Supporting Information

Characterizing the Dynamics of the Leader-Linker Interaction in the Glycine Riboswitch with Site-Directed Spin-Labeling

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Materials and Methods

Synthetic RNA preparation. Synthetic RNA fragments, 20 nucleotides in length, were purchased from Dharmacon (Pittsburgh, PA) with site-specific modifications of the phosphodiester backbone to phosphorothioate moieties. Sequences for the designed oligomers are shown in Table S1 along with other nucleic acid sequences (described in following sections). The protecting group, 2'-O-bis(2-acetoxyethoxy)methyl, also known as ACE, which is synthetically incorporated into each 2'OH position within the RNA fragment was removed according to the vendor's instruction via an acid catalyzed hydrolysis of the orthoester groups. RNAs were incubated for 30 minutes with the provided acetic acid buffer at pH 3.8, then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) (PCA) (Fisher Scientific, Pittsburgh, PA), ethanol precipitated, and dissolved in water. RNA concentrations were determined by measuring UV absorbance at 260 nm using a Cary WinUV 50 spectrophotometer (Varian Instruments, Walnut Creek, CA) in a quartz cuvette (Agilent Technologies, Santa Clara, CA). Extinction coefficients of the RNAs were calculated by OligoCalc.[1]

Table S1. Synthetic RNA oligonucleotide, DNA splint, and *in vitro* transcribed RNA sequences used for this study. Asterisks indicate sites of phosphorothioate modification. Deoxynucleotides are used prior to the modified site to prevent strand scission as described in the followed protocol.[2]

Modified site and location	Nucleic acid sequence 5' to 3'
Site 1 at 5' end	dU*UGUUCCGUUGAAGACUGCA
Site 2 at duplex	UUGdU*UCCGUUGAAGACUGCA
Site 3 at internal loop	UUGUUCCGdU*UGAAGACUGCA
DNA Splint	CTGGTTAACAACCACTCTCCTGCAGTCTTCAACGGAACAA
In vitro transcribed RNA	GGAGAGUGGUUGUUAACCAGAUUUUAACAUCUGAGCCAAA UAACCCGCCGAAGAAGUAAAUCUUUCAGGUGCAUUAUUCU UAGCCAUAUAUUGGCAACGAAUAAGCGAGGACUGUAGUUG GAGGAACCUCUGGAGAGAACCGUUUAAUCGGUCGCCGAAG GAGCAAGCUCUGCGCAUAUGCAGAGUGAAACUCUCAGGCA AAAGGACAGAGG

Site-directed spin labeling of synthetic RNA. After removal of the protecting groups and prior to splinted ligation, synthetic RNA oligomers containing phosphorothioate modifications were spin labeled with R5 in accordance with a previously published protocol.[2] The R5 spin label was also prepared following the same protocol using the R5 precursor that was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario). After overnight incubation of the RNA with R5, the RNA was PCA extracted, ethanol precipitated, and dissolved in Tris-EDTA (TE) buffer (Fisher Scientific, Pittsburgh, PA) before concentration determination and ligation.[2]

In vitro transcribed RNA preparation. Plasmid DNA containing the remaining sequence of the full length VC glycine riboswitch was prepared via *in vitro* transcription according to the published protocols.[3] DNA primers from Integrated DNA Technologies (Commercial Park, IA) were used to amplify the region of interest by PCR. Double digestion with *Eco*RI and *Hin*dIII (New England Biolabs, Ipswich, MA) was then performed using the supplied buffers at 37 °C for 1 hour. Plasmid containing the 212 nucleotide riboswitch DNA sequence was generated by ligating the double digested insert into the pUC19 vector using T4 DNA ligase and the supplied buffer at 16 °C with overnight incubation (New England Biolabs, Ipswich, MA). The ligated plasmid was chemically transformed into JM109 cells, amplified, miniprepped with Qiagen Miniprep kits (Qiagen, Valencia, CA), and sequenced by Genewiz, Inc. (South Plainfield, NJ). Qiagen Maxiprep kits (Qiagen, Valencia, CA) were used to isolate milligram quantities of the plasmid DNA according to the vendor's supplied protocol.

Linearization of the plasmid was achieved through EarI digestion using the supplied buffer (New England Biolabs, Ipswich, MA) at 37 °C with overnight incubation. Large scale *in vitro* transcription of the riboswitch was performed with in-house made T7 RNA polymerase according to the published protocols.[3] The transcription mixture was incubated with 1 mM $CaCl_2$ and RNase-free DNase I (Promega, Madison, WI) at a concentration of 0.5 units per μg of the DNA plasmid at 37 °C for 30 minutes. The RNA transcript was then purified by 6-8% polyacrylamide gel electrophoresis (PAGE), excised under UV shadowing, electroeluted into TE buffer, PCA extracted, ethanol precipitated, and resuspended in TE buffer prior to concentration determination via UV absorbance at 260 nm. Using these methods, approximately 14 mg of transcribed RNA was obtained.

Splinted ligation and preparation of transcribed RNA. T4 DNA ligase recognizes nicked double stranded substrates and produces a phosphodiester bond between the 3' OH group of an RNA acceptor and the 5' monophosphate of an RNA donor.[4] In our ligation scheme, the 20 nucleotide synthetic RNA containing a 3' OH was used as the acceptor fragment and the 212 nucleotide RNA containing a 5' monophosphate was used as the donor. A 40 nucleotide DNA oligomer, with sequence complementary to both RNAs, was used as the splint to form a ternary DNA/RNA-RNA complex with the two RNA fragments. This non-native DNA/RNA-RNA substrate causes inefficient turnover of T4 DNA ligase, thus requiring stoichiometric quantities of the enzyme. Consequently, we obtained T4 DNA ligase plasmid from the Herschlag lab as a

gracious gift, and expressed and purified the recombinant protein according to published procedures.[5,6]

As the *in vitro* transcribed RNA contains a 5' triphosphate, to prepare it for ligation, dephosphorylation and subsequent monophosphorylation were performed. RNA was dephosphorylated by treatment with fast alkaline phosphatase, also known as FastAP (Thermo Scientific, Waltham, MA). Treatment of the RNA was performed at 37 °C for 3 hours followed by inactivation of FastAP by heating at 75 ˚C for 5 minutes. Subsequent to dephosphorylation the RNA was monophosphorylated by incubating with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) at 37 ˚C for 1 hour. Finally, PCA extraction and ethanol precipitation was performed to obtain monophosphorylated RNA suitable for splinted ligation.

Optimization of splinted ligation yield. Splinted ligation is performed in two steps that include annealing of RNAs to the DNA splint and ligation with T4 DNA ligase. Conditions in both steps that can influence the yield of ligated product have been previously published.[4,7] Our determination of percent yield was calculated by comparing the initial mole quantity of RNA used in the ligation reaction, to the final mole quantity of full length, ligated and purified riboswitch RNA products obtained. For the three ligated riboswitch constructs reported in this work, the average percent yield was 43%. Optimization of our ligation yields was achieved by elongating the DNA splint from 20 to 40 nucleotides and also by varying the ratio of synthetic RNA: transcribed RNA: DNA splint (details are in the following section and sequences are shown in Table S1).

Splinted ligation and purification of spin labeled riboswitch RNA. Annealing of the ternary complex was achieved by mixing spin labeled synthetic RNA, monophosphorylated transcribed RNA, and DNA splint; respectively, the optimized mole ratio was 4:1:3.5 in the presence of 0.5M NaCl. The total scale of transcribed RNA used was 7-10 nmoles (1 nmole per reaction tube). The mixture was then heated at 95 °C for 5 minutes and equilibrated to room temperature in a heat block. To each reaction tube containing the annealed complex, 10X ligase buffer (New England Biolabs, Ipswich, MA), stoichiometric T4 DNA ligase, and 0.2 units per 1 μl of RNasin (Promega, Madison, WI) was added. The final concentration of NaCl after addition of ligation reagents was 0.05M. Ligation was performed at 30 °C for 3 hours. A small scale 8% denaturing PAGE was used to visualize the resulting products of ligation as shown in Figure S1. A large scale 6% denaturing PAGE was then used to purify the RNA allowing for the 232 nucleotide ligated product to be excised from the gel and electroeluted overnight at 4 °C in prepared TE buffer. The RNA was PCA extracted, ethanol precipitated, and dissolved in water for EPR sample preparations. Concentration was determined as previously described.

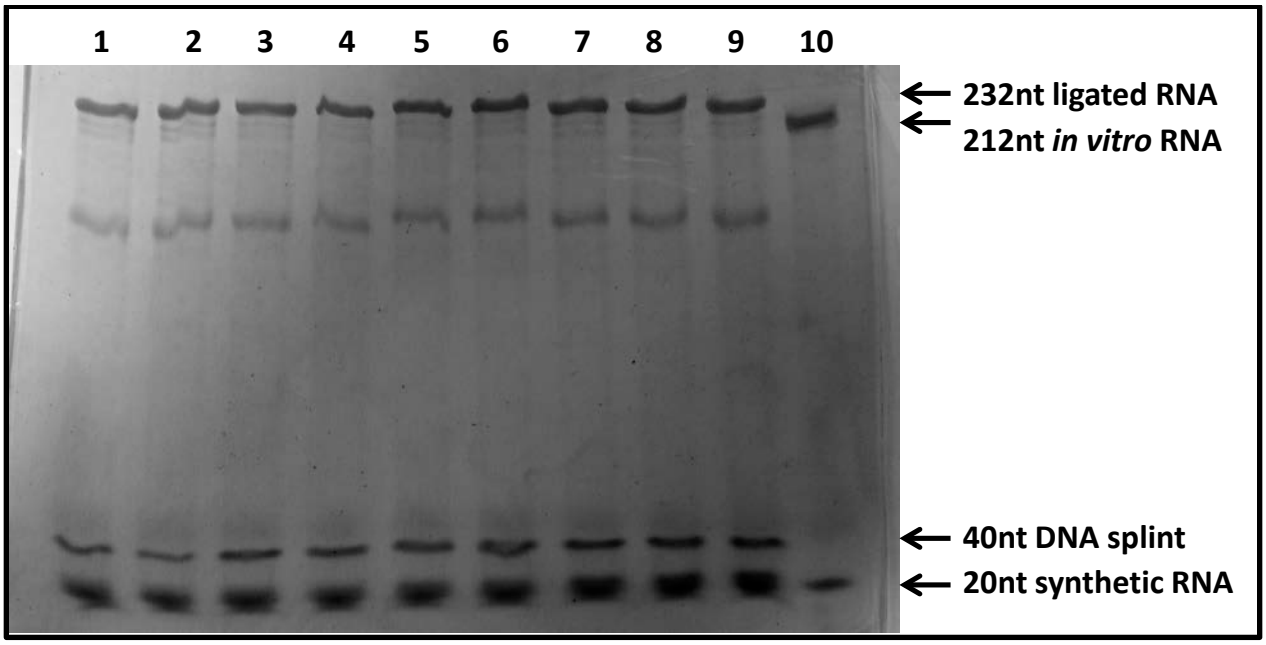

Figure S1. Small scale, 8% denaturing PAGE gel showing the resulting products of the splinted ligation reaction. Lanes 1-9 represent identical reaction tubes each containing 1 nanomole of transcribed RNA with all other reagents as described in materials and methods. Lane 10 is 212 nucleotide *in vitro* transcribed RNA.

CW X-band EPR spectra and EPR data analysis. X-band (9.5 GHz) EPR spectra were collected on a Bruker ER200 spectrometer using a loop gap resonator (Medical Advances, Milwaukee, WI). All spectra were obtained with 2mW incident microwave power, as 100 Gauss (G) scans, with optimized 100 kHz field modulation amplitude, and are reported as the average of 4 scans. Experiments at variable temperatures were performed by surrounding the loop gap resonator with a quartz Dewar (Wilmad-Labglass, Vineland, NJ). Temperature control was achieved by passing nitrogen gas through a copper coil that was submerged in a recirculating water bath (Thermo Scientific, Waltham, MA). For each of the three riboswitch constructs, varying EPR samples were prepared as follows: RNA in water only, RNA in the presence of 100 mM KCl, 5 mM $MgCl_2$, 100 mM KCl with 5 mM $MgCl_2$, 5mM $MgCl_2$ with 5 mM glycine, and 100 mM KCl with both 5 mM $MgCl_2$ and 5 mM glycine (Fisher Scientific, Pittsburgh, PA). In all samples, the concentration of RNA was approximately 100 μM. Samples of 5 μL were loaded into 0.6mm I.D. x 0.85mm O.D. glass capillary tubes, (Fiber Optic Center, New Bedford, MA) which were sealed at one end. EPR for each sample was performed at 10 °C, 15 °C, and 25 °C. Reported spectra are double integral area-normalized and plotted with intensities scaled accordingly.

Baseline correction and normalization of spectra were performed using Labview based software, which was graciously provided by Dr. Christian Altenbach and Dr. Wayne Hubbell. These programs were also used for analysis of $h_{(0)}$ and $h_{(-1)}$ to generate empirical plots including those shown in Figure 3B, Figure S2, and Figure S3. Based upon triplicate measurements, the standard

deviation of measurements is one third the size of the data points and statistically relevant differences were analyzed using the T-test as shown in Figure S4.

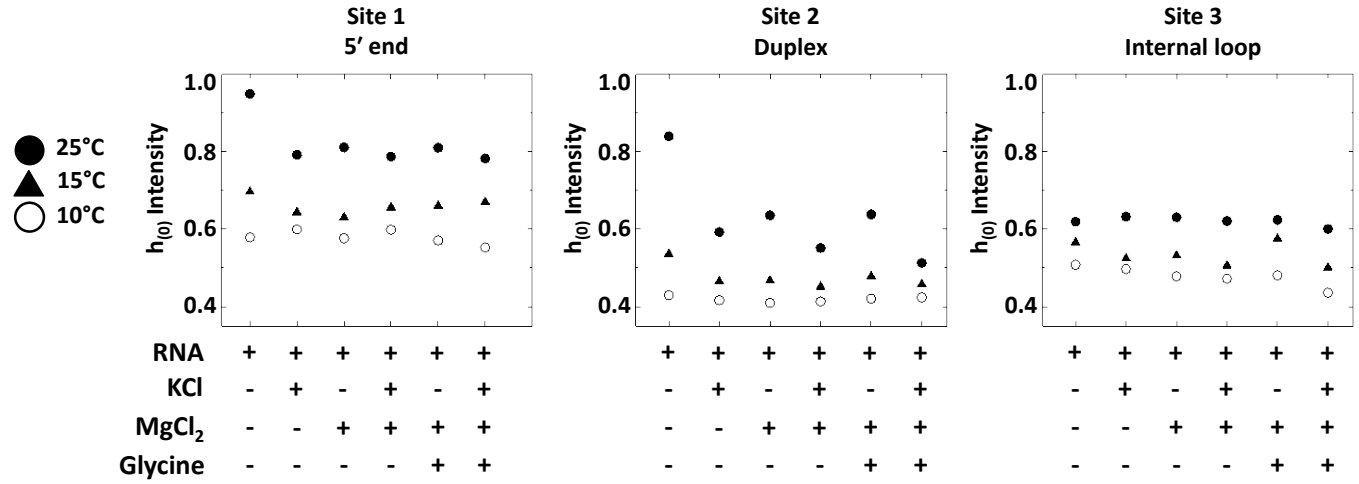

Figure S2. Plots of normalized $h_{(0)}$ intensity for R5 labeled sites of *VC* riboswitch RNA(100 μM) at three different temperatures and in the presence or absence of 100 mM KCl, 5 mM $MgCl_2$, and 5 mM glycine. Error bars are not shown on graphs as they are smaller than the data points.

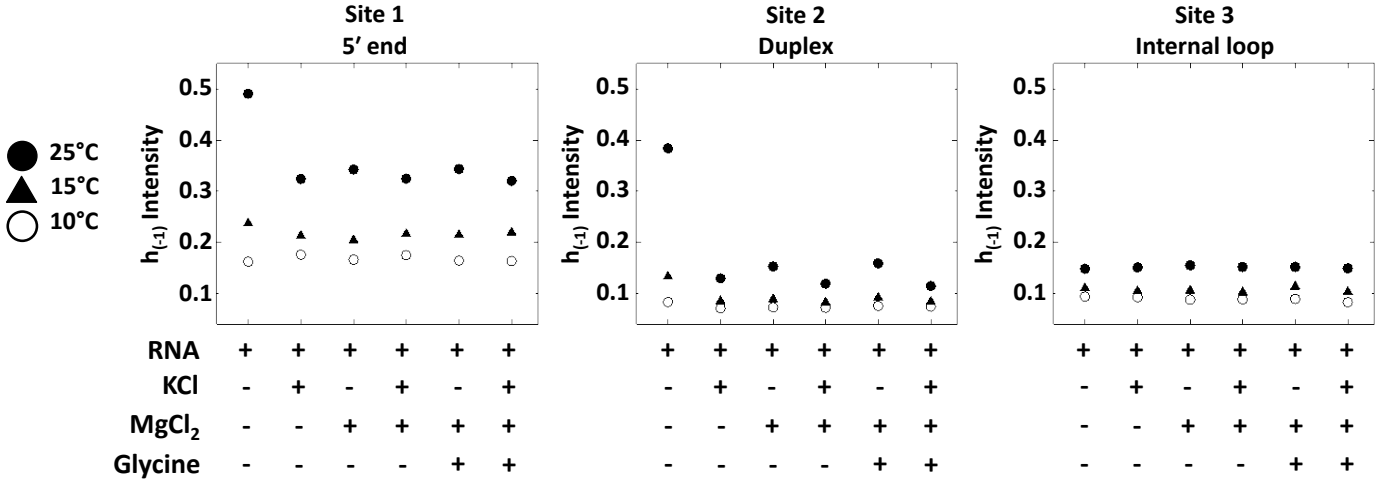

Figure S3. Plots of normalized $h_{(-1)}$ intensity for R5 labeled sites of *VC* riboswitch RNA(100 μM) at three different temperatures and in the presence or absence of 100 mM KCl, 5 mM $MgCl_2$, and 5 mM glycine. Error bars are not shown on graphs as they are smaller than the data points.

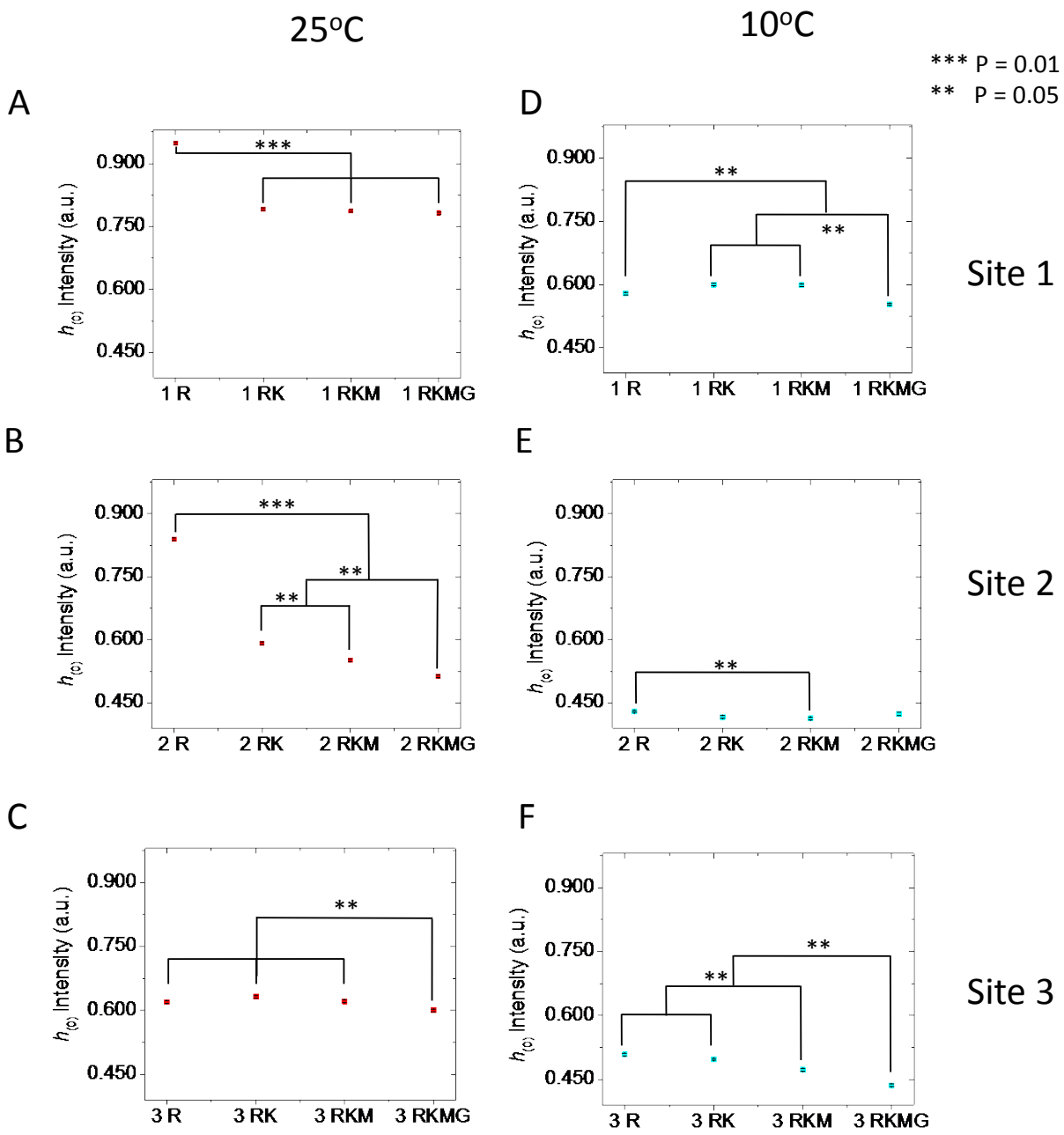

Figure S4. Graphs showing statistically relevant differences in normalized $h_{(0)}$ intensity for R5 labeled sites of *VC* riboswitch RNA (100 μM) at 25 °C (**A,B,C**) and 10 °C (**D,E,F**) for site 1, site 2 and site 3; respectively. Error bars shown within data points represent the standard deviations from triplicate measurements.

In-line probing control assays. To show that spin label attachment does not alter the function of the glycine riboswitch, in-line probing was performed on all three spin-labeled glycine riboswitch constructs. The obtained glycine binding affinities (K_d) are similar to those reported for the wild-type VC glycine riboswitch (5.0 ± 4.0 μM).[8] The K_d is 8.2 ± 3.3 μM for site 1 (VCLDR5PS1); the K_d is 6.6 ± 3.5 μM for site 2 (VCLDR5PS2); the K_d is 4.9 ± 3.2 μM for site 3 (VCLDR5PS3).

In-line probing assays were carried out similar to previously reported procedures.[9,10] Briefly, ~50 kcpm ^{32}P labeled RNA transcript was incubated at 25 ^{o}C for ~48 h in 50 mM Tris-HCl (pH 8.3), 20 mM $MgCl_2$, 100 mM KCl under various glycine concentrations. After incubation, spontaneously cleaved RNA fragments were resolved by 8% denaturing PAGE, dried, exposed to PhosphorImager screens (GE Healthcare Life Sciences, Piscataway, NJ), and quantified by ImageQuant (Molecular Dynamics) (GE Healthcare Life Sciences, Piscataway, NJ). For the VC glycine riboswitch constructs, the following regions were quantified: U74 (r2), A121-G123 (r3), G146 (r5), G170-A172 (r6), C177-A178 (r7). To control for loading differences, the band intensities were normalized to invariable cleavage bands (U156-U161). K_d values of the individual glycine-perturbed regions were determined by non-linear regression fitting of each plot using the following equation in KaleidaGraph software v3.09 (Synergy Software, Reading, PA): fraction bound = $m_1 \times [gly]^n/(K_d{}^n + [gly]^n) + m_2$,[10] where n represents Hill coefficient. The reported binding affinities were calculated as average K_d values obtained from the individual glycine-perturbed regions in two independent trials with curve coefficients better than 0.97.

In-line probing results for each labeled site shown in Figures S5-7 include dPAGE gel images and curve fittings for determination of binding affinities. Regions 6, 3, 2 (designated as r2, r3, r6) and the invariable cleavage loading control band 161 are labeled on the gel images. NR is a no reaction control, NG represents no glycine, and increasing glycine concentration is indicated for each gel. The NR control reactions exhibit more degradation compared to wild-type VCLD,[8] which may be due to the presence of the spin label radical. The graphs plot normalized bound fraction versus glycine concentration. The error bar of each data point is calculated as the standard error between two independent trials.

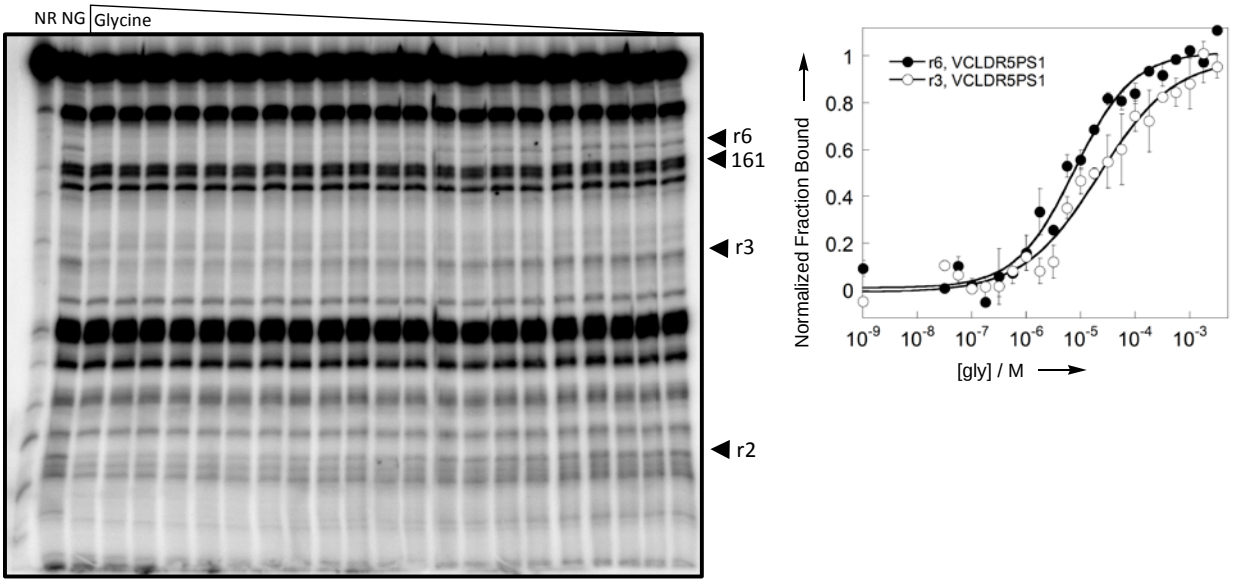

Figure S5. (Left) Denaturing polyacrylamide gel electrophoresis results for in-line probing of the *Vibrio cholerae* glycine riboswitch (VCLDR5PS1), labeled with the R5 spin label at site 1 at the 5' end. (Right) The corresponding semi-log plot for determination of the dissociation constants. Solid lines through data represent non-linear regression curve fitting and error bars represent standard error between two independent trials.

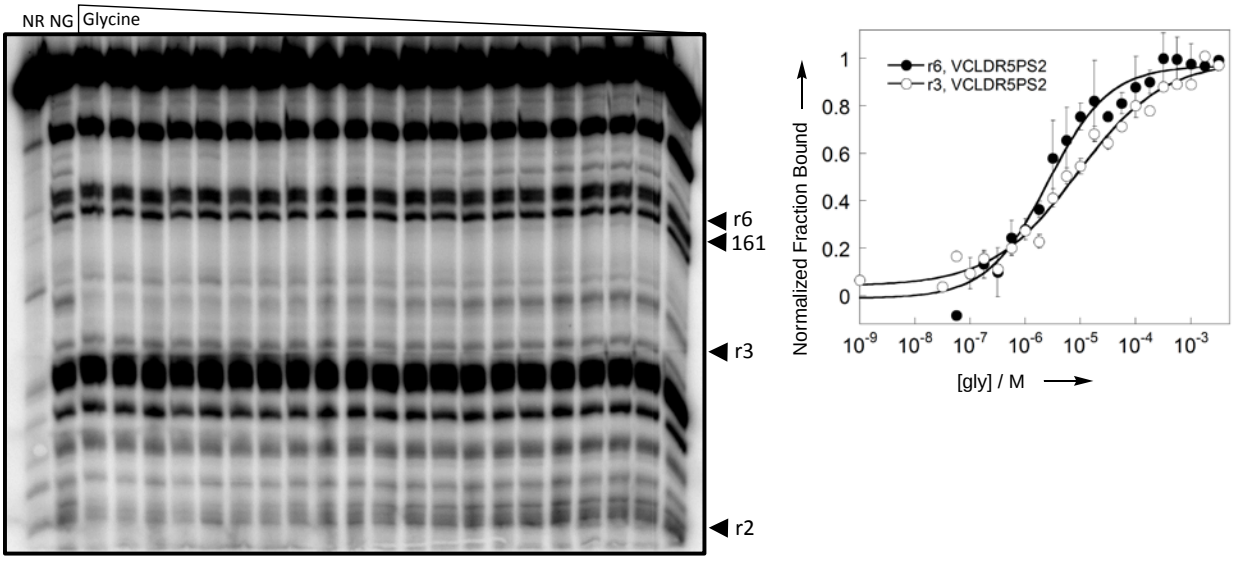

Figure S6. (Left) Denaturing polyacrylamide gel electrophoresis results for in-line probing of the *Vibrio cholerae* glycine riboswitch (VCLDR5PS2), labeled with the R5 spin label at site 2 within the duplex. (Right) The corresponding semi-log plot for determination of the dissociation constants. Solid lines through data represent non-linear regression curve fitting and error bars represent standard error between two independent trials.

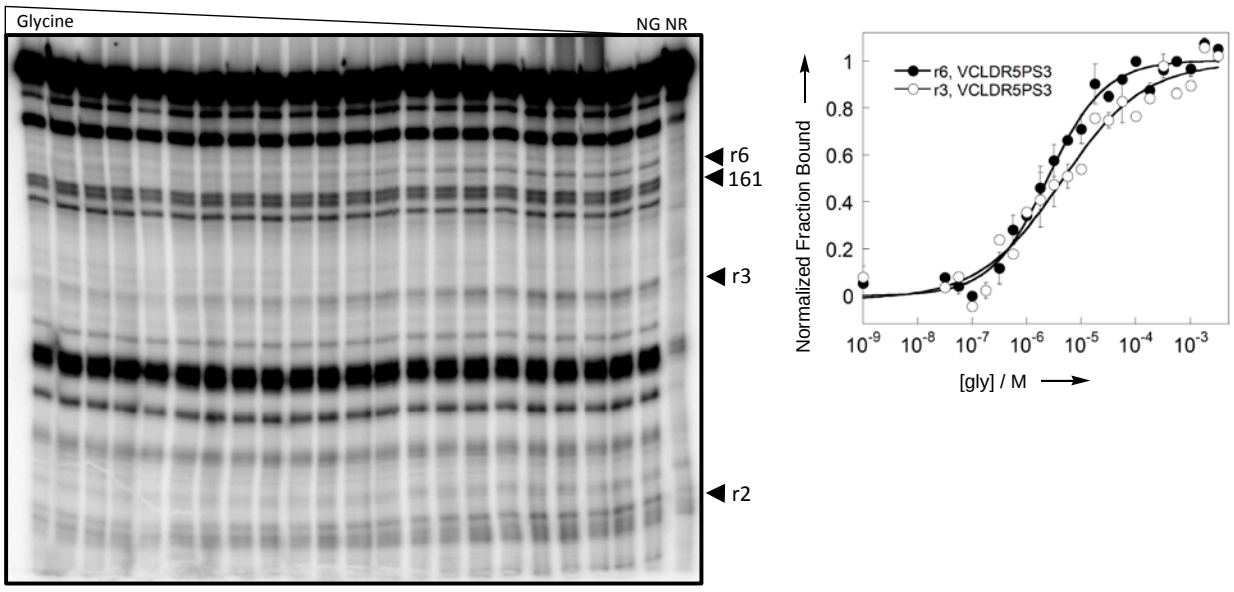

Figure S7. (Left) Denaturing polyacrylamide gel electrophoresis results for in-line probing of the *Vibrio cholerae* glycine riboswitch (VCLDR5PS3), labeled with the R5 spin label at site 3 at the internal loop. (Right) The corresponding semi-log plot for determination of the dissociation constants. Solid lines through data represent non-linear regression curve fitting and error bars represent standard error between two independent trials.

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