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Chemical engineering of the peptidyl transferase center reveals an important role of the 2′-hydroxyl group of A2451

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ABSTRACT

The main enzymatic reaction of the large ribosomal subunit is peptide bond formation. Ribosome crystallography showed that A2451 of 23S rRNA makes the closest approach to the attacking amino group of aminoacyl-tRNA. Mutations of A2451 had relatively small effects on transpeptidation and failed to unequivocally identify the crucial functional group(s). Here, we employed an *in vitro* reconstitution system for chemical engineering the peptidyl transferase center by introducing non-natural nucleosides at position A2451. This allowed us to investigate the peptidyl transfer reaction performed by a ribosome that contained a modified nucleoside at the active site. The main finding is that ribosomes carrying a 2′-deoxyribose at A2451 showed a compromised peptidyl transferase activity. In variance, adenine base modifications and even the removal of the entire nucleobase at A2451 had only little impact on peptide bond formation, as long as the 2′-hydroxyl was present. This implicates a functional or structural role of the 2′-hydroxyl group at A2451 for transpeptidation.

INTRODUCTION

The ribosome, a multifunctional ribonucleo-protein complex, assembles polypeptides according to the genetic information encoded by mRNAs. Functions of the ribosome in protein synthesis are complex and involve different types of activities critical for decoding of the genetic information, assembling of polypeptide chains, and the proper targeting and release of the synthesized protein. However, there are only two chemical reactions crucial for protein synthesis involving formation or breaking of covalent bonds that are directly catalyzed by the ribosome: peptide bond formation and peptide release of the nascent polypeptide from tRNA. Both of these reactions take place in the active site of the ribosomal peptidyl transferase center (PTC)—a catalytic center located in the large ribosomal subunit.

The recent advancement in ribosome crystallography provided a detailed view of the structural organization of the peptidyl transferase active site and confirmed the notion that the PTC is an RNA enzyme (1,2). Investigation of complexes of the *Haloarcula marismortui* large ribosomal subunit with analogs of the peptidyl transferase substrates showed that the cavity of the PTC, which accommodates donor (peptidyl-tRNA) and acceptor (aminoacyl-tRNA) substrates of the peptidyl transfer reaction, is decorated by several conserved 23S rRNA residues. These residues are therefore of potential functional significance and can be involved in substrate positioning and/or chemical catalysis of transpeptidation. The close proximity of the N3 of A2451 to the putative position of the tetrahedral carbon center of the transition state intermediate drew a lot of attention to this nucleotide, and made the nucleobase of this residue one of the most ‘suspicious’ in 23S rRNA for directly participating in peptide bond formation (1).

Despite the structural identification of the potential molecular players involved in forging a peptide bond, the detailed mechanism of catalysis remains controversial and far from being understood (3–5). Mutational studies failed to unequivocally identify functional groups crucial for peptide bond formation. Base substitutions at several conserved nucleotides in the PTC active site, including A2451, had relatively minor effects on the peptidyl transfer reaction (6–10). Although somewhat different on a quantitative scale, all mutational

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studies were similar on a qualitative level: ribosomes with base changes at A2451 retain significant peptidyl transferase activity. These data lead to a notion that the contribution of chemical catalysis involving the A2451 base, or other nearby 23S rRNA residues, to the overall rate of peptide bond formation is relatively small, leaving substrate orientation as the main source of catalytic power provided by the ribosome.

While mutational analysis did not appear to support direct implication of the A2451 base in catalysis of peptide bond formation, it also could not explicitly rule it out. Neither of the mutations at A2451 would entirely eliminate the hydrogen bonding capacity of a nucleotide base at this position: replacements of A by G, C or U would put a hydrogen bond acceptor (N3 for G and O2 for C or U) in approximately the same place as the N3 of A (11). Therefore, the role of substrate alignment and/or activation played by the base of A2451 could potentially be achieved by any of the three other bases as well. In addition to N3 of A2451, two other groups, the 2′-hydroxyl of A2451 and the 2′-hydroxyl of the terminal adenosine of P-site bound peptidyl-tRNA, were seen within hydrogen bonding distance to the attacking α-amino group of the acceptor substrate. These groups could therefore promote peptide bond formation by orienting or activating the α-amino group for its attack onto the carbonyl carbon of the peptidyl-tRNA ester bond (1,11).

The level of chemical engineering that can be achieved by conventional mutational studies of 23S rRNA is fairly limited because at any particular position, it is restricted to only three possible mutations that can be incorporated by RNA polymerase. At the same time, the complexity and size of 23S rRNA renders many of the molecular tools successfully used to study smaller ribozymes inapplicable. In particular, it is extremely difficult to modify the chemical nature of a specific residue within a chemically homogeneous RNA molecule containing thousands of nucleotides. To overcome this obstacle, we have re-engineered the *in vitro* reconstitution system of *Thermus aquaticus* large ribosomal subunits (12) to make possible the assembly of ribosomal subunits from a combination of *in vitro* transcribed and chemically synthesized rRNA fragments. The centerpiece of this approach is the use of circularly permuted 23S rRNA (cp-23S rRNA) as the major rRNA component for the *in vitro* assembly. This new approach, which we named 'gapped-cp-reconstitution', allows placing a wide range of chemically altered rRNA residues at desired positions within the large ribosomal subunit. Investigation of the performance of the chemically engineered peptidyl transferase enzyme revealed the importance of the ribose 2′-hydroxyl group at position 2451 of 23S rRNA for promoting peptide bond formation.

MATERIALS AND METHODS

tRNA

Formyl-[^{3}H]Met-tRNA (45 000 c.p.m./pmol) was prepared by aminoacylation of *E.coli* $tRNA^{fMet}$ in a buffer containing 20 mM HEPES/KOH (pH 7.6), 8 mM $MgAc_2$, 150 mM NH_4Cl, 4 mM β-mercaptoethanol, 2 mM spermidine, 0.05 mM spermine as described in (13). *N*-acetyl-[^{3}H]Phe-tRNA (1000 or 15 000 c.p.m./pmol) was prepared as described previously (14). Formyl-[^{3}H]Met-tRNA and *N*-acetyl-[^{3}H]Phe-tRNA were purified using a reverse phase C4 HPLC column (Vydac) in buffer containing 50 mM $MgAc_2$ and 50 mM NH_4Ac (pH 6.0) with a linear gradient from 0 to 25% ethanol.

Construction of circularly permuted 23S rRNA

The circularly permuted gene product used for *in vitro* transcription of the cp-23S rRNA was produced by PCR using a plasmid (pCPTaq23S) carrying tandemly repeated 23S rRNA genes from *T.aquaticus*. This plasmid was created by the subsequent cloning of two 23S rRNA gene copies into pBR322. For the first copy, the T7 RNA promoter, which is present in the parental plasmid (pT7TA), containing the cloned 23S rRNA gene (12), was removed and a BamH1 site at the 5′ end of the gene was introduced by PCR. This 23S rRNA gene was then cloned as a BamH1/Bsp119l fragment into pBR322. For the second copy of the 23S rRNA gene, the parental plasmid pT7TA was altered so that the Bsp119l site at the 3′ end of the gene was removed by Bsp119l digestion and subsequent filling up of the overhangs by Klenow polymerase. After blunt-end re-ligation, PCR on this plasmid was employed to remove the T7 RNA promoter and to concomitantly create a new Bsp119l site at the 5′ end of the gene. This second 23S rRNA gene construct was excised by Bsp119l/EcoRI digestion and cloned into the pBR322 vector that already contained the first copy of the 23S rRNA gene. The resulting plasmid contained two copies of the 23S rRNA gene that were ligated tail to head via the Bsp119l site thereby covalently connecting the 3′ and 5′ ends of the two genes. This plasmid was subsequently used as PCR template using the forward primer <u>TAATACGACTCACTATA</u>GG**G**AGCGTCCACAGCGGCG, which contained the T7 promoter sequence (underlined), and the reverse primer **G**TAACTTTTATCCGTTGA. Two additional Gs following the T7 promoter were introduced during PCR to ensure efficient transcription initiation. The position corresponding to the new 5′ end of the cp-23S rRNA at G2468 (*E.coli* nomenclature) is indicated in bold in the forward primer, while the position defining the new 3′ end (C2440) is bold in the reverse primer. The 2897 kb PCR product was purified and cp-23S rRNA was transcribed and purified as described previously (12).

Reconstitution of 50S subunits

50S subunits were assembled by using 10 pmol (0.24 A_{260} units) *in vitro* transcribed cp-23S rRNA, 10 pmol (0.01 A_{260} units) 5S rRNA, total 50S subunit proteins (0.29 equivalent units) and 20 pmol synthetic RNA CCCGGGGAUA<u>X</u>CAGGCUGAUCUCCCC in the presence of the macrolide antibiotic RU69874 (0.5 mM) in a total volume of 12.9 µl reconstitution buffer 20 mM Tris–HCl, pH 7.4, 4 mM $MgCl_2$, 400 mM NH_4Cl, 5 mM β-mercaptoethanol, 0.2 mM EDTA (12,15). The nucleotide corresponding to A2451 of 23S rRNA in the synthetic RNA oligonucleotide is indicated (<u>X</u>). The *in vitro* reconstitution procedure was performed essentially as described in (12), with the exception that the $MgCl_2$ concentration during the last incubation step was adjusted to 30 mM. The *in vitro* reconstituted 50S subunits were reassociated with 4 pmol native *T.aquaticus* 30S subunits as previously described (6).

Synthetic RNA oligonucleotides

Non-modified 26 nt RNAs as well as those containing 2′-deoxyadenosine, a deoxy-abasic site analog, inosine, purine, 2-aminopurine, 2,6-diaminopurine or the C3-linker were purchased from *Dharmacon*. The phosphoramidite building blocks of 1-methylinosine, N^6-methyladenosine, N^6,N^6-dimethyladenosine were synthesized following published procedures (16). The phosphoramidite of the ribose-abasic site analog was obtained from *Glen Research*. The phosphoramidite of 3-deazaadenosine was synthesized in eleven steps starting from inosine via a 5-amino-4-imidazolecarboxamide (AICA) riboside derivative. Details of the synthesis will be described elsewhere (K. Lang and R. Micura, manuscript in preparation). The modified building blocks were incorporated into RNA by solid-phase synthesis using the 2′-*O*-[(triisopropylsilyl)oxy]methyl (TOM) methodology (17–19). Deprotection and purification of the oligoribonucleotides was performed as described previously (20). All oligoribonucleotides were analyzed and confirmed by MALDI-TOF mass spectrometry.

Peptide bond formation

For the puromycin reaction, 10 pmol of 70S ribosomes were programmed with 60 pmol synthetic mRNA AAGGAG-AUAUAACA**AUG**UAAU (*Dharmacon*) containing a Shine–Dalgarno sequence (underlined) and a unique AUG codon (bold) and incubated with 0.3–3 pmol formyl-[^{3}H]Met-tRNA^{fMet} for 15 min at 37°C. The peptidyl transferase reaction was initiated by the addition of 2 mM puromycin and performed in 34 µl of the final buffer containing 7 mM Tris–HCl, pH 7.5, 11.8 mM HEPES/KOH pH 7.5, 11.6 mM $MgCl_2$, 167 mM NH_4Cl, 2.5 mM spermidine, 0.03 mM spermine, 4.1 mM β-mercaptoethanol and 0.07 mM EDTA at 37°C for the indicated periods of time. Reaction products were extracted into ethyl acetate and quantified by liquid scintillation counting as described in (12). In antibiotic inhibition experiments, 200 µM sparsomycin was added before puromycin addition. In all transpeptidation reactions, the background values (amount of product formed in samples containing only native *T.aquaticus* 30S subunits) were subtracted from all experimental data points. Under the applied conditions, peptide bond formation is a single turnover event. Chase experiments with deacylated tRNA^{Phe} (in 33-fold excess over the donor *N*-acetyl-[H^3]Phe-tRNA) added simultaneously with puromycin did not interfere with the measured reaction rates in poly(U) programmed 70S containing reconstituted 50S. However, addition of deacylated tRNA^{Phe} during the *N*-acetyl-[H^3]Phe-tRNA binding step completely inhibited transpeptidation (data not shown).

The reaction with CC-puromycin was carried out with gapped-cp-reconstituted 50S assembled from 10 pmol cp-23S rRNA containing the wt RNA 26mer or its deoxy-adenosine or deoxy-abasic 2451 analogs. The assay employed 3 pmol of *N*-acetyl-Phe-tRNA and 4 pmol [^{32}P]-end-labeled pCpCp-puromycin (*Dharmacon*), and was performed as described in (6). The radioactive species were separated by gel electrophoresis and quantified using the Molecular Dynamics Storm PhosphorImager.

Sucrose gradient analysis

Gapped-cp-reconstituted 50S subunits assembled from 40 pmol cp-23S rRNA and the 26mer containing the wild-type sequence, dA or the deoxy-abasic analog at A2451 were layered over a 10–40% sucrose gradient prepared in reconstitution buffer and centrifuged using a Beckmann SW-41 rotor as described (12).

RF1-mediated peptidyl-tRNA hydrolysis

Ten pmol 70S ribosomes containing gapped-cp-reconstituted 50S subunits were programmed with 250 pmol synthetic mRNA UUCAUGUAA (*Dharmacon*) and incubated with 0.3 pmol formyl-[^{3}H]Met-tRNA for 15 min at 30°C in 17.9 µl of the P-site binding buffer described in (9). The peptidyl-tRNA hydrolysis reaction was initiated by the addition of 15 pmol *T.thermophilus* RF1 (21) and performed at 25°C in a final volume of 35.8 µl as described in (9). The incubation time, 30 min, corresponded to the endpoint of the reaction catalyzed by reconstituted wild-type ribosomes. The reaction was stopped and quantified by liquid scintillation counting as described in (9).

Chase experiments with deacylated tRNA$^{f\,Met}$ (in 33-fold excess over f[^{3}H]Met-tRNA) using 70S ribosomes containing reconstituted 50S subunits revealed that peptidyl-tRNA hydrolysis is a single turnover reaction since the rate was not affected when excess of deacylated tRNA$^{f\,Met}$ was added simultaneously with RF1. In contrast, addition of excess deacylated tRNA$^{f\,Met}$ during f[^{3}H]Met-tRNA binding competed efficiently with RF1-mediated f[^{3}H]Met release (data not shown).

RESULTS

Use of circularly permuted 23S rRNA for 50S assembly

In order to site-specifically position modified nucleosides within the 23S rRNA, we developed a 'gapped-cp-reconstitution' approach which expands the potential of the previously described *in vitro* reconstitution system of *T.aquaticus* 50S subunits (12). As the major rRNA component for the *in vitro* assembly, we employed a circularly permuted 23S rRNA (cp-23S rRNA) whose natural 5′ and 3′ ends are covalently connected and new ends have been introduced elsewhere in the molecule. For chemical engineering of the PTC, a cp-23S rRNA was designed so that its 5′ end corresponds to position 2468 (*E.coli* nomenclature is used here and throughout the manuscript) and its 3′ end to 2440 (Figure 1A). This cp-23S rRNA places a gap of 26 nt in the PTC, including the conspicuous residue A2451. The missing 23S rRNA segment was chemically synthesized and used in combination with the cp-23S rRNA to assemble large ribosomal subunits *in vitro*. The reconstituted 50S subunits were reassociated with native 30S subunits from *T.aquaticus* and tested in a peptidyl transferase assay. Limiting amount of peptidyl-tRNA analog formyl-Met-tRNA was bound to the P-site and the reaction was initiated by the addition of saturating amounts of puromycin as an acceptor. Subunits assembled from cp-23S rRNA and the compensating RNA oligonucleotide showed

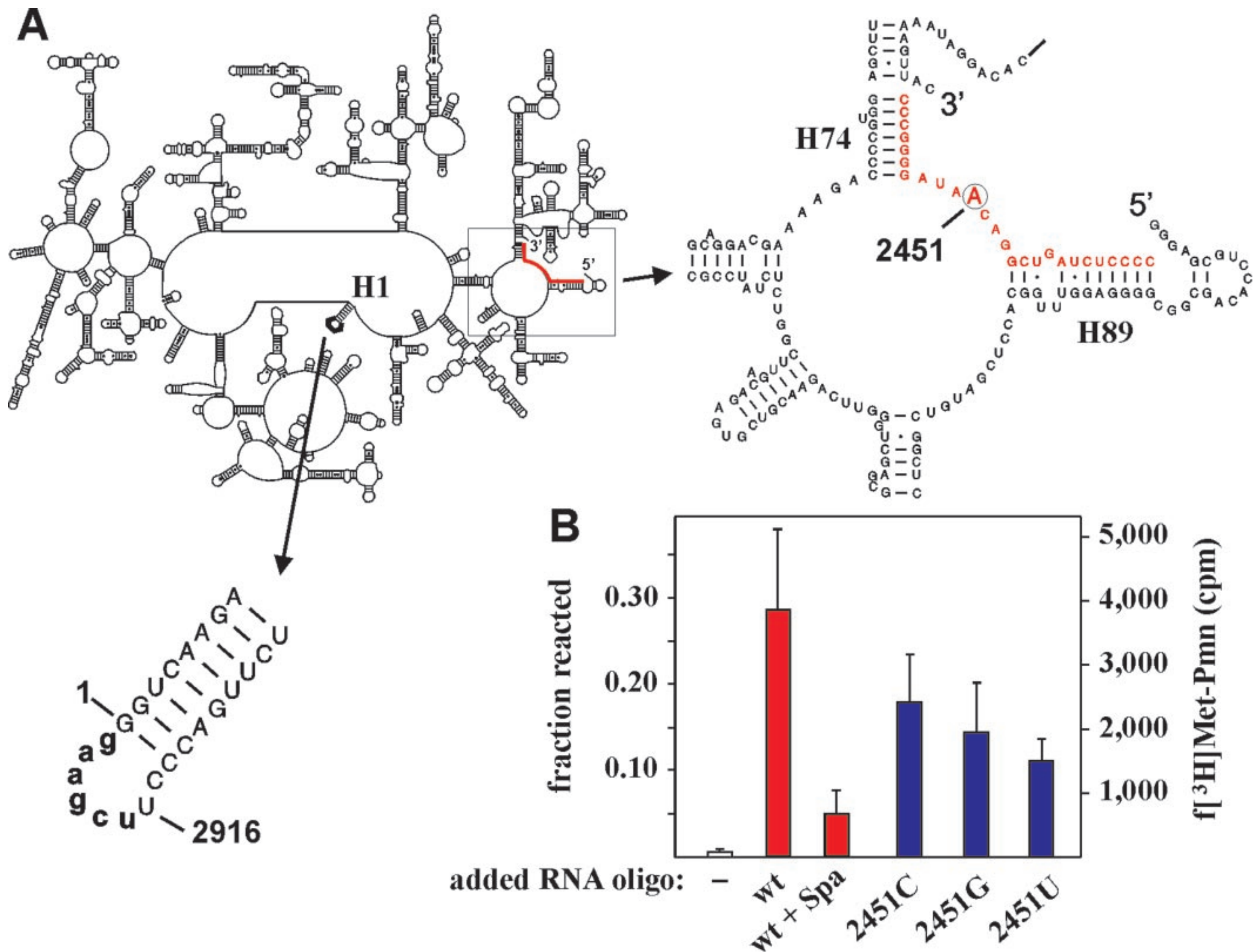


Figure 1. Structural and functional characteristics of reconstituted 50S subunits containing circularly permuted (cp) 23S rRNA (gapped-cp-reconstitution). (**A**) Schematic representation of the secondary structure of the cp-23S rRNA. The natural 5′ and 3′ ends of 23S rRNA, which form helix 1 (H1) in the native 50S subunit, were covalently connected (bold black line) and new endpoints at positions 2468 and 2440 (5′, 3′) in helices 89 (H89) and 74 (H74) have been introduced to generate the cp-23S rRNA used in this study. The sequence of helix 1 of the cp-23S rRNA is shown whereas the natural ends of native 23S rRNA are indicated by numbers. The additional nucleotides that were added during clone construction are in bold lower case letters. The chemically synthesized 26 nt long RNA fragment, which compensates for the missing 23S rRNA segment in the peptidyl transferase center (boxed), is shown in red. (**B**) The peptidyl transferase activity of ribosomes composed of gapped-cp-reconstituted 50S subunits and native *T.aquaticus* 30S subunits. Product yields in the absence of the compensating 26mer (−) or in the presence of fragments containing the wild-type sequence (wt; red bar) or carrying base changes (C, G, U; blue bars) at the position corresponding to A2451 of 23S rRNA are indicated. Yield of product formation of 50S carrying the wild-type sequence in the presence of 200 µM sparsomycin (+ Spa) are shown. In all cases, the mean and the SD values of the fraction of f[^{3}H]Met-tRNA that reacted with puromycin after 240 min of incubation are shown.

considerable peptidyl transferase activity and at the endpoint of the reaction (240 min) ~30% of peptidyl-tRNA was converted into the reaction product, fMet-puromycin. Control experiments with excess of deacylated tRNA added simultaneously with puromycin demonstrated that the peptidyl transfer reaction catalyzed by gapped-cp-reconstituted ribosomes is a single turnover event (data not shown). Therefore, the incomplete conversion of the substrate indicates that only a fraction of the reconstituted ribosomes was catalytically active. Nevertheless, in these ribosomes, product formation could be readily inhibited by the peptidyl transferase inhibitor sparsomycin (Figure 1B). This reveals the general integrity of the PTC and suggests that the short RNA fragment probably binds to its natural site within the PTC restoring its functionally competent structure. Importantly, it was necessary for the supplementing RNA fragment to be present from the very beginning of the reconstitution procedure. Addition of the RNA fragment at a later point did not yield active 50S particles (data not shown). Ribosomes composed of the cp-23S rRNA missing the 26 residues long RNA fragment of the PTC showed essentially no peptidyl transfer activity (Figure 1B).

'Natural' mutations at A2451 do not eliminate peptidyl transferase activity

Previous studies showed that mutations at A2451 only moderately affect peptidyl transferase activity (6,7). In accordance with these findings, ribosomes carrying the 26mer with all three base changes at 2451 showed <3-fold reduced product yields after 240 min of incubation (Figure 1B). It, therefore,

appears that the experimental system used here recapitulates at the qualitative level the functional activity of the PTC as seen previously with reconstituted mutant ribosomes that contained full-length 23S rRNA.

Modifications at the A2451 nucleobase have minor effects on peptide bond formation

The possibility to introduce non-natural nucleotide analogs into a synthetic RNA oligonucleotide allowed us to study the effects of fine changes at the nucleobase at position 2451 on peptidyl transfer activity. In total, we investigated eight different nucleoside analogs carrying base alterations. Modifications were introduced at the N1, C2, N3 or C6 position of adenine (Figure 2A). Particularly interesting was the 3-deaza A2451 where the nitrogen at position 3 was changed to a carbon, which renders this group unsuited for general acid/base catalysis. In our assay, however, none of the introduced base modifications at A2451 had a severe impact on the initial rates of transpeptidation (Figure 2C). Neither do they affect significantly the product yield at the endpoints (Figure 2B). Even attaching rather bulky groups to the base of A2451, such as N^6,N^6-dimethyl adenosine, had only minor consequences on the peptidyl transfer. Significantly, even ribosomes in which the entire base was removed, thereby creating a ribose-abasic site at position 2451 of 23S rRNA, retained almost full peptide bond formation activity compared to gapped-cp-reconstituted 50S containing the wt A2451 (Figure 2B and C). These data suggest that neither the nature, nor the general presence of a nucleobase at position 2451 is critical to form a peptide bond.

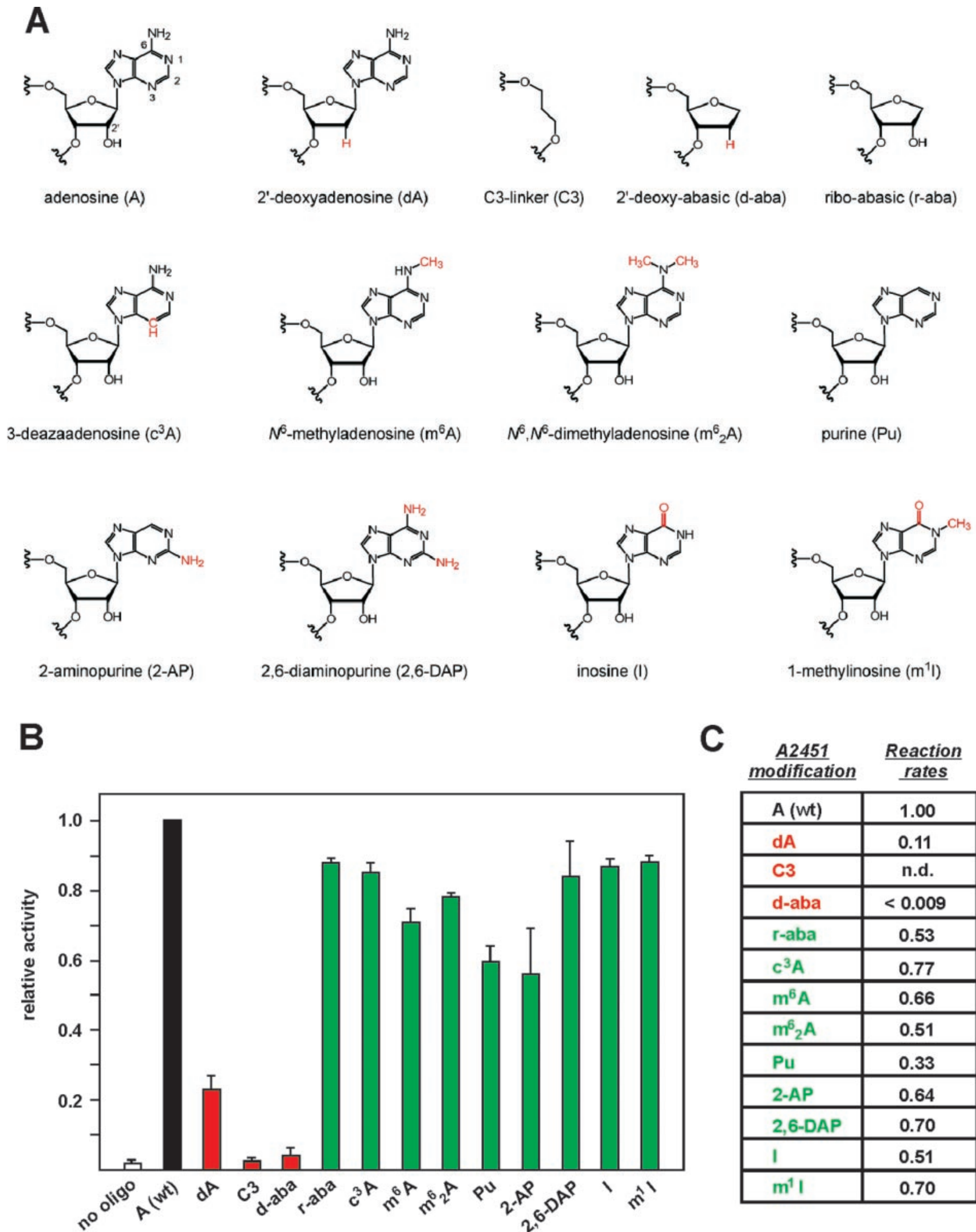
Removal of the A2451 ribose 2′-hydroxyl inhibits peptide bond formation

In addition to alterations in the nucleobase, the gapped-cp-reconstitution also allows to study transpeptidation in ribosomes that carry a chemically modified ribose. Compared to base modifications, alterations at the ribose at position 2451 more significantly compromised peptide bond formation. Removing the 2′-hydroxyl group of the abasic-site analog, thereby creating a deoxy-abasic site at 2451, almost completely deprived the ribosome of peptidyl transferase activity. This was in clear contrast to ribosomes with the 2451 ribo-abasic site, which remained almost fully active (Figure 2B). The difference in measured peptidyl transferase rates between ribosomes containing the deoxy- and the ribo-abasic site at 2451 is at least 60-fold (Figure 2C). This is striking, given the fact that the chemical composition of the PTCs of these ribosomes differ only by a single oxygen atom. Introducing a deoxy adenosine nucleoside at position 2451, thereby adding back the entire adenine base to the deoxy-abasic analog, recovered some peptidyl transferase activity though it remained lower than with any of the tested ribo-nucleobase analogs (Figures 2 and 3A). This indicates that the ribose 2′-hydroxyl at A2451 is not only in close proximity to the active site (11), but also hints at its potential functional importance for transpeptidation. In view of this result, it was not surprising that placing a C3-linker at A2451, where most of the ribose was deleted and three carbon atoms function as a placeholder for the nucleotide backbone, was not compatible with an active PTC (Figure 2).

To investigate if the reduced activities seen with 2′-deoxyribose-modified ribosomes are the result of a less efficient P-site tRNA binding, we increased the peptidyl-tRNA concentration 10-fold. This change in peptidyl-tRNA concentration did not markedly affect the yield of product formation or the relative rates of the reaction catalyzed by the 2451 2′-deoxyribose-modified ribosomes (data not shown) thus ruling out tRNA binding effects.

It has been shown recently that the minimal A-site substrate puromycin may behave differently than aminoacyl-tRNA in peptide bond formation employing mutant ribosomes (10). In this study, base changes at active site nucleotides, including A2451, resulted in strong defects in catalysis with puromycin while essentially no effects were seen with aminoacyl-tRNA as acceptor. This indicates that puromycin is hypersensitive to base changes in the PTC. In our system however, we do not see strong effects on transpeptidation with any of the tested modified-bases at A2451 in the puromycin assay. To investigate whether the reduced peptidyl transferase activities of the 2451 2′-deoxyribose-modified ribosomes (Figures 2 and 3) also result from a potential puromycin hypersensitivity, we re-tested the reactivity with a more natural A-site substrate. We used the donor *N*-acetyl-Phe-tRNA in combination with [^{32}P]pCpCp-puromycin, both present in excess to reconstituted ribosomes. pCpCp-puromycin mimics better than puromycin, the universally conserved CCA end of aminoacyl-tRNA with the cytidine residues making additional contacts with the PTC (1,11,22,23). This results in increased binding and acceptor activities of pCpCp-puromycin compared to puromycin (24). In spite of the extending contacts of this acceptor substrate with the ribosome, both 2451 2′-deoxyribose-substituted ribosomes showed a decrease in peptidyl transferase activity similar to what we observed previously in the standard puromycin assay (Figure 4). These results show that the reduced transpeptidation activities seen with ribosomes containing 2′-deoxyribose modifications at position 2451 were apparent in two different peptidyl transferase assays, and did not appear to critically depend on the nature of the A-site substrate used.

Though the results of chemical engineering of A2451 pointed at the importance of the ribose 2′-hydroxyl for peptide bond synthesis, the reduced activities of the modified 50S subunits could potentially stem from a less efficient *in vitro* assembly. Sucrose gradient profiles did not reveal significant differences in the overall reconstitution efficiency between 50S containing the wt A2451, the 2′-deoxy-A, or the deoxy-abasic analog in the PTC (Figure 3B). The ribosomal PTC catalyzes two major reactions, namely peptide bond formation and peptide release. Importantly, ribosomes containing the 2′-deoxy-A or the deoxy-abasic site analog at position 2451, substitutions that showed strong defects in peptide bond formation, had almost unimpaired peptide release activities. Both types of ribose 2′-deoxy-modified ribosomes reached essentially the same endpoint of product formation compared to ribosomes with ribo-A at position 2451 (Table 1). Since both transpeptidation and peptide release are single turnover reactions under the applied conditions (see Materials and Methods), these findings indicate that comparable fractions of wild-type or modified gapped-cp-reconstituted 50S subunits have a functionally competent PTC. This re-emphasizes the notion that the negative effects



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 2. Peptidyl transferase activity of ribosomes containing non-natural nucleoside analogs at A2451. (**A**) Chemical structures of all the tested nucleoside analogs. The introduced chemical modifications are depicted in red. (**B**) Product yields in the absence of the compensating 26mer (no oligo) or in the presence of fragments containing the wild-type sequence (wt; black bar), ribose sugar modifications (red bars) or carrying base modifications (green bars) at the position corresponding to A2451 of 23S rRNA are indicated. The yield of f-Met-puromycin formed with gapped-cp-reconstituted ribosomes carrying the wt 26mer after 240 min of incubation was taken as 1.0. Values shown represent the mean of at least three independent experiments. (**C**) Initial rates of peptide bond formation catalyzed by A2451-modified ribosomes. The initial rates were determined from experimental points in the linear range of the reactions within the first 30 min of incubation (with the exception of the deoxy-abasic ribosome, where the first time point that could be measured was at 240 min). The rates were normalized to the rate of reconstituted ribosomes containing the synthetic wild-type (wt) RNA fragment (A). In all cases, the background values (amount of product formed in reactions containing only native 30S subunits) were subtracted from all experimental data points.

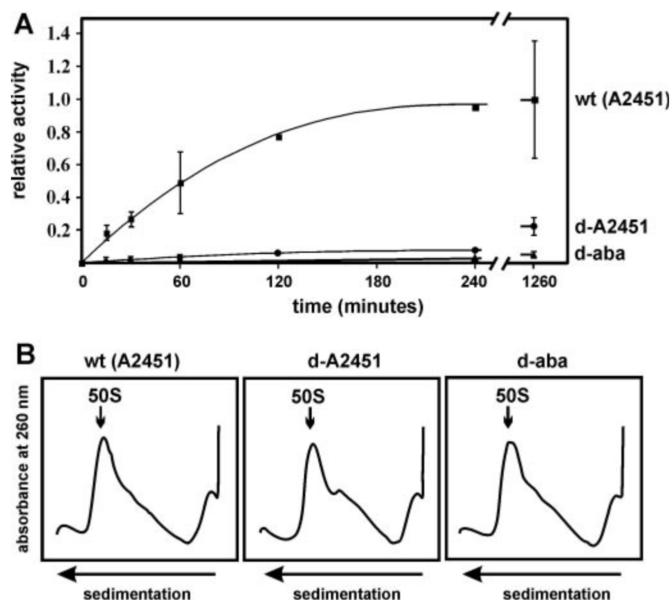


Figure 3. Peptidyl transferase activity of ribosomes containing 2′-deoxyribose modifications at A2451. (**A**) Time course of peptide bond formation promoted by ribosomes containing 50S reconstituted from cp-23S rRNA and the compensating 26mer with either adenosine (wt), 2′-deoxyadenosine (d-A2451), or a deoxy-abasic site analog (d-aba) at a position corresponding to A2451 of 23S rRNA. The curves represent the mean of 2–3 independent time course experiments, whereas the amount of product formed at the endpoint of the reaction catalyzed by ribosomes containing the wild-type 26mer was taken as 1.0. (**B**) Sucrose gradient analysis of gapped-cp-reconstituted large ribosomal subunits carrying adenosine (wt), 2′-deoxyadenosine (dA) or the deoxy-abasic analog (d-aba) at 2451. Vertical arrows indicate the mobility of native 50S subunits.

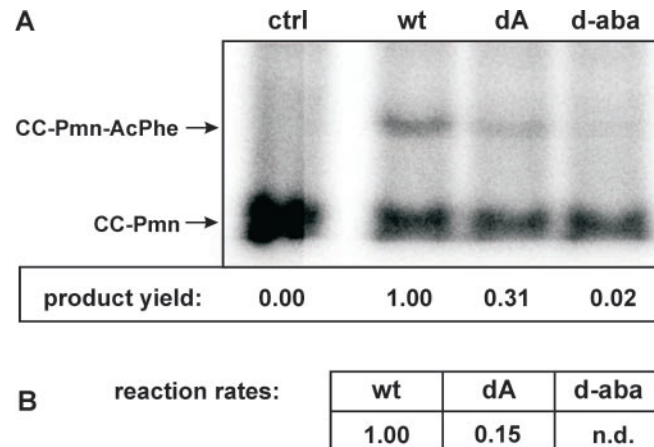


Figure 4. Peptidyl transferase activities of gapped-cp-reconstituted subunits containing 2′-deoxyribose modifications at A2451 using CC-puromycin as acceptor substrate. (**A**) The reaction between *N*-acetyl-Phe-tRNA and [^{32}P]CC-puromycin was carried out for 120 min, a time point that corresponded to the endpoint of the reaction catalyzed by reconstituted wild-type large subunits. The product *N*-acetyl-Phe-CC-puromycin (CC-Pmn-AcPhe) was resolved from CC-puromycin (CC-Pmn) by gel electrophoresis (6). The control reaction (ctrl) contained the whole reaction mixture except ribosomal particles. The relative yields of product formation by gapped-cp-reconstituted subunits containing adenosine (wt), 2′-deoxyadenosine (dA), or the deoxy-abasic analog (d-aba) at 2451 are shown below the gel. (**B**) The initial rates of peptide bond formation catalyzed by the wt or the deoxy-A2451-modified large ribosomal subunit were determined from experimental points within the first 45 min of incubation. During this incubation time, no product formation with ribosomal particles carrying the deoxy-abasic site analog at position 2451 could be measured (n.d.). The rates were normalized to the rate of reconstituted subunits containing the synthetic wild-type RNA fragment (wt).

Table 1. RF1-mediated peptide release activities of gapped-cp-reconstituted *T.aquaticus* ribosomes carrying nucleoside modifications at A2451 of 23S rRNA

	RF1-mediated hydrolysis[a]	Fraction formyl-Met released[b]
A2451 (wt)	100	0.27 ± 0.01
d-A2451	79 ± 4	0.22 ± 0.01
d-aba 2451	92 ± 6	0.25 ± 0.02

[a]Peptide release activity of ribosomes containing 50S subunits carrying the wild-type (wt) 26mer was taken as 100%. The incubation time, 30 min, corresponded to the endpoint of the reaction catalyzed by reconstituted wild-type ribosomes. Values shown represent an average of two independent experiments using RNA fragments carrying either the wt, the deoxy (d-A2451) or the deoxy-abasic (d-aba 2451) nucleoside analog at a position corresponding to A2451 of 23S rRNA.
[b]The fraction of f-[^{3}H]Met released from f-[^{3}H]Met-tRNA after 30 min of incubation.

Table 2. Peptidyl transferase activities of gapped-cp-reconstituted *T.aquaticus* ribosomes carrying nucleoside modifications at A2450 or A2453 of 23S rRNA

23S rRNA position	Modification	Reaction rates[a]
wt	none	1.00
A2450	dA	1.29
	d-aba	0.36
	r-aba	0.61
A2453	dA	1.23
	d-aba	n.d.
	r-aba	0.53

[a]The peptidyl transfer rate of ribosomes containing 50S subunits carrying the wild-type (wt) 26mer was taken as 1.00 and compared to the rates of ribosomes carrying the deoxy adenine (dA), the deoxy-abasic site (d-aba) or the ribose-abasic site analog (r-aba) at positions A2450 or A2453. The initial rates were determined from experimental points in the linear range of the reactions within the first 30 min of incubation. The d-aba 2453 modification was not tested (n.d.).

of removing the 2′-hydroxyl at A2451 are specific for transpeptidation.

To test whether the removal of a ribose 2′-hydroxyl group or the complete nucleobase had similar negative effects at other conserved residues in the PTC, we tested the peptidyl transferase activities of ribosomes containing a deoxy adenosine, a deoxy-abasic or a ribose-abasic site at positions A2450 or A2453. At both sites, the deoxy-adenosine modifications were tolerated and peptide bond formation proceeded with rates comparable to the wild-type control (Table 2). Furthermore, introduction of ribose-abasic analogs at residues 2450 or 2453 had only relatively small effects. In clear contrast to ribosomes with deoxy-abasic 2451, ribosomes with a deoxy-abasic site at 2450 were at least 40-fold more active in transpeptidation and readily formed the products of the peptidyl transferase reaction (compare Table 2 with Figure 2C). These results suggest that the inhibition of the peptidyl transferase activity by introducing a 2′-deoxyribose at position A2451 of 23S rRNA are specific and re-emphasize a special role of this ribose 2′-hydroxyl in functions of the PTC.

DISCUSSION

In this study, we used circularly permuted 23S rRNA (cp-23S rRNA) for engineering the chemical make-up of the ribosomal PTC. The generation of the cp-23S rRNA is possible due to the proximity of the natural 5′ and 3′ ends of mature 23S rRNA in the tertiary structure of the ribosome. The 5′ and 3′ ends of natural 23S rRNA are base-paired to each other forming helix 1 of 23S rRNA (25). Helix 1 is located on the solvent side of the 50S ribosomal subunit, far from known functional centers. Therefore, covalent connection of the 23S rRNA ends is unlikely to cause major structural changes in the large ribosomal subunit.

The 'gapped-cp-reconstitution' of the large ribosomal subunit used here for engineering the chemical environment of one of the key nucleotides in the PTC, A2451, employed a cp-23S rRNA that lacked a 26 nt long RNA fragment encompassing A2451. The missing rRNA segment was chemically synthesized to either contain the wild-type sequence, natural mutations or non-natural nucleoside analogs at position A2451 of 23S rRNA. The synthetic RNA fragment was provided *in trans* to the *in vitro* reconstitution of 50S ribosomal subunits. Base-pairing with the complementary sites of the cp-23S rRNA transcript locks the synthetic rRNA piece in its native place thus restoring the structure of 23S rRNA (Figure 1A). The main benefit of using cp-23S rRNA in these experiments is that it allows the reconstitution of the large ribosomal subunit from two rather than three pieces of rRNA. Using three or more rRNA fragments markedly reduces the efficiency of the *in vitro* assembly procedure [(26), N. Polacek and A. Mankin, unpublished data, 2005]. The obvious advantage of the gapped-cp-reconstitution system is that it allows to incorporate non-natural nucleosides at desired sites within 23S rRNA. This technique significantly expands the chemical repertoire of nucleotide residues that can be placed at specific 23S rRNA sites. In this work, we substituted functional groups of the nitrogen base or the ribose of A2451 and tested the effects on the ability of the ribosome to catalyze peptide bond formation.

Our major finding is that the removal of the ribose 2′-hydroxyl group at A2451 reproducibly reduces the ribosomes' ability to promote peptide bond formation in a standard puromycin assay. Subsequent removal of the base, thereby creating a deoxy-abasic site, almost completely depleted the ribosome of its ability to catalyze peptidyl transfer (Figures 2B and 3A). Notably, placing a ribose-abasic instead of the deoxy-abasic site at 2451, thereby reintroducing a single hydroxyl group to the ribose 2′ position, stimulates the reaction rate at least 60-fold, thereby restoring near wild-type levels of peptidyl transferase activity (Figure 2B). This indicates that the base at A2451 does not contribute significantly to transpeptidation in our assay system. In support of this, all the tested base modifications at A2451 had relatively minor impact on the performance of the PTC (Figure 2). This was the case for placing bulky groups at the N1 (1-methyl inosine), the C2 (e.g. 2,6-diaminopurine) or the C6 position (e.g. N^6,N^6-dimethyl adenosine) as well as for changing an allegedly functional nitrogen atom at position 3 to a carbon (3-deaza adenosine). To confirm these findings in a peptidyl transfer assay employing a more natural A-site substrate, we replaced puromycin by CC-puromycin. The two cytidine residues enable this acceptor substrate to make additional important hydrogen bonding interactions with the A-loop of the PTC (1,11,22,23) thus enhancing its resemblance to the natural acceptor substrate (10,24). Using this assay, the negative effects of removing the ribose 2′-hydroxyl group at A2451 remained apparent and similar rate reductions compared to the puromycin reaction were observed (Figure 4). Taken together, these results are compatible with the notion that this 2′-hydroxyl is of functional or structural significance for transpeptidation. Although the theoretical possibility remains that the observed differences in activity is the result of a less efficient subunit reconstitution with the 2′-deoxyadenosine or the deoxy-abasic site analog at 2451, this scenario seems unlikely. Our observation that 'wild-type' or 'modified' ribosomes showed indistinguishable sucrose gradient profiles argue against major reconstitution defects (Figure 3B). This conclusion is further supported by the results of the peptide release assay where the deoxy-A2451 and the 2451 deoxy-abasic versions of the reconstituted ribosomes showed similar product yields at the reaction endpoint (Table 1) compared to the wild-type control. In a single turnover reaction with incomplete conversion of the substrate into the product, the portion of the reacted substrate shows the active fraction of the enzyme. Therefore, the results of the release assay demonstrate that comparable fractions of catalytically active 50S subunits were assembled with the wild-type RNA 26mer, or its deoxy-A or deoxy-abasic versions.

Our findings reveal a possible functional or structural importance of the 2′-hydroxyl group of A2451 for peptide bond formation. This interpretation is compatible with recent *in vivo* data showing that snoRNA-guided methylation of the 2′-hydroxyl group at A2451 resulted in a lethal growth phenotype in yeast (27). In the crystallographic structure of the PTC, this hydroxyl group was found to be in hydrogen bonding distance to the reactive amino group of the A-site substrate (11). The ribose 2′-hydroxyl of 2451 may therefore potentially help to orient the attacking amino group of the acceptor substrate. Earlier theoretical studies as well as more recent experimental work suggest that peptide bond formation is mainly driven by positioning of the two reactants, the α-amino group from the A-site aminoacyl-tRNA and the carbonyl carbon from the P-site peptidyl-tRNA (1,6–8,10,28,29). The role and contribution of chemical catalysis in the overall scheme of events that take place during the formation of a new peptide bond is less clear. Recently, a model was proposed in which the 2′-hydroxyl of the terminal nucleotide of the P-tRNA is directly involved in substrate-assisted catalysis of the peptide bond (30). This model is compatible with the notion that the main role of the ribosome to promote efficient peptide bond formation is to provide the molecular template where the two reactants can be properly aligned. The 2′-hydroxyl of A2451 of 23S rRNA might be one of the critical groups to fulfill this task. The unperturbed p*K*a of the 2′-hydroxyl of RNA is far from neutral, ∼13 (31). Therefore, its direct participation in general acid–base catalysis is unlikely. However, by analogy with the model proposed for the ribose 2′-hydroxyl of the terminal A of P-tRNA, it may possibly serve as a hydrogen bond acceptor in the transition state intermediate (30,32,33) or be involved in metal ion coordination similar to catalytically important nucleotides in other ribozymes (34). We cannot exclude, however, that the removal of the 2′-hydroxyl at A2451 may somehow disturb the functionally competent

conformation of the active site, therefore affecting accurate placing of the reaction substrates in the PTC.

We would like to note that the gapped-cp-reconstitution inherits some intrinsic limitations of the 'standard' *in vitro* reconstitution systems. One of the major shortcomings is that peptide bond synthesis does not proceed at rates comparable to that found *in vivo*. Therefore, our system does not allow the measurement of subtle changes in peptidyl transferase activity, but is applicable to pinpoint crucial groups in the PTC whose replacement or removal significantly affects the overall rate of transpeptidation, such as the ribose 2′-hydroxyl at A2451.

In conclusion, we have developed an experimental approach, the gapped-cp-reconstitution of the large ribosomal subunit, which makes it possible to place modified nucleotides at specific positions within 23S rRNA. By this approach, we showed that removal of the ribose 2′-hydroxyl group at A2451 of 23S rRNA interfered with peptide bond formation. In contrast, all base modifications and even the deletion of the entire nucleobase were largely tolerated, as long as the 2′-hydroxyl remained in place. Our data suggest that the ribose 2′-hydroxyl at position A2451 plays an important role in the catalytic activity of the PTC.

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