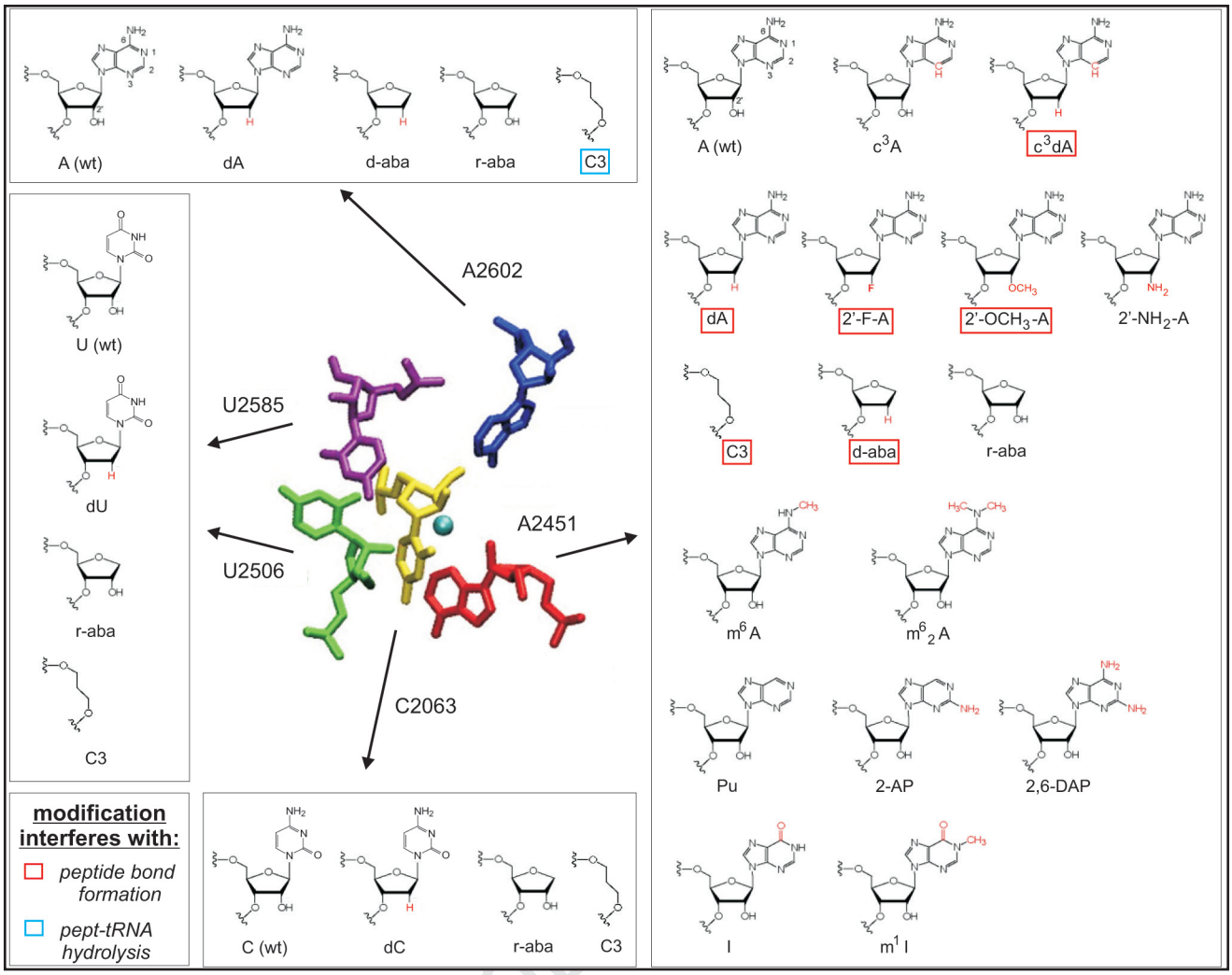

Figure 3. Atomic mutagenesis of the ribosomal peptidyl transferase center. The inner core 23S rRNA residues (center) have been substituted by the depicted nucleotide analogs using the gapped-cp-reconstitution approach.[42] The green sphere marks the location of the nitrogen atom of the attacking α-amino group of aa-tRNA. The view of the three-dimensional architecture of the PTC was generated from pdb file 1FG0.[10] The 50S particles carrying the respective non-natural nucleotide analogs were tested for their ability to catalyze peptide bond formation and pept-tRNA hydrolysis, respectively.[42,43,54,58] Introduced nucleotide analogs that interfered significantly with efficient peptide bond formation or pept-tRNA hydrolysis are highlighted by red and blue rectangles, respectively. dN: 2'-deoxy-nucleotide; c^3A: 3-deaza-adenosine; c^3dA: 3-deaza-2'deoxy-adenosine; 2'-F-A: 2'-fluoro-adenosine; 2'-OCH_3-A: 2'-O-methyl-adenosine; 2'-NH_2-A: 2'-amino-adenosine; C3: C3-linker where most of the ribose is deleted and three C-atoms serve as place holder for the RNA backbone; d-aba: deoxy-abasic analog; r-abasic: ribose-abasic analog; m^6A: N^6-methyladenosine; $m^6{}_2$A: N^6,N^6-dimethyladenosine; Pu: purine; 2-AP: 2-aminopurine; 2,6-DAP: 2,6-diaminopurine; I: inosine; m^1I: 1-methylinosine.

water molecule.[7,45,48-50] However, it has been shown that pept-tRNA hydrolysis can be efficiently triggered even in the absence of a class I RF by replacing it with an A-site bound deacylated tRNA,[7,51,52] thus hinting at an RNA-catalyzed reaction (Fig. 5A).

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, both in the context of in vitro assembled 50S particles[52] as well as in vivo-derived mutant ribosomes.[34] Based on the results of mutational studies, we have proposed a model in which the class I RF triggers peptide release by repositioning A2602 in the PTC so that its nucleobase can coordinate and possibly activate a water molecule for the attack onto the ester carbonyl carbon of the pept-tRNA.[52] The structural flexibility and the central location of A2602 in the PTC are compatible with this proposed role.[10,23,53] In this scenario the GGQ motif of the RF functions to promote this crucial conformational change at A2602 which in turn allows the adenine base to coordinate the hydrolytic water molecule. The most likely group on the nucleobase that could fulfill this task is the N1 position. In order to test this hypothesis we have recently applied the 'atomic mutagenesis' approach described above to introduce non-natural nucleotide analogs at A2602 and all other inner core residues (Fig. 3). To our surprise we showed that removal of the entire adenine base at position 2602 (by introducing an abasic nucleotide analog) did not interfere with pept-tRNA hydrolysis activity[54] (Fig. 5B). The severe reductions seen before in ribosomes carrying base changes at A2602 are therefore likely the result of a conformationally distorted PTC that trapped the active site in a non-productive state for RF1-mediated pept-tRNA hydrolysis.[34,52] This active site residue probably requires a certain amount of structural flexibility for full peptide

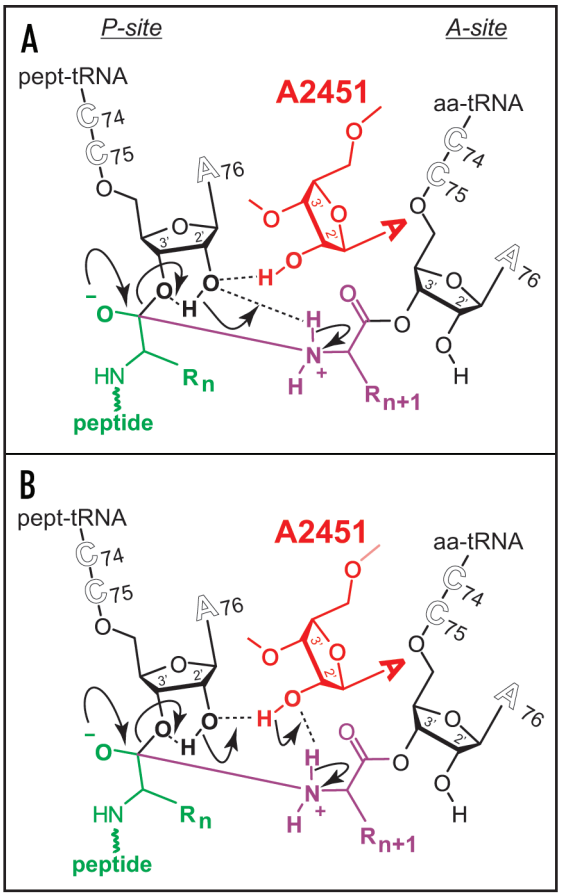

Figure 4. Model for the mechanism of amide bond formation proposing a role for 23S rRNA A2451. A2451 of 23S rRNA (red), peptidyl moiety of pept-tRNA in the P-site (green), and aminoacyl-moiety of A-site bound aa-tRNA (purple); (A) The hydrogen bonding interaction between the A2451 2'-OH and the pept-tRNA A76 2'-O bond assists in P-site tRNA A76 ribose positioning and in suppression of spontaneous intramolecular transesterification.[58] 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 six-atom 'proton-shuttle' mechanism.[36-38] (B) Alternatively, peptide bonds can also be formed via an 8-membered cyclic arrangement. The A2451 2'-OH is in close vicinity to the attacking α-amino group and can, in principle, participate in an eight-atom 'proton-shuttle' mechanism by acting as hydrogen donor and hydrogen acceptor simultaneously.[58]

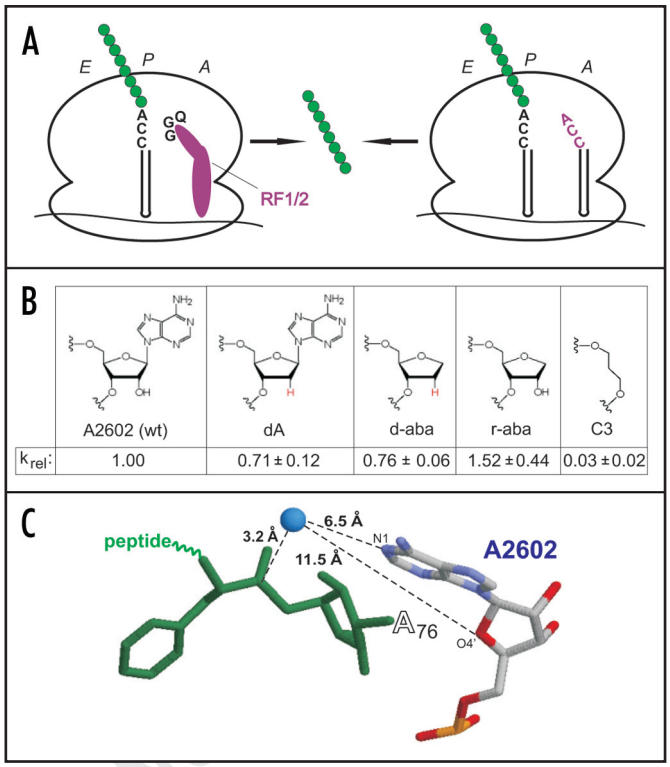

Figure 5. Ribosome catalyzed peptidyl-tRNA hydrolysis. (A) pept-tRNA hydrolysis and release of the peptidyl chain (green) from P-site bound pept-tRNA is triggered by an A-site bound class I release factor (RF1 or RF2 in bacteria). The 3' CCA tRNA ends and the universally conserved GGQ peptide mini motif 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 the pept-hydrolysis reaction (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.[54] (C) The three-dimensional representation highlights the distances between the ribose O4' position of A2602 and the position of the oxygen atom of the A-site bound CC-hydroxypromycin, which marks the position from where the hydrolytic water molecule (blue sphere) is supposed to launch its attack onto the ester carbonyl carbon of pept-tRNA. Additionally, the distances between this putative water and the ester carbonyl carbon of pept-tRNA as well as the adenine N1 position of A2602 are indicated. The figure was prepared using the coordinates of pdb 1VQN.[44]

release activity which is obviously not necessarily the case when an incorrect base is attached to the ribose. 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 strongly 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 (Fig. 3). It is noteworthy that removal of the 2'-OH at A2451, which was shown to be so crucial for catalyzing peptide bond formation, had an only very mild effect on the rate of pept-tRNA hydrolysis.[54]

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?[52] 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 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, appears to be too large (11.5 Å) for the direct coordination of the hydrolytic water (Fig. 5C). However it is theoretically possible that structural water molecules could functionally replace the missing adenine base in the context of ribosomes carrying the abasic nucleotide analog at 2602. Evidence for such water 'wires' that can shuffle protons over longer distances has recently been presented for smaller ribozymes (reviewed in ref. 55). Even though we can not completely discard the possibility of direct water coordination by A2602 during the peptide release reaction, 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. For this switching function the nucleobase at 2602 is not actually required, however, an intact ribose moiety appears to be strictly necessary. It is conceivable that the prime function of the A2602 switch is to guide or channel the hydrolytic water into the catalytic center for optimal pept-tRNA hydrolysis. An alternative function for A2602 might be the coordination of a water molecule that stabilizes the carbonyl oxyanion that is formed during the transition state of the reaction (Fig. 2). Such a water molecule has been observed in crystallographic structures and in molecular dynamics simulations to be held in place by the N1 and/or N6 of the adenine base at A2602 and the 2'-OH of A2584.[39,56] However, removing these two putative interaction partners of this 'oxyanion water' individually did not influence the rate of pept-tRNA hydrolysis[54] or peptide bond synthesis significantly (refs. 34 and 52 and our unpublished data).

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[42,43] 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 (Fig. 3), leaving the 2'-OH of A76 of P-tRNA, which plays an important role in peptide bond formation,[36-38] 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 recently presented that highlights the importance of the A76 2'-OH in activating the water possibly also via the 6-membered proton shuffle (Fig. 2).[56] In these computer simulations the glutamine of the GGQ motif 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. Experimental support for such a scenario was recently published suggesting that the RF has two important contributions to pept-tRNA hydrolysis. First, docking of the GGQ motif into the active site activates the PTC via an induced fit, and second, the glutamine of the GGQ specifically selects water as the nucleophile and positions it for the attack onto the pet-tRNA ester carbonyl carbon.[57] Aqvist and co-workers concluded that the PTC catalyzes its two chemical reactions using a common mechanism.[56] 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 (Fig. 4), it obviously does not have a comparable functional relevance for the mechanism of pept-tRNA hydrolysis.[54] (ii) If the primary role of A2602 is to position the GGQ motif for optimal pept-tRNA hydrolysis it follows that this would also be the role of this nucleotide 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. 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.[34,52,54] We conclude that the 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, RF1 was exclusively employed in almost all PTC mutagenesis studies to date.[34,42,52,54,57] In summary, it appears that our mechanistic understanding of pept-tRNA hydrolysis is still limited and there is work ahead of us until we reach similar insight into this fundamental biological reaction as we have attained for peptide bond formation.

Conclusion and 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[9,10,39] and recently also of 70S particles,[25] enormous progress has been made in combination with biochemical work (reviewed in ref. 41). Now we are at a point where we no longer 'fight' about fundamentalistic questions such as to whether or not the RNA or the r-proteins promote catalysis. The ribosome community has moved towards a rather advanced stage to now discuss where and how single protons move in order to facilitate the RNA-catalyzed transpeptidation reaction. 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.[55] In the recent 50S structures[39] as well as in molecular dynamics simulations[40,56] 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.

One of the big open questions in the field of ribosome research is why evolution has selected so many universally conserved nucleotides in the inner core of the PTC, when standard[31,32,34,52] and atomic mutagenesis studies[42,43,54] have shown that the nucleobases are not actually critical for catalysis? Of course these studies have been performed exclusively in vitro and only single model reactions have been investigated. So it can be legitimately argued 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 a bacterial organism that was viable, indicating that mutations at this pivotal site are in principle compatible with cellular life.[35] So why is the evolutionary pressure to conserve the nucleobases obviously so strong, even though in vitro test have revealed that only RNA backbone groups are crucial for the catalytic reactions (the A2451 2'-OH for transpeptidation, the ribose ring at A2602 for pept-tRNA hydrolysis and the P-tRNA A76 2'-OH probably for both reactions)? Maybe the nucleobase identities have been selected so rigorously by nature to create an active site environment that allows all 20 (or actually 22) natural amino acids to smoothly accommodate into the catalytic crevice of the 50S subunit and pass the PTC unhindered on their way out of the ribosome via the exit tunnel.

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