Role of a ribosomal RNA phosphate oxygen during the EF-G–triggered GTP hydrolysis

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Elongation factor-catalyzed GTP hydrolysis is a key reaction during the ribosomal elongation cycle. Recent crystal structures of G proteins, such as elongation factor G (EF-G) bound to the ribosome, as well as many biochemical studies, provide evidence that the direct interaction of translational GTPases (trGTPases) with the sarcin-ricin loop (SRL) of ribosomal RNA (rRNA) is pivotal for hydrolysis. However, the precise mechanism remains elusive and is intensively debated. Based on the close proximity of the phosphate oxygen of A2662 of the SRL to the supposedly catalytic histidine of EF-G (His87), we probed this interaction by an atomic mutagenesis approach. We individually replaced either of the two nonbridging phosphate oxygens at A2662 with a methyl group by the introduction of a methylphosphonate instead of the natural phosphate in fully functional, reconstituted bacterial ribosomes. Our major finding was that only one of the two resulting diastereomers, the S methylphosphonate, was compatible with efficient GTase activation on EF-G. The same trend was observed for a second trGTase, namely EF4 (LepA). In addition, we provide evidence that the negative charge of the A2662 phosphate group must be retained for uncompromised activity in GTP hydrolysis. In summary, our data strongly corroborate that the nonbridging phosphate oxygen at A2662 positions His84 in its catalytic conformation, thus enabling it to act as a general base by subtracting a proton from the hydrolytic water that attacks the γ-phosphate of GTP (12). This model stimulated an intense scientific discussion in the field (22, 23), because it is not fully compatible with certain biochemical, genetic (24), structural (25), and molecular dynamics simulation (26) data.

To shed light on the possible function of the A2662 phosphate oxygen for activation of trGTPases, we applied here an atomic mutagenesis approach (27). The core of this technique is based on the in vitro reconstitution of functional 50S ribosomal particles, we show that a nonbridging phosphate oxygen of rRNA is critical in ribosome association. Although it has been shown by structural and biochemical studies that the sarcin-ricin loop of ribosomal RNA (rRNA) closely approaches the supposedly catalytic His of elongation factor (EF)-G and EF-Tu and thereby may contribute to GTP hydrolysis, the exact mechanism of activation remains unclear and is a matter of controversial debate. Using the atomic mutagenesis approach that allows incorporation of nonnatural 23S rRNA nucleotides into 70S particles, we show that a nonbridging phosphate oxygen of rRNA is key for GTP hydrolysis of translational GTPases. To date, this is the second rRNA backbone residue shown to be critical in ribosome function.

Significance

Translational GTPases are key players in ribosomal protein synthesis. Their intrinsic GTase activity is low and is stimulated by ribosome association. Although it has been shown by structural and biochemical studies that the sarcin-ricin loop of ribosomal RNA (rRNA) closely approaches the supposedly catalytic His of elongation factor (EF)-G and EF-Tu and thereby may contribute to GTP hydrolysis, the exact mechanism of activation remains unclear and is a matter of controversial debate. Using the atomic mutagenesis approach that allows incorporation of nonnatural 23S rRNA nucleotides into 70S particles, we show that a nonbridging phosphate oxygen of rRNA is key for GTP hydrolysis of translational GTPases. To date, this is the second rRNA backbone residue shown to be critical in ribosome function.


The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4Y27).

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Here we applied this method for the first time, to our knowledge, to address the role of the nonbridging phosphate oxygen at A2662 in affecting the acid-base behavior of the conserved His87 and its consequences on GTP hydrolysis.

The methylphosphonate modification generates chirality at the phosphor atom and results in two possible diastereomeric RNAs (Rp and Sp; Cahn–Ingold–Prelog nomenclature is used throughout the manuscript) (28). Importantly, our experimental design allowed individual access to either of the two diastereomers (Fig. 2). Solid-phase synthesis first provided a diastereomeric mixture of the short 8-nt methylphosphonate oligonucleotide covering the sequence from C2658 to A2665, followed by separation of the two diastereomeric strands using reversed-phase HPLC (Fig. 2). Then, templated enzymatic ligation to achieve the required 45-nt SRL stem-loop segment was conducted with either of the two RNA diastereomers and proceeded in high yields (Fig. 2B).

To verify the structural integrity of the methylphosphonate modification that was used in combination with a 2′-deoxy or a 2′-methyl group at A2662 to avoid strand cleavage (31), we performed imino proton 1H NMR spectroscopy of a corresponding 27-nt SRL RNA, namely 5′-UGC UCC UAG UAC-3′ (Fig. 3). Both diastereomers showed nearly identical spectra compared with the WT RNA with the natural phosphodiester moiety (Fig. 3B). This observation unequivocally confirmed proper folding into the hairpin in aqueous buffer solution. Additionally, the stereochernical assignment of the two diastereomeric RNAs was tentatively achieved based on nuclear Overhauser enhancement (NOE) patterns observed in a NOESY 1H,1H NMR spectrum (Fig. 3D).

To obtain even more detailed structural insights, we put significant efforts into crystallography and indeed succeeded in solving the crystal structure of the 2′-OCH3-G2661–mP-A2662 Rp diastereomer of the 27-nt SRL RNA at 1.0 Å resolution (Fig. 4 and Table S1). The high-resolution structure reveals that the methylphosphonate adopts a similar conformation as observed for the specific phosphate in the natural RNA (RMSD of 0.08 Å with the unmodified isolated SRL (Fig. 4 and Fig. S1) and RMSD of 0.84 Å within the context of the 70S ribosome, PDB 4YBB (34) (Fig. S2)). The methyl group of the Rp methylphosphonate at A2662 is directed outward and coincides with the orientation of the native proSP nonbridging oxygen.

Taken together, our NMR spectroscopic and X-ray crystallographic analysis provided strong evidence for the structural integrity of the methylphosphonate modification, which represented a fundamental prerequisite for the functional studies described below.
A2662 Methylphosphonate Does Not Affect Overall 50S Assembly.

The synthetic strand was subsequently placed into the 50S ribosomal subunit via the atomic mutagenesis procedure (27). First, we addressed the correct assembly of reconstituted ribosomes, carrying either the WT SRL sequence or one of the methylphosphonate stereoisomers by testing their ability to catalyze peptide bond formation via the puromycin reaction. Results of the puromycin assay showed that both the synthetic SRL and its methylphosphonate analog at A2662 (0.001 s−1) (Fig. 5B). Within the first 15 min. Ribosomes carrying the methylphosphonate in the $S_P$ configuration were still able to stimulate EF-G GTP hydrolysis, although hydrolysis was reduced compared with the WT control (Fig. 5A). We determined the initial rate of GTP hydrolysis, which was 0.068 s−1 and therefore about eightfold reduced compared with reconstituted ribosomes harboring the WT SRL oligo (0.535 s−1). These data are consistent with our structural data of the SRL, indicating that in the $S_P$ stereoisomer the methyl group points toward the inside of the SRL loop (Figs. 3A and 4), whereas the remaining nonbridging phosphate oxygen is directed outward and faces the catalytic center of EF-G. It can thus potentially activate the conserved His87. Ribosomes carrying the methylphosphonate in the $R_P$ configuration did not stimulate EF-G GTP hydrolysis significantly above background and revealed a drastically 37-fold reduced GTPase rate (Fig. 5A). In our previous study using the same in vitro reconstitution construct as here (Fig. 1B), we demonstrated that EF-G binding to T. aquaticus ribosomes was not affected by manipulations or ablation of the SRL (15), thus excluding binding defects as cause of the observed differences in EF-G GTPase. These results clearly demonstrate that the nonbridging phosphate oxygen of A2662 in the pro$S_P$ configuration plays a critical role in stimulating EF-G GTP hydrolysis.

As it was proposed that the mechanism of GTPase stimulation by the ribosome and GTP hydrolysis is conserved for all tGTPases (12), we tested ribosomes carrying the methylphosphonate modification for their ability to stimulate GTP hydrolysis of EF4 (LepA). The overall GTPase activity of EF4 in our assay was markedly lower, about fourfold reduced, compared with EF-G. In accordance with the EF-G data above, essentially no EF4 GTP hydrolysis was observed in the presence of ribosomes carrying the $R_P$ stereoisomer of the methylphosphonate analog at A2662 (0.001 s−1) (Fig. 5B).

Only One A2662 Methylphosphonate Diastereomer ($R_P$) Interferes with GTPase Activation. To test the modified ribosomes for stimulation of EF-G GTP hydrolysis, we performed an uncoupled GTPase assay (15). We used radioactively labeled GTP and followed GTP hydrolysis over time. GTP hydrolysis in the presence of ribosomes lacking the complementing SRL RNA oligonucleotide was considered as background and subtracted from all values. Ribosomes carrying the unmodified (WT) SRL sequence very efficiently stimulated EF-G GTP hydrolysis (Fig. 5). Nearly all GTP, present in the reaction, was hydrolyzed by EF-G.
HPO$_4^{2-}$ pK$_a$ 6.8 (36); or GMP pK$_a$ 6.2 and GMPS pK$_a$ 5.0 (37) and thus, it is not surprising that the thiophosphate analog of A2662 was active in the EF-G GTPase assay (Fig. 6A and Fig. S4A).

More precisely, both thiophosphate diastereomers could stimulate GTP hydrolysis as efficiently as the WT control. These data support the conclusion that the negative charge of the backbone moiety between SRL G2661 and A2662 represents a crucial determinant in the mechanistic scenario of GTPase activation.

Elimination of the Negative Charge at the A2662 Renders GTP Hydrolysis pH Dependent. It has been shown before that GTP hydrolysis by EF-Tu is pH independent (in the range between pH 6.5 and 8.5) and therefore a primary role of His84 (EF-Tu) as a general base in GTP hydrolysis was excluded (24). However, molecular simulation experiments lead to the suggestion that removal of the negative charge at the phosphate backbone at position A2662 would render the reaction pH dependent (38). Consequently, we decided to test ribosomes carrying the methylphosphonate in the $S_P$ configuration in activation of EF-G GTP hydrolysis at different pH values between 7.4 and 6.0. In parallel, we tested ribosomes carrying the WT SRL sequence, for which we expected pH independence as demonstrated previously for EF-Tu and ribosomes composed of natural 50S (24). It has been shown previously that within this pH range both the elongation factor and the ribosome stay intact (39, 40). In our hands, reconstituted ribosomes harboring the WT SRL fragment efficiently stimulated EF-G GTP hydrolysis at all pH tested; only at the lowest pH 6.0 did we observe a slight decrease in the reaction rate (Fig. 6B and Fig. S4B). Modified ribosomes containing a methylphosphonate in the $S_P$ configuration at position A2662, however, did not efficiently stimulate GTP hydrolysis at lower pH values, and the relative reaction rates dropped markedly compared with the WT control (Fig. 6B). Thus, substituting the nonbridging oxygen by a methyl group and concomitantly eliminating the negative charge on the other hand, the $S_P$ diastereomer was able to trigger EF4 GTPase, albeit to a lesser degree than the unmodified WT SRL fragment (Fig. 5B).

In sum, these results demonstrate that the nonbridging phosphate oxygen in the pro$S_P$ configuration that points toward the key GTPase residue His87 (see refs. 12 and 20 and our own structural data, Figs. 3A and 4) is essential for stimulation of GTP hydrolysis by EF-G and EF4.

A2662 Thiophosphate Diastereomers Both Have WT Activities in GTPase Hydrolysis. For an additional reference experiment, we substituted the nonbridging oxygens of the original phosphate at position A2662 by sulfur atoms (Fig. 1C, Right). SRL strands with both thiophosphate configurations were individually synthesized using the same strategy as elaborated for the methylphosphonates described above (Fig. 2). Subsequently, these RNAs were incorporated into the 50S ribosomal subunit. Like a phosphodiester group in the RNA backbone, the thiophosphate analog is charged, and therefore, its chemical properties are much closer to the original phosphate compared with the methylphosphonate. Its pK$_a$ value is estimated to be decreased by only about one unit [compare, for example, HSP3$_2$ pK$_a$ 5.5 and $S_P$ configuration at position A2662 by sulfur atoms (Fig. 1C, Right). SRL strands with both thiophosphate configurations were individually synthesized using the same strategy as elaborated for the methylphosphonates described above (Fig. 2). Subsequently, these RNAs were incorporated into the 50S ribosomal subunit. Like a phosphodiester group in the RNA backbone, the thiophosphate analog is charged, and therefore, its chemical properties are much closer to the original phosphate compared with the methylphosphonate. Its pK$_a$ value is estimated to be decreased by only about one unit [compare, for example, HSP3$_2$ pK$_a$ 5.5 and

Fig. 4. X-ray structure of a G2661-A2662 methylphosphonate-modified 27 nt SRL RNA at 1.0-Å resolution. (A) Overlay of modified and unmodified (53) RNA (PDB ID codes 4Y27 and 3DVZ) shows that the two structures are nearly identical, and also at the site of modification (RMSD, 0.08 Å). The R$_p$ methylphosphonate at A2662 is shown in green. (B) The 2Fo-Fc electron density map is shown contoured at the 1.0σ level around residues G2659 and A2662, clearly showing the density corresponding to the methylphosphonate modification on the latter residue. Two surrounding water molecules are depicted with red spheres. RNA sequence: 5′-UGCUCCUAGUACGA-3′.

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Fig. 5. Uncoupled EF-G GTPase activities using reconstituted ribosomes with the A2662 methylphosphonate SRL modification. (A) Representative time course of ribosome-dependent uncoupled EF-G GTP hydrolysis, using reconstituted ribosomes (without or with WT complementing RNA oligomer). (Top) Products were separated via TLC and visualized by phosphor imaging. GTP hydrolysis was quantified using Aida image analysis software. (Middle) Means and the SD of three independent experiments. Rates of GTP hydrolysis [k$_p$] were calculated from the first three time points, for relative GTP hydrolysis rates (k$_p$/k$_r$). WT hydrolysis rate was set to 1 (Bottom). (B) Uncoupled EF4 GTPase activity stimulated by reconstituted ribosomes. Experiments and analysis were performed as described above for EF-G. Modified SRL oligomers contained the 2′-OCH$_3$-G2661-mP-A2662 unit.
at A2662 renders the EF-G promoted GTP hydrolysis reaction pH sensitive.

Discussion

trGTPases possess low intrinsic GTP hydrolysis activity, which is, however, substantially stimulated by ribosome association, suggesting that a part of the ribosome is involved in catalysis or in some conformational arrangements in the trGTPases required for GTP hydrolysis. It has been shown by structural and biochemical data that residues G2655, A2660, G2661, and A2662 (9, 10, 12) of the SRL are involved in binding of EF-Tu or EF-G to the ribosome, suggesting they may also contribute to GTPase activation. Using the atomic mutagenesis approach, the nucleobase exocyclic amino group of A2660 was identified as critical for EF-G GTPase activation; however, the exact contribution of this residue remained unclear (15). One possible scenario is that it contributes to the correct arrangement of the catalytic center of trGTPases by stacking interactions, which is emphasized by a recent crystal structure of EF-G on the ribosome, where A2660 was suggested to stack on Arg660 of EF-G (18).

Another residue of the 23S rRNA, which was proposed to be involved in GTPase activation, is the nonbridging phosphate oxygen of A2662. Based on the structure of EF-Tu on the ribosome, Vorhees et al. suggested a mechanism for GTP hydrolysis. In this model, His84 acts as a general base catalyst and is activated by the phosphate oxygen of A2662 (12). However, as GTP hydrolysis is pH independent, one may exclude that His84 functions as a general base catalyst (24). Furthermore, the fact that the conserved histidine is surrounded by several negative charges indicates its pKₐ to be elevated and thus its side chain to be protonated at physiological pH (41). Nevertheless, the proposed general base mechanism continues to be intensively discussed and the role of the SRL, and the conserved histidine remains unclear (22, 23). Recent studies favor a model where GTP hydrolysis is activated via substrate-assisted catalysis, thereby highlighting the importance of functional groups on the GTP substrate for the phosphoryl transfer mechanism (41, 42). However, the contribution of the nonbridging phosphate oxygen at position 2662 of rRNA to GTP hydrolysis is still unclear.

Here we addressed this open point by using the atomic mutagenesis approach to site specifically modify the phosphate backbone of the rRNA. Thereby, we demonstrated that one of the nonbridging phosphate oxygen (proSP) at A2662 is involved in activation of EF-G GTP hydrolysis, as its replacement with a methyl group results in ribosomes severely hampered in triggering EF-G GTPase (Fig. 5). On the other hand, ribosomes harboring the SP diastereomer of the methylphosphonate-modified SRL, which renders the residual nonbridging oxygen in proper orientation, directed toward the crucial histidine (Fig. 3), still possess substantial activity in triggering GTP hydrolysis. This observation is evidence that the mere introduction of a methyl group at this position of the SRL does not globally disrupt RNA architecture (Fig. 4A and Fig. S1); thus, the observed effects with the two diastereomers can be considered as specific. Nevertheless, the active stereoisomer (SP) did not stimulate EF-G GTPase hydrolysis as efficiently as ribosomes carrying a regular phosphodiester bridge between positions G2661 and A2662. It is possible that replacing the nonbridging proSP oxygen by the more bulky methyl group renders the SRL structure less compact. In any case, this replacement unsets one original hydrogen bond, between the proSP oxygen and the exocyclic amino group of G2659. Also, the NMR spectra indicated that rotation of the methyl group (SP diastereomer) is slightly hindered at low temperature (Fig. 3C, Lower) compared with rotation of the methyl group of the corresponding proR oxygen (Fig. 3C, Upper). Thus, it cannot be completely excluded that introducing a methyl group in the SP configuration may slightly affect the functionally competent loop conformation for efficiently triggering GTP hydrolysis.

A more likely and favored explanation for the reactive, but eightfold reduced, activity of the SP configurated methylphosphonate is the following: the introduction of the methyl group instead of the nonbridging oxygen removes a negative charge at the A2662 backbone. This charge, however, is substantial for the interaction with His87, enabling the formation of a very strong hydrogen bond. Removing the charge at the nonbridging oxygen (SP diastereomer) is consistent with weakening these interactions and hence reducing activity for GTP hydrolysis. Likewise, total replacement of the nonbridging oxygen by a methyl group (R₈ diastereomer) is consistent with abrogating these interactions and consequently abolishing substrate hydrolysis.

The negative charge of the phosphate may contribute to increase the pKₐ of the catalytic histidine and thus enable protonation...
of the imidazole nitrogen as previously proposed by computer simulations (41). The protonated histidine could then donate this proton to form a hydrogen bond to the catalytic water. In this scenario, the water is then ideally positioned in a way that allows the γ-phosphate of GTP to subtract a water proton. This substrate-generated hydroxide ion subsequently attacks the γ-phosphate, leading to GTP hydrolysis (Fig. 7). In the A2662 methylphosphonate-modified ribosome, the active S₅ stereoisomer is still able to hydrogen bond to the active site histidine; however, due to the absence of the negative charge at the S₅ backbone, the pKₐ shift at the catalytic histidine appears not to be as substantial as in ribosomes carrying a phosphodiestere bond (Fig. 7). This catalytic scenario and the significance of the negative charge at A2662 are compatible with the phosphorothioate-modified S₅, because both stereoisomers carry a negative charge and trigger GTPase as efficiently as the WT control (Fig. 6). This mechanistic model is also compatible with recent in silico approaches that predict the catalytic histidine (His84 of Escherichia coli EF-Tu) to be protonated and to be part of an allosteric repositioning of active site residues (38). The authors suggest that for such an allosteric model, the electrostatic interaction between the positively charged histidine and the negatively charged phosphate oxygen at A2662 is important, and they predict that the removal of the latter charge would render GTP hydrolysis pH dependent. Our data are fully compatible with this prediction because the elimination of the negative charge at A2662 results in a GTP hydrolysis reaction that is inhibited at low pH values compared with the WT control (Fig. 6). Although our data contribute to the understanding of the role of the nonbridging phosphate oxygen at A2662 of the S₅ for ribosome-triggered GTP hydrolysis, it does not yet allow a complete mechanistic model to be drawn. Very recently another residue, namely an aspartate residue (Asp21 of EF-Tu), has been shown to be crucial for stimulation of GTP hydrolysis by EF-Tu on cognate decoding, whereas it is dispensable for intrinsic GTPase activity (42). It was suggested that basal GTP hydrolysis of trGTPases does cause the elimination of the negative charge at 3′-OCH₃-G methyl phosphonamide from ChemoGenes, dG, and 2′-OCH₃-G methyl phosphonamidates from ChemGenes and Glen Research, and polystyrene support from GE Healthcare (Custom Support, 80 μmol/g, PS 200). All oligonucleotides were synthesized on an ABI 394 Nucleic Acid Synthesizer following standard methods: detritylation (80 s) with dichloroacetic acid/1,2-dichloroethane (4/96); coupling (2 min for standard amidites and 6 min for methylphosphonamidites) with phosphorimidates/acetonitrile (0.3 M × 360 μL) and benzylthiophosphate/tetrazole/acetonitrile (0.3 M × 360 μL); capping (3 × 0.4 min, Cap A/Cap B = 1/1) with Cap A: 4-(dimethylamino)pyridine in acetonitrile (0.5 M) and Cap B: AcO/C/Sym-collidine/acetonitrile (2/3/5); and oxidation (1.0 min) with iodosylbenzene (20 mM) in tetrahydrofuran (THF)/pyridine/H₂O (35/10/5). The solutions of amidites and sym-collidine/acetonitrile (2/3/5); and oxidation (1.0 min) with iodosylbenzene (20 mM) in tetrahydrofuran, the corresponding 2′-OCH₃-G phosphonamidate was dissolved in tetrahydrofuran, the corresponding 2′-OCH₃-G phosphoramidate was applied in acetonitrile. DNA Thiophosphate Synthesis. For the synthesis of thiophosphate-containing RNA, automated phosphoramidite chemistry was applied as described above. Sulfur was introduced manually using phenylacetyl disulfide (PADS) in analogy to ref. 47. Deprotection of Methylphosphonate-Modified RNA. The solid support was treated with 0.5 mL of a mixture of concentrated aqueous ammonium hydroxide and 1 M ethanethiol (10/44/5; 3 vol/vol/vol) for 3 min at room temperature. Then, 0.5 mL ethylenediamine was added, and the mixture was filtered or 5 h at room temperature. The supernatant was filtered into a 100-mL round-bottomed flask, and the solid support was washed twice with ethanol/water (1/1 (vol/vol)). The supernatant and the washings were combined, and triethylammonium bicarbonate (TEAB) (1 M, 1 mL) was added. The solution was evaporated, and a clear oil was obtained. To remove the 2′-silyl protecting groups, the resulting oil was treated with tetraethylammonium fluorid trihydrate (TEAF·3 H₂O) in THF (1 M, 1 mL), and the suspension was agitated at 37 °C overnight. The reaction was quenched by the addition of triethylammonium acetate (TEAA) (1 M, pH 7.4, 1 mL). The volume of the solution was reduced, and the solution was desalted with a size exclusion column (GE Healthcare, HiPrep 26/10 Desalting; 2.6 × 10 cm; Sephadex G25) eluting with H₂O, and the collected fraction was evaporated to dryness and dissolved in 1 mL H₂O. Analysis of the crude RNA after deprotection was performed by anion-exchange chromatography on a Dionex DNA PAC PA-100 column (4 × 250 mm) at 80 °C. Flow rate, 1 mL/min; eluent A, 25 mM Tris HCl (pH 8.0), 6 mM urea; eluent B, 25 mM Tris HCl (pH 8.0), 0.5 M NaClO₄, 6.0 mM urea; gradient, 0-60% B in A within 45 min or 0–40% B in 30 min for short sequences up to 15 nucleotides; UV detection, 260 nm. Deprotection of Thiophosphate-Modified RNA. Deprotection was conducted as for unmodified RNA following standard procedures described in ref. 48. Purification of the Methylphosphonate- and Thiophosphate-Modified RNA. Crude RNA products were purified on a semipreparative Dionex DNA PAC PA-100 column (9 × 250 mm) at 80 °C with a flow rate of 2 mL/min. Fractions containing RNA were loaded on a C18 SepPak Plus cartridge (Waters/Millipore), washed with 0.1–0.15 M (Et₃NH)₂HCO₃, H₂O, and eluted with H₂O/CH₃CN (1/1). RNA-containing fractions were lyophilized. Analysis of the quality of purified RNA was performed by UV photometric analysis of oligonucleotide solutions. Separation of Methylphosphonate- and Thiophosphate-Containing RNA Diastereomers. RNA diastereomers were separated on a reversed-phase GE Healthcare Resource RPC (3 mL; 6.4 × 130 μL) column (40 °C) with a flow rate of 2 mL/min; eluent A, 0.1 M ammonium acetate, pH 7.0; eluent B, acetonitrile (HPLC grade); gradient, 0-4% B in A within 45 min for sequences up to 30 nucleotides; UV detection, 260 nm. Fractions containing RNA were loaded on a C18 SepPak Plus cartridge (Waters/Millipore), washed with 0.1–0.15 M (Et₃NH)₂HCO₃, H₂O, and eluted with H₂O/CH₃CN (1/1). RNA-containing fractions were evaporated, and the residue was dissolved in H₂O and lyophilized. Analysis of the RNA diastereomers after separation was performed by reverse-phase chromatography on a Xbridge C18 column (130 Å, 5.0 μm, 4.6 × 150 mm) at 40 °C. Flow rate, 1 mL/min; eluent A, 0.1 M ammonium acetate, pH 7.0; eluent B, acetonitrile (HPLC grade); gradient, 3-5% B in A within 25 min; UV detection, 260 nm. SLM methylphosphate stereoc hemistry was assigned by NMR spectroscopy (Fig. 3); SLM thiophosphate stereochemistry was tentatively assigned based on their retention time by reversed-phase column chromatography according to ref. 49. Enzymatic Ligation of Diastereomerically Pure Methylphosphonate- and Thiophosphate-Containing RNA. Enzymatic ligation experiments using T4 DNA ligase (Fig. 2) were performed with the separated RNA diastereomers in analogy to a previously published procedure (50). MS of Modified RNA. All experiments were performed on Finnigan LC Advantage Max LCQ Trap instrument connected to an Amphenol Etten micro LC system. RNA sequences were analyzed in the negative-ion mode with a potential of −4 kV applied to the spray needle. LC: sample (200 pmol) RNA dissolved in 30 μL of 20 mM EDTA solution; average injection volume: 30 μL; column (Waters XTerraMS, C18 2.5 μm, 1.0 × 50 mm) at 21 °C; flow rate, 30 μL/min; eluent A,
X-Ray Crystallography. The 27-nt SRL hairpin modified with methylphosphonate at the G2661–A2662 step and a 2′-OCH3 group on the G2661 residue was chemically synthesized. The diastereomers were separated by reversed-phase column chromatography, and the Rp diastereomer was used for crystallization. RNA was dissolved in a buffer made with 1 mM Na EDTA, pH 8.0, and 1 mM Tris HCl, pH 8.0, at a 350 μM concentration. The RNA sample was heated at 55 °C for 10 min and cooled to 25 °C by switching off the heating block. Crystals were grown for 2 wk at 20 °C by mixing one volume of the RNA sample with one volume of a crystallization buffer made with 2.5 M ammonium sulfate, 10 mM magnesium acetate, and 50 mM 2-(N-morpholino)ethanesulfonic acid (Mes), pH 5.6. Crystals were cryoprotected for about 5 min in a reservoir solution containing 15% (vol/vol) glycerol and 3.0 M ammonium sulfate and flash-frozen in liquid ethane for data collection. X-ray diffraction data were collected using a fixed slicing strategy (0.2° oscillation) using the xRaya beamline at the SLS synchrotron (S1) Data were processed by molecular replacement using an unmodified SRL structure as a search model (Protein Data Bank (PDB) id code 3DVZ (35) and MOLREP (54) and refined with the PHENIX package (55). The model was built using Coot (56).

Reconstitution of 50S Subunits. Generation of the circularly permuted (cp) 23S rRNA, subsequent in vitro reconstitution of the 50S particles, and reassociation with native 50S subunits were done as previously described (15, 27, 43). The plasmid pUC19–cp2685–2639, encoding the cp2685–2639 RNA under the control of the T7 promoter, was linearized using Nael. The plasmidized RNA was used as template for in vitro transcription. For generation of pUC19–cp2685–2639, the 23S was PCR amplified from pCPTaq23 (35) (using the following primer: EcoRI–T7–2585 5′–CCGAATTCGGATCCTAATACGACTCACTATAGGTTTCC–3′) and HindIII–NsiI–Taq2639 5′–CCCCAAGCT–GCAGTGCTTCCCTGGGACTA–3′ and HindIII–Taq2639 5′–CCCAAGCT–TATGCATCAAGCCTCTGGGGCGGTG–3′. The purified PCR product was cloned into pUC19 via the EcoRI and HindIII restriction sites. Upon transformation of E. coli DH5α, the transformants were selected on agar plates containing kanamycin (50 μg/ml) and X-gal (50 mg/ml). The positive transformants were picked, grown, and then used to generate plasmids. 2 μl of the plasmid solution containing a single copy of the cp23S rRNA (adenosine at position 278 or 298 K (Fig. 3). The size of the data matrices were 2,048 × 300 complex data points, the number of scans was 144, the interscan delay was 1.5 s, and the mixing time was set to 150 ms, resulting in a total measuring time of 20 h for each spectrum.

The NOESY 1H, 1H-NMR spectra were acquired on a Bruker Avance II 600 MHz spectrometer equipped with a proton-optimized triple resonance NMR ‘inverse’ probe (TCl Cryoprobe Prodigy 5 mm). The NOESY spectra were acquired at 278 or 298 K (Fig. 3). To compensate the missing RNA sequence in the cp-23S RNA, the following RNA oligos were added during in vitro reconstitution: 2640–2684 5′–GGGGGGGGCGUUCCGUGAGA-G2661–A2662–GGACCGGAAGGAGCG–CACCUCU–3′, with the following modifications—dG2661–mp–A2662 (both diastereomers); 2′-OCH2–G2661–mp–A2662 (both stereoisomers); and G2661–thio–A2662 (both stereoisomers); for control experiments—dG2661, 2′-OCH2-G2661. The G2644U mutation is facilitated by SRL strand annealing to cp-23S RNA.

Porurycin Reaction. The peptidyl transferase assay was carried out as previously described in ref. 35 using 1 pmol N-acetyl-[3H]-Phe-trna55 (15,000 cpn/pmol) as P-site substrate and 2 μM peumycin as acceptor substrate.

Multiple-Turnover EF-G GTP Hydrolysis. For the uncoupled GTP hydrolysis assay, reconstituted ribosomes (200 pmol cp2685–2639 23S RNA, 200 pmol RNA oligo 2640–2684, and 2 pmol E. coli tRNA) were used as template for in vitro transcription. For generation of pUC19-cp2685–2639 the nucleotide was centrifuged for 20 min at −80 °C and centrifuged for 20 min at 18,000 × g at 4 °C, and the ribosomal pellet was resuspended in 5 μl GTPase reaction buffer (10.4 mM Tris-HCl, pH 7.4, 208 mM NaCl, 7.8 mM MgCl2, 0.1 mM EDTA, 3.12 mM spermidine, and 2.6 mM 2-mercaptoethanol). The following reaction was performed in GTPase reaction buffer: 0.7 μM deacetylated tRNA55 (bound to the site of reconstitution of the ribosomes in the presence of 26 μg poly(U) for 5 min at 37 °C. The reaction was initiated by addition of 50 μM (125I)GTP (6,000 Ci/mmol, 10 mCi/mL; Perkin Elmer) and 1.14 μM his-tagged EF-G from Thermus thermophilus in a total volume of 13.4 μL and incubated at 37 °C. At the indicated time points, 2 μL was withdrawn from the reaction and terminated by the addition of one volume of 20% (vol/vol) formic acid. Hydrolysis was performed by TLC in 0.5 M KCl, 0.1 M NaCl, 10 μM EDTA, pH 6.6, for 15 min at 37 °C. The TLC Polygram CEL300 PE(UV) plates were dried, exposed for 1–2 h, and scanned at the phosphorimager (FujiFilm). GTP hydrolysis in the absence of any complementing RNA oligonucleotide was considered as background, coming from slight contaminations of the 30S with native 50S and was subtracted from all experimental points. For the pH dependency assay, reactions were performed as described above; however, GTPase re-action buffer contained 10.4 mM Tris-HCl, pH 7.0, 6.5, and 6.0.

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Koch et al.


