Supplemental Experimental Procedures

RNA preparation for crystallization

For the structures bound to **2**, **3**, **4** and thiamine, plasmid pTB13 (Edwards and Ferré-D'Amaré, 2006) which encodes 83 nucleotides of the TPP riboswitch aptamer domain preceded by a hammerhead ribozyme and followed by a VS ribozyme substrate stem-loop, was used to generate RNA. For the structure bound to **1**, a pIDT-TPP plasmid, with an insert encoding 80 nucleotides of the *E. coli thiM* TPP riboswitch aptamer domain described previously (Serganov et al., 2006), followed by a HDV ribozyme, purchased from IDT in the pIDTSmart (Amp) vector, was used to generate RNA. RNA was transcribed with T7 RNA polymerase either from *Bam*HI-linearized pTB13 plasmid or from PCR-amplified pTB13 or pIDT-TPP plasmid essentially as described (Ferré-D'Amaré and Doudna, 1996; Xiao et al., 2008) . The pIDT-TPP PCR product was designed to include a 61 nucleotide extension at the end of the HDV ribozyme to facilitate purification. The transcripts were purified by electrophoresis (8-12% polyacrylamide, 8 M urea gels), either passively eluted or electroeluted with an EluTrap Electroelution System (Whatman) overnight into 1X TBE buffer, desalted, concentrated by ultrafiltration, and stored either in water or in 0.1 mM EDTA.

RNA preparation for SAXS

Point mutations at residue 72 of the TPP riboswitch were introduced to plasmid pTB13 using the QuikChange site-directed mutagenesis kit (Agilent). RNA transcribed from PCR templates of plasmid pTB13 (for wild-type RNA), pTB13-G72A (for G72A RNA) and pTB13-G72U (for G72U RNA) was purified as described above.

RNA preparation for ITC

G72U mutant RNA transcribed from PCR templates of plasmid pTB13-G72U was purified as described above for crystallography.

RNA preparation for SHAPE analysis

DNA templates for the aptamer domain of the *E. coli* TPP riboswitch and its corresponding mutants, each embedded within 5' and 3' structure cassette flanking sequences, were generated by PCR (Wilkinson et al., 2006). RNAs were generated 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, 50 μL PCR-generated template, 0.1 g/L T7 RNA polymerase, 37 °C; 4 h]. RNAs were purified by denaturing polyacrylamide gel electrophoresis (8% polyacrylamide, 7 M urea gels) and recovered overnight by passive elution and ethanol precipitation. The purified RNAs were resuspended in 50 μL 1x TE and stored at -20 °C.

SAXS data collection and analysis

RNA was folded by equilibration in 1X SAXS buffer [50 mM Tris-HCl (pH 8.1), 0.1 M KCl, 10 mM NaCl, 1.5 mM $MgCl_2$] and fractionated on Superdex 75 or Superdex 200 size-exclusion chromatography columns (GE Life Sciences) to remove aggregates and dimers. Samples were concentrated by washing 3 times in 0.5 mL 10,000 MWCO Amicon centrifugal ultrafiltration

devices (Millipore). RNA samples were washed with 1X SAXS buffer (ligand-free) or 1X SAXS buffer supplemented with 100 μM TPP, 1.5 mM thiamine, or 2 mM fragment **2**. All RNA was filtered with Amicon Ultrafree MC spin filters (Millipore) prior to use.

All SAXS experiments were performed at the 12-ID-B beamline at the APS. Buffer samples were made from the same solutions used to wash the RNA samples. Solutions were under continuous flow during exposure to X-radiation. Twenty 1-second exposures were separately collected for the buffer and sample. All RNA samples in identical buffers were collected in a series; buffer samples were collected before and after each set of samples and checked for agreement to ensure consistent experimental conditions over of sample collection.

Scattering data were reduced to a one-dimensional scattering plot using IGOR PRO (WaveMetrics) software. Data were separately averaged for each solution followed by subtraction of the buffer scattering of the sample. The *q*-range used for data analysis was generally between 0.05 and 0.3 $Å^{-1}$. Reported R_g values were calculated from a Guinier plot in the *q*-range such that q_{max} x R_g ~ 1.3. Errors are estimated based on experiments repeated with multiple samples. The GNOM software (Svergun, 1992), part of the PRIMUS package (Konarev et al., 2003), was used to calculate *P*(*r*) plots. *P*(*r*) plots were normalized to *I*(0) to account for differences in concentration.

Isothermal titration calorimetry

One milligram of RNA was folded in 500 μL of 1x ITC buffer [50 mM Tris-HCl (pH 8.1), 0.1 M KCl, 10 mM NaCl, 10 mM $MgCl_2$] and filtered with an Amicon Ultrafree MC spin filter (Millipore). The RNA was washed 3 times with 1x ITC buffer with an 0.5 mL 10,000 MWCO Amicon spin column (Millipore). Fragment **2** and TPP were dissolved in 1x ITC buffer to a concentration of 3 mM. Measurements were carried out by titrating ligand into the RNA (~300 μL; 100 μM) placed in the sample cell using one injection of 0.2 μL followed by 16 injections of 2.35 μL each, with 180 sec intervals between injections; the reference power was 6 $\mu cal/s^{-1}$. Thermograms were analyzed with Origin (MicroCal) or with SEDPHAT and NITPIC (Keller et al., 2012; Schuck, 2000).

SHAPE analysis

RNA constructs (5 pmol in 5 μL 1/2x TE) were heated at 95 °C for 2 min, cooled on ice for 3 min, treated with 3 μL 3.3x 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 mL; 50 μM TPP or 20 mM fragment **2**) or sterile water was added and incubated at 37 °C for 20 min. After incubation, 9 μL of the folded RNA (+/- ligand) was treated with 1 μL 80 mM 1M7 (1-methyl-7-nitroisatoic anhydride; in anhydrous DMSO) (Mortimer and Weeks, 2007) and incubated at 37 °C for 70 sec. No-reagent control reactions were performed with 1 μL neat DMSO. The RNA was recovered by ethanol precipitation and resuspended in 10 μL 1/2x TE. Primer extension for RNA from the 1M7-modified reaction, the control reaction, and for dideoxy sequencing ladders was performed as described (Steen et al., 2010). cDNA fragments were separated by capillary electrophoresis

using an Applied Biosystems 3500 Genetic Analyzer DNA sequencing instrument. Raw capillary electrophoresis traces were analyzed using QuSHAPE (Karabiber et al., 2013).

Supplemental References

Edwards, T.E., and Ferré-D'Amaré, A.R. (2006). Crystal structures of the *thi*-box riboswitch bound to thiamine pyrophosphate analogs reveal adaptive RNA-small molecule recognition. Structure *14*, 1459–1468.

Ferré-D'Amaré, A.R., and Doudna, J.A. (1996). Use of *cis*- and *trans*-ribozymes to remove 5' and 3' heterogeneities from milligrams of in vitro transcribed RNA. Nucleic Acids Res *24*, 977–978.

Karabiber, F., McGinnis, J.L., Favorov, O.V., and Weeks, K.M. (2013). QuShape: rapid, accurate, and best-practices quantification of nucleic acid probing information, resolved by capillary electrophoresis. RNA *19*, 63–73.

Keller, S., Vargas, C., Zhao, H., Piszczek, G., Brautigam, C.A., and Schuck, P. (2012). High-precision isothermal titration calorimetry with automated peak-shape analysis. Anal Chem *84*, 5066–5073.

Konarev, P.V., Volkov, V.V., Sokolova, A.V., Koch, M.H.J., and Svergun, D.I. (2003). PRIMUS: a Windows PC-based system for small-angle scattering data analysis. J Appl Crystallogr *36*, 1277–1282.

Mortimer, S.A., and Weeks, K.M. (2007). A fast-acting reagent for accurate analysis of RNA secondary and tertiary structure by SHAPE chemistry. J Am Chem Soc *129*, 4144–4145.

Schuck, P. (2000). Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. Biophys J *78*, 1606–1619.

Serganov, A., Polonskaia, A., Phan, A.T., Breaker, R.R., and Patel, D.J. (2006). Structural basis for gene regulation by a thiamine pyrophosphate-sensing riboswitch. Nature *441*, 1167–1171.

Steen, K.-A., Malhotra, A., and Weeks, K.M. (2010). Selective 2′-hydroxyl acylation analyzed by protection from exoribonuclease. J Am Chem Soc *132*, 9940–9943.

Svergun, D.I. (1992). Determination of the regularization parameter in indirect-transform methods using perceptual criteria. J Appl Crystallogr *25*, 495–503.

Wilkinson, K.A., Merino, E.J., and Weeks, K.M. (2006). Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution. Nat Protoc *1*, 1610–1616.

Xiao, H., Edwards, T.E., and Ferré-D'Amaré, A.R. (2008). Structural basis for specific, high-affinity tetracycline binding by an in vitro evolved aptamer and artificial riboswitch. Chem Biol *15*, 1125–1137.