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The Ribosomal Peptidyl Transferase Center: Structure, Function, Evolution, Inhibition

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ABSTRACT The ribosomal peptidyl transferase center (PTC) resides in the large ribosomal subunit and catalyzes the two principal chemical reactions of protein synthesis: peptide bond formation and peptide release. The catalytic mechanisms employed and their inhibition by antibiotics have been in the focus of molecular and structural biologists for decades. With the elucidation of atomic structures of the large ribosomal subunit at the dawn of the new millennium, these questions gained a new level of molecular significance. The crystallographic structures compellingly confirmed that peptidyl transferase is an RNA enzyme. This places the ribosome on the list of naturally occurring riboyzmes that outlived the transition from the pre-biotic RNA World to contemporary biology. Biochemical, genetic and structural evidence highlight the role of the ribosome as an entropic catalyst that accelerates peptide bond formation primarily by substrate positioning. At the same time, peptide release should more strongly depend on chemical catalysis likely involving an rRNA group of the PTC. The PTC is characterized by the most pronounced accumulation of universally conserved rRNA nucleotides in the entire ribosome. Thus, it came as a surprise that recent findings revealed an unexpected high level of variation in the mode of antibiotic binding to the PTC of ribosomes from different organisms.

KEYWORDS ribosome, ribozyme, peptide bond formation, peptide release, antibiotics, RNA World

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INTRODUCTION

The ribosomal peptidyl transferase center (PTC) is the catalytic heart of the ribosome and plays a fundamental role in protein synthesis. It is a part of the large ribosomal subunit (50S in eubacterial ribosomes), a complex dynamic ribo-nucleoprotein ensemble with a molecular weight of approximately 1.8 MD. The primary function of the ribosomal PTC is to covalently link amino acids via peptide bonds into polypeptides. The peptidyl transferase reaction involves aminolysis by the α-amino group of the A-site aminoacyl-tRNA of the ester bond that links the nascent peptide to the 3′ hydroxyl of the 3′ terminal ribose of the P-site tRNA. In the first step, the α-NH_3^+ group is deprotonated to yield the nucleophilic NH_2 group. The proton in this step is likely to be accepted by a water molecule. Subsequently, a nucleophilic attack of the α-amino group of

aminoacyl-tRNA occurs on the electrophilic carbonyl group of peptidyl-tRNA. This leads to the formation of a short-lived tetrahedral reaction intermediate. The transition state decomposes by donating a proton back to the leaving oxygen to yield the reaction products deacylated tRNA at the P-site and peptidyl-tRNA (elongated by one amino acid) at the A-site (Figure 1A). This reaction is performed by the ribosome with an astounding speed of ~15 to 50 peptide bonds per second (Katunin *et al.*, 2002).

The second principle chemical reaction that takes place in the PTC is peptidyl-tRNA hydrolysis, which is required for termination of translation and release of the fully assembled polypeptide from the ribosome. The termination reaction involves the transfer of the peptidyl moiety of P-site located peptidyl-tRNA to a water molecule (Tate & Brown, 1992). In the course of the reaction, the nucleophilic attack of an activated water molecule in the PTC acceptor site onto the carbonyl carbon of the peptidyl-tRNA ester leads to formation of a tetrahedral intermediate. A proton from the water is subsequently transferred to the 2′(3′)-hydroxyl of the 3′-terminal adenosine of peptidyl-tRNA, breaking the ester bond between the peptide and tRNA (Figure 1C). From a chemical point of view, peptide release is a more challenging reaction than transpeptidation because hydrolysis of the ester bond is driven by a significantly less nucleophilic water oxygen. In this scenario, it is expected that the ribosome-catalyzed reaction of peptide release should involve specific coordination and activation of the water molecule, possibly by general base chemistry. The catalytic rate constant of peptide release has been estimated in an *in vitro* translation assay to be 0.5 to 1.5 per second (Zavialov *et al.*, 2002) and is therefore clearly slower than transpeptidation. The switch of the mode of action of the PTC from amino acid polymerization to peptidyl-tRNA hydrolysis is triggered by class I release factors, which bind in response to an mRNA stop codon in the decoding A-site at the small ribosomal subunit.

Both of these fundamental chemical reactions of protein synthesis are targeted by numerous, naturally occurring antibiotics. Even though the ribosome, with a molecular weight of ~ 2.7 MD, offers many potential drug target sites, only a few sites have been selected by nature for antibiotic action, with the PTC being one

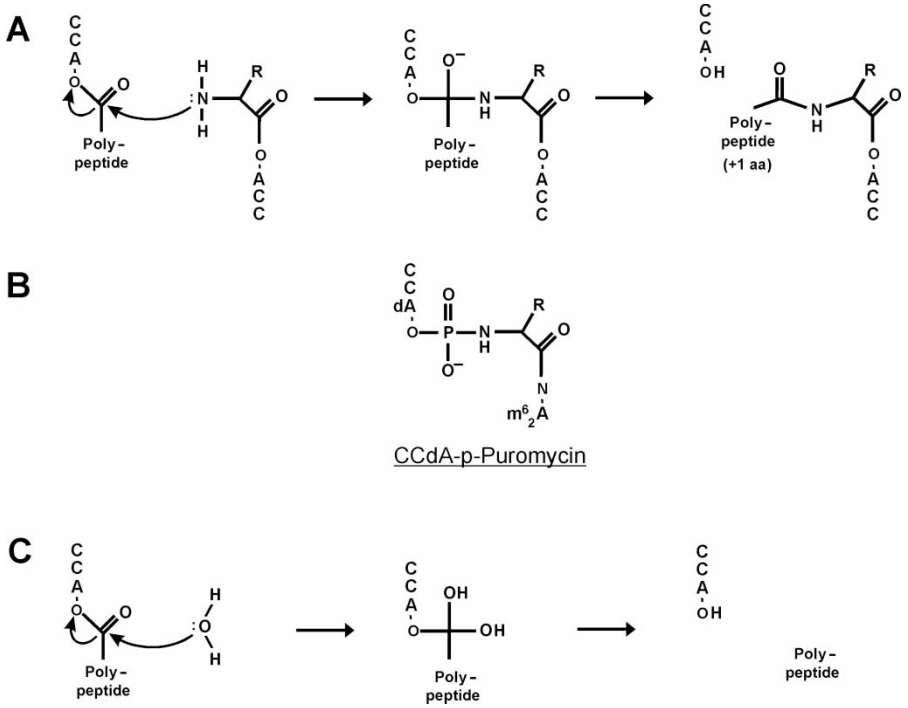


FIGURE 1 **Chemistry of peptide bond formation and peptidyl-tRNA hydrolysis. (*A*) During peptide bond formation, the α-amino group of aminoacyl-tRNA in the A-site attacks the carbonyl carbon of P-site bound peptidyl tRNA. The tetrahedral transition state decomposes into the reaction products, deacylated tRNA at the P-site and peptidyl-tRNA carrying an additional amino acid (+1 aa) at the A-site. For clarity, only the CCA-ends of both tRNAs are depicted here. (*B*) The transition state mimic, CCdA-p-puromycin (Welch *et al.*, 1995), that was used in crystallographic studies to identify the PTC (Nissen *et al.*, 2000) is shown underneath the authentic reaction intermediate. $m^6{}_2A$ depicts the N6,N6-dimethyladenosine of puromycin. (*C*) In peptide release, the carbonyl carbon of P-site located peptidyl-tRNA is attacked by an activated water molecule which leads to peptidyl-tRNA hydrolysis and polypeptide release.**

of the most prominent. Equipped with the now available atomic structures of the large ribosomal subunit and the huge body of biochemical evidence that has accumulated over the past 45 years, we nevertheless are just starting to understand the range of functional capacity of the ribosomal PTC and its role in antibiotic resistance.

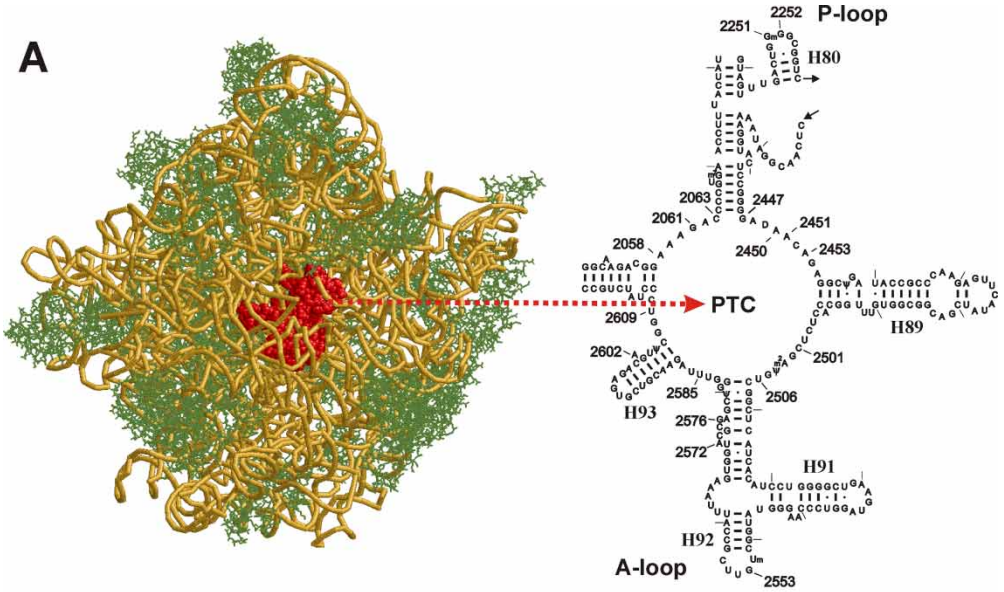
MOLECULAR ANATOMY OF THE PTC

Ribosomal RNA accounts for two thirds of the ribosome's weight. For a number of years, rRNA was viewed primarily as a scaffold for binding ribosomal proteins that were presumed to carry out major functions of the ribosome. However, as early as in 1968, Francis Crick speculated that functions of rRNA extend beyond that of a simple skeleton for organizing the protein "flesh" of the ribosome. Crick proposed that the protoribosome, an allegedly important enzyme in the RNA World, was composed entirely of RNA and relied on RNA functions to fulfill its catalytic duties in amino acid polymerization (Crick, 1968). At the time, such an assumption was quite radical, since not a single RNA enzyme was known and it was therefore considered by many as a ribocentric fantasy of a celebrated pioneer of nucleic acids structure rather than a serious scientific hypothesis of any significant value for understanding functions of the ribosome. And so the main emphasis in pinpointing the catalytic components of the PTC in the 1970s and early 1980s was focused on identifying a protein or a combination of ribosomal proteins that catalyze peptide bond formation, a rather futile task as we now realize.

Meanwhile, the RNA tide was rising. Through a dedicated effort of several laboratories, biochemical and genetic evidence of the pivotal role of rRNA in the functioning of the PTC was accumulating. Mutational, footprinting, and crosslinking studies consistently implicated the 23S rRNA segment structure, which became known as the central loop of domain V, in peptidyl transferase activity (Figure 2) (reviewed in: Barta & Halama, 1996; Green & Noller, 1997; Noller, 1993a; Polacek, 2001). The concept of the PTC as a ribozyme was finally sealed when the high resolution crystallographic structure of the large ribosomal subunit from the archaeon *Haloarcula marismortuii* was unveiled in 2000 (Nissen *et al.*, 2000). The absence of ribosomal proteins in the PTC active site confirmed Crick's prophetic vision and placed the ribosome, the mother of all protein-enzymes, as the key entry on the list of naturally occurring ribozymes (reviewed in Doudna & Cech, 2002).

The high resolution structural data obtained by soaking analogs of substrates and products of the peptidyl transfer reaction into crystals of 50S subunits were in excellent agreement with genetic and biochemical data and showed that the PTC active site is located at the bottom of a large cleft at the interface side of the large ribosomal subunit underneath the central protuberance (Ban *et al.*, 2000; Harms *et al.*, 2001) (Figure 2). The peptidyl transferase (PT) cavity is densely packed and decorated with nucleotides of the central loop of domain V of 23S rRNA (Figures 2 and 3). No ribosomal protein comes to the reactive center of the transition state intermediate closer than 18 Å in the *H. marismortui* structure. The closest approaches are made by long "tails" of ribosomal proteins L2, L3, L4 and L10e (L16 equivalent) that extend into the core of the ribosome from their globular domains located on the solvent side of the 50S subunit (Nissen *et al.*, 2000). At least two of these proteins (L2 and L3) appear to be indispensable for the PTC functions in the bacterial ribosome (Hampl *et al.*, 1981; Khaitovich *et al.*, 1999a; Khaitovich *et al.*, 1999b). In the crystallographic structure of a eubacterial ribosome, the flexible N-terminus of protein L27 was seen fairly close to the PTC, and biochemical data indicate even closer proximity to the active site in solution (Harms *et al.*, 2001; Colca *et al.*, 2003; Maguire *et al.*, 2005). However, protein L27 is dispensable for cell survival and, thus, for the ribosome-catalyzed peptide bond formation (Wower *et al.*, 1998). Although the role of some ribosomal proteins in the organization and possibly the function of the PTC cannot be completely excluded (reviewed in Khaitovich & Mankin, 2000), neither (at least in cytoplasmic ribosomes of the three phylogenetic domains) appears to be directly involved in catalysis. The long, positively charged extensions of ribosomal proteins may serve to hold together rRNA components of the PTC (Nissen *et al.*, 2000), which explains the lack of catalytic activity of protein-free 23S rRNA (Khaitovich *et al.*, 1999a; Khaitovich *et al.*, 1999b). The lack to demonstration that isolated 23S rRNA can catalyze peptide bond formation is probably the last 'missing link' in the chain of arguments that picture modern rRNA as a descendant of the prebiotic RNA-only ribosome. Nevertheless, there is a clear consensus that peptide bond formation is essentially an rRNA-driven reaction and that the ribosomal

A



B

23S rRNA position	Functional implication
A2058	- entrance to the nascent peptide tunnel
A2059	- entrance to the nascent peptide tunnel
G2061	- member of the hydrogen bonding network that orients A2451
C2063	- forms base pair with A2450 with putative near-neutral pKa
G2251	- forms base pair with C75 of P-site tRNA
G2252	- forms base pair with C74 of P-site tRNA
G2447	- member of the hydrogen bonding network that orients A2451
A2450	- A-minor interaction with A76 of P-site tRNA - member of the hydrogen bonding network that orients A2451 - forms base pair with C2063 with putative near-neutral pKa
A2451	- N3 of the base and 2'-OH of the ribose are in hydrogen bonding distance to the α-amino group of aminoacyl-tRNA - removal of the 2'-OH markedly inhibits transpeptidation, while all nucleobase modifications have little effect
A2453	- forms base pair with C2499 with putative near-neutral pKa
C2499	- forms base pair with A2453 with putative near-neutral pKa
C2501	- A-minor interaction with A76 of P-site tRNA
U2506	- A-minor interaction with A76 of A-site tRNA
G2553	- forms base pair with C75 of A-site tRNA
A2572	- located at a sharp bend of helix 90; putative conformational flexibility
U2583	- A-minor interaction with A76 of A-site tRNA
U2585	- the second (after A2602) most flexible residue in the PTC - located at a hydrogen bonding distance to the ribose 2'-OH of A76 of the acceptor substrate
A2602	- sandwiched between both A- and P-tRNA CCA ends - conformationally most flexible residue in the PTC - mutations strongly interfere with peptide release

FIGURE 2 The ribosomal peptidyl transferase center (PTC). (*A*) The crystallographic structure of the *H. marismortui* large ribosomal subunit viewed from the interface side (*left*). The 23S rRNA and 5S rRNA are shown as gold ribbons and ribosomal proteins are in green. The PTC is highlighted by nucleotides of the 23S rRNA peptidyl transferase loop and shown in red spacefill. The figure was generated from the pdb file 1JJ2 (Klein *et al.*, 2001). Secondary structure diagram of the central loop of domain V of *E. coli* 23S rRNA (Cannone *et al.*, 2002) and selected neighboring structure elements are shown on the right. Numbers of nucleotides and 23S rRNA helices (*H*) discussed in the text are provided. (*B*) Functional implications of selected 23S rRNA nucleotides (*E. coli* numbering) in activities of the PTC.

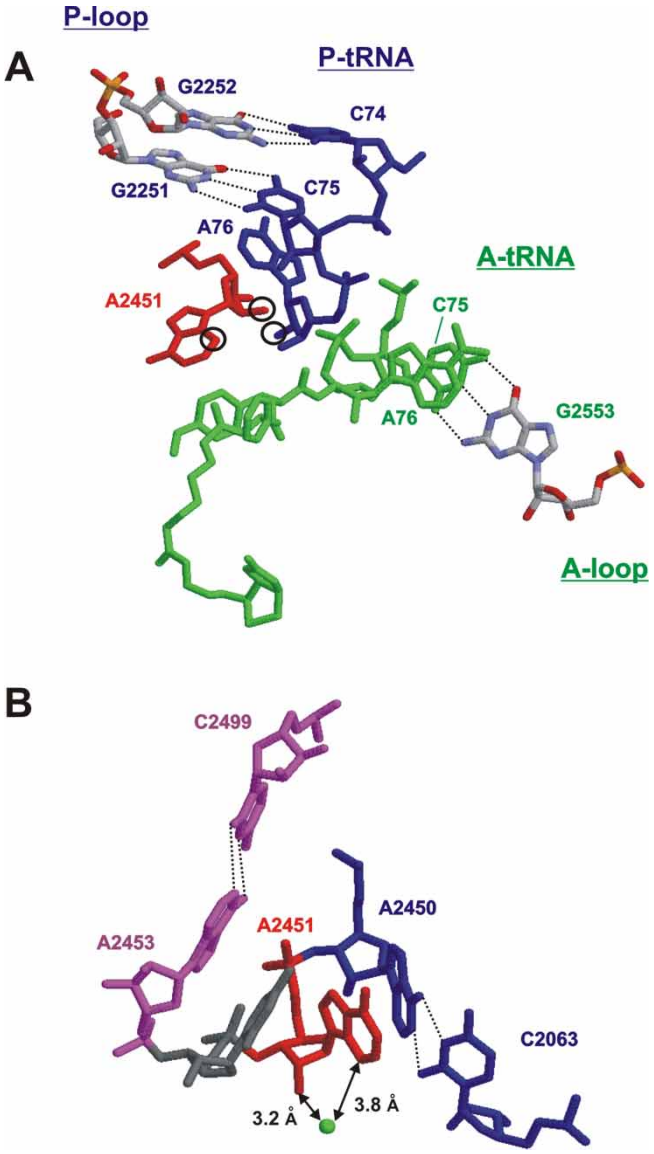


FIGURE 3 High-resolution structures of the ribosomal PTC. (*A*) Interaction of the acceptor ends of ribosome-bound substrate analogs with the PTC. Following peptide bond formation, the deacylated product (CCA) (*blue*) base pairs with 23S rRNA residues G2251-52 of the P-loop. The peptidyl product (C-puromycin-phenylalanine-caproic acid-biotin) (*green*) is fixed by interactions with the 23S rRNA A-loop via a base-pairing with G2553. The A-minor interactions of A76 of both A- and P-tRNA are not shown for clarity. The 23S rRNA nucleotide A2451 makes the closest approach to the attacking amino group (Nissen *et al.*, 2000) and is shown in red. The three groups located in hydrogen bonding distance to the α-amino group of aminacyl-tRNA (Nissen *et al.*, 2000; Hansen *et al.*, 2002a), namely the N3 and the ribose 2′-hydroxyl of A2451 as well as the 2′-hydroxyl of A76 of P-tRNA, are encircled. (*B*) Alternative ionizable groups in the PTC. The two non-Watson-Crick base pairs (A2450:C2063 and A2453:C2499) whose formations have been proposed to depend on adenine N1 protonation (Katunin *et al.*, 2002) are shown in blue and magenta, respectively. The distance between the nitrogen atom of the attacking amino group of an aminoacyl-tRNA analog (*green sphere*) and the N3 or the ribose 2′-hydroxyl of A2451 (red) are indicated by arrows. (*A*) and (*B*) were generated from pdb files 1KQS (Schmeing *et al.*, 2002) and 1FG0 (Nissen *et al.*, 2000), respectively.

proteins are likely needed to assist the formation and maintenance of a catalytically active rRNA fold or may fulfill auxiliary roles in substrate placement in the PTC.

The acceptor arms of A- and P-site tRNA moieties of the PTC acceptor and donor substrates reach deep into the cleft at the 50S interface side, where their universally conserved CCA ends are oriented and held in place by interactions with 23S rRNA (Yusupov *et al.*, 2001). In the P-site, C74 and C75 of the tRNA base-pair to G2252 and G2251 of the 23S rRNA P-loop (Samaha *et al.*, 1995). The CCA end of A-site tRNA is fixed by pairing C75 with G2553 of the 23S rRNA A-loop (Figure 3A) (Kim & Green, 1999; Khaitovich & Mankin, 2000). 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 (Nissen *et al.*, 2000; Nissen *et al.*, 2001; Hansen *et al.*, 2002a). Crystallographic structures of archaeal (*H. marismortui*) and bacterial (*Deinococcus radiodurans*) 50S subunits showed fairly similar interactions of small substrate analogs with the PTC (Nissen *et al.*, 2000; Bashan *et al.*, 2003). However, the larger PT substrates, such as the puromycin-containing minihelix (Bashan *et al.*, 2003), were positioned in a slightly different way in *D. radiodurans* 50S subunits, compared to the respective *H. marismortui* complexes (Hansen *et al.*, 2002a). It was suggested that the difference in the binding modes of larger PTC substrates is the consequence of their idiosyncratic interactions with more remote RNA and protein elements of the peptidyl transferase cavity (Yusupov *et al.*, 2001; Agmon *et al.*, 2004). It is possible, however that neither of the currently available complexes accurately depict the outer-shell contacts of tRNA with the PTC: the exact orientation of peptidyl- and aminoacyl-tRNAs relative to the 50S subunit and the PT cavity should be influenced by interaction of the anticodon stem-loop of tRNA with mRNA and the small ribosomal subunit. However, the resolution of crystalline complexes of tRNA with the 70S ribosome is not yet high enough to see fine details of tRNA-ribosome contacts (Yusupov *et al.*, 2001).

Though high-resolution views of a catalytically competent PTC with simultaneously bound genuine donor and acceptor tRNA substrates are still missing, their mutual orientation can be roughly deduced from structures of complexes carrying minimal substrate analogs and reaction products (Nissen *et al.*, 2000; Hansen *et al.*,

2002b; Schmeing *et al.*, 2002; Bashan *et al.*, 2003). The acceptor ends of the A- and P-site bound tRNAs esterified with peptidyl- or aminoacyl residues meet at the bottom of the funnel-shaped active site crater directly above the entrance to the nascent peptide exit tunnel. In the nontranslating large ribosomal subunit, the PT cavity is hollow except for the bases of nucleotides A2602 and U2585, which bulge into its center. The orientation of these two universally conserved residues depends on the functional state of the ribosome and the nature of the bound substrate, suggesting functional relevance of these nucleotides, as will be discussed later. In the structures of *H. marismortui* 50S subunits with bound reaction substrates, the universally conserved A2451 of 23S rRNA makes the closest approach to the α-amino group of aminoacyl-tRNA (Nissen *et al.*, 2000; Hansen *et al.*, 2002a). The N3 position of the adenine base and the ribose 2′-hydroxyl of A2451 are seen within hydrogen bonding distance (<4 Å) from the attacking α-amino group (Figure 3). Therefore, A2451 was initially considered the most likely candidate for a catalytic nucleotide in the PTC. Its placement in the active site is achieved by a hydrogen bonding network involving other highly conserved residues of the peptidyl transferase loop, such as G2447, A2450, and G2061 (Nissen *et al.*, 2000). Several other bases were also proposed to be directly involved in the complex functions of the peptidyl transferase active site. Two nonconventional pairs, A2453-C2499 and A2450-C2063 located in the vicinity of A2451 (Figure 3B), are presumed to posses a near-neutral pKa and, thus, could potentially account for the pH dependence of the peptidyl transfer reaction (see below) (Muth *et al.*, 2001; Katunin *et al.*, 2002). Significantly, no electron density for a potential catalytic metal ion in close enough vicinity of peptide bond formation has been seen in any of the crystal structures of *H. marismortui* 50S subunit or its complexes with substrate analogs (Klein *et al.*, 2004; Steitz, 2005). The sole metal ion observed in the inner PT cavity was a potassium ion that seems to help coordinating the orientation of the active site residues A2451, G2447, and G2061 (Nissen *et al.*, 2000). In the structure of *D. radiodurans* 50S subunit complexed with an A-site substrate analog, electron density that can be attributed to hydrated Mg^{2+} ions was reported (Bashan *et al.*, 2003). The CCA termini of tRNA substrates bound in ribosomal P- and A-sites are rotated by approximately 180° relative to each other. This rotational transition of tRNA CCA upon transition form A- into P-site correlates with a twofold symmetry of rRNA in the PTC center that was noticed by Yonath and colleagues (reviewed in Baram & Yonath, 2005). The symmetry involves the RNA backbone fold and nucleotide orientation rather than nucleotide identities. The two residues that define the position of the axes of this twofold symmetry in the PTC are the aforementioned A2602 and U2585 (Bashan *et al.*, 2003).

CATALYSIS OF PEPTIDE BOND FORMATION

The PTC Is a Ribozyme

Although by the late 1980s it became clear that rRNA is intimately involved in functions of the PTC, the involvement of ribosomal proteins in catalysis was difficult to rule out (Khaitovich *et al.*, 1999a; and reviewed in Cooperman *et al.*, 1995; Khaitovich & Mankin, 2000). It was ribosome crystallography that settled the issue. The lack of proteins in the vicinity of the reactive center and the demonstration that peptide bond synthesis can occur in the crystalline state without ribosomal proteins wagging their "tails" into the PTC of *H. marismortui* 50S subunits revealed that 23S rRNA plays the central role in catalysis of amino acid polymerization (Nissen *et al.*, 2000; Schmeing *et al.*, 2002).

RNA enzymes can exhibit catalytic power comparable to protein enzymes (Emilsson *et al.*, 2003). Nevertheless, compared with protein enzymes, ribozymes are generally rather poor prospects as catalysts owing to the limited repertoire of RNA functional groups which ionize at the neutral pH range. This was apparently one of the important factors that drove the transition from a hypothetic RNA World, where RNA was the main provider of biochemical catalysis, to the modern cell, where the majority of chemical reactions are accelerated by protein enzymes. In this respect, it is remarkable that while proteins have taken over most of the RNA catalytic duties, one of the key biochemical reactions in the cell–polymerization of amino acids into polypeptides–remains a prerogative of rRNA!

What chemical resources does the PTC possess for catalysis and how does rRNA accelerate the two principal chemical reactions that take place in the 50S subunit, peptide bond synthesis and peptide release? What is the mode of catalysis? How conserved is this rRNA enzyme and how has it evolved?

Forging a Peptide Bond: Is There Anything Beyond Entropic Catalysis?

Two major components determine the catalytic power of an enzyme. All enzymes bind the substrates and arrange them in a favorable configuration, thereby lowering the entropy of the catalyzed reaction. In addition, functional groups of the enzyme can affect the chemistry of the reaction by acting, for example, as a general acid/base or by stabilizing the transition state via electrostatic interactions. The relative contribution of the components to the overall catalysis can differ substantially in different enzymatic systems.

The formation of a peptide bond does not require additional energy besides that stored in an ester bond linking the peptidyl residue to the tRNA 3′-hydroxyl group. Creation of this bond is "energized" by ATP hydrolysis that drives aminoacylation of a tRNA by the respective aminoacyl-tRNA synthetase. While hydrolysis of 1 mol of ATP releases about 8 kcal, energy which is almost completely transferred to the aminoacyl-tRNA, only about 0.5 kcal/mol is required for amide bond formation (Krayevsky & Kukhanova, 1979). Primary amines are rather strong nucleophiles, and aminolysis of an ester is a favorable reaction that can occur spontaneously. However, the uncatalyzed reaction (extrapolated from model reactions) occurs very slowly in solution, with about 1 peptide bond formation per 30 hours (Nierhaus *et al.*, 1980; and reviewed in Wilson *et al.*, 2002). The ribosome accelerates the rate of peptide bond formation approximately 10^5 to 10^7-fold compared with the uncatalyzed reaction (Nierhaus *et al.*, 1980; Sievers *et al.*, 2004). It is obvious that the entropic component plays a critical role in the way the ribosome promotes peptide bond formation. The major reduction of the reaction entropy comes from locking tRNA substrates in the proper orientation in the P- and A-sites of the ribosome. Multiple interactions of tRNA molecules with RNA and protein components of large and small ribosomal subunits hold tRNA molecules in place (Samaha *et al.*, 1995; von Ahsen & Noller, 1995; Bocchetta *et al.*, 1998; Kim & Green, 1999; Wimberly *et al.*, 2000; Yusupov *et al.*, 2001; Bashan *et al.*, 2003). The function of the PTC, then, is to precisely juxtaposition the universal CCA ends of the peptidyl-tRNA and aminoacyl-tRNA in the PT cavity primarily by establishing critical interactions with several conserved 23S rRNA nucleotides. Watson-Crick base-pairing of C74 and C75 of peptidyl-tRNA with G2253 and G2252, respectively, located in the so-called P-loop and an A-minor interaction of A76 with A2450-C2501 fixes the position of the donor substrate (Figure 3A) (Samaha *et al.*, 1995; Nissen *et al.*, 2000). Base pairing of C75 of aminoacyl-tRNA with G2553 of the 23S rRNA A-loop and A-minor interaction of A76 with U2506-G2583 holds the acceptor substrate in place (Kim & Green, 1999; Khaitovich & Mankin, 2000; Nissen *et al.*, 2000). The limited number of substrate analogs studied so far does not allow a definite conclusion whether interactions of PTC components with the side chains of amino acids that esterify the donor and acceptor substrates play any role in substrate coordination. However, possible hydrogen bonding interactions with several yet to be determined functional rRNA groups in the PTC active site probably orients the α-amino group of aminoacyl-tRNA for an efficient attack upon the carbonyl carbon atom of the peptidyl-tRNA ester bond. Theoretical calculations and model experiments suggest that substrate alignment alone could be sufficient for the observed rate enhancement of peptide bond formation achieved by the ribosome (Krayevsky & Kukhanova, 1979; Nierhaus *et al.*, 1980; Sievers *et al.*, 2004). Therefore, it is most likely that the major acceleration factor provided by the ribosome for peptide bond formation is entropic.

An important question is whether the ribosome also uses other means, such as chemical catalysis, in addition to substrate alignment to further boost the rate of peptidyl transfer. The strongest argument in favor of chemical catalysis playing a role in the PTC functions came from early biochemical data showing that the rate of transferring a peptidyl residue from a donor substrate to puromycin, catalyzed by *Escherichia coli* ribosome, shows strong pH dependence. The reaction rate increases with increasing pH between 6 and 8.5, with an apparent pKa of 7.5 (Maden & Monro, 1968; Pestka, 1972a). These experiments indicated the presence of a titratable group whose protonation status affects the overall rate of peptidyl transfer. Subsequent experiments by Rich and coworkers (Fahnestock *et al.*, 1970) showed that the replacement of puromycin's α-amino group with a hydroxyl neither eliminated the reaction nor prevented its pH dependence. This suggested that the titratable group belongs to the *E. coli* ribosome, rather than to the acceptor substrate. Though these experiments could not clearly differentiate between the effect of pH on substrate binding, conformational transitions in the ribosome, or the chemical step of the

reaction, the near-neutral pKa of the reaction led to the proposal that a general base catalysis might be involved (Pestka, 1972a). More recent fast kinetic data confirmed the pH dependence of the chemical step of the reaction and the existence of a single ionizable group with a pKa of 7.5 in *E. coli* ribosomes, whose protonation affects the rate of the puromycin reaction (Katunin *et al.*, 2002).

The discussion on the mechanism of catalysis of peptide bond formation was fueled by high resolution crystallographic structures of the complexes of large ribosomal subunits with various peptidyl transferase substrates and inhibitors. Arguably, the most provocative of all was the complex of the *H. marismortui* 50S subunit with CCdAp-puromycin, an analog of the peptidyl transfer transition state intermediate (Welch *et al.*, 1995). In CCdAp-puromycin, the tetrahedral carbon atom and the oxyanion of the transition state were mimicked by a phosphate engaged in a phosphoamide bond with the hydroxymethyl-tyrosine of puromycin (Figure 1B). The CCdA side of the inhibitor formed interactions with the PT P-site (including base-paring of cytosines with G2252, G2253 in the P-loop), while the puromycin moiety was apparently 'properly' positioned in the A-site. Though functional groups of four nucleotides (C2063, A2451, U2585, and A2602) were within approximately a 5 Å distance from the attacking α-amino group or the putative position of the tetrahedral carbon center of the transition state intermediate, it was the base of A2451 that originally received the highest prominence and spurred a series of studies on the possible functional role of this residue. The original model put forward by Steitz, Moore, and colleages (Nissen *et al.*, 2000) suggested that the N3 of A2451 was the long sought general acid/base catalyst in the PTC. The model predicted that the N3 abstracts a proton from the attacking amino group, then, in its protonated form, contributes to stabilizing the negative charge on the transition state intermediate, thereby acting as an oxyanion hole, and finally donates the proton to the leaving ribose 3′-hydroxyl of deacylated tRNA. For the N3 of A2451 to be able to play the assigned role as a general acid/base catalyst, its pKa, which in solution is below 1, had to be shifted by more than 6 pH units. Though not unprecedented, such a significant pKa shift is generally not a trivial task. A hydrogen bonding network involving several conserved nucleotides of the PTC (G2447, G2061 and A2450) was proposed to provide charge relay that could elevate the pKa of the N3 of A2451. Furthermore, the suspected elevated pKa of the A2451 N3 initially gained biochemical support from pH-dependent dimethyl sulfate probing data (Muth *et al.*, 2000).

Though many aspects of the initial model of Steitz, Moore, and colleagues (Nissen *et al.*, 2000) did not survive the scrutiny of subsequent experimental testing, this model was extremely stimulating for the entire ribosome field and provided an important framework for the investigation of PTC functions. Studies stimulated by the original proposal were directed to test whether the pKa of the N3 of A2451 is indeed elevated as predicted by the model, whether the charge relay required for such a pKa shift does exist, and whether the pH dependence of the peptidyl transfer reaction is indicative of general base/acid catalysis.

Experimental Testing of the Catalytic Mode of the PTC

One of the arguments in favor of an unusual pKa of A2451 came from its hydrogen bonding distance of 3.4 Å from the nonbridging oxygen atom of the phosphate of CCdAp-puromycin inhibitor seen in *H. marismortui* crystals grown at pH 5.8. Formation of a hydrogen bond between these partners requires that one of them is protonated. A similar interaction was thought to stabilize the oxyanion of the transition state intermediate. However, studies by Strobel and colleagues (Parnell *et al.*, 2002) showed that the binding affinity of CCdAp-puromycin to the PTC of *E. coli* ribosomes was independent of pH, a finding that was inconsistent with the stabilization of a transition intermediate by the protonated A2451 or by any other ribosomal group. Reevaluation of the crystallographic data confirmed this view since the oxyanion of the tetrahedral intermediate actually points away from A2451 and interacts with a water molecule instead (Hansen *et al.*, 2002a; and reviewed in Steitz, 2005). Thus, protonation of the N3 of A2451, even if it takes place, was certainly not required for stabilization of the PT reaction intermediate, at least to the extent that CCdAp-puromycin correctly mimics the transition state.

The most direct approach to assess the importance and functions of A2451 in catalysis was, of course, to mutate it and to test the activity of the mutant PTC. Unfortunately, mutations of A2451 and several other neighboring residues are lethal in *E. coli* (Muth *et al.*, 2000; Thompson *et al.*, 2001), thus making it difficult to obtain pure populations of mutant ribosomes. Therefore, indirect approaches were used to

study the effect of mutations on PT catalysis. Mutations at A2451 or at the neighboring active site residues C2063, G2447, U2585, or A2602 in the context of *in vitro* reconstituted 50S ribosomal subunits of *Thermus aquaticus* or *Bacillus stearothermophilus* had only moderate effects on the rate of transpeptidation, which was reduced only several-fold (Polacek, 2001; Thompson *et al.*, 2001; Polacek *et al.*, 2003). Even combining two mutations that confer *in vivo* lethality in one 23S rRNA molecule (A2451U/A2602G) did not eliminate ribosomal transpeptidation activity (Polacek *et al.*, 2002). An elegant approach based on selective inhibition of wild-type ribosomes by antibiotics in a mixed ribosome population, originally developed by Garrett and coworkers (Leviev *et al.*, 1995), was applied by Dahlberg's lab to test the activity of A2451 mutant *E. coli* ribosomes assembled *in vivo* (Thompson *et al.*, 2001). Consistent with results obtained in reconstitution systems, the mutant PTC was alive for the most part, arguing against a direct catalytic involvement of the A2451 base and, as a result, against the importance of the proposed base/acid catalytic model for the overall rate of transpeptidation. Though coherent, these data were not entirely conclusive because reconstituted ribosomes catalyze peptide bond formation rather slowly, and the assays that were used examined the overall rate of the reaction rather than the rate of its chemical step. Despite these concerns, these findings were subsequently qualitatively confirmed in fast kinetic experiments employing *in vivo*-derived ribosomes which could directly measure the rate of the chemical step of the reaction. With puromycin as an acceptor substrate, the catalytic rate of the *E. coli* A2451U mutant decreased about 150-fold relative to wild-type (Katunin *et al.*, 2002; Youngman *et al.*, 2004). However, the A2451 mutations produced almost no rate reduction when two full-length tRNA substrates were used in fast kinetic transpeptidation assays (Youngman *et al.*, 2004; Beringer *et al.*, 2005). The latter results do not support the catalytic scheme utilizing A2451 as a general base/acid.

The charge relay mechanism that was proposed to shift the pKa of the N3 of A2451 was tested by mutating G2447–one of the hubs in the proposed hydrogen-bonding network (Nissen *et al.*, 2000). Mutations of G2447 to other nucleotides commonly had only small growth effects on Gram-positive and Gram-negative bacteria (Shinabarger, 1999; Thompson *et al.*, 2001; Xiong *et al.*, 2001; Sander *et al.*, 2002). Base changes at G2447 resulted in only modest PT activity decreases (Polacek *et al.*, 2001; Thompson *et al.*, 2001) and failed to eliminate the pH dependence of the PT reaction (Beringer *et al.*, 2003). All of these data questioned the existence of the proposed charged relay scheme. In the absence of a charge relay, the putative elevated pKa of A2451 became less credible. In addition, RNA probing studies showed that A2451 exhibits pH-dependent changes in dimethyl sulfate reactivity only in an inactive conformation of the *E. coli* ribosome (Bayfield *et al.*, 2001). Furthermore, the dimethyl sulfate modification pattern of A2451 seen in *E. coli* was not observed in other species (Xiong *et al.*, 2001; Muth *et al.*, 2001). Most importantly, the pH-dependent changes in reactivity were not confined to A2451 as was initially thought, but were seen at several PTC nucleotides, an observation consistent with a general pH-dependent conformational change in the PTC (Miskin *et al.*, 1968; Bayfield *et al.*, 2001; Xiong *et al.*, 2001; Beringer *et al.*, 2005).

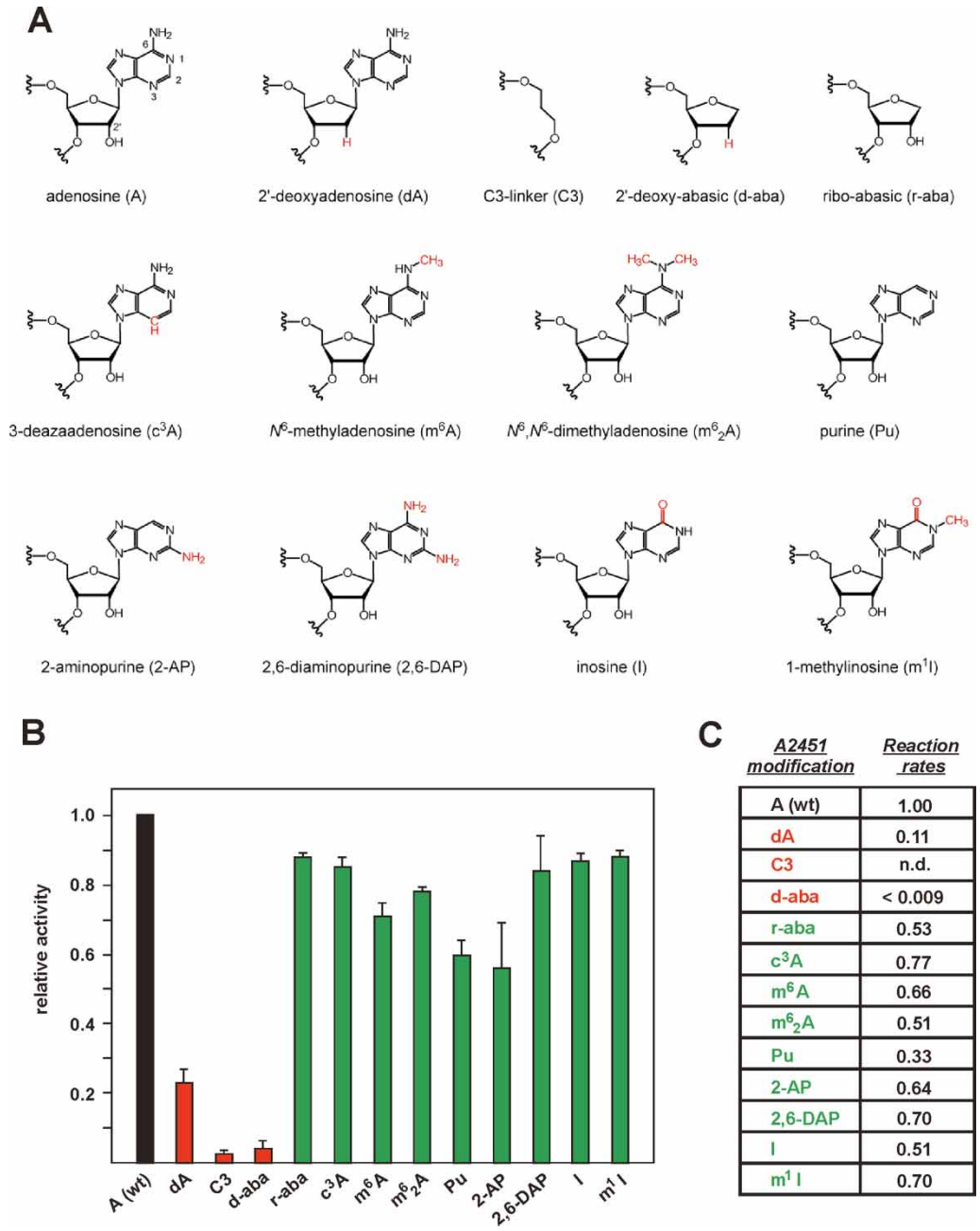
The pH dependence of the transpeptidation reaction was initially seen as an argument in favor of general base/acid catalysis (Pestka, 1972a). The ionizable group could potentially belong to the sought-after catalytic rRNA residue. Rapid kinetic analysis of the puromycin reaction showed the existence of two ionizable groups with pKas 6.9 and 7.5 which accounted for acceleration of the transpeptidation rate with pH increase (Katunin *et al.*, 2002). Deconvolution of the kinetic curves and experiments with hydroxy-puromycin attributed the pKa of 6.9 to the α-amino group of puromycin whereas the group with pKa of 7.5 belonged to the *E. coli* ribosome. Intriguingly, ribosomes carrying the A2451U mutation have lost the pH dependence, suggesting that either A2451 itself or one of the neighboring nucleotides carries the ionizable ribosomal group. Since, in the absence of charge relay, it was difficult to expect that any group of A2451 would have a near-neutral pKa, other possibilities were considered. Rodnina and colleagues have proposed two attractive candidates, namely the A-C pairs A2450-C2063 and A2453-C2499 (Figure 3B). These 23S rRNA base pairs are located close to A2451 and were viewed as possible pH sensors because formation of the A-C wobble pair depends on protonation of the N1 of adenine (Katunin *et al.*, 2002; Bayfield *et al.*, 2004). Mutant ribosomes in which the A2450-C2063 base pair was changed to a pH-independent G2450-U2063 showed an approximately 200-fold reduced peptidyl transferase activity (Hesslein *et al.*, 2004), but were only ~1.4-fold down in a cell free translation system (Bayfield *et al.*,

2004). However, since the G:U mutation disrupts several tertiary interactions inside the PTC, which can explain the observed rate reductions, the unequivocal assignment of A2450 as the active site residue with the neutral pKa could not conclusively be made (Hesslein *et al.*, 2004). The other potentially important base pair, A2453-C2499, does show a near-neutral pKa and could explain the pH dependence of the PT reaction in *E. coli* ribosomes, but its lack of conservation makes it unlikely to contribute fundamentally to catalysis (Bayfield *et al.*, 2004). Consistent with these findings, a recent chemical mutagenesis study revealed that the complete removal of the nitrogen bases from the nucleotides A2450 or A2453 did not significantly inhibit transpeptidation rates, implying that neither of these A:C base pairs are crucial for peptide bond formation (Erlacher *et al.*, 2005).

Further studies showed that the pH dependence of transpeptidation may after all not be a strong argument in favor of an evolutionarily conserved acid/base catalytic mechanism but rather indicate species-specific conformational transitions in the ribosome. Traditionally, most of the kinetic experiments have been carried out with the ribosomes of *E. coli.* Tendency to use a single experimental model does not allow for distinguishing species-specific peculiarities from the universal principles. This became apparent when fast kinetic experiments were carried out with ribosomes of another bacterial species, *Mycobacterium smegmatis* (Beringer *et al.*, 2005). The A2451U mutation, which is lethal in *E. coli*, turned out to be viable in *M. smegmatis* (as well as in mouse mitochondria [Kearsey & Craig, 1981]), re-emphasizing the notion that the base identity at this highly conserved residue is not absolutely critical for peptide bond synthesis. Kinetic measurements carried out with wild type and mutant *M. smegmatis* ribosomes showed that the ribosome-specific pKa of the reaction is shifted from 7.5 (as seen in *E. coli*) to 8.0. Furthermore, though the A2451U mutation slowed the reaction in *M. smegmatis*, in contrast to *E. coli* it did not eliminate the ionizable group. Therefore, it became clear that in *M. smegmatis*, A2451 was not responsible for the pH dependence of the reaction, which put the last nail into the model of general acid/base catalysis of peptidyl transfer involving adenine 2451 as a catalytic base. It appears that ionization of one (not necessarily conserved) group in rRNA, or even in a ribosomal protein, accounts for the conformational rearrangement of the PTC.

Chemical Engineering of the PTC Active Site

Even though the adenine base at position A2451 of 23S rRNA does not appear to be involved in general acid/base catalysis of transpeptidation, the nucleotide is clearly positioned right in the heart of the PT active site, and its base or ribose can be involved in coordination of the α-amino group for attack on the peptidyl-tRNA ester bond. In fact, in *H. marismortui* 50S subunit complexes with the aminoacyl-tRNA analogs, the adenine N3 of the A2451 and the 2′-hydroxyl group of the nucleotide's ribose were within hydrogen bonding distance to the α-amino group of aminoacyl-tRNA (Figure 3) (Nissen *et al.*, 2000; Hansen *et al.*, 2002a). More detailed examination of the possible functions of A2451 is complicated by the limited mutational capacity of RNA. Thus, neither of the mutations at A2451 would entirely eliminate the hydrogen bonding potential of a nucleotide base at this position: replacements of A by G, C, or U would put a proton acceptor (N3 for G and O2 for C or U) in approximately the same place as the N3 of A (Nissen *et al.*, 2000; Hansen *et al.*, 2002a). Additionally, in all "natural" mutants, the ribose 2′-hydroxyl remained untouched, underscoring the requirement for more delicate chemical engineering techniques. These problems were partly overcome by a modified version of the *in vitro* reconstitution approach that allowed the incorporation of nonnatural nucleoside analogs at desired positions of 23S rRNA in the *T. aquaticus* large ribosomal subunit. Introducing chemical modifications at the base at position 2451, including those which eliminated the hydrogen bonding capabilities at N1 or N3 of adenine, or even the deletion of the entire nucleobase only marginally affected the reaction rates. In dramatic contrast, the removal of the ribose 2′-hydroxyl group at A2451 markedly inhibited peptide bond formation (Figure 4) (Erlacher *et al.*, 2005). Thus, the ribose 2′-hydroxyl at position A2451 appears to play an important role in the catalytic activity of the PTC and promotes transpeptidation probably by coordinating the attacking α-amino group. The universally conserved A2451 may then play a role as a molecular trigger that senses the substrates in the PT active site and properly aligns its ribose 2′-hydroxyl group for coordinating the attacking α–amino group. This model is compatible with the reported mobility of A2451 upon ligand binding and its importance for interactions with PT substrates (Moazed & Noller, 1989; Bochetta *et al.*,



A2451 modification	Reaction rates
A (wt)	1.00
dA	0.11
C3	n.d.
d-aba	< 0.009
r-aba	0.53
c^3A	0.77
m^6A	0.66
$m^6{}_2A$	0.51
Pu	0.33
2-AP	0.64
2,6-DAP	0.70
I	0.51
m^1I	0.70

FIGURE 4 **Peptidyl transferase activity of ribosomes containing non-natural nucleoside analogs at A2451. (*A*) Chemical structures of the tested modified nucleoside analogs. The introduced chemical modifications at A2451 of 23S rRNA are depicted in red. (*B*) Product yields of f-Met-puromycin formed on *T. aquaticus* ribosomes containing reconstituted 50S subunits carrying the wild type sequence (*wt; black bar*), ribose sugar modifications (*red bars*), or base modifications (*green bars*) at the position corresponding to A2451 of 23S rRNA. (*C*) Relative initial rates of peptide bond formation catalyzed by A2451 modified ribosomes. The rates were normalized to the rate of reconstituted ribosomes containing the unmodified A2451 (*A* (*wt*)). This figure was taken from (Erlacher *et al.*, 2005) with permission of Oxford University Press.**

1998). Alternatively, the 2′-hydroxyl group of A2451 could be crucial for metal ion coordination or for providing the functionally competent conformation of the active site that allows accurate placing of the reaction substrates in the PTC.

Another important ribose 2′-hydroxyl group that appears to play a significant function in peptidyl transfer is the ribose 2′-hydroxyl at A76 of the peptidyl-tRNA. In the composite crystallographic model based on structures of the large subunit with bound P- or A-site substrates, this hydroxyl is placed close enough to the attacking α-amino group to form a hydrogen bond with it (Figure 3A). Indeed, removal of the ribose 2′-hydroxyl of the P-site tRNA substrate markedly

hampers peptidyl transferase activity (Hecht *et al.*, 1974; Dorner *et al.*, 2003), which is compatible with its significance for peptide bond catalysis. In fast kinetic experiments employing full-length tRNA substrates the rate reduction (10^5- to 10^6-fold) observed upon removal of the A76 2′-hydroxyl of the P-site substrate or its replacement with fluorine was larger than would be expected if this hydroxyl was simply involved in hydrogen bonding needed for orienting the attacking nucleophile (Weinger *et al.*, 2004). This led to the proposal that the A76 2′-hydroxyl of the peptidyl-tRNA may participate in substrate-assisted catalysis. It was suggested that this 2′-hydroxyl might promote peptide bond formation by acting as a general acid and/or base or by coordinating a catalytic metal ion, such as Mg^{2+} or K^+. However, the extent by which substrate-assisted catalysis contributes to peptide bond synthesis and how it relates to the proposed role of the ribosome as an entropy trap (Sievers *et al.*, 2004), remains to be determined.

CATALYSIS OF PEPTIDE RELEASE

Translation Termination—Who is the Catalyst?

When the synthesis of a polypeptide is completed, it is released from peptidyl-tRNA. This is accomplished by hydrolysis of the ester bond connecting the newly synthesized polypeptide to the P-site bound tRNA. This is the same ester bond that is attacked by the α-amino group of the aminoacyl-tRNA in the reaction of peptide bond formation. In peptide release, however, the nucleophile that attacks the carbonyl carbon of the ester is most likely an activated water molecule instead of the α-amino group of the aminoacyl-tRNA (Figure 1C).

Early studies showed that antibiotics that inhibited peptide bond formation also interfered with peptide release, showing that both reactions are a function of the ribosomal PTC (Vogel *et al.*, 1969; Caskey *et al.*, 1971; Tate & Brown, 1992). The change in the mode of operation of the PTC (from peptide bond synthesis to ester hydrolysis) occurs in response to the binding of a class I release factors to the ribosomal A-site. Biochemical and cryo-electron microscopy studies showed that when the release factor binds to the ribosome in response to the presence of a stop codon in the decoding center, the tip of domain III of the factor (which harbors the conserved GGQ motif) reaches into the heart of the PTC and by some means promotes the hydrolysis of the peptidyl-tRNA ester bond (Frolova *et al.*, 1999; Wilson *et al.*, 2000; Rawat *et al.*, 2003; Scarlett *et al.*, 2003; Klaholz *et al.*, 2004). What happens in the PTC in response to the release factor binding, what the role of the GGQ motif is, and which functional groups are involved in the coordination and activation of the water molecule remain unknown. Proposed models suggest that the GGQ motif directly participates in peptidyl-tRNA hydrolysis by coordinating the water molecule (Frolova *et al.*, 1999; Song *et al.*, 2000; Vestergaard *et al.*, 2001; Zavialov *et al.*, 2002; Mora *et al.*, 2003). However, mutational studies do not seem to support direct participation of the GGQ sequence in catalyzing peptide release (Seit Nebi *et al.*, 2000; Seit-Nebi *et al.*, 2001; Zavialov *et al.*, 2002).

As early as in 1971, Caskey & colleagues (1971) have shown that the ribosome can be "tricked" into hydrolyzing the peptidyl-tRNA ester bond even in the absence of a release factor if the reaction is carryed out in the presence of 30% acetone and an A-site-bound deacylated tRNA. This was a strong argument in favor of the ribosome being the main provider of catalytic power for peptide release. Therefore, the reaction of peptide release has often been viewed as a modified version of the peptidyl transfer reaction (Maden & Monro, 1968; Vogel *et al.*, 1969; Caskey *et al.*, 1971; Tate & Brown, 1992).

Peptide Release is the Function of rRNA

In variance with the reactive primary amine of aminoacyl-tRNA, water is a relatively weak nucleophile. Indeed, spontaneous peptidyl-tRNA hydrolysis only occurs slowly with a rate of about 1 per 14 hours (Zavialov et al., 2002). The ribosome-catalyzed reaction of peptide release should, therefore, involve activation of the water molecule, possibly by general base chemistry to yield the observed termination rates of 0.5 to 1.5 per second (Zavialov *et al.*, 2002). Alteration or elimination of a putative catalytic ribosome residue is expected to affect the rate of peptide release much more severely as compared with the mild effects of rRNA mutations on the reaction of transpeptidation. Experiments with *in vitro* reconstituted *T. aquaticus* large ribosomal subunits, and later, with affinity-tag purified *E. coli* ribosomes, showed that A2602 of 23S rRNA may be one of the critical components of the reaction for peptide release. Whereas mutations of the active site residues C2063, A2451, U2585, and U2506 had

only moderate effects on either of the reactions, substitution of A2602 with C or its deletion dramatically reduced the ribosome's ability to promote peptide release but had little effect on transpeptidation (Polacek *et al.*, 2003; Youngman *et al.*, 2004). Essentially identical effects of the A2602 mutations were seen in a release factor-independent assay in which the peptidyl-tRNA hydrolyzing activity of the PTC was activated by A-site-bound deacylated tRNA and 30% acetone (Caskey *et al.*, 1971; Polacek *et al.*, 2003). Thus, A2602 appears to be one of the major players (from the ribosome side) in the catalysis of peptide release. In experiments with *E. coli* ribosomes, mutations of U2585 also reduced the rate of peptide release, though to a smaller extent (approximately 40-fold compared with 350-fold for the A2602C mutation) (Youngman *et al.*, 2004), indicating a possible additional contribution of U2585 for the peptide release mechanism. The importance of an A-site bound deacylated tRNA for the factor-independent peptide release (Polacek *et al.*, 2003) suggests that its contacts with A-site residues, possibly the A-loop, are also important for the formation of the PTC structure competent for peptidyl-tRNA hydrolysis. One of the release factor functions could be in establishing such a conformation in the absence of the A-site bound tRNA.

Based on the results of mutational studies, a model was proposed in which the class I release factor triggers peptide release by reorienting A2602 in the PTC so that it can coordinate and possibly activate a water molecule for the attack onto the carbonyl carbon atom of the ester bond of the peptidyl-tRNA (Figure 5). The structural flexibility and the central location of A2602 in the PTC are compatible with this proposed role (Nissen *et al.*, 2000; Bashan *et al.*, 2003; and reviewed in Baram & Yonath, 2005). The repositioning of A2602 for peptide release can potentially be coordinated with the movement of U2585, the second most flexible nucleotide in the PTC (Schmeing *et al.*, 2002).

The universally conserved GGQ motif of the class 1 release factors, which reaches into the PTC, may coordinate A2602 in the orientation required for the catalysis of ester bond hydrolysis. In this scenario, the release factor plays a regulatory or trigger function in peptide release while the chemistry of the reaction is primarily driven by the ribosome itself–compatible with the results of the factor-free peptide release experiments (Caskey *et al.*, 1971). Therefore, it appears that termination may be yet another reaction of protein synthesis that is possibly inherent to the rRNA and may be a relic

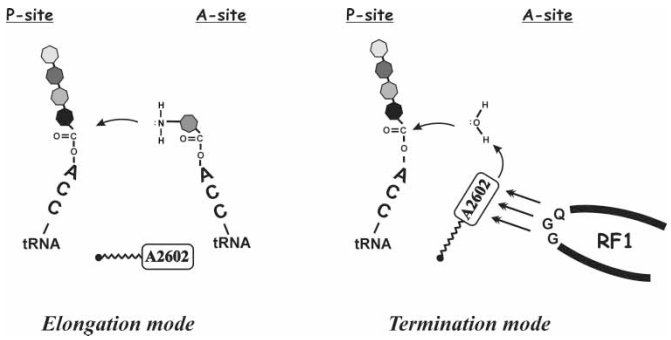


FIGURE 5 **The putative conformational switch at A2602 as a trigger for changing the mode of activity of the PTC. Orientation of A2602 during translation elongation allows for proper positioning of peptidyl- and aminoacyl-tRNAs in the PTC that makes peptidyl transfer and a new peptide bond formation possible. Binding of the class 1 release factor (*RF1*) in response to the presence of a stop codon in the decoding site re-orients A2602, probably involving the universally conserved GGQ motive of the RF. This places A2602 in a position where its reactive groups can potentially activate a water molecule, thus facilitating its nucleophilic attack on the carbonyl carbon atom of the peptidyl-tRNA ester bond and therefore accelerating the rate of peptidyl-tRNA hydrolysis.**

of the RNA World. The assisting protein factors probably evolved to fine-tune and enhance the ribosome performance in the tightly regulated process of protein synthesis in modern cells.

An important question that has not yet been directly addressed experimentally is the nature of the chemical group in the PTC that activates the water molecule or possibly stabilizes the oxyanion at the transition state. The distinct effects of mutations on the peptidyl transfer and peptide release and the somewhat different antibiotic sensitivity profiles of the two reactions (Caskey *et al.*, 1971; Polacek *et al.*, 2003) suggest that the exact configuration of the PTC functional groups differs during peptidyl transfer, when aminoacyl-tRNA is present in the A-site, compared with peptide release, when a release factor occupies the A-site. In the "release conformation," A2602 (and/or possibly U2585) may establish interactions that would allow them to serve as a general base or to form electrostatic interactions with the transition state intermediate.

CONFORMATIONAL FLEXIBILITY OF THE PTC

Irrespective of a possible role of substrate-assisted catalysis in peptide bond formation (Weinger *et al.*, 2004), the main (and possibly the sole) function of the PTC in peptide bond formation is the proper alignment of the reaction components. Yet, during the peptide release reaction, the mode of action of the PTC must

change and the ribosome needs to become a chemical catalyst to probably activate a water molecule for hydrolysis of the peptidyl-tRNA ester bond.

Therefore, the PTC cannot be a mere rigid mold for the positioning of peptidyl- and aminoacyl-tRNAs. Rather, it presents a flexible and sensitive environment whose precise chemical make-up may be achieved through some kind of induced fit in response to the binding of the reaction substrates or auxiliary factors. The flexibility of the PTC, may be affected by various factors. Thus, certain mutations in the PTC, as well as binding of some PT inhibitors that affect accuracy of translation, may prevent conformational transitions that are required for communication between the PTC and the decoding center in the small ribosomal subunit (Weiss-Brummer *et al.*, 1995; O'Connor *et al.*, 1995; Thompson *et al.*, 2001; Thompson *et al.*, 2002). The communication signal is likely to be transmitted to and from the PTC in the form of a conformational change. The general plasticity of the PTC structure has long been known (Miskin *et al.*, 1968; Miskin *et al.*, 1970). Chemical probing showed that the accessibility of several bases alters in response to changes in pH or cation concentrations (Bayfield *et al.*, 2001; Muth *et al.*, 2001; Xiong *et al.*, 2001), indicating structural transitions inside the PTC. This general flexibility of the PTC may easily account for the earlier discussed pH-dependence of the puromycin reaction: ionization of a specific functional group in the PTC may trigger conformational transitions in the PTC structure that, through their effect on the substrate alignment, will affect the rate of the reaction.

Crystallographic and biochemical data revealed several PTC positions that appear to contribute to the general and local conformation of the PTC structure. The universally conserved A2602 is the most mobile of all the nucleotides in the PTC (Moazed & Noller, 1989; Nissen *et al.*, 2000; Bashan *et al.*, 2003; Duarte *et al.*, 2003). Its orientation differs dramatically in different crystalline complexes and its accessibility to chemical modification is affected by both A- and P-site bound substrates (Moazed & Noller, 1989). The movement of A2602 was proposed to be part of the mechanism that guides the rotational movement of the CCA end of tRNA during its translocation from the A- to P-site (Bashan *et al.*, 2003). However, A2602 may also be a sensor of the nature of the PT substrates that can adjust the mode of action accordingly, as might be happening during switching of the PTC activity to the peptide release mode (Polacek *et al.*, 2003). Another nucleotide whose position varies significantly in different crystalline complexes is U2585. Upon binding of the PT substrates, U2585 reorients and comes into close proximity to them. Its O4 was seen at a hydrogen-bonding distance to the 2′-hydroxyl of the A-site substrate (Schmeing *et al.*, 2002) in the *H. marismortui* 50S subunit and close to A76 of P-site bound peptidyl-tRNA analog complexed to 50S subunit of *D. radiodurans* (Bashan *et al.*, 2003). Orientation of U2585 is affected by several antibiotics, for example, streptogramins A, which could explain their inhibitory effect on peptide bond formation (Harms *et al.*, 2004). Accessibility of U2585 to carbodiimide modification, as well as the reactivity of the neighboring U2584 to modification with dimethyl sulfate are sensitive to pH and the presence of the P-site substrate in ribosomes of *E. coli* and *M. smegmatis* (Moazed & Noller, 1989; Bayfield *et al.*, 2001; Beringer *et al.*, 2005). The mutation of A2451 to U in *M. smegmatis* results in dramatic changes in accessibility to chemical probes of several other PTC residues, including U2585, U2506, A2060, and A2572. This indicates the sensitivity of the overall conformation of the PTC cavity to the identity and most likely spatial placement of individual nucleotides (Beringer *et al.*, 2005). Among the residues affected by the A2451U mutation, A2572 deserves special attention. It is located in an elbow of helix 90, which brings the A-loop into contact with the aminoacyl-tRNA CCA-end. Helix 90 bends sharply at A2572 and the accessibility of the adenine base to dimethyl sulfate is affected by active/inactive transition in *E. coli* ribosomes (Bayfield *et al.*, 2001). The rRNA backbone at this residue was shown to be sensitive to Pb^{2+} cleavage, a structural probing technique that targets unconstrained nucleotides, which provides additional evidence for the flexible nature of this position (Polacek & Barta, 1998). It is therefore possible that A2572 either senses or fine-tunes the spatial orientation of the A-loop and of the A-loop-bound acceptor substrate in the PTC cavity.

THE PTC AS AN ANTIBIOTIC TARGET

The Benefit of an RNA Target

Activities of the ribosomal PTC are critical for protein synthesis and, thus, cell well being. A number of antibiotics that inhibit growth of microorganisms do so by binding to the PTC and inhibiting its activity. The PTC is by far the most "popular" antibiotic target in

the cell. Inhibitors of the peptidyl transferase activity are plenty and diverse. They represent the largest group among natural antibiotics (reviewed in: Vazquez, 1975; Cundliffe, 1981) (Figure 6). The RNA-based design is certainly one of the key reasons why PTC has been repeatedly "chosen" in the course of evolution as a preferred target for the interspecies warfare: it is difficult to find good defense against drugs acting upon rRNA. One of the most effective defense mechanisms used in "antibiotic wars" is a mutation of the antibiotic target site. Such mutations should prevent drug binding but preserve the function of the active site. Owing to redundancy of rRNA genes in most species, a spontaneous mutation in one of the *rrn* alleles can render only a fraction of cellular ribosomes resistant to an antibiotic. Additional mutations or gene conversion are usually required to attain sufficient levels of mutant ribosomes in the cell (Lobritz *et al.*, 2003), therefore slowing the development of resistance. Furthermore, RNA targets in general offer fewer mutational options to choose from—3 possible RNA mutations in comparison with 19 available for protein targets—which makes it more difficult to find a compromise between functional integrity and reduced antibiotic binding.

The drugs that inhibit the PTC functions interact with rRNA, which forms the PT cavity as well as the upper segment of the nascent peptide exit tunnel. The sites of antibiotic action have initially been mapped using biochemical and genetic methods (reviewed in Cundliffe, 1990). The recent crystallographic structures of ribosome-antibiotic complexes provided critical insights into the orientation of the drug molecule in the ribosome and atomic interactions that contribute to the drug binding. Since chemical structures of the drugs acting upon the PTC are very different, it is not surprising that different classes of antibiotics form idiosyncratic sets of contacts with rRNA of the PTC. What is surprising is that the binding sites of so many different antibiotics overlap so closely (Figure 7A–C), indicating that the evolutionary effort was specifically dedicated to select compounds that interact with a rather confined region in the ribosome structure.

Why Do Antibiotics Like the PTC?

It is not immediately clear what makes the PTC so special as an antibiotic target. The functional importance of the PTC is certainly a factor, but interference with the activity of other functional sites in the ribosome can inhibit translation equally well. Thiostrepton, a drug that acts upon the GTPase-associated center of the large ribosomal subunit, or evernimicin, an antibiotic that interacts with the helices 89 and 91 of 23S rRNA and prevents binding of translation factors, are as efficient in inhibiting protein synthesis as the drugs that inhibit peptidyl transfer (Thompson *et al.*, 1988; Egebjerg *et al.*, 1989; Belova *et al.*, 2001). Nevertheless, with only a few exceptions, the majority of the compounds that inhibit the activities of the large ribosomal subunit act upon the PTC. An additional factor that may contribute to the evolutionary attractiveness of the PTC as an antibiotic target is that the identity of many rRNA nucleotides in the PTC are important for efficient translation and, thus, for the fitness of the cell. Even though most of the mutations in the PTC do not have dramatic effects on model reactions *in vitro*, they certainly negatively affect protein synthesis and, as a result, confer deleterious or lethal phenotypes consistent with their evolutionary conservation (O'Connor & Dahlberg, 1993; Gregory *et al.*, 1994; Porse & Garrett, 1995). Therefore, many potential resistance mutations have high fitness costs and would decrease the success of the target organism in competition with the antibiotic-producing rival. This dilemma is taken advantage of by the antibiotic blasticidin S, which base-pairs to G2252 or G2253 of the PTC P-loop (Porse & Garrett, 1995; Hansen *et al.*, 2003). Mutations of any of these residues are lethal for the cell (Lieberman & Dahlberg, 1994; Samaha *et al.*, 1995) since correct positioning of the P-site tRNA inside the PTC would be severely affected (Figure 3A). Only a low level of blasticidin S resistance can be attained by mutations of nucleotides contacting other functional groups of the drug (Porse & Garrett, 1995; Hansen *et al.*, 2003). The functional importance of the nucleotide identity in the PTC is likely the reason why most of the producers of PTC-targeting drugs escape suicide by either rapidly exporting the drug or by postranscriptionally modifying the RNA residues involved in the drug binding rather then by mutating the site of the drug action in its own ribosome (reviewed in Cundliffe, 1989).

The small radii of curvature of the PTC cavity and of the nascent peptide exit tunnel provide structural advantages for antibiotic binding: drugs binding in the structural pockets or highly curved surfaces bury larger surface areas and can establish additional interactions that increase antibiotic affinity. A clear example of this comes from studies of macrolides,

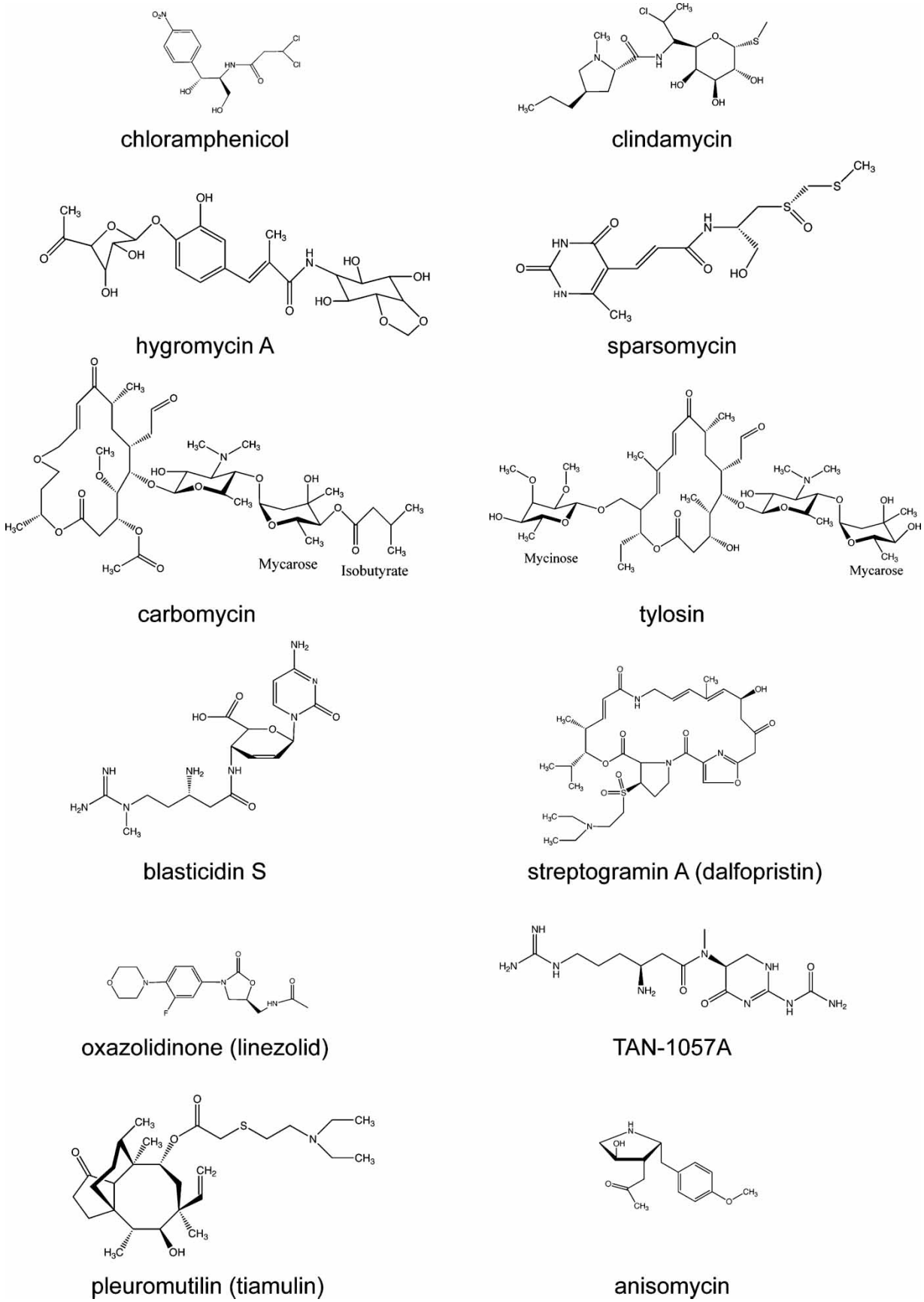


FIGURE 6 A variety of chemical structures of antibiotics, inhibitors of peptide bond formation (an arbitrary selection).

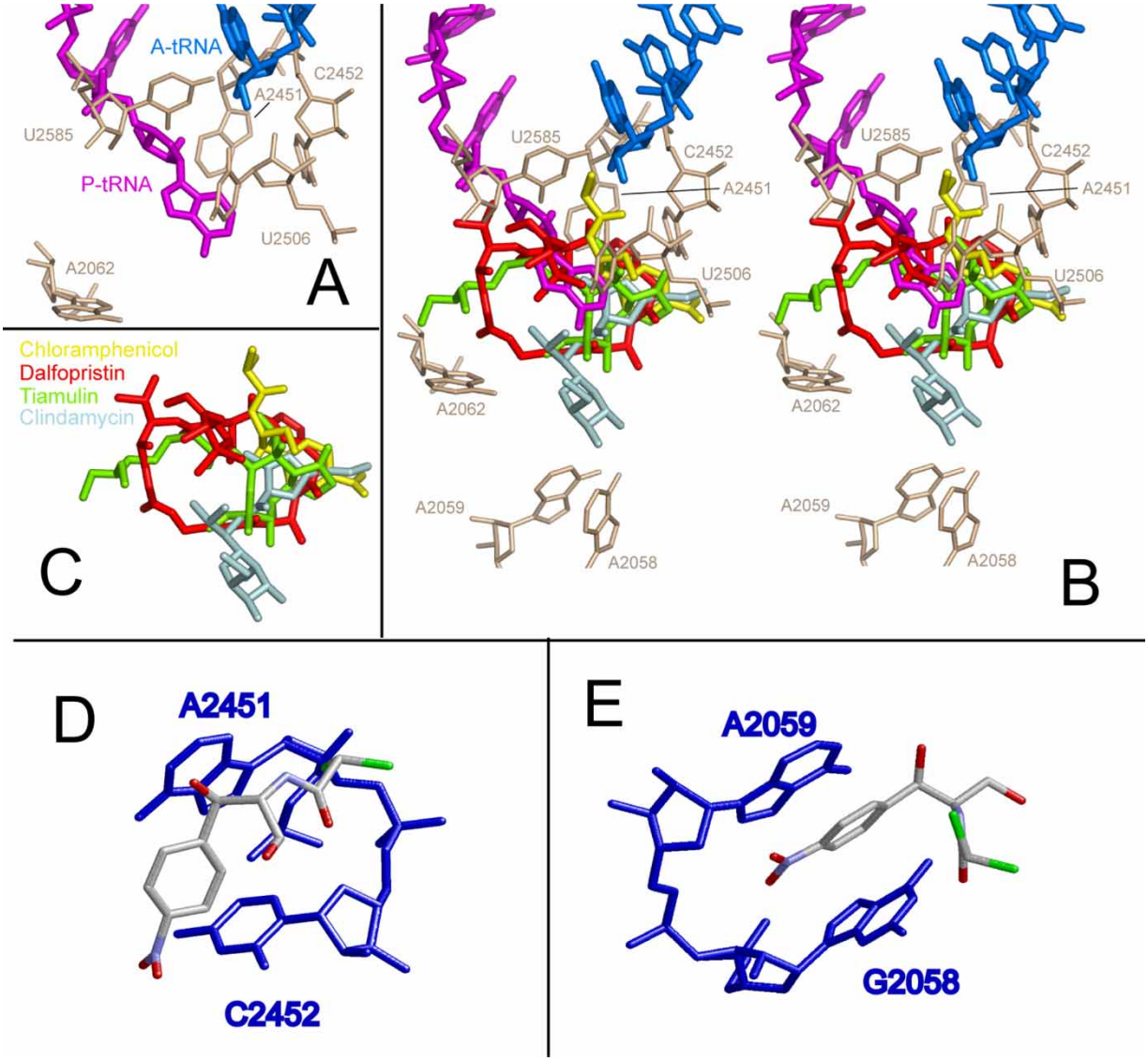


FIGURE 7 Interaction of antibiotics with the PTC (A–D—*D. radiodurans*; E—H. *marismortui*). (*A*) Placement of the 3′ termini of P- and A-site bound tRNA analogs in the PTC. (*B*) Stereoview of relative positions of P- and A-site bound tRNA analogs and several antibiotics, inhibitors of peptide bond formation within the PTC. tRNA analogs are shown with the same colors and in the same orientation as in (*A*). Shown antibiotics are chloramphenicol—*gold*, streptogramin A (dalfopristin)—*red*, pleuromutlin (tiamulin)—*green*, and lincosamide (clindamycin)—*cyan*. 23S rRNA residues are depicted in beige and numbered according to *E. coli* nomenclature. (*C*) Relative orientation of antibiotics within their binding site in the PTC. (*D, E*) Differences in the interaction of chloramphenicol with the PTC of bacterial and archaeal ribosomes. (*D*) Binding of chloramphenicol (*gray*) to the 2451/2452 crevice (*blue*) in a bacterial (*D. radiodurans*) PTC. (*E*) Binding of chloramphenicol to the 2058/2059 crevice in an archaeal (*H. marismortui*) PTC. Note that in *H. marismortui* position 2058 is occupied by guanine, while in most bacteria it is adenine.

drugs whose structure is represented by a substituted 14- to 16-member lactone ring. Macrolides, such as erythromycin, azithromycin, carbomycin, tylosin, and others, bind at the upper portion of the exit tunnel and can protrude their appendages into the PT cavity. A mycarose-containing disaccharide tentacle at position 5 of the 16-member lactone ring of carbomycin, tylosin, and spiramycin reaches into the PTC cavity (Poulsen *et al.*, 2000) and the isobutyrate extension of carbomycin inhibits peptidyl transfer by competing with binding of the A-site substrates (Hornig *et al.*, 1987; Hansen *et al.*, 2002b). The lactone of all macrolides establishes important interactions with the tunnel wall, and groups of the desosamine sugar residue are involved in hydrogen bonding to 23S rRNA nucleotides 2058/2059. However, tylosin, which contains a mycinose sugar at position 14 of the lactone ring, also reaches *across* the tunnel and establishes additional interactions with helix 35 of domain II of 23S rRNA, which contributes to the overall affinity of tylosin for the ribosome

(Hansen *et al.*, 2002b). Similar cross-tunnel interactions are apparently used by the newer generation of 14-member ring macrolides, the ketolides (Xiong *et al.*, 1999; Hansen *et al.*, 1999; Garza-Ramos *et al.*, 2002; Schlunzen *et al.*, 2003; Xiong *et al.*, 2005). Were the surface of the tunnel less curved, establishing of such a second site interaction would be more problematic.

Other idiosyncratic structural features of the PTC play important roles in antibiotic binding, as revealed by the example of chloramphenicol, a natural compound that is produced by *Streptomyces venezuelae*. Chloramphenicol is a classic peptidyl transferase inhibitor which binds tightly (with the affinity in a micromolar range) to ribosomes of Gram-positive and Gram-negative bacteria (Wolfe and Hahn, 1965; Fernandez-Munoz and Vazquez, 1973; Nierhaus & Nierhaus, 1973). At higher drug concentrations, a second chloramphenicol molecule might bind to the ribosome with much lower affinity ($K_D = 0.2$ mM) (Lessard & Pestka, 1972; Contreras & Vazquez, 1977; Das *et al.*, 1996;). Footprinting and mutational studies identified the PTC as a site of chloramphenicol action (Blanc *et al.*, 1981; Ettayebi *et al.*, 1985; Moazed & Noller, 1987; Vester & Garrett, 1988; Mankin & Garrett, 1991; Douthwaite, 1992). This finding was confirmed by crystallographic studies of chloramphenicol complexed to the bacterial 50S subunit of *D. radiodurans* (Schlunzen *et al.*, 2001). In the *D. radiodurans* crystal structure, chloramphenicol is bound at the A-site where it establishes hydrophobic and hydrogen bonding interactions with several PTC nucleotides, including A2451 and C2452. Binding of chloramphenicol at this site is compatible with mutational and footprinting data and can easily account for its well-documented interference with the placement of A-site substrates (Rodriguez-Fonseca *et al.*, 1995; Kirillov *et al.*, 1997). Chloramphenicol also inhibits the PT activity of archaeal ribosomes (Mankin & Garrett, 1991; Rodriguez-Fonseca *et al.*, 1995). Because of the general conservation of the PT structure, it was expected that, in archaea, the drug would bind to the same site that it uses in the ribosomes of bacteria. Surprisingly, in the crystals of the large ribosomal subunit of the archaeon *H. marismortui*, chloramphenicol was found to intercalate between bases of nucleotides 2058 and 2059 at the entrance to the exit tunnel −13Å *away* from the site used in the bacterial ribosome (Hansen *et al.*, 2003)! It is difficult to imagine that the structure of chloramphenicol has been evolutionarily optimized to enable the drug to bind to two different ribosomal sites. Assuming that the reported structures of *D. radiodurans* and *H. marismortui* 50S complexes with chloramphenicol are correct, a more realistic scenario is that the drug has been selected for binding to the bacterial ribosome site around positions 2451/2452, while its binding to the archaeal ribosome is fortuitous. What is amazing, however, is that the site of inadvertent drug binding in the aerchaeal ribosome, which can potentially be *anywhere* in the ribosome is located only 13 Å away from the site of drug action in bacterial ribosomes. To account for this astonishing fact, Steitz and colleagues (Hansen *et al.*, 2003) pointed to the structural similarity of the bacterial (2451/2452) and archaeal (2058/2059) chloramphenicol sites. In both cases, the drug inserts into characteristic hydrophobic clefts formed by the adjacent bases (2451/2452 or 2058/2059) that are splayed apart owing to the stacking interaction of one of the bases with the flanking base pair (Figure 7 D, E). What is unclear, however, is whether these structures are unique to the PTC (which would explain the special attractiveness of the PTC for antibiotic binding) or whether similar structures are present elsewhere in the ribosome (which would leave the question of chloramphenicol specificity open).

Both drug-binding crevices exist in bacterial and archaeal ribosomes and are generally available for chloramphenicol binding. However, the difference in rRNA sequences between archaea and bacteria (for example the presence of G2058 in archaea as opposed to A2058 in bacteria) is apparently sufficient to affect the shape and size of the crevices significantly enough to re-direct drug binding to the alternative site. Nevertheless, both crevices are generally available for drug binding and while chloramphenicol binds to the 2058/2059 pocket in *H. marismortui*, anisomycin, a functional analog of chloramphenicol with considerable structural similarity to it, binds to the 2451/2452 crevice (Hansen *et al.*, 2003).

Several other PT inhibitors interact with either one or another of the same crevices, even though the mode of interaction is antibiotic specific (Hansen *et al.*, 2003). The generally hydrophobic interiors of these crevices attract hydrophobic groups of the antibiotics. Since both crevices are located in functionally important sites–at the PT active site (the 2451/2452 crevice) and at the tunnel entrance (the 2058/2059 crevice)–and binding of a drug at either of the two should interfere with translation, it is possible that the general "logic" of evolutionary selection of many antibiotics was to "find"

compounds that are bestowed with non-polar groups that can bind at these pockets.

Principles of Binding and Action of the PTC Inhibitors

Crystallographic examination of complexes of several antibiotics with 50S subunits of *D. radiodurans* and *H. marismortui* showed that binding of drugs to rRNA in the PTC relies on generally similar types of interaction that are exploited to a different degree by different inhibitors. Hydrophobic and stacking interactions between aromatic and other non-polar groups of antibiotics with rRNA bases support binding of chloramphenicol, anisomycin, telithromycin, and several other drugs (Berisio *et al.*, 2003; Hansen *et al.*, 2003; Schlunzen *et al.*, 2003; Tu *et al.*, 2005). Hydrophobic interactions between methyl groups of the lactone ring of macrolides and the surface of the exit tunnel account for a significant fraction of the binding energy of these drugs in *H. marismortui* (Hansen *et al.*, 2002b; Tu *et al.*, 2005) (though not in *D. radiodurans* (Schlunzen *et al.*, 2001; Schlunzen *et al.*, 2003)). Specificity of drug binding and precise orientation of the drug molecule in its site are tightly governed by a hydrogen bonding network that involves edges of RNA bases, ribose hydroxyls and phosphates (Schlunzen *et al.*, 2001; Hansen *et al.*, 2003; Harms *et al.*, 2004; Tu *et al.*, 2005). Mutations of nucleotides whose bases are engaged in hydrogen bonding with the drug often lead to drug resistance. The proximity and juxtaposition of the C6 acetaldehyde group of 16-member ring macrolides with N6 of A2062 in *H. marismortui* and the existence of a continuous electron density that connects these groups in the crystal structure led to a proposal that they form a reversible covalent bond. Its thermodynamic contribution to the affinity of 16-member ring macrolides remains to be elucidated (Hansen *et al.*, 2002b).

The most common mechanism of action of the PT-targeting antibiotics is preventing the correct positioning of acceptor or donor substrates in the PT active site. With the exception of blasticidin S, which disrupts interaction of the CCA end of peptidyl-tRNA with the P-loop, this is commonly achieved by invasion of the drug into the space belonging to the amino acid residues that esterify the A76 ribose of A- or P-site bound tRNAs. The clash with the bound antibiotic molecule displaces a substrate from its optimal orientation for nucleophilic attack of the α-amino group of the acceptor onto carbonyl carbon atom of the donor–consistent with the entropic mode of catalysis (see earlier). tRNA binding relies on multiple interactions involving contacts directly within the PT active site as well as with the outer shell RNA and protein residues (Yusupov *et al.*, 2001; Youngman *et al.*, 2004). It appears, however, that the binding of small molecules only directly in the active site of the PT enzyme produces enough distortion in substrate placement to severely interfere with peptide bond formation. None of the known drugs disrupt the outer-shell contacts of tRNA with the large ribosomal subunit.

Interference with the placement of the A-site substrate is a more common mode of action of the PT inhibitors. The 2451/2452 hydrophobic crevice discussed earlier is involved in interaction with side chains of amino acids of the A-site-bound acceptor substrates (Moazed & Noller, 1989; Hansen *et al.*, 2002a; Bashan *et al.*, 2003). Several antibiotics interacting with this crevice or coming in close proximity to it (chloramphenicol, sparsomycin, carbomycin, clindamycin, tiamulin in bacteria, and anisomycin in archaea) commonly inhibit placement of the PT substrates in the A-site (Poulsen et al., 2001; Schlunzen *et al.*, 2001; Hansen *et al.*, 2002a; Hansen *et al.*, 2003; Schlunzen *et al.*, 2004). Drugs that bridge A- and P-sites may affect placement of both donor and acceptor substrates (clindamycin, streptogramin A, tiamulin) (Celma *et al.*, 1970; Celma *et al.*, 1971; Schlunzen *et al.*, 2001; Schlunzen *et al.*, 2004; Tu *et al.*, 2005). Such antibiotics tend to bind closer to the 2058/2059 crevice at the entrance to the nascent peptide tunnel and as a result, their binding is usually negatively affected by the presence of a long nascent peptide. Many such drugs (macrolides, streptogramins B, lincosamides, tiamulin and others) preferentially inhibit early rounds of peptide bond formation and cause polysome decomposition (Cundliffe, 1969; Pestka, 1972b; Pestka, 1974; Contreras & Vazquez, 1977; Dornhelm & Hogenauer, 1978; Tenson *et al.*, 2003).

Similar But Not the Same

High conservation of the rRNA sequence in the PTC was always taken as an indication that the PTC three-dimensional structure is also highly conserved and, consequently, that the binding of antibiotics to ribosomes of different species should be very similar. A number of facts indicate that this view may be only partially true. One indication of a certain degree of species specificity

of the PTC structure comes from comparison of spectra of antibiotic resistance mutations in different organisms. The most notorious example is represented by mutations conferring resistance to linezolid, a representative of oxazolidinones–a new family of clinically approved drugs that target the PTC of bacteria and archaea (Shinabarger *et al.*, 1997). The predominant oxazolidinone resistance mutations in Gram-positive bacteria are G2576U and (more rarely) G2447U (Shinabarger, 1999; Prystowsky *et al.*, 2001; Sander *et al.*, 2002). In contrast, the only known mutation that provides fairly high levels of linezolid resistance in *E. coli* is G2032A (Xiong *et al.*, 2000; Thompson *et al.*, 2002). Yet in the archaeon *H. halobium*, the most prominent resistant mutations were C2452U, A2453G, U2500C and U2504C (Kloss *et al.*, 1999). There is no doubt that such a difference reflects a polymorphism of the PTC center in ribosomes of different species that leads either to a different mode of binding of the drug to different ribosomes (as observed for chloramphenicol in bacteria and archaea; discussed earlier) or to a significantly different fitness cost of equivalent mutations in different species. This observation calls for certain caution in translating results obtained with one organism to another species. Similarly, we should be careful not to over-generalize the implications of crystallographic structures of ribosome-antibiotic complexes that are currently available for only one bacterial (*D. radiodurans*) and only one archaeal (*H. marismortui*) organism. In fact, even data obtained with these two experimental models sometimes differ so dramatically that it makes one wonder which of the two structures, if any, reflects interaction of the drugs with the ribosomes of pathogenic bacteria. (After all, for all practical reasons, it is the binding of the drug to the ribosomes of pathogens that we want to understand best!) While some of the contradictions of the *D. radiodurans* and *H. marismortui* structures could result from modeling errors, the others could reflect authentic differences in binding of antibiotics to different ribosomes. As an example, a side chain of telithromycin, a ketolide drug binding near the entrance of the nascent peptide tunnel, is extended in the *D. radiodurans* crystallographic structure but is folded over the lactone ring in *H. marismortui* (Berisio, 2003; Schlunzen *et al.*, 2003; Tu *et al.*, 2005). Chemical probing of the corresponding ribosome-antibiotic complexes is in agreement with the published structures and supports differential interaction modes of the telithromycin side chain with *D. radiodurans* and *H. marismortui* ribosomes (Xiong & Mankin, unpublished data). However, neither of the available crystallographic models account for specific interactions of telithromycin with the ribosomes of *E. coli* or *Staphylococcus aureus* that are seen in probing experiments (Hansen *et al.*, 1999; Xiong *et al.*, 1999). Similarly, the earlier discussed example of differential binding of chloramphenicol to ribosomes of archaea and bacteria also attests to the species-specificity of drug-ribosome interactions.

While distinctive interactions of antibiotics with the ribosomes of different species complicate the study of drug binding to the bacterial targets, this same specificity provides foundation for the key principle of antibiotic action–selectivity. Since the detailed structures of eukaryotic ribosomes are yet to be determined, the drug specificity can currently be rationalized primarily on the bases of a nucleotide polymorphism of rRNA constituting the PTC of eukaryotic and bacterial ribosomes. Some nucleotide changes that convey antibiotic resistance in bacteria are present in the wild-type sequence of the PTC rRNA in human ribosomes and may account for the insensitivity of the latter to inhibition by clinical antibiotics. The A at position 2058 of 23S rRNA favors binding of macrolides and lincosamides to bacterial ribosomes whereas the G present at this position in cytoplasmic and mitochondrial ribosomes in human cells renders them impervious to these drugs (Vester & Douthwaite, 2001). The mutation of C2609 to U in bacteria renders them resistant to ketolides–newer macrolide derivatives (Garza-Ramos *et al.*, 2002). The presence of U2609 in human cytoplasmic ribosomes may thus contribute to the selective action of the drug on bacterial, but not human ribosomes. Similarly, the 2057/2611 base pair (G/C in many bacterial pathogens; A/U in human cytoplasmic and mitochondrial ribosomes) can modulate resistance to some macrolides (Pfister *et al.*, 2005). In many other cases, however, (chloramphenicol, oxazolidinones, etc.) the nucleotides that constitute the drug binding site in bacterial ribosome are conserved in human ribosomes which makes it more difficult to explain why these antibiotics exhibit specific antibacterial effects. One possibility is that less conserved rRNA residues of the PTC may affect the orientation of the conserved bases in the antibiotic binding site and therefore modulate the affinity of the drugs as well as the spectrum of resistance mutations (Xiong *et al.*, 2000; Sander *et al.*, 2002). Such a possibility is supported by the observed difference in orientation of nucleobases in the crystal structures of

archaeal (*H. marismortui*) and bacterial (*D. radiodurans*) ribosomes (Ban *et al.*, 2000; Harms *et al.*, 2001).

The detailed view of how drugs interact with the PTC of ribosomes of model organisms provided by genetic, biochemical, and most importantly crystallographic studies revealed some key principles of interactions of peptidyl transferase-directed inhibitors with rRNA in the PTC. The general principles of inhibition of PT by small molecules binding in the PT active site are beginning to emerge. We also learned that interaction of drugs with ribosomes of different species can be highly unique and differ between even closely related species. Therefore, before this knowledge can be effectively applied for the development of better antibiotics, it is important to understand how drugs interact with the ribosomes of pathogens, or at least with ribosomes of one or two other bacteria. Fortunately, new bacterial structures are apparently on the way (J. Cate, personal communication; J. Sutcliffe, personal communication) and will hopefully yield important information on the general features of drug-ribosome interactions. Structure-assisted design of PTC-targeting selective drugs will further benefit tremendously from the knowledge of the detailed structure of cytoplasmic and mitochondrial ribosomes of human cells.

THE EVOLUTION OF THE PTC

It is assumed that the life on Earth has its roots in the RNA World, a hypothetical pre-biotic era that existed about 3.8 billion years ago, when biologically relevant reactions relied on RNA catalysis (White, 1976; Gilbert, 1986). The RNA World theory and its implication for the origin of life gained support and prominence with the discovery of RNA enzymes in contemporary organisms (Cech *et al.*, 1981; Guerrier-Takada *et al.*, 1983). Subsequently, the list of naturally occurring ribozymes increased (see Doudna & Cech, 2002, for review), even though the chemical reactions they carry out are relatively simple and focus primarily on phosphoryl transfer reactions that require the activation of a ribose 2′-hydroxyl group or a water molecule for nucleophilic attack on the phosphordiester backbone of RNA molecules. The discovery that the ribosome is a ribozyme provided the strongest support yet for the RNA World hypothesis (Steitz & Moore, 2003).

Functions of the two ribosomal subunits are very distinct. The small subunit deals exclusively with an RNA template (mRNA) on which it assembles complementary RNA entities (tRNA anticodons). The large subunit, in contrast, deals primarily with amino acids that are activated by esterification to the tRNA 3′-hydroxyl of the terminal ribose. In principle, the large subunit (the PTC) "does not care" whether the aminoacyl-tRNA is "correct"—meaning whether or not its anticodon is complementary to the mRNA codon in the decoding center. As long as aminoacyl-tRNA is properly accommodated in the P-site, the reaction of transpeptidation catalyzed by the PTC will rapidly take place. In bearing with this, the PTC can catalyze reactions between substrates that contain RNA moieties much smaller than full-size tRNAs (Monro *et al.*, 1968). Thus, it is likely that catalysis of amino acid polymerization evolved independently, and probably preceded template-dependent protein synthesis (Moore, 1993).

In the RNA World, what was the selective advantage for the host to have a proto-ribosome that "learned" to produce peptides and proteins? It is highly unlikely that the first catalyzed polypeptides themselves had any significant enzymatic functions. Noller (2004) therefore proposed that the driving force for the selection of primitive protein synthesis was to enlarge the structural and, hence, functional repertoire of RNA. It has been amply demonstrated that the interaction of low-molecular weight molecules with RNA can lead the establishment of specific RNA folds that are not attainable without the ligand. This concept of ligand-induced RNA conformational changes obviously survived the transition from the RNA World to the contemporary DNA-RNA-protein world and represents the basic principle of riboswitch elements found in certain prokaryal mRNAs (Mandal & Breaker, 2004). Davies (1990) speculated that some antibiotics, or other primordial "low-molecular weight effectors," might be the remnants of the first rRNA-binding peptides that helped the RNA-based proto-ribosome to function. Therefore the first translation system that produced "functional" peptides did not evolve to pave an exit path out of the RNA world, but rather "aimed at" improving the properties of RNA molecules and ribozymes in the pre-protein world.

The distinctive features of the modern ribosome are its mammoth size and enormous structural complexity. The molecular weight of the ribosome exceeds 2.5 million daltons and the particle comprises at least three large rRNA molecules and more than 50 different ribosomal proteins. It is unimaginable that the

ribosome, which contains rRNAs of thousands of nucleotides in length, evolved in a single evolutionary step. Instead, individual ribosomal RNA protodomains, possibly endowed with their specific functions, might have evolved separately in the RNA World. To function as whole, they did not even need to be parts of the same RNA molecule. Even in the modern world, rRNA fragments in some species can assemble into a functional ribosome without being covalently linked into a continuous rRNA (Boer & Gray, 1988; Schnare & Gray, 1990). However, the need for synchronization of the production and assembly of protodomains apparently favored their association (possibly via RNA ligation) into longer RNA molecules. Higher-order complexes could have been formed that might have added functional diversity and sophistication to such a hypothetical proto-ribosome (Noller, 1993b).

As pointed out earlier, from a chemical point of view, transpeptidation is a rather simple reaction and can be catalyzed by RNA or protein enzymes much smaller and simpler than the large subunit of the modern ribosome (Welch *et al.*, 1997; Zhang & Cech, 1997; Tamura & Schimmel, 2001). Catalysis of peptide bond formation *per se* does not require the PTC to be a part of as large and complex a particle as the modern large ribosomal subunit. In fact, 50S-like particles that have lost many ribosomal proteins retain peptidyl transferase activity (Noller *et al.*, 1992; Khaitovich *et al.*, 1999a). Even the highly conserved 5S rRNA and large domains of 23S rRNA can be deleted without the loss of the ribosome's ability to catalyze the peptidyl transfer (Dohme & Nierhaus, 1976; Khaitovich & Mankin, 1999; Khaitovich *et al.*, 1999a). Similar conclusions likely also apply to peptide release, though more detailed investigations of structural requirements for the catalysis of the peptidyl-tRNA hydrolysis must be carried out. That rRNAs do not have to be so large in order to guarantee protein synthesis is further demonstrated by some animal mitochondrial ribosomes that contain severely reduced rRNA molecules (*e.g.*, *C. elegans*). It is of note however, that mitochondrial ribosomes have a higher number of ribosomal proteins that supposedly compensate for the rRNA loss (O'Brien T *et al.*, 2005). Since the level of complexity reached by the modern-day ribosome is not fundamentally required for catalyzing the peptidyl transfer or peptide release reactions, the need for it is likely dictated by other ribosomal functions, such as translocation, communication with the small subunit, interaction with chaperones and the membrane translocon, and other activities that require dynamic and well orchestrated interactions with various ligands and protein factors.

In vitro evolution can generate small RNA molecules that are capable of binding a transpeptidation transition state analog, binding an inhibitor of peptide bond formation, or even catalyzing peptide bond formation (Burke *et al.*, 1997; Welch *et al.*, 1997; Zhang & Cech, 1997; Zhang & Cech, 1998). It is remarkable that some of these *in vitro* selected RNA molecules exhibit certain structural similarities to the multi-branched central loop of domain V of 23S rRNA known to build the core of the PTC (reviewed in Polacek, 2001). For example, twenty-three nucleotides of the catalytic core of the *in vitro* selected peptidyl transferase ribozyme share 70% base identity to 23S rRNA that appear to be present in the same local secondary structure context (Zhang & Cech, 1998). It is intriguing that in all these different *in vitro* evolution experiments, the sequence resembling the rRNA segment of the PTC 2451 region of 23S rRNA has been selected from random RNA pools despite the different selective pressures. Does it mean that only very few RNA solutions exist for the problem of catalyzing peptide bond formation and that we are really lucky that one of these solutions has been found by the primordial ribosome?

In vitro selection experiments demonstrate that RNA can catalyze peptide synthesis without the help of proteins. However, in strict terms, this result does not prove that the rRNA was able to do it on its own. An important link that is still missing in the RNA World theory is the demonstration that rRNA from a modern ribosome, or an RNA molecule that is very similar to the modern rRNA, can catalyze amino acid polymerization. All attempts to prepare protein-free and catalytically active rRNA from the modern ribosome thus far have been unsuccessful (Khaitovich *et al.*, 1999b). Apparently, rRNA has been co-evolving with ribosomal proteins for too long to still "remember" that it could do the job without protein crutches. An obvious way to overcome such amnesia is to carry out the retro-evolution of modern 23S rRNA to select for (hopefully) a few rRNA mutations that would render rRNA able to catalyze peptidyl transfer in the absence of ribosomal proteins. Experimental approaches that have appeared in recent years, including *in vitro* reconstitution of functionally active large ribosomal subunits from circularly permuted 23S rRNA (Erlacher *et al.*, 2005), provide new ways to attack this problem.

We would like to end this section and this review with a citation of David Lilley (Lilley, 2003) with which we whole-heartedly agree: "Ultimately, the finest achievement of the RNA World was probably the creation of proteins. These then took over most of the catalytic functions, leaving the ribosome as the most permanent monument to a heroic era."

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