Supplementary Information

Conformational capture of the SAM-II riboswitch

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1. Supplementary Methods

1.1. Preparation of RNA

1.1.1. Solid-phase synthesis of oligoribonucleotides

All oligonucleotides were synthesized on Pharmacia instruments (Gene Assembler Plus) following DNA/RNA standard methods on 1 µmol synthesis scale. For an overview on oligoribonucleotides synthesized for this study see Supplementary Table 1.

*Automated synthesis cycle.* Detritylation (2.0 min): dichloroacetic acid / 1,2-dichloroethane (4/96); coupling (3.0 min): phosphoramidites / acetonitrile (0.1 M x 120 µL) were activated by benzylthiotetrazole / acetonitrile (0.3 M x 360 µL); capping (3 x 0.4 min): A: Ac₂O / sym-collidine / acetonitrile (20/30/50), B: 4-(N,N-dimethylamino)pyridine / acetonitrile (0.5 M), A/B = 1/1; oxidation (1.0 min): I₂ (10 mM) in acetone / sym-collidine / H₂O (10/1/5). For RNA containing 5-aminoallyl uridine, 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 (TOM) standard nucleoside phosphoramidites were obtained from ChemGenes. 5'-Biotin phosphoramidite was purchased from GlenResearch. (2'-O-t-Butylidemethylsilyl-3'-O-[(diisopropylamino)(2-cyanoethoxy)phosphino]-5'-O-(4,4'-dimethoxytrityl)-5-[E-3-(trifluoroacetamido)-1-propenyl]uridine (5'-aminoallyl uridine phosphoramidite) was purchased from Berry&Associates. N⁴-Acetyl-2'-O-(tert-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-5-fluoro cytidine phosphoramidite was synthesized according to reference (1). ¹⁵N⁴-Acetyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-[(triisopropylsilyl)oxy]methyl cytidine phosphoramidite was synthesized according to reference (2). 5'-O-(4,4'-dimethoxytrityl)-2'-O-[(triisopropylsilyl)oxy]methyl-2-aminopurine (Ap) phosphoramidite was synthesized according to reference (3). The phosphorsphoramidite building block allowing for 5'-phosphorylation was synthesized according to references (4). Solid supports for RNA synthesis were purchased from GE Healthcare (Custom Primer Supports: riboC Ac 80, riboA 80, riboG 80, riboU 80). For synthesis of 3'-phosphate-modified oligoribonucleotides a solid support was prepared according to references (5) and (6) or obtained from commercial sources (GE Healthcare (Custom Primer Support, 3'-phosphate 100s, 17-5214-49).

1.1.2. Deprotection of oligoribonucleotides

Oligoribonucleotides (from 1 µmol synthesis scale) were deprotected by using CH₃NH₂ in EtOH (8 M, 0.65 mL) and CH₃NH₂ in H₂O (40%, 0.65 mL) at room temperature for 6-8 h. After complete evaporation of the solution, the 2'-O-TOM protecting groups were removed by treatment with tetrabutylammonium fluoride trihydrate (TBAF·3H₂O) 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 (TEAA) (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 x 10 cm; Sephadex G25). The crude RNA was eluted with H₂O, evaporated to dryness and dissolved in 1.0 mL of nanopure water.

1.1.3. 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 x 250 mm) at 80°C (60°C for the 5-aminoallyl uridine RNA variants). Flow rate: 1 mL/min; eluant A: 25 mM Tris-HCl pH 8.0, 6 M urea; eluant B: 25 mM Tris-HCl pH 8.0, 0.5 M NaClO₄, 6 M urea; gradient: 0-60% B in A within 45 min; UV-detection at 260 nm.

Crude RNA products (trityl-off) were purified on a semi-preparative Dionex DNAPac100 column (9 x 250 mm) at 80°C (60°C for 5-aminoallyl uridine containing RNA). For the amount of crude product obtained by 1 µmol scale synthesis, we typically used 5 to 8 individual runs in order to avoid overloading of the column. Flow rate: 2 mL/min; gradient: 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 (Et₃NH)₄HCO₃⁻ and H₂O, eluted with H₂O/CH₃CN 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 LC system (negative-ion mode with a potential of -4 kV applied to the spray needle). LC: Sample (200 pmol of oligonucleotide dissolved in 30 µL of 20 mM EDTA solution; average injection volume: 30 µL); column (Xterra® MS, C18 2.5 µm; 1.0 x 50 mm) at 21°C; flow rate: 100 µL/min; eluant A: 8.6 mM TEA, 100 mM 1,1,1,3,3,3-hexafluoro-2-propanol in H₂O (pH 8.0); eluant B: methanol; gradient: 0-100% B in A within 30 min; UV detection at 254 nm.

1.2. NMR spectroscopy

¹H NMR imino proton spectra were recorded on a Varian Unity 500 MHz instrument and applied a selective excitation refocusing sequence employing selective pulses shaped according to the G4 (excitation; 2.62 ms, RF amplitude 1.74 kHz) and REBURP (refocusing; 1.4 ms, RF amplitude 4.47 kHz) profile, respectively (7-9). Both shaped pulses were centered at 13 ppm.

¹⁹F NMR spectra without ¹H-decoupling were recorded at a frequency of 470.3 MHz on a Varian Inova 500 MHz NMR spectrometer equipped with a 5 mm Indirect Detection PFG probe. Typical experimental parameters were chosen as follows: spectral width 14 kHz, ¹⁹F
excitation pulse 12.4 µs, acquisition time 2.0 s, relaxation delay 2.0 s, number of scans 2048.

$^{19}$F resonances were referenced relative to external CCl$_3$F.

Typical NMR sample preparation:

- Free RNA: the RNA (triethyl ammonium salts) was lyophilized and dissolved in water, the corresponding amount of arsenate buffer solution (50 mM, pH 6.5) and D$_2$O to a total volume of 500 µL.
- RNA/SAM complex: the aliquots of a 25 mM aqueous solution of SAM and a 0.3 mM solution of MgCl$_2$ were directly added to the NMR sample of the free RNA.

Final concentrations were as indicated in the corresponding Figure captions. All samples were heated to 90°C for one minute, then rapidly cooled in an ice bath and equilibrated to room temperature for 15 min before measurements.

1.3. Steady-state fluorescence spectroscopy

All experiments were performed on a Cary Eclipse spectrometer (Varian, Palo Alto, USA) equipped with a peltier block, a magnetic stirring device, and a RX2000 stopped-flow apparatus (Applied Photophysics Ltd., Leatherhead, UK).

1.3.1. Binding affinities for Ap SAM-II variants

Using quartz cuvettes equipped with a small stir bar, RNA samples were prepared in 0.5 µM concentration in a total volume of 1 mL of buffer (50 mM KMOPS pH 7.5, 100 mM KCl, 2 mM MgCl$_2$). The samples were heated to 90°C for 2 min, allowed to cool to room temperature, and held at 20°C in the peltier controlled sample holder. Then, SAM was manually pipetted in 1 µL aliquots in a way not to exceed a total volume increase of 2%. The solution was stirred during each titration step and allowed to equilibrate for at least 15 min before data collection. Spectra were recorded from 320 to 500 nm using the following instrumental parameters: excitation wavelength, 308 nm; increments, 1 nm; scan rate, 120 nm/min; slit widths, 10 nm.

The apparent binding constants $K_D$ were determined by following the increase in fluorescence after each titration step via integration of the area between 330 and 450 nm. Changes in fluorescence ($F - F_0$) were normalized to the maximum fluorescence measured in the absence of SAM. The measurement for each titration step was repeated at least two times and the mean of the normalized fluorescence intensity was plotted against the SAM concentration. Data were fit using a $K_D$ quadratic equation solution for 1:1 stoichiometry (10).

\[
\frac{(F - F_0)}{(F_f - F_0)} = \frac{K_D + [\text{SAM}]_{\text{tot}} + [\text{RNA}]_{\text{tot}} - \sqrt{(K_D + [\text{SAM}]_{\text{tot}} + [\text{RNA}]_{\text{tot}})^2 - 4[\text{SAM}]_{\text{tot}}[\text{RNA}]_{\text{tot}}}}{2[\text{RNA}]_{\text{tot}}}
\]
Where $F_0$ corresponds to initial fluorescence; $F_f$ corresponds to final fluorescence; $[\text{RNA}]_{\text{tot}}$ corresponds to the total Ap-RNA concentration; $[\text{SAM}]_{\text{tot}}$ corresponds to the total SAM concentration for each titration step. The final $K_D$ value is the arithmetic mean, determined from two independent titration experiments.

1.3.2. Binding affinities for the Cy3/Cy5 variants

Sample preparation of both the Cy3/Cy5 labeled construct and the Cy3-only labeled construct was performed as described above for the Ap variants. The efficiency of energy transfer was measured from the induced emission of the acceptor (Cy5) according to reference (11):

For the Cy3/Cy5 labeled construct, spectra were recorded in a range between 1) 550 to 750 nm while exciting at 548 nm and between 2) 650 to 750 nm while exciting at 646 nm using the following instrumental parameters: increments, 1 nm; scan rate, 120 nm/min; slit widths, 10 nm. For the Cy3-only labeled construct, spectra were recorded in a range between 550 to 750 nm while exciting at 548 nm using the same instrumental parameters.

The donor (Cy3) contribution from each energy transfer is removed by subtracting the Cy3-only emission spectrum from the emission spectrum of the Cy3/Cy5 labeled construct at every wavelength. The resulting spectrum is divided by the integrated intensity between 657 and 710 nm of the emission spectrum excited at 646 nm of the same Cy3/Cy5 labeled sample.

The apparent binding constants $K_D$ were determined by following the increase in fluorescence after each titration step via integration of the area between 630 and 720 nm. Changes in fluorescence ($F - F_0$) were normalized to the maximum fluorescence measured in the absence of SAM. The measurement for each titration step was repeated at least two times and the mean of the normalized fluorescence intensity was plotted against the SAM concentration. Data were fit using a $K_D$ quadratic equation solution for 1:1 stoichiometry, reference (10). The final $K_D$ value was the arithmetic mean, determined from two independent titration experiments.

1.3.3. Rate constants

Rate constants $k$ for individual riboswitch variants (A14Ap, U34Ap, A41Ap, Cy3/Cy5) were measured under pseudo-first-order conditions with SAM in excess over RNA. Stock solutions were prepared for each Ap and Cy3/Cy5 variant (concentration $c_{\text{RNA}} = 0.6 \, \mu\text{M}$ in 50 mM KMOOPS pH 7.5, 100 mM KCl, 2 mM MgCl$_2$) and for SAM (concentration $c_{\text{SAM}} = 1.2$ to 13.2 $\mu$M in 50 mM KMOOPS pH 7.5, 100 mM KCl, 2 mM MgCl$_2$). Mixing equal volumes of these
stock solutions via the stopped-flow apparatus resulted in a final concentration of 0.3 µM RNA and of 0.6 to 6.6 µM SAM. Spectra were recorded at 20°C using the following instrumental parameters for the Ap variants: excitation wavelength, 308 nm; emission wavelength, 372 nm; increment of data point collection, 0.05 s; slit widths, 10 nm. For the Cy3/Cy5 variant, the following instrumental parameters were used: excitation wavelength, 548 nm; emission wavelength, 646 nm; increment of data point collection, 0.05 s; slit widths, 10 nm. The stopped-flow fluorescence data were fit to a single-exponential equation:

\[ F = A_1 + A_2 e^{k't} \]

- \( A_1 \) = final fluorescence
- \( A_2 e^{k't} \) = change in fluorescence over time (t) at the observed rate \( k' \).

The measurement for each concentration was repeated at least three times and the mean of the observed rates \( k' \) was plotted against SAM concentration to obtain the rate constant \( k \) from the slope of the plot. The final rate constant \( k \) value is an arithmetic mean, determined from two independent stopped-flow measurements. All data processing was performed using Kaleidagraph software (Synergy Software, Reading, UK).
1.4. Supplementary References


Supplementary Table 1. Oligoribonucleotides prepared by chemical solid-phase synthesis.

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2. Supplementary Results

Supplementary Fig. 1. Construction of a Cy3/Cy5 labeled SAM-II variant. (a) Concept of synthesis, labeling and enzymatic ligation. (b) Anion exchange (AE) HPLC trace of enzymatic ligation using T4 DNA ligase and a DNA splint at the start (0 h) and after completion (9 h). The product (retention time = 37.3 min) was purified by AE-HPLC. (c) LC-ESI mass spectrum of purified ligation product. See Supplementary Fig. 2 for the chemical structures of fluorophores and linkers used.
Supplementary Fig. 2. Chemical structures of labels, fluorophores and linker. (a) 5-Aminoallyluridine in RNA (5-aaU). (b) N-Hydroxysuccinimide esters of the cyanine dyes Cy3 and Cy5 used for attachment to 5-aaU labeled RNA fragments. (c) Biotin and linker moiety at the 5'-end of the RNA used in the smFRET experiments.
Supplementary Fig. 3. Fluorescence behavior of riboswitch Ap variants. (a) Structure of SAM (left) and SAH (right); (b) Fluorescence response of the U34Ap SAM-II variant upon addition of 5 µM SAM (left) and 5 µM SAH (right); start conditions: cRNA = 0.5 µM, 50 mM KMOPS, 100 mM KCl, 2 mM MgCl₂, pH 7.5, 20°C; additions were performed manually (with an average operational time of ~4 s); (c) Same as (b) for A41Ap SAM-II variant; (d) Same as (b) for A14Ap SAM-II variant; (e) Same as (b) for Cy3/Cy5 SAM-II variant.
Supplementary Fig. 4. Kinetics of Ap SAM-II variants – Part I. Stopped-flow fluorescence spectroscopy was used to monitor the kinetics of SAM-II riboswitch complex formation (exampled for the U34Ap variant here). The figure shows examples of one dataset for different SAM concentrations (ranging from 1.2 to 6.6 µM) with single-exponential curve fits. The determined $k'$ values are plotted against the concentration of SAM and subjected to a linear fit. The slope of the plot yields the rate constant $k_{on}$. The final rate constant $k_{on}$ value is an arithmetic mean, determined from two independent stopped-flow measurements.

Conditions: $c_{RNA} = 0.3$ µM, 50 mM KMOPS pH 7.0, 100 mM KCl, 2 mM MgCl$_2$, 293 K. Further details are available in Supplementary Methods section.
Supplementary Fig. 5. Kinetics of Ap SAM-II variants – Part II. Plot of observed rate $k'$ versus ligand concentration $c$ for three different SAM-II Ap variants and the Cy3/Cy5 variant as indicated. Observed rates were determined under pseudo-first-order conditions from at least two independent stopped-flow measurements. The slope of the plot yields the rate constant $k_{on}$ (values are the mean and s.d. of at least two independent experiments).
Supplementary Fig. 6. Access to the binding pocket of the SAM-II riboswitch. Different views on the P2b/L1/P2a region (a-c); coordinates according to pdb 2QWY (Gilbert, S.D., Rambo, R.P., Van Tyne, D. & Batey, R.T. Structure of the SAM-II riboswitch bound to S-adenosylmethionine. Nat. Struct. Mol. Biol. 15, 177-182 (2008)).
Supplementary Fig. 7. NMR spectra of 5-fluoro-C16 labeled SAM-II riboswitch. (a) Schematics of the SAM-II pseudoknot with the crucial Watson-Crick base pair C16:G50 highlighted in blue. (b) Structure of the potential base pair of 19F-labeled cytidine-16 and G50. (c) 19F NMR spectra of the 5-F-C16 labeled RNA in the absence of ligand and absence of Mg2+ (top), after addition of Mg2+ (middle), and after addition of SAM (bottom); (d) 1H-NMR imino proton spectra the 5-F-C16 labeled RNA in the absence of ligand and Mg2+ (top), after addition of Mg2+ (middle), and after addition of SAM (bottom). Conditions for (c) and (d): \( c_{RNA} = 0.2 \text{ mM}, c_{SAM} = 0.4 \text{ mM}, c_{Mg} = 2.0 \text{ mM}, 25 \text{ mM Na}_2\text{HAsO}_4, \text{ pH} 7.0, \text{ H}_2\text{O}/\text{D}_2\text{O} 9:1, 298 \text{ K} \); (e) 19F NMR spectra of the 5-F-C16 labeled RNA in the absence of ligand and presence of Mg2+ at different temperatures as indicated. Conditions: \( c_{RNA} = 0.2 \text{ mM}, c_{Mg} = 2.0 \text{ mM}, 25 \text{ mM Na}_2\text{HAsO}_4, \text{ pH} 7.0, \text{ H}_2\text{O}/\text{D}_2\text{O} 9:1 \).