Supporting Information

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SI Materials and Methods

All oligoribonucleotides were chemically synthesized following the descriptions in refs. 1–4 with slight modifications as outlined below.

Solid-phase synthesis of oligoribonucleotides. All oligonucleotides were synthesized on Applied Biosystems instruments (ABI 392) following DNA/RNA standard synthesis cycles.

Detritylation (1.8 min) used dichloroacetic acid/1,2-dichloroethane (4/96); coupling (2.0 min) was as follows: phosphoramidites/acetonitrile (0.1 M × 120 μL) were activated by benzylthiotetrazole/acetonitrile (0.3 M × 360 μL); capping (0.25 min) used solutions A and B: A—acetic anhydride/sym-collidine/acetonitrile (20/30/50); B—4-(dimethylamino)pyridine/acetonitrile (0.5 M), A/B = 1/1; oxidation (0.33 min) used I_2 (20 mM) in tetrahydrofuran/pyridine/H_2O (7/2/1). For 5-aminoallyl-uridine (^{5aa}U) and 2′-*O*-aminopropyl-cytidine ($^{2'propyl}$C) sequences, the following mild capping solutions were used: A—0.2 M phenoxyacetic anhydride in THF; B—0.2 M *N*-methylimidazole and 0.2 M sym-collidine in THF. Acetonitrile, solutions of amidites, and tetrazole were dried over activated molecular sieves overnight.

2′-O-[(Triisopropylsilyl)oxy]methyl(2′-O-TOM)-protected ribonucleoside phosphoramidites (5) and 2′-*O*-methyl ribonucleoside phosphoramidites were obtained from Glen Research or ChemGenes. 2′-*O*-aminopropyl-cytidine phosphoramidite was purchased from ChemGenes, 2′-*O*-propargyl-adenosine ($^{2'prop}$A) phosphoramidite from Jena Bioscience, 5′-biotin phosphoramidite, and protected biotin serinol phosphoramidite were purchased from Glen Research. 5-Aminoallyl-uridine (^{5aa}U) phosphoramidite was purchased from Berry & Associates. All solid supports for RNA synthesis were purchased from GE Healthcare (Custom Primer Supports: riboA 80, dA 80).

Deprotection of oligonucleotides. RNA oligonucleotides were deprotected by using CH_3NH_2 in ethanol (8 M, 0.65 mL) and CH_3NH_2 in H_2O [40% (wt/vol), 0.65 mL] for 4–6 h at 35 °C. After complete evaporation of the solution, the 2′-*O*-TOM protecting groups were removed by treatment with tetrabutylammonium fluoride trihydrate (TBAF·3H_2O) in THF (1 M, 1.0–1.5 mL) for at least 14 h at 37 °C. The reaction was quenched by addition of triethylammonium acetate (1 M, pH 7.0, 1.0–1.5 mL). The volume of the solution was reduced to 0.8 mL, and the solution was loaded on a GE Healthcare HiPrep 26/10 desalting column (2.6 × 10 cm; Sephadex G25). The crude RNA was eluted with H_2O, evaporated to dryness, and dissolved in 1.0 mL of nanopure water.

2′-*O*-methyl RNA oligonucleotides were deprotected by using CH_3NH_2 in H_2O [40% (wt/vol), 0.65 mL] and ammonia in H_2O [30–33% (wt/vol), 0.65 mL] for 10 min at room temperature and for 15 min at 65 °C. The solution was evaporated to dryness, and the crude 2′-*O*-methyl RNA was dissolved in 1.0 mL of nanopure water.

Analysis, purification, and mass spectrometry of oligoribonucleotides. Analysis of crude oligonucleotides after deprotection was performed by anion-exchange chromatography on a Dionex DNAPac100 column (4 × 250 mm) at 80 °C (60 °C for 5-aminoallyl-uridine and 2′-*O*-aminopropyl-cytidine RNA variants). Flow rate was 1 mL/min; eluant A was composed of 25 mM Tris·HCl, pH 8.0, and 6 M urea; eluant B was composed of 25 mM Tris·HCl, pH 8.0, 0.5 M $NaClO_4$, and 6 M urea; gradient was 0–60% B in A within 45 min; UV detection was at 260 nm.

Crude RNA products [4,4′-Dimethoxytrityl (DMT) off] were purified on a semipreparative Dionex DNAPac100 column (9 × 250 mm) at 80 °C (60 °C for 5-aminoallyl-uridine and 2′-*O*-aminopropyl-cytidine sequences). Flow rate was 2 mL/min, and gradient was Δ12–22% B in A within 20 min. Fractions containing oligonucleotide were loaded on a C18 SepPak cartridge (Waters/Millipore), washed with 0.1 M triethylammonium bicarbonate and H_2O, eluted with H_2O/CH_3CN 1/1, and lyophilized to dryness.

The purified oligonucleotides were characterized by mass spectrometry on a Finnigan LCQ Advantage MAX ion trap instrumentation connected to an Amersham Ettan micro liquid chromatography (LC) system (negative-ion mode with a potential of −4 kV applied to the spray needle). LC included 200 pmol of oligonucleotide dissolved in 30 μL of 20 mM EDTA solution (average injection volume: 30 μL); column (XterraMS, C18 2.5 μm; 1.0 × 50 mm) at 21 °C; flow rate was 100 μL/min. Eluant A consisted of 8.6 mM triethylamine and 100 mM 1,1,1,3,3,3-hexafluoro-2-propanol in H_2O (pH 8.0); eluant B consisted of methanol. Gradient was 0–100% B in A within 30 min, and UV detection was at 254 nm.

Preparation of Cy3-Cy5–labeled RNA. Materials used included (Sulfo-) Cy3 and (Sulfo-) Cy5 NHS Ester purchased from GE Healthcare or Lumiprobe. DMSO was dried over activated molecular sieves. Labeling was performed as described in ref. 5 with slight modifications as described: Dye-NHS ester (1 mg; ∼1,200 nmol) was dissolved in anhydrous DMSO (500 μL). Lyophilized RNA (20 nmol) containing 5-aminoallyl-uridine or 2′-*O*-aminopropyl-cytidine modification was dissolved in labeling buffer (25 mM phosphate buffer, pH 8.0), and nanopure water was added to reach a fraction of 55% (vol/vol) (49 μL) of the intended final reaction volume (89 μL) with a final concentration of c_{RNA} of 225 μM. The corresponding volume of the dye-NHS ester solution [45% (vol/vol)] (40 μL) was added to the RNA solution (to reach a concentration of c_{Dye} = 1,124 μM in the final reaction volume). The reaction mixture was gently tumbled on a shaker for 5 h at room temperature in the dark.

Product purification was achieved by precipitation with 2.5 equivalents of reaction volumes containing absolute ethanol and 1/5 equivalents of reaction volumes containing 1 M sodium acetate for 30 min at −20 °C and centrifuged for 30 min at 4 °C at 13,000 × *g* to remove the excess of unreacted and hydrolyzed dye. The pellets were dried under air and high vacuum. The dried pellets were resuspended in water and purified by anion-exchange chromatography on a Dionex DNAPac100 column (9 × 250 mm) at 60 °C. Flow rate was 2 mL/min, and gradient was Δ12–22% B in A within 20 min; UV detection was at a wavelength λ of 260 nm (RNA), 548 nm (Cy3), and 646 nm (Cy5). Fractions containing labeled oligonucleotide were loaded on a C18 SepPak cartridge (Waters/Millipore), washed with 0.1 M triethylammonium bicarbonate and H_2O, eluted with H_2O/CH_3CN 1/1, and lyophilized to dryness.

Click labeling was performed with the following materials: Sulfo-Cy3 azide (1 mg; ∼1,800 nmol), purchased from Lumiprobe, was dissolved in H_2O (180 μL). Lyophilized RNA (20 nmol) containing 2′-*O*-propargyl-adenosine modification was dissolved in 3 μL acetonitrile (20% of the intended final reaction volume), 100 nmol azide dye (10 μL), 300 nmol sodium ascorbate, and 300 nmol copper sulfate to give a final reaction volume of 15 μL. The reaction mixture was gently tumbled on a shaker for 2 h at room temperature under argon atmosphere. The reaction mixture was directly purified by anion-exchange chromatography on a Dionex DNAPac100 column (9 × 250 mm) at 80 °C.

Enzymatic ligation. Enzymatic ligations were performed as described in refs. 6 and 7. The use of T4 DNA ligase requires a double-stranded ternary substrate formed by a 5′-phosphorylated

RNA donor, a single-stranded RNA acceptor with a free 3′-OH group, and a splint oligonucleotide. The following fragments were used (for the corresponding modifications and their positions, see main text): 45-nt RNA acceptor strand for the 81-nt RNA sequences (WT/14–87, WT/24–68, WT/29–62, A69G/24–68): 5′-ACG ACU CGG GGU GCC CUU CUG CGU GAA GGC UGA GAA AUA CCC GUA-3′; 36-nt RNA donor strand for the 81-nt RNA sequences (WT/14–87, WT/24–68, WT/29–62, A69G/24–68): 5′-p UCA CCU GAU CUG GAU AAU GCC AGC GUA GGG AAG UCA-3′; 45-nt RNA acceptor strand for the 82-nt RNA sequence (WT^{P1stab}/24–68): 5′-CGG ACU CGG GGU GCC CUU CUG CGU GAA GGC UGA GAA AUA CCC GUA-3′; 37-nt RNA donor strand for the 82-nt RNA sequence (WT^{P1stab}/24–68): 5′-p UCA CCU GAU CUG GAU AAU GCC AGC GUA GGG AAG UCC G-3′; Splint 18-nt 2′-*O*-methyl-RNA: 5′-UCA GGU GAU ACG GGU AUdA-3′.

The 45-nt RNA acceptor strand for WT/41–55 containing the 2′-*O*-propargyl-adenosine-41 for click chemistry was ligated from two fragments: 16-nt RNA acceptor strand: 5′-ACG ACU CGG GGU GCC C-3′; 29-nt RNA donor strand: 5′-p UUC UGC GUG AAG GCU G($^{2'prop}$A41)G AAA UAC CCG UA-3′; Splint 30-nt DNA: 5′-AGC CTT CAC GCA GAA GGG CAC CCC GAG TCG-3′; 36-nt RNA donor strand for WT/41–55: 5′-p U (^{5aa}U55)A CCU GAU CUG GAU AAU GCC AGC GUA GGG AAG UCA-3′.

Ligation reactions were first performed on an analytical scale (0.4 nmol) before proceeding to a preparative scale (5–12 nmol). T4 DNA ligase was purchased from Fermentas (5 U/μL). Optimal ligation conditions were the following: 10 μM for each RNA fragment, final ligase concentration of 0.5 U/μL in a final volume of 0.5–1.2 mL; 3 h at 35 °C for 81- and 82-nt RNA sequence (WT/14–87, WT/24–68, WT/29–62, A69G/24–68, WT/41–55, WT^{P1stab}/24–68); 5 h at 37 °C for 45-nt RNA sequence (WT/41–55). Analysis of the ligation reaction and purification of the ligation products were performed by anion-exchange chromatography. Liquid chromatography electrospray ionization mass spectrometry (LC ESI MS) was used for characterization of the HPLC-purified RNA. The yield of the thiamine pyrophosphate (TPP) riboswitch aptamer was higher than 30% after purification by anion-exchange chromatography.

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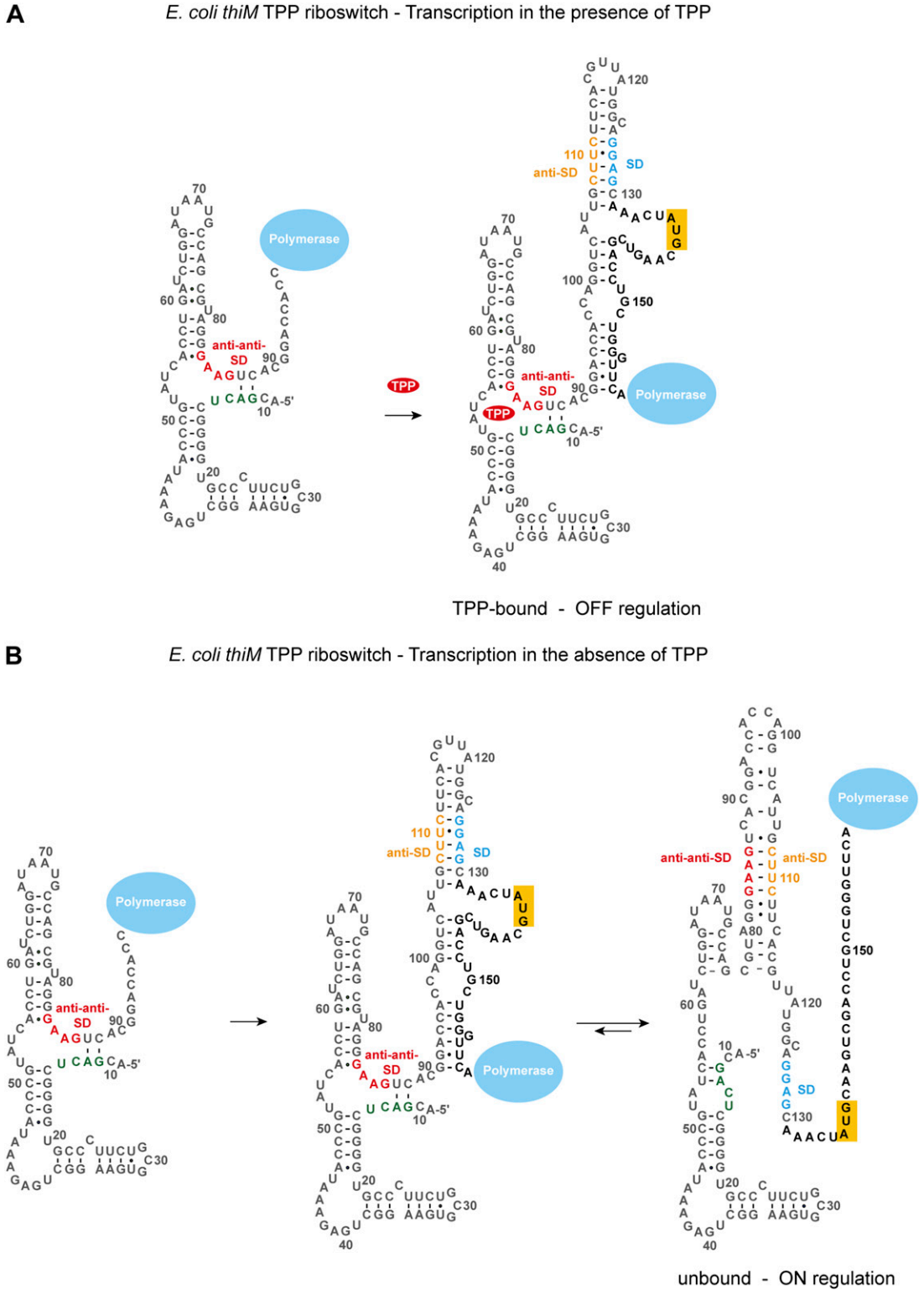

Fig. S1. Secondary structure model for cotranscriptional folding of the *Escherichia coli thiM* riboswitch in the presence (*A*) and absence (*B*) of TPP.

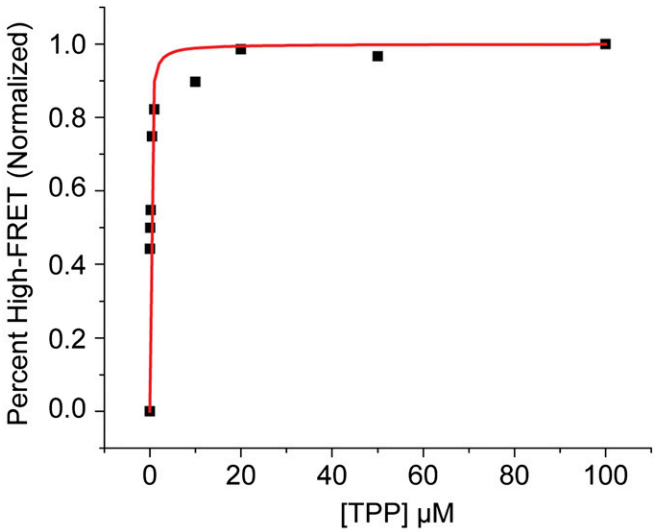

Fig. S2. Dye-labeled TPP riboswitch (WT/24–68) binds TPP with nanomolar affinity. The affinity of TPP for the WT/24–68–labeled construct was estimated by titrating TPP from 0 to 100 μM in the presence of 2 mM Mg^{2+} and quantifying the change in population of fully folded riboswitch molecules [high-fluorescence resonance energy transfer (FRET) state occupancy] as a function of TPP concentration using single-molecule FRET measurements. Shown (black squares) is the average high-FRET state occupancy value (total area under the curve) obtained by fitting population FRET histograms to three Gaussian distributions (low-, intermediate-, and high-FRET) over three independent experiments. Each value was normalized to the percentage of high-FRET state occupancy observed at 100 μM TPP. The apparent dissociation constant was determined by fitting these data points to the equation $y = y_{max}(x/x + K_d)$. The estimated K_d value from these fitting procedures (fit shown in red) was 115 nM (5).