Supporting Information

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SI Text

SI Materials and Methods. ***RNA solid-phase synthesis.*** 2′-*O*-TOM standard nucleoside phosphoramidites were obtained from GlenResearch or ChemGenes. 2′-*O*-*tert*.-Butyldimethylsilyl-5′-*O*-(4,4′-dimethoxytrityl)-5-fluoro uridine phosphoramidite was purchased from ChemGenes. 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[(triisopropylsilyl)oxy]methyl-2-aminopurine (Ap) phosphoramidite was synthesized according to reference (1). All solid supports for RNA synthesis were purchased from GE Healthcare (Custom Primer Supports: riboC Ac 80, riboA 80, riboG 80, riboU 80).

Solid-phase RNA synthesis protocol: Detritylation (2.0 min): dichloroacetic acid/1,2-dichloroethane (4/96); coupling (3.0 min): phosphoramidites/acetonitrile (0.1 M × 120 μL) were activated by benzylthiotetrazole/acetonitrile (0.3 M × 360 μL); capping (3 × 0.4 min): A: Ac_2O/sym-collidine/acetonitrile (20/30/50), B: 4-(dimethylamino)pyridine/acetonitrile (0.5 M), A/B = 1/1; oxidation (1.0 min): I_2 (10 mM) in acetonitrile/sym-collidine/H_2O (10/1/5). Acetonitrile, solutions of amidites and tetrazole were dried over activated molecular sieves overnight.

Enzymatic ligation. Enzymatic ligations were performed along the lines described in reference (2). 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: 5′-AGA UGU GCU AGC AAA ACC AUC UUU AAA AAA CU(Ap) G-3′ (acceptor strand 34 nt RNA), 5′-p ACU UGG GGU GCA AGU CCC CUU UUU UAU p-3′ (donor strand for the 61 nt RNA variant), 5′-p ACU UGG GGU GCA AGU CCC CUU UUU UAU UGC UUA AAU p-3′ (donor strand for the 70 nt RNA variant); 5′-ACT TGC ACC CCA AGT CTA GTT TTT TAA AGA-3′ (splint DNA). For optimization of ligation conditions, ligation reactions were first performed on an analytical scale (0.2 to 1 nmol) with different lengths of DNA splint before proceeding on preparative scale (20 nmol). T4 DNA ligase was purchased from *Fermentas* 5 U/μL. Optimal ligation conditions: 10 μM for each RNA fragment, 12 μM for the 30 nt DNA splint and a final ligase concentration of 0,5 U/μL in a total volume of 2 ml; 22 h at 39 °C.

Analysis of the ligation reaction and purification of the ligation products were performed by anion exchange chromatography. LC ESI MS was used for characterization of the HPLC-purified RNA. The yield of the full-length $preQ_1$ riboswitch was >40% after purification by anion exchange chromatography (61 nt: 10.5 nmol, OD^{260} ~ 7.2; 70 nt: 9.0 nmol, OD^{260} ~ 7.2).

Native gel shift assay. RNA dimerization behavior and ligand binding were investigated by a native gel shift assay with unlabeled and ^{32}P-labeled RNA (approximately 25,000 cpm for each lane). The RNA samples in absence or presence of the ligand $preQ_1$ (3) in 10 μL loading buffer (50 mM KMOPS pH 7.0, 100 mM KCl, 3 mM $MgCl_2$, and 5% glycerol) were prepared in two different ways: (*A*) Heat shock (*hs*): the sample was heated to 90 °C for 1 min, then rapidly cooled in an ice bath and equilibrated to room temperature for 15 min. (*B*) Annealing (*an*) the sample was heated to 90 °C for 1 min, then incubated for 15 min at room temperature. The reaction mixture was then resolved on a 12% polyacrylamide gel (C = 2.5%). The nondenaturing gel was run for approximately 5 h (approximately 180 V at 4 °C or room temperature). The unlabeled RNA and RNA–ligand complex were visualized by methylene blue staining (staining solution: methylene blue 0.02% in H_2O). ^{32}P-labeled RNA was analyzed using a phosphor imaging screen. Final concentrations of RNA and $preQ_1$ were as indicated in the corresponding figure captions.

Chemical and enzymatic probing. Lead(II)-induced cleavages and enzymatic probing were used to study the secondary structure of the different RNA sequences either free or in complex with $preQ_1$. For detection of the cleavages 5′ end-labeled RNA was chosen (4–7).

5′ End-labeling of RNA. RNA (15 pmol) was 5′ end-labeled using 3 μL of [γ-^{32}P]ATP (6000 Ci/mmol, 500 μCi), 1 μL T4 polynucleotide kinase PNK (*Fermentas*— 10 U/μL), 8 μL H_2O, 1.5 μL 10 × PNK buffer (0.5 M Tris-HCl pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, and 1 mM EDTA). The reaction was incubated for 30 min at 37 °C and then stopped with 1 μL 140 mM EDTA. The labeled RNA was purified by electrophoresis on a 8% polyacrylamide gel (C = 3.6%; 7 M urea). After PAGE purification, labeled RNA was eluted from gel slices with 200 μL of 0.2 M NaCl overnight at 4 °C. The RNA was then precipitated and diluted in 20 μL water. The activity of the RNA was measured on a scintillation counter.

Lead(II)-induced cleavages. All reactions were performed in a total volume of 20 μL. Labeled RNA (1 μL, approximately 50,000 cpm) and unlabeled RNA (3 μL, 45 pmol) were heated to 90 °C for one minute, and then rapidly cooled in an ice bath. The RNA samples were renatured in the presence of 13 μL water and 2 μL 10 × buffer (0.5 M KMOPS pH 7.0, 1 M KCl, 20 mM Mg^{2+}). The reactions were performed without or with 12.5 μM $preQ_1$ (1 μL of 250 μM $preQ_1$ stock solution). Hydrolysis was initiated with 2 μL of a freshly prepared 10 mM (20 mM) aqueous lead(II)-acetate solution (1–2 mM final concentration; lead(II)-actetat trihydrate purchased from Sigma–Aldrich) for 15 min at 20 °C. The reactions were stopped by adding 6 μL of 140 mM EDTA, precipitated with 70 μL of 0.3 mM NaOAc in 80% ethanol and 1 μL glycogen (10 μg/μL) for 20 min at −20 °C and centrifuged for 20 min at 4 °C at 13,000 rpm.

Enzymatic probing. All reactions were performed in a total volume of 20 μL. Appropriate dilutions of enzymes were prepared in dilution buffer (50 mM Tris-HCl pH 7.0, 50% glycerol) just before use. The reactions were performed without or with 12.5 μM $preQ_1$ (1 μL of 250 μM $preQ_1$ stock solution). *RNase S1*: 2 μL *S1* (10 U/μL, *Promega*— 95 U/μL); 2 μL of (0.5 M KMOPS pH 7.0, 1 M KCl, 100 mM $MgCl_2$, 10 mM $ZnCl_2$); 10 min at 20 °C (*RNase S1* is specific for unpaired regions). *RNase V1*: 2 μL *V1* (0.01 or 0.04 U/μL, *Ambion*— 0.1 U/μL); 2 μL of 10 × reaction buffer (0.5 M KMOPS pH 7.0, 1 M KCl, 100 mM $MgCl_2$); 10 min at 20 °C (*RNase V1* is specific for double-stranded regions). The reactions were stopped and precipitated by adding 30 μL of a commercially available precipitation/inactivation buffer (Ambion), 70 μL of 0.3 mM NaOAc in 80% ethanol and 1 μL glycogen (10 μg/μL).

Incubation control and ladder for cleavage assignments. Incubation controls C in the absence or presence of $preQ_1$ were always performed to detect non specific cleavages. In these controls, lead(II)-acetate or the enzyme were replaced by nanopure H_2O. The *RNase T1* ladder was carried out with labeled RNA (1 μL, approximately 50,000 cpm), unlabeled RNA (3 μL, 45 pmol), 6 μL of RNA loading buffer (0.02% xylene cyanol, 0.02% bromophenol blue in 8 M urea—preincubation at 50 °C for 5 min) and 2 μL of *RNase T1* (0.5 U/μL, *Fermentas*— 1000 U/μL) for 10 min at 50 °C. The T1 reaction was directly loaded on the gel. For the alkaline ladder labeled RNA (2 μL, ~100,000 cpm) and unlabeled RNA (6 μL, 90 pmol) were

incubated at 90 °C for 15 min in the presence of 23 μL of ladder buffer (100 mM Na_2CO_3, 100 mM Na_2CO_3, pH 9.0). The alkaline reaction was stopped and precipitated by adding 115 μL of 0.3 mM NaOAc in 80% ethanol and 1 μL glycogen (10 μg/μL).

The pellets were washed with 70% cold ethanol and dissolved in 8 μL (C and H in 10 μL) of loading buffer (0.02% xylene cyanol, 0.02% bromophenol blue in 8 M urea). The end-labeled RNA fragments were sized by electrophoresis on a 15% polyacrylamide gel (C = 3.3%; 7 M urea; 1 × TBE; approximately 80 min at 40 W). The 15% PAGE was transferred without drying on a plastic film and analyzed using a phosphor imaging screen (overnight exposure at −20 °C).

Fluorescence spectroscopy—Binding affinities. Data were fit using a K_D quadratic equation solution for 1∶1 stoichiometry.

$$\frac{F - F_0}{F_f - F_0} = (K_D + [\mathrm{preQ_1}]_{tot} + [\mathrm{RNA}]_{tot} + \{(K_D + [\mathrm{preQ_1}]_{tot} + [\mathrm{RNA}]_{tot})^2 - 4[\mathrm{preQ_1}]_{tot}[\mathrm{RNA}]_{tot}\}^{-2})/2[\mathrm{RNA}]_{tot}$$

F_0 (initial fluorescence), F_f (final fluorescence), $[\mathrm{RNA}]_{tot}$ (total Ap-RNA concentration), $[\mathrm{preQ_1}]_{tot}$ (total $\mathrm{preQ_1}$ concentration for each titration step).

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Fig. S1. Chemical and enzymatic probing of a full-length riboswitch domain (54 nt) by lead(II)-acetate, *RNase S1* and *RNase V1* in the absence (–) and presence (+) of a 4-fold excess of $\mathrm{preQ_1}$. *C* designates control reactions without and with $\mathrm{preQ_1}$. T1 and H designate *RNase T1* and alkaline hydrolysis ladders, respectively. For further details see *SI Materials and Methods*.

Fig. S2. ^1H NMR spectroscopic analysis of the 61 nt U37-^{5F}U preQ_1 riboswitch. (*A*) Secondary structure model (see also Fig. 3 in the main text). (*B*) ^1H NMR imino proton spectra of the 61 nt U37-^{5F}U variant before and after addition of preQ_1 and Mg^{2+} ions. Conditions: $c_{RNA} = 0.2$ mM, $c_{preQ_1} = 0.2–0.4$ mM, $c_{Mg} = 3.0$ mM, 25 mM Na_2HAsO_4, pH 6.5, 298 K, H_2O/D_2O 9/1.

Fig. S3. Aminopurine (Ap) modified aptamer. (*A*) Schematics of ligand-induced structure rearrangement of the A33Ap preQ_1 aptamer. (*B*) ^1H NMR imino proton spectra of the 34 nt A33Ap variant before and after addition of preQ_1. Conditions: $c_{RNA} = 0.2$ mM, $c_{preQ_1} = 0.4$ mM, $c_{Mg} = 2.0$ mM, 25 mM Na_2HAsO_4, pH 6.5, 298 K, H_2O/D_2O 9/1.

Fig. S4. Stopped-flow fluorescence spectroscopy was used to monitor the kinetics of $preQ_1$/aptamer complex formation (A33Ap 34 nt variant). The figure shows examples of one dataset for different $preQ_1$ concentrations (ranging from 2 to 11 μM) with single-exponential curve fits. The determined k' values are plotted against the concentration of $preQ_1$ and subjected to a linear fit. The slope of the plot yields the rate constant k. The final rate constant k value is an arithmetic mean, determined from three independent stopped-flow measurements. Conditions: $c_{RNA} = 0.5$ μM, 50 mM KMOPS pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 298 K. Further details are available in *SI Text*.

Fig. S5. RNA dimerization behavior and ligand sensitivity was investigated by native gel shift assays and structure probing. (*A*) Aptamer dimerization; native gel at 4 °C with RNA reference sequences (c_{RNA} = 25 μM). The RNA concentration of the wild-type (wt) aptamer ranges from 25 μM to 200 μM. (M) monomer, (D) dimer. (*B*) Aptamer dimerization; same as A, but rt. (*C*) Aptamer dimerization; native gel at 25 °C with ^{32}P-labeled 34 nt wt RNA (c_{RNA} = 0.25 μM–20 μM). (*D*) Ligand responsiveness to RNA (c_{RNA} = 100 μM) with varying length analyzed by native gel shifts at rt [lanes 2(hs), 3(an), 5(hs), 6(an), 8(hs), 9(an), 11(hs), 12(an), 14(hs), 15(an)]; wt refers to the native sequence depicted in Fig. 1; mut refers to the U9A/A33G mutant. A fast migrating band occurs upon preQ$_1$ addition, representing the ligand/aptamer complex (indicated as C). Heat shock (hs), annealing (an), monomer (M), dimer (D). (*E*) Chemical and enzymatic probing of the 34 nt wild type aptamer and the mutant variant (U9C/A33G shown in green color) by lead(II)-acetate, *RNase S1* and *RNase V1* in the absence (–) and presence (+) of a 4-fold excess of preQ$_1$. C designates control reactions without lead(II)-acetate and preQ$_1$. T1 and H designate *RNase T1* and alkaline hydrolysis ladders, respectively. Further details available in *SI Text*.

Fig. S6. Stopped-flow fluorescence spectroscopy was used to monitor the kinetics of $preQ_1$/aptamer complex formation (A33Ap 61 nt variant). The figure shows examples of one dataset for different $preQ_1$ concentrations (ranging from 2 to 11 μM) with single-exponential curve fits. The determined k' values are plotted against the concentration of $preQ_1$ and subjected to a linear fit. The slope of the plot yields the rate constant k. The final rate constant k value is an arithmetic mean, determined from three independent stopped-flow measurements. Conditions: $c_{RNA} = 0.5$ μM, 50 mM KMOPS pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 298 K. Further details available in *SI Text*.

Fig. S7. Stopped-flow fluorescence spectroscopy was used to monitor the kinetics of $preQ_1$/aptamer complex formation (A33Ap 70 nt variant). The figure shows examples of one dataset for different $preQ_1$ concentrations (ranging from 2 to 11 μM) with single-exponential curve fits. The determined k' values are plotted against the concentration of $preQ_1$ and subjected to a linear fit. The slope of the plot yields the rate constant k. The final rate constant k value is an arithmetic mean, determined from three independent stopped-flow measurements. Conditions: $c_{RNA} = 0.5$ μM, 50 mM KMOPS pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 298 K. Further details available in *SI Text*.

Table S1. Thermodynamic and kinetic parameters of riboswitch–ligand binding

Riboswitch class	*Domain*	$K_{D,298\,K}$ [nM]	$k\text{-}on_{298\,K}$ $[M^{-1}s^{-1}]$	$k\text{-}off_{298\,K}$ $[s^{-1}]$	*Lit.*
		Transcriptional control			
$PreQ_1$ *F. nucleatum*	aptamer	283	6.02×10^5	0.170	*
	full-length (61 nt)	208	1.26×10^5	0.026	*
	full-length (70 nt)	261	7.26×10^4	0.019	*
Adenine pbuE *B. subtilis*	aptamer	574	2.63×10^5	0.151	(1)
	full-length	not responsive in vitro			(1)
FMN ribD *B. subtilis*	aptamer	12	1.97×10^5	0.002	(2)
	full-length†	not responsive in vitro			(2), †
cyGG tfoX *V. cholerae*	aptamer	0.011‡	1.67×10^4	1.8×10^{-7}	(3, 4)
		Translational control			
Adenine add *V. vulnif.*	aptamer	440	3.75×10^4	0.017	(5)
	full-length	1550	4.15×10^4	0.064	(5, 6)
TPP thiM *E. coli*	aptamer	495	8.66×10^4	0.043	(7)
	full-length	420	8.13×10^4	0.034	(7)

One definition of thermodynamic/kinetic control refers to the transcriptional time between completion of the aptamer by the RNA polymerase and its progression to the termination decision point (ΔtRNAP) (2). If it is comparable to or longer than $1/k_{off}$ the switch will approximate equilibrium control. However, if ΔtRNAP is much smaller than $1/k_{off}$ the switch is under kinetic control. In the thermodynamically controlled *F. nucleatum* $preQ_1$ regulation scenario discussed in the main text, this would mean that pausing at the U-rich sequence (positions 54–70) in the order of a few seconds were required.

*This study.

†The construct used in ref. 2 contained 244 nucleotides and is close to the full-length domain (244 *ribD*) (ref. 2 and Fig. 3): the *ribD* aptamer comprises 165 nucleotides, the complete full-length domain comprises 263 nucleotides.

‡No temperature specified in ref. 4.

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