Supporting Information for:

Selective 2′-Hydroxyl Acylation Analyzed by Protection from Exoribonuclease

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Experimental Procedures

Screening of Candidate RNases. Recombinant RNase R enzymes from *Escherichia coli*,[1] *Mycoplasma genitalium*[2] and *Aquifex aeolicus* were evaluated to identify an enzyme that efficiently and non-specifically degrades RNA (including highly structured RNA), is quantitatively inhibited by 2'-*O*-adducts, and can be fully and permanently inactivated. All three enzymes efficiently degraded structured RNA. The *E. coli* and *M. genitalium* enzymes are additionally readily inactivated by a simple heating step (95 °C, 3 min) whereas the *A. aeolicus* enzyme is very difficult to heat inactivate. 2'-*O*-adducts caused significantly stronger stops with the *M. genitalium* enzyme than with the *E. coli* version. For these reasons, we focused on using the *M. genitalium* enzyme for detection of covalent adducts in RNA. RNase R from *M. genitalium* was purified as described[2] and used for all subsequent experiments. Enzyme purification should be performed using equipment separate from that used for RNA-based research.

Heat Inactivation of RNase R. RNase R enzymes require Mg^{2+} for activity and can be immediately inactivated by addition of excess EDTA. The enzyme is also permanently inactivated by heat denaturation. To assess the thermal inactivation of *M. genitalium* RNase R, individual 1 µL aliquots of the enzyme (1.5 µg/µL stock) were incubated at 95 °C [in 20 mM Tris (pH 8.0), 100 mM KCl]. Each aliquot was removed at time points ranging from 15 s to 10 min and placed on ice. To the heat-treated RNase, 8 µL of 5′-[^{32}P]-labeled RNA in the same buffer containing 0.25 mM $MgCl_2$ were added. Reactions were subjected to the standard exoribonuclease digestion step (see below; incubation at 50 °C for 30 min) and resolved on a 10% denaturing polyacrylamide gel. RNase R activity was quantified as the fraction of remaining full length RNA. The half-life of *M. genitalium* RNase R is 18 sec at 95 °C. Although the exonuclease-detected SHAPE experiment does not require that the enzyme be inactivated, we recommend heating all RNase R-containing solutions for 3 min at 95 °C (~10 half-lives) to prevent introduction of RNase to other RNA experiments in the laboratory.

RNAs. DNA templates for the aptamer domain of the *Escherichia coli* thiamine pyrophosphate (TPP) riboswitch[3] both with and without 5′ and 3′ structure cassette flanking sequences[4] were generated by PCR [1 mL; 20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 250 nM each forward and reverse primer (IDT), 40 nM template (IDT), and 0.025 units/µL Taq polymerase; denaturation at 95 °C, 45 s; annealing at 55 °C, 30 s; elongation at 72 °C, 1 min; 35 cycles]. The PCR product was recovered by ethanol precipitation and resuspended in 200 µL TE [10 mM Tris (pH 8.0), 1 mM EDTA]. RNA constructs were synthesized by *in vitro* transcription [1 mL; 40 mM Tris (pH 8.0), 10 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 0.01% (v/v) Triton X-100, 4% (w/v) poly(ethylene) glycol 8000, 2 mM each NTP, 40 µL PCR-generated template, 0.1 mg/mL T7 RNA polymerase; 37 °C; 4 h]. Phosphorothioate-containing RNAs for sequencing were synthesized with the same protocol in 100 µL volumes using 10 µL PCR-generated template and contained 0.2 mM guanosine or cytidine α-thiotriphosphate (Glen Research). RNAs were purified by denaturing polyacrylamide gel electrophoresis (8% polyacrylamide, 7 M urea, 29:1 acrylamide:bisacrylamide, 0.4 mm × 28.5 cm × 23 cm; 32 W, 1.5 h), excised from the gel, recovered by overnight passive elution at 4 °C, and precipitation with ethanol. Purified RNAs were resuspended in TE and stored at -20 °C.

Purified RNAs were 5′-[^{32}P]-radiolabeled by: (1) dephosphorylation [300 μL; 50 mM Tris (pH 8.5), 0.1 mM EDTA, 10 μM RNA (TPP RNA) or 1 μM (phosphorothioate-containing TPP RNA), 300 units SUPERase-In (Ambion), 200 units alkaline phosphatase (Roche); 50 °C; 1 h]; (2) phenol:chloroform:isoamyl alcohol extraction, ethanol precipitation, and resuspension in TE (storage at -20 °C); and (3) treatment with T4 polynucleotide kinase [20 μL; 80 pmol dephosphorylated RNA, 70 mM Tris (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 2 μL T4 polynucleotide kinase (NEB, 10,000 units/mL), 80 μCi [γ-^{32}P]-ATP; 37 °C; 30 min]. Phosphorothioate RNA radiolabeling reactions contained 20-30 pmol RNA, 1 μL T4 polynucleotide kinase (10,000 units/mL), and 20 μCi [γ-^{32}P]-ATP. Radiolabeled RNAs were purified by denaturing (8%) gel electrophoresis, excised from the gel, and recovered by overnight passive elution at 4 °C. The purified 5′-[^{32}P]-labeled RNAs were precipitated with ethanol, resuspended in 10 mM HEPES (pH 8.0), and stored at -20 °C.

Structure-Selective RNA Modification. Unlabeled RNA with flanking sequences (5 pmol) or 5′-[^{32}P]-labeled RNA with and without flanking sequences (~1 pmol) in 5 μL 1/2× TE (for modified RNAs analyzed by primer extension) or sterile water (for modified RNAs analyzed by RNase R degradation) was heated at 95 °C for 2 min, cooled on ice, treated with 3 μL 3.3× folding buffer [333 mM HEPES (pH 8.0), 333 mM NaCl, 33.3 mM $MgCl_2$], and incubated at 37 °C for 10 min. The ligand (1 μL; 50 μM TPP) or sterile water was added and incubated at 37 °C for 20 min. After incubation, 9 μL of the folded RNA (+/– TPP) was added to 1 μL 80 mM 1M7 (in DMSO)[5] and incubated at 37 °C for 2 min. No-reagent control reactions were performed with 1 μL neat DMSO. The RNA was recovered by ethanol precipitation. For the 5′-[^{32}P]-labeled RNA, a five-fold molar excess of EDTA was added to chelate Mg^{2+} before ethanol precipitation. Unlabeled RNA was resuspended in 10 μL 1/2x TE and 5′-[^{32}P]-labeled RNA was resuspended in 8 μL sterile water after being washed twice with 70% ethanol.

RNase R Digestion. 5′-[^{32}P]-labeled RNA from the 1M7 or kethoxal modification reaction (8 μL) was supplemented with 1 μL 10× reaction buffer [200 mM Tris (pH 8.0), 1 M KCl, 2.5 mM $MgCl_2$] and 1 μL *M. genitalium* RNase R[2] (4.5 μg/μL) and incubated at 50 °C for 30 min. [Note: *M. genitalium* RNase R activity is very sensitive to Mg^{2+} concentration.] The RNase R enzyme was inactivated by the addition of 1 μL 100 mM EDTA followed by incubation at 95 °C for 3 min. The RNA fragments were recovered by precipitation with 2.5 vol ethanol plus 1 vol isopropanol, washed once with 70% ethanol, and resuspended in 2 μL sterile water and 7 μL stop dye [96% formamide, 1 mM EDTA (pH 8.0), bromophenol blue and xylene cyanol]. RNA fragments were separated by denaturing polyacrylamide gel electrophoresis (10% polyacrylamide, 29:1 acrylamide:bisacrylamide, 7 M urea, 1× TBE, 0.75 mm × 31 cm × 38.5 cm; 70 W) and visualized by phosphorimaging. Fragments were run for both 1.5 and 3.5-4 h to resolve most nucleotide positions.

Kethoxal Modification. 5′-[^{32}P]-labeled RNA (1 μL) in 15 μL sterile water was heated at 95 °C for 2 min, cooled on ice, mixed with 2 μL 1 M HEPES (pH 8.0), and incubated at 70 °C for 3 min. The RNA was then treated with 2 μL 20 mM kethoxal (in sterile water, from USB) and incubated at 70 °C for 5 min.[6] The reaction was quenched with 20 μL 10 mM boric acid followed by ethanol precipitation, washed twice with 70% ethanol, and resuspended in 8 μL sterile water.

Cleavage of Phosphorothioate-Containing RNA. 5′-[^{32}P]-labeled phosphorothioate-containing RNA was treated with 3 μL 85 mM iodine in ethanol for 3 min at room temperature.[7] The reaction was quenched with stop dye, and used directly for sequencing.

Primer Extension. The general procedure has been outlined previously.[4,5] DNA primers were 5′-end labeled with VIC or NED fluorophores (from Applied Biosystems). Unlabeled RNA from the 1M7 modification reaction (10 μL) was added to a fluorescently labeled DNA primer (5′-VIC-labeled GAA CCG GAC CGA AGC CCG; 3 μL, 0.3 μM) and allowed to anneal at 65 °C for 6 min and then cooled on ice. Reverse transcription buffer [6 μL; 167 mM Tris (pH 8.3), 250 mM KCl, 10 mM $MgCl_2$, 1.67 mM each dNTP] and Superscript III (1 μL, 200 units) were added and incubated at 45 °C for 2 min, 52 °C for

20 min then 65 °C for 5 min. The reactions were quenched with 4 μL 50 mM EDTA. The cDNAs were recovered by ethanol precipitation, washed twice with 70% ethanol, dried in a SpeedVac for 5 min, and resuspended in 10 μL deionized formamide. Dideoxy sequencing ladders were produced using unlabeled, unmodified RNA, annealing a 5′-NED-labeled fluorescently labeled DNA primer (3 μL, 0.3 μM), and by adding 1 μL 2′,3′-dideoxycytosine (10 mM) triphosphate before addition of Superscript III. cDNA fragments were separated by capillary electrophoresis using an Applied Biosystems 3130 DNA sequencing instrument.

Data Analysis. Band intensities visualized by gel electrophoresis were quantified using SAFA (Semi-Automated Footprinting Analysis)[8] and raw capillary electrophoresis traces were analyzed using SHAPEFinder.[9] SHAPE reactivity profiles for both the RNase R degradation- and primer extension-detected experiments were obtained by subtracting the no-reagent background from the (+) reaction intensities. All data sets were normalized by excluding the top 2% of the reactive nucleotides, averaging the next 10% of reactive nucleotides, and then dividing all intensities by this averaged value.

RNase R Structure Model. A homology model for the *M. genitalium* RNase R was generated using I-TASSER[10-12] with 2ix0[13] and 2vnu[14] as the template structures. The center-most model (by RMSD) is shown in Figure 1B. The RNA strand is from the 2ix1[13] and 2vnu structures; the adenosine residue visualized in the 2ix1 structure at position N-4 was changed to guanosine. RNase R likely uses a two Mg^{2+} mechanism. Only one Mg^{2+} ion has been visualized crystallographically and is shown, along with its coordinating aspartic acid residues, in Figure 1B.

Images were composed with Pymol (Delano Scientific). The secondary structure for the (+) ligand TPP riboswitch aptamer domain was adapted from Lescoute and Westhof.[15]

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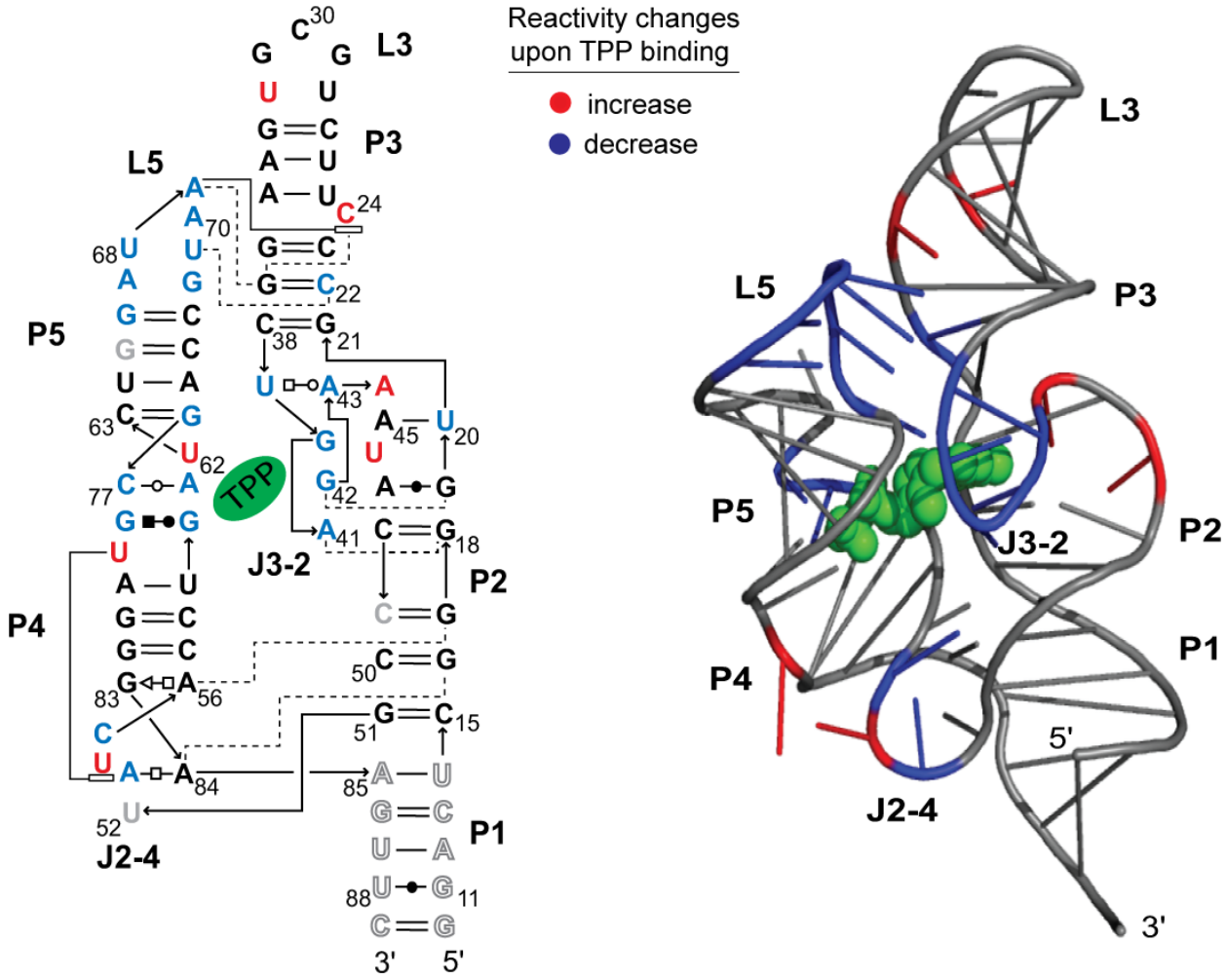

Supporting Figure 1. Base-pairing and tertiary structure of the ligand-bound TPP riboswitch showing structural features whose constituent nucleotides increase (red) or decrease (blue) in SHAPE reactivity when TPP binds.