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The Role of 23S Ribosomal RNA Residue A2451 in Peptide Bond Synthesis Revealed by Atomic Mutagenesis

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SUMMARY

Peptide bond formation is a fundamental reaction in biology, catalyzed by the ribosomal peptidyl-transferase ribozyme. Although all active-site 23S ribosomal RNA nucleotides are universally conserved, atomic mutagenesis suggests that these nucleobases do not carry functional groups directly involved in peptide bond formation. Instead, a single ribose 2′-hydroxyl group at A2451 was identified to be of pivotal importance. Here, we altered the chemical characteristics by replacing its 2′-hydroxyl with selected functional groups and demonstrate that hydrogen donor capability is essential for transpeptidation. We propose that the A2451-2′-hydroxyl directly hydrogen bonds to the P-site tRNA-A76 ribose. This promotes an effective A76 ribose C2′-endo conformation to support amide synthesis via a proton shuttle mechanism. Simultaneously, the direct interaction of A2451 with A76 renders the intramolecular transesterification of the peptide from the 3′- to 2′-oxygen unfeasible, thus promoting effective peptide bond synthesis.

INTRODUCTION

The genome's mRNAs are translated into proteins by the ribosome, a multifunctional ribonucleoprotein particle of >2.7 MDa. The central chemical reaction, peptide bond formation that links amino acids into polypeptide chains (Figure 1) is performed by the peptidyl transferase center (PTC), which is located at the interface side of the large ribosomal subunit. The means by which the PTC catalyzes this fundamental biological reaction has been a subject of intense discussion over the past decades.

After realizing that the ribosome is the enzyme that forges peptide bonds in the mid 1950s, efforts began to experimentally identify the crucial ribosomal protein that confers this catalytic power—a rather futile task, as we now realize (reviewed by Barta and Halama, 1996). At that time, only protein enzymes were known in nature; thus, it was reasonable to assume that the ribosome also uses amino acid functional group(s) of ribosomal proteins for catalysis. With the identification of the first RNA enzymes in the early 1980s, the pendulum swung toward ribosomal RNA (rRNA)-mediated catalysis on the ribosome (Noller, 1993). It reached its peak at the dawn of the new millennium, when the first high-resolution crystal structures of the large 50S ribosomal subunit were presented revealing the PTC to be devoid of any ribosomal proteins, thus confirming peptidyl transferase to be an RNA enzyme (Harms et al., 2001; Nissen et al., 2000). Moreover, a catalytic model for amide bond formation was proposed in which the N3 position of the universally conserved adenine base at position 2451 of 23S rRNA (*Escherichia coli* nomenclature is used here and throughout the manuscript) was predicted to function as a key group in a general acid-base mechanism (Nissen et al., 2000). A2451 is one of the five universally conserved active-site rRNA residues that comprise the catalytic core of the PTC (Figure 2A). Subsequent biochemical and genetic studies, however, did not seem to support a crucial role of the nucleobase at A2451 for catalysis (Bayfield et al., 2001; Beringer et al., 2005; Polacek et al., 2001; Thompson et al., 2001; Xiong et al., 2001; Youngman et al., 2004). Furthermore, evidence was presented suggesting that general acid-base chemistry is unlikely to be used by the PTC to forge peptide bonds (Bieling et al., 2006).

The pendulum shifted again when it was suggested that the PTC may not, in fact, provide any specific functional group for catalysis, but merely serves as an entropy trap that places and orients the two tRNA substrates (peptidyl-tRNA and aminoacyl-tRNA at the P and A sites, respectively) optimally for spontaneous peptide bond formation (Sievers et al., 2004; Yonath, 2003), a scenario compatible with an earlier proposal (Nierhaus et al., 1980). Subsequently, a critical functional group was identified; however, it was not provided by the PTC, but, rather, resided on one of the substrates—namely, the 2′-hydroxyl group at the terminal adenosine of the P-site tRNA (Dorner et al., 2003, 2002; Weinger et al., 2004). This tRNA 2′-OH was proposed to be essential for transpeptidation in a so-called "substrate-assisted catalysis" (Weinger et al., 2004), thus reducing the role of the PTC during peptide bond formation to a merely passive stage for the main actors, the tRNA reaction substrates. Additionally, a novel nucleotide analog interference technique that allows individual functional groups within the 23S rRNA to be modified revealed a single-ribose 2′-OH at position A2451 of the PTC to be crucial for peptide bond synthesis (Erlacher et al., 2005, 2006). Subsequent molecular dynamics simulations confirmed these

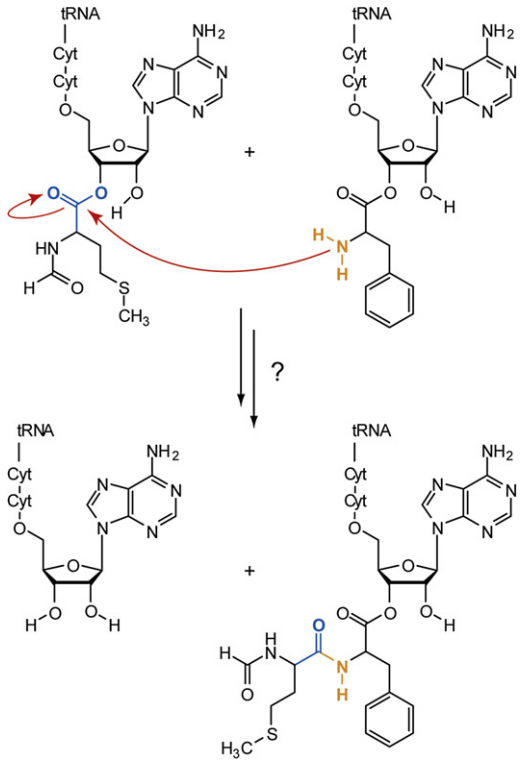

Figure 1. Ribosomal Protein Synthesis
Amide bond formation is the fundamental chemical reaction catalyzed by the PTC of the ribosome. The PTC possesses ribozyme activity: Attack of the α-amino group of the amino acid-charged A-site tRNA (orange) at the ester carbonyl carbon of the methionyl- or peptidyl-tRNA in the P site (blue) is supported to yield the amide linkage. The present study reveals details of this mechanism.

findings (Trobro and Aqvist, 2005). All currently available data point to the view that these two fundamental ribose 2′-OH groups, one residing in the PTC (at A2451) and the other on the P-tRNA (at A76), are both essential to facilitate efficient amide bond synthesis, yet the interrelationship between the two functional groups is not yet understood.

The present work addresses the important question as to the specific role played by the ribosomal A2451 2′-OH moiety during transpeptidation. By applying an in vitro reconstitution approach, we obtained ribosomes with functional group replacements at the ribose C2′ of A2451. We were able to demonstrate that, within the limits of our assay system (see Experimental Procedures), hydrogen *donor* activity of the original hydroxyl group is a stringent requirement of the PTC for amide bond formation. In accordance with these experimental data, we present a novel model illustrating how the interaction between the ribose 2′-OH groups of A2451 and P-tRNA is essential to the mechanism of ribosomal peptide synthesis.

RESULTS

Concept

In the present study, we investigated the role of A2451 2′-OH in terms of its ability to function as a hydrogen donor and/or

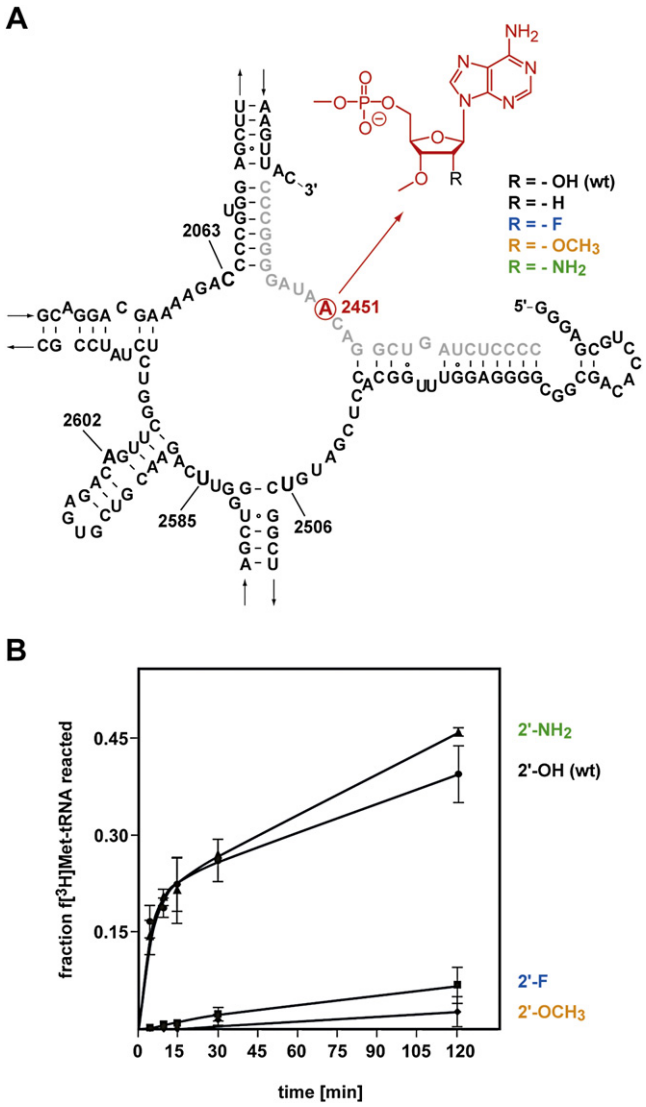

Figure 2. Atomic Mutagenesis of the Ribosomal PTC
(A) Secondary structure of the PTC of gapped-cp-reconstituted 50S subunits showing the new endpoints of the circularly permuted 23S rRNA at positions 2468 and 2440 (5′ and 3′, respectively). The chemically synthesized 26 nucleotide RNA, which compensates for the missing rRNA segment, is shown in gray. The five universally conserved residues that build the inner core of the active site are indicated by the nucleotide numbers with *E. coli* nomenclature. Residue A2451 is highlighted in red, and the different chemical groups that were introduced at the ribose 2′ position are depicted next to the chemical structure.
(B) Peptidyl transferase activity of ribosomes containing ribose 2′ modifications at A2451. Time course of peptide bond formation promoted by ribosomes containing gapped-cp-reconstituted 50S carrying adenosine (2′-OH, wt; circles), 2′-amino adenosine (2′-NH_2; triangles), 2′-fluoro adenosine (2′-F; squares), or 2′-O-methyl adenosine (2′-OCH_3; diamonds) at the position corresponding to A2451 of 23S rRNA. In all cases, the background values (amount of product formation in reactions containing 50S subunits that were reconstituted in the absence of the synthetic RNA fragments) were subtracted from all experimental data points, and the fraction of the 0.3 pmol input f-[^{3}H]Met-tRNA that reacted is indicated. Curves represent the mean and standard deviation of at least three independent time course experiments.

hydrogen acceptor. We considered 2′-fluoro (2′-F) A2451 and 2′-O-methyl (2′-OCH_3) A2451 ribosome variants appropriate to determine if the hydrogen atom of the original 2′-OH is crucially involved in donating its proton. If this were the case, we hypothesized that, for ribosomes with A2451 2′-F or 2′-OCH_3 substitutions—functional groups that lack any hydrogen donor activity—peptide bond formation activity would be significantly reduced. On the other hand, if the 2′-OH of A2451 were replaced by a 2′-amino (2′-NH_2) group, hydrogen donor capability is retained and, therefore, the activity in amide bond formation should also be maintained.

In order to introduce these nonnatural nucleotide analogs into the 23S rRNA, we applied a recently developed in vitro reconstitution technique for 50S subunits (Erlacher et al., 2005). A key feature of this approach is the use of circularly permuted 23S rRNA transcripts that place the novel 5′ and 3′ ends close to the PTC, thus leaving a sequence gap of 26 nucleotides within the active site (Figure 2A). The missing RNA segment was then provided in *trans* during reconstitution as a chemically synthesized RNA fragment containing the desired nucleotide analog at position A2451.

A2451 2′-F Ribosome Variant Lacking Hydrogen Donor Properties

We first introduced 2′-F-adenosine at position 2451 of the PTC (Figure 2A). This nucleoside adopts—like the natural adenosine—a C3′-endo sugar pucker conformation and retains the potential to act as a hydrogen acceptor at the 2′-position, but lacks any hydrogen donor properties. We used puromycin as an A-site substrate to show that ribosomes containing chemically modified 50S subunits carrying 2′-F-A2451 were clearly hampered in catalyzing peptidyl transfer from a P-site-bound formyl-Met-tRNA (Figure 2B and see Table S1 in the Supplemental Data available with this article online). Only after prolonged incubation times could minute amounts of reaction product be detected. This indicates that a functional group with hydrogen acceptor capabilities is not sufficient for effective peptide bond formation. In a control experiment, full peptidyl-tRNA hydrolysis activity was retained by ribosomes harboring the 2′-F-A2451 modification (Figure S1). Peptidyl-tRNA hydrolysis is the second chemical reaction of protein synthesis catalyzed by the PTC, and occurs during the termination phase, resulting in release of the nascent polypeptide chain (reviewed by Polacek and Mankin, 2005). Since both these PTC-catalyzed reactions are single-turnover events under the conditions used (Erlacher et al., 2005), full activity in peptidyl-tRNA hydrolysis thus indicates that the introduced ribose modification does not affect the overall assembly efficiency of the active site. In another experiment, we introduced a 2′-methoxy group at position 2451 (2′-OCH_3-A2451). Again, this functional group lacking hydrogen donor potential severely inhibited transpeptidation activity (Figure 2B), but remained active in peptidyl-tRNA hydrolysis (data not shown), consistent with the outcome of the 2′-F-A2451 modification.

A2451 2′-NH_2 Ribosome Variant Providing both Hydrogen Donor and Acceptor Properties

Adenosine was used as the initial building block to synthesize 2′-amino-2′-deoxyadenosine in nine steps as a 2′-*N*-phthaloyl protected phosphoramidite derivative and incorporated into the RNA oligonucleotide by solid-phase synthesis (see Experimental Procedures). Like its natural 2′-OH counterpart, the amino modification provides both hydrogen acceptor and hydrogen donor propensities. When we introduced 2′-amino adenosine at position 2451 of the PTC (2′-NH_2-A2451), the chemically modified 50S subunits were completely active in peptidyl transfer (Figure 2B), with rates comparable to reconstituted 50S particles carrying the native 2′-OH (Table S1). We stress that the transpeptidation rates of A2451 2′-OH versus 2′-NH_2 reconstituted ribosomes did not change relative to each other as a function of pH between 5.5 and 8.5 (Figure S2). The absence of rate effects of peptide bond formation on pH change is in good agreement with recent work by Steitz, Strobel, and coworkers (Schmeing et al., 2005a, 2005b; Strobel and Cochrane, 2007; Weinger and Strobel, 2006), revealing that the inner-most critical groups for peptide bond formation (the amino nucleophile as well as the 3′-O leaving group) are completely shielded from the solvent. Thus, changes in the overall pH are not expected to greatly affect the reaction rates.

The A2451 2′-O-H ••• O(2′) A76 Hydrogen Bond Assists in P-site tRNA A76 Ribose Positioning

The ribosome crystal structure with A- and P-site substrates at 2.4 Å resolution shows that the 2′-oxygen atom of A2451 forms a more or less equilateral triangle with the nitrogen of the α-amino nucleophile and the 2′-oxygen atom of P-site tRNA A76 (Figure 3A; Schmeing et al., 2005b), with an average distance of 2.9 Å between each member (Figure 4A, Table S2). In accordance with our experimental finding that A2451 2′-OH acts as a hydrogen donor, we propose that the H atom of this hydroxyl group is involved in hydrogen bond formation. Following classical configuration and conformation analysis, two arrangements are conceivable. In the first, the A2451 2′-OH accepts the lone pair of the sp^3-hybridized nitrogen of the A-site aminoacyl-tRNA (A2451 2′-O-H ••• NH_2 amino acid). We consider this conformation nonproductive, since the lone pair of the amino group is required for nucleophilic attack at the ester carbonyl carbon of the tRNA peptidyl ester in the P site. The second possibility is the formation of a hydrogen bond between A2451 2′-OH involving one lone pair of the sp^3-hybridized 2′-oxygen of tRNA A76 in the P site (A2451 2′-O-H ••• O(2′) A76) (Figure 3A, dotted line in magenta; Figure S3). For such an arrangement, at least two rationales can be given. On the one hand, formation of A2451 2′-O-H ••• O(2′) A76 assists the positioning of the tRNA terminus: it is remarkable that the ribose of the terminal adenosine of P-site peptidyl-tRNA adopts a C2′-endo conformation in the crystal structure, since this conformation is usually less favorable than the C3′-endo for RNA nucleosides. In this respect, the proposed hydrogen bond would support the observed unfavorable C2′-endo conformation. On the other hand, formation of A2451 2′-O-H ••• O(2′) A76 would be expected to hamper spontaneous intramolecular transesterification. The peptidyl moiety attached at the ribose 3′-O has the potential to migrate to the 2′-O of A76. This isomerization reaction is very prominent for free tRNAs and is catalyzed by general bases (Figure 5A). On the ribosome, such a spontaneous transesterification would interfere with rapid peptide bond formation. It therefore makes sense for the ribosomal PTC to support an architecture of A- and P-site tRNA

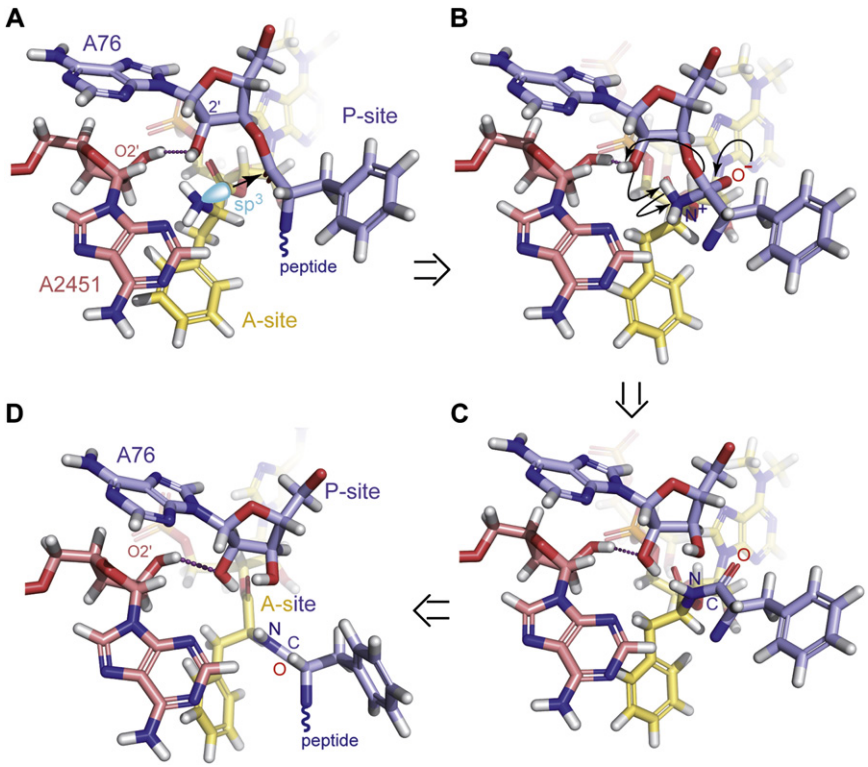

Figure 3. Model for Ribosomal Amide Synthesis Proposing a Role for A2451

A2451 of 23S rRNA (red), A76 of peptidyl-tRNA (P site; blue), and aminoacyl-tRNA (A site; yellow).

(A) A2451 2′-OH provides its hydrogen to form a hydrogen bond with the ribose 2′-O of tRNA-A76 in the P site (dotted line in magenta) and, thereby, properly positions A76 2′-OH for proton shuttling (note: A76 adopts C2′-endo conformation). The lone pair of the sp^3 hybridized α-amino group (cyan) is ready to attack the ester carbonyl carbon (black arrow); PDB code: 1VQN (Schmeing et al., 2005b); 50S with CCA-phenylalanine-caproic-acid-biotin as P-site substrate and CC-hydroxypuromycin as A-site substrate (Schmeing et al., 2005b).

(B) The respective hydrogen bond (dotted line in magenta) is retained after attack of the α-amino nucleophile when the tetrahedral intermediate has formed: A76 2′-OH is positioned to simultaneously donate its proton to the lone pair of A76 3′-O and to receive the proton from the positively charged nitrogen by its own lone pair (PDB code: 1VQ7 [Schmeing et al., 2005b]; 50S with transition state analog "DCA" [Schmeing et al., 2005b]). Black arrows indicate pairwise electron movement for proton shuttling (see also Figure 6).

(C) Formal outcome of proton shuttling showing the newly formed and broken bonds; coordinates refer to (B). Note that the amide bond is arbitrarily shown with sp^2-hybridized N, C, and O in a not-yet planar conformation.

(D) PTC with peptide product. The amide bond has relaxed into a planar conformation (PDB code: 1KQS [Schmeing et al., 2002]; 50S with CC-puromycin-peptide in the A site and deacylated CCA at the P site).

termini that avoids spontaneous transesterification (Figure 5B). In the proposed geometry, rotation around the C2′-O2′ bond is restricted in a manner such that the oxygen lone pairs cannot be positioned to allow nucleophilic attack on the neighboring ester carbonyl carbon (see Figure 5C).

DISCUSSION

The presently accepted model for peptide bond formation is based on the crystal structures of the 50S subunit from *Haloarcula marismortui* (Hma), with the peptidyl- and aminoacyl-tRNA analogs CCA-phenylalanine-caproic acid-biotin (CCApcb) and CC-hydroxypuromycin (CChPmn) bound to the P and A sites, respectively (PDB code: 1VQN) (Schmeing et al., 2005b), and on structures of the ribosomal PTC in complex with transition state mimics (PDB code: 1VQ7) or peptide products (PDB code: 1KQS). In conjunction with biochemical data, these structures indicate a significant role of the 2′-OH group of P-site tRNA A76 to serve as a "proton shuttle" in catalysis of peptide bond formation (Dorner et al., 2003, 2002; Schmeing et al., 2005a, 2005b; Weinger et al., 2004). The 2′-OH of A76 is in direct proximity to the attacking α-amino nucleophile as well as to the 3′-*O* leaving group; thus, mechanisms have been suggested that involve concerted proton transfer from the α-amino to the A76 3′-*O* based on the simple fact that the amine must lose a proton and the 3′-oxygen must obtain one (Bayryamov et al., 2007; Dorner et al., 2003, 2002; Weinger et al., 2004). These mechanisms share a common theme, in that six crucial atoms are employed in a conformational arrangement which allows proton shuttling without significant charge generation on either the A76 2′-OH or the A76 3′-OH leaving group. The requirement not to invoke protonation or deprotonation of these hydroxyl groups (at neutral pH) originates from the pK_a of ~12 of vicinal diols.

However, how does A2451 2′-OH of 23S rRNA participate in ribosomal peptide bond formation? We showed previously that a deoxyribose at 23S rRNA residue A2451 clearly reduced the rate of peptide bond formation with minimal (puromycin, CpCp-puromycin) or full-length (aminoacyl-tRNA) A-site substrates (Erlacher et al., 2005, 2006). Recently, we observed an identical effect in model in vitro translation reactions, where the aminoacyl-tRNA is bound to the ribosome as ternary complex with EF-Tu and GTP (M.E., A. Chirkova, N.P., unpublished data), thus demonstrating that the inhibitory effect of the 2′-deoxy modification at A2451 is independent of the nature of delivery of the A-site substrate. This finding further supports a potential involvement of the A2451 2′-OH in the chemistry of the peptidyl transferase reaction. Additionally, molecular dynamics calculations predicted a significant energetic contribution of the A2451 2′-OH to transition-state stabilization of ~3 kcal/mol, which equates to a rate reduction of almost 1000-fold in the absence of this pivotal hydroxyl group (Trobro and Aqvist, 2005). However, deoxyribose substitution alone provides no information about the precise

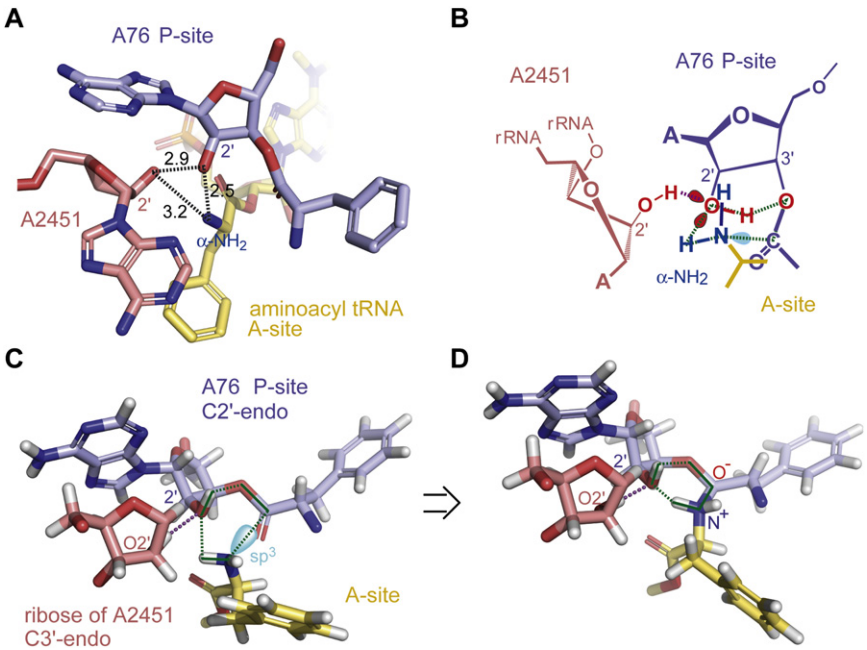

Figure 4. Cyclic Conformational Arrangement for Ribosomal Peptide Bond Formation

(A) Distance analysis based on PDB code: 1VQN (ground state). The dashed lines in black highlight the distances of the triangle formed by the 2′-oxygen atom of A2451, the nitrogen of the α-amino nucleophile, and the 2′-oxygen atom of A76; the distance values are given in Ångstroms.

(B) Representation of the conformational arrangement by chemical formulas referring to the ground state.

(C) The α-amino group of the aminoacyl moiety (A-site aa) of the A-tRNA is ready for attack at the ester carbon (coordinates based on PDB code: 1VQN; ground state): the six membered conformational arrangement of the atoms that are going to be involved in the proton shuttle is indicated by green straight and dotted lines.

(D) Intermediate state after attack of the α-amino group (coordinates based on PDB code: 1VQ7); the cyclic conformational arrangement is indicated as in (C).

chemical contribution of a particular 2′-OH. Therefore, in the present study, to investigate in detail the role of the A2451 2′-OH of 23S rRNA during transpeptidation, we introduced selected modifications at the ribose 2′ carbon that severely influence hydrogen bonding capability. Our observation that efficient peptide bond formation is only achieved when the 2′ functional group of A2451 is endowed with hydrogen donor propensity, necessitates a reanalysis of existing models to address the key question as to how the hydrogen donor activity of 2′-OH of A2451 fits into a mechanistically solid, chemical scenario.

The experimental data presented here support the conclusion that the 2′-OH of A2451 donates its proton to establish a direct hydrogen bond with the P-tRNA ribose 2′-OH at position 76. Our analysis of the 50S crystal structure with both A- and P-site substrates, and our experimental data, support a mechanistic model in which the hydrogen bond A2451 2′-O-H ••• O(2′) A76 occupies one lone pair of the P-tRNA A76 2′-O, and the other lone pair is positioned toward the amino nucleophile (Figure 3A; Figure S3). Once the tetrahedral intermediate has been formed (Figure 3B), A76 2′-O can easily receive the hydrogen from the positively charged ammonium group in a proton shuttle mechanism involving six atoms, as highlighted in Figure 6 and Figure S4. We point out that the distance between 2′-O A2451 and 2′-O A76 is retained within the limits for hydrogen bonding, as deduced from crystal structures of the PTC in complex with transition-state analogs (Table S2). In other words, by donating its proton to the A76 2′-O of P-tRNA, A2451 helps to stabilize the six membered ring system of the proposed proton shuttle in its productive conformation (Figure 4). Proton shuttling results in the nascent amide bond (Figure 3C), which requires further relaxation to obtain planarity (Figure 3D; Schmeing et al., 2002).

In principle, the question can be raised as to whether the A2451 2′-OH might also be directly involved in the proton shuttle. At first sight, the expansion of the above-discussed, six membered cyclic arrangement to an eight membered one involving the A2451 2′-OH moiety is plausible (Figure S5). A2451 2′-OH would function as both hydrogen donor (to A76 of P-tRNA) and hydrogen acceptor (from the α-amino nucleophile). However, the crystal structure of the PTC with A- and P-site substrates does not support simultaneous formation of the A2451 2′-O-H ••• O(2′) A76 hydrogen bond together with that of A2451 2′-HO ••• H-NH (A-site aminoacyl tRNA), simply because of a severe distortion of the tetrahedral geometry of the sp^3-hybridized 2′-oxygen. Moreover, in the crystal structure of the PTC with transition-state analogs, the distance between A2451 2′-HO ••• H-NH becomes too large for a typical hydrogen bond. Both arguments render the eight membered ring proton shuttle less likely, although it cannot be completely excluded—in particular, when formation and breakage of bonds would deviate from an ideal, simultaneous process.

Additionally, direct interaction of A2451 with A76 stabilizes a particular ribose conformation at the terminal adenosine of P-tRNA that would impede spontaneous transesterification of the growing peptide chain from the productive 3′ to its nonproductive 2′ position (Figure 5). This appears to be significant, since the rate of spontaneous transesterification has been investigated in several contexts, and values reported in the literature range from 4.10^3 s^{-1} to 5 s^{-1} (Griffin et al., 1966; Sprinzl, 2006; Taiji et al., 1981, 1983). These rates are faster than, or at least in the same order of magnitude as, the rate of amino acid incorporation during in vivo protein synthesis (~20 s^{-1}), so that the effect of spontaneous transesterification must be considered throughout the translation process.

SIGNIFICANCE

By using an atomic mutagenesis approach to investigate all the 23S rRNA residues that compose the inner core of the peptidyl transferase center, we identified a single functional

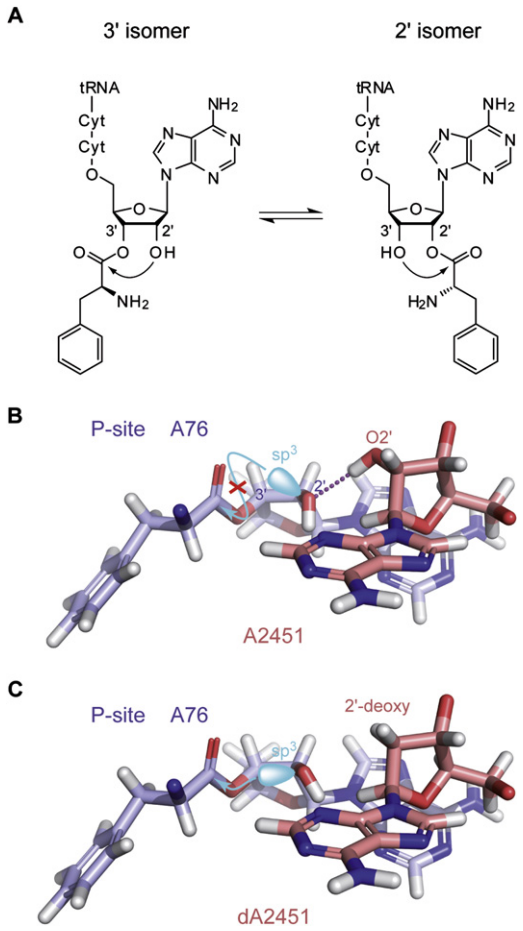

Figure 5. Transesterification Is a Spontaneous, Base-Catalyzed, Intramolecular Reaction Observed for Free Aminoacylated tRNAs in Solution that Results from Attack of the Ribose 2′-OH at the Neighboring 3′-O Ester Carbonyl Carbon and Vice Versa

(A) A dynamic equilibrium is obtained for 2′- and 3′-aminoacylated derivatives, with an isomeric ratio of about 1:1.

(B) The A2451 2′-O-H ••• O(2′) A76 hydrogen bond is proposed to suppress transesterification, since rotation around the A76 C(2′)-O(2′) becomes restricted and, therefore, the O(2′) lone pair is unable to attack the ester group.

(C) If the hydrogen bond of A2451 2′-O-H ••• O(2′) A76 were lacking, rotation around C(2′)-O(2′) would cause the lone pair to be a potential nucleophile for transesterification (note that additional minor conformational changes concerning the ester and ribose sugar pucker are required for a mechanistically correct attack at the carbon center) (Bürgi et al., 1974, 1973).

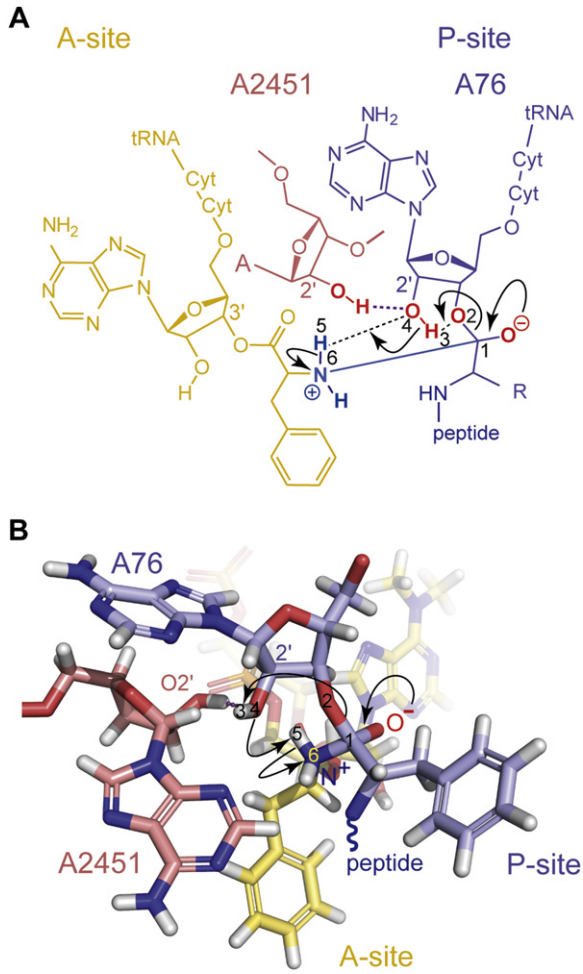

Figure 6. A Tetrahedral Intermediate, Seen in Atom Number 1, Is Formed after Attack of the α-Amino Nucleophile at the Ester Carbonyl Carbon

Proton shuttling is proposed to occur at the intermediate state to finally result in the desired amide bond; (A) Presentation by chemical formulas and (B) correlation to the three-dimensional structure of the PTC in complex with the tetrahedral intermediate. Proton shuttling involves six atoms (see numbers) in a ring-like conformational arrangement that respects characteristic bond angles and lone pair geometries of the individual atoms (for further details see Figure 4).

group with crucial importance for peptide bond catalysis—namely, the ribose 2′-OH at A2451. This ribose 2′ group needs to maintain hydrogen donor characteristics in order to promote effective amide bond formation. By combining these novel findings with biochemical and structural data that have accumulated over the last 2 decades highlighting the importance of the P-tRNA ribose 2′-OH at A76 (Bashan et al., 2003; Dorner et al., 2003, 2002; Quiggle et al., 1981; Schmeing et al., 2005a, 2005b; Sprinzl, 2006; Weinger et al., 2004), we were able to propose a novel, comprehensive model for ribosomal peptide bond formation. We propose that the A2451 2′-hydroxyl directly assists in positioning the P-site tRNA-A76 ribose via hydrogen bond formation. This can promote an effective A76 ribose C2′-endo conformation to support amide synthesis via a proton shuttle mechanism. This synergistic approach appreciates the concept of "substrate-assisted catalysis" (Weinger et al., 2004), and combines with it the strict functional requirement of the ribose 2′ group at A2451 of 23S rRNA to possess hydrogen donor capability.

EXPERIMENTAL PROCEDURES

Chemical Synthesis

The synthesis of N^6-acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-deoxy-2′-*N*-phthaloyl-3′-*O*-[(*N*,*N*-diisopropylamino)-(β-cyanoethoxy)phosphino]adenosine proceeded in nine steps (Figure 7), and will be reported in detail in a future article (currently unpublished data). A part of the synthetic pathway followed the lines

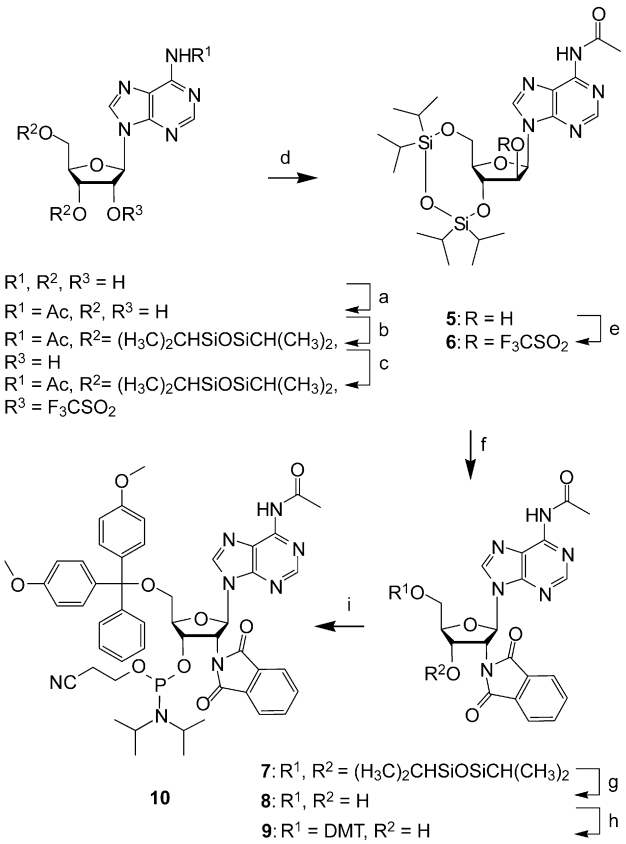

Figure 7. Synthesis of 2′-*N*-Phthaloyl Protected 2′-Deoxy-2′-Aminoadenosine Phosphoramidite Building Block 10

The synthesis of N^6-acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-deoxy-2′-*N*-phthaloyl-3′-*O*-[(*N*,*N*-diisopropylamino)-(β-cyanoethoxy)phosphino]adenosine proceeded in nine steps: (a) i. 10 eq TMS-Cl in pyridine, 0°C to room temperature (rt), 14 hr; ii. 1.5 eq CH_3COCl in pyridine/acetonitrile, 0°C, 1 hr; iii. 10 eq CH_3COOH in methanol, −20°C, 2 d, 70%; (b) 2.3 eq TBDS-Cl_2 in pyridine, rt, 5 hr, 90%; (c) 1.5 eq DMAP, 1.5 eq Tf-Cl in CH_2Cl_2, rt, 30 min, 95%; (d) 1.5 eq $(iPr)_2NEt$, 5 eq $CF_3COO^-K^+$, 2 eq, 18-crown-6-ether in toluene, 80°C, 14 hr, 85%; (e) 1.5 eq DMAP, 1.5 eq Tf-Cl in CH_2Cl_2, rt, 30 min, 95%; (f) 1.5 eq potassium pthalimide in DMF, 35°C, 12 hr, 70%; (g) 3.2 eq TEA, 2.8 eq TEA•HF in CH_2Cl_2, rt, 6 hr, 85%; (h) 1.5 eq DMT-Cl, rt. 20 hr, 60%; (i) 5.9 eq $(iPr)_2NEt$, 5 eq 1-methylimidazol, 2 eq CEP-Cl, rt, 2 hr, 90%. CEP-Cl = 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite; DMAP = 4-dimethylaminopyridin; DMF = *N*,*N*-dimethylformamide; DMT-Cl = 4,4′-dimethoxytrityl chloride; $(iPr)_2NEt$ = *N*-ethyldiisopropylamine; TBDS-Cl_2 = 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane; TEA = triethylamine; TEA•HF = triethylamine trihydrofluoride; Tf-Cl = trifluoromethanesulfonylchloride; TMS-Cl = chlorotrimethylsilane.

of a previously published procedure by Beigelman and coworkers (Karpeisky et al., 2002). The synthesis of 2′-F-, 2′-NH_2-, and 2′-OCH_3-derivatized oligoribonucleotides was performed according to previously reported protocols (Höbartner et al., 2002, 2004; Lang et al., 2007; Micura, 2002; Moroder et al., 2006).

In Vitro Reconstitution of 50S Subunits

Gapped-cp-reconstituted 50S subunits for investigating A2451 were assembled in vitro and reassociated with native *Thermus aquaticus* 30S subunits as described previously (Erlacher et al., 2005). The circularly permuted 23S rRNA transcript used was generated by covalently connecting the natural 23S rRNA ends and introducing novel 5′ and 3′ ends at positions 2468 and 2440, respectively. The missing RNA fragment that compensates for the introduced 26 residue-long sequence gap was provided in *trans* during reconstitution as chemically synthesized RNA oligonucleotide, and either contained the wild-type sequence or the desired ribose modifications at residue A2451.

Assaying PTC-Catalyzed Reactions

The puromycin reaction was employed to measure transpeptidation activities of the gapped-cp-reconstituted 50S subunits carrying nucleoside analogs in the PTC. Therefore, 10 pmol reconstituted 70S were programmed with 60 pmol synthetic mRNA containing a unique AUG codon and incubated with 0.3 pmol formyl-[^{3}H]Met-$tRNA^{fMet}$ (30,000 cpm/pmol) in order to fill the P site. Puromycin was added to a final concentration of 2 mM, the peptidyl transferase reaction was performed, and the reaction product identified as described previously (Erlacher et al., 2005). Release factor 1-mediated peptidyl-tRNA hydrolysis reactions employing gapped-cp-reconstituted particles were performed as described previously (Amort et al., 2007). Even though the gapped-cp-reconstitution procedure has the advantage of performing atomic mutagenesis studies in the ribosome, its intrinsic limitations have to be considered (Amort et al., 2007; Erlacher et al., 2005, 2006). Most importantly, peptide bond formation in these 50S subunits proceeds at a rate of ~0.12 min^{-1}, which is about 400-fold slower than in comparable assays with the same reaction substrates, but employing native 70S ribosomes (Katunin et al., 2002). Therefore, this experimental system is not applicable for measurement of subtle changes in transpeptidation, but has turned out to be valuable in pinpointing crucial groups in the PTC, the chemical modification of which significantly hampers the reaction.

Modeling

Modeling of hydrogen atoms, modification of structures, as well as visualization and figure preparation were performed with PyMol (DeLano, 2002; http://www.pymol.org).

SUPPLEMENTAL DATA

Supplemental Data, including five additional figures and two additional tables, are available with this article online at http://www.chembiol.com/cgi/content/full/15/5/485/DC1/.

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