Supporting Information

Modified pyrimidines specifically bind the purine riboswitch

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Materials and Methods

RNA Synthesis and Preparation. RNA was transcribed from a PCR amplified template using in vitro transcription. Oligonucleotides for the PCR reaction were obtained from Integrated DNA Technologies, Inc. The template was a DNA fragment encoding the guanine riboswitch aptamer domain carrying a C74U mutation¹. The DNA template was constructed by PCR in a reaction that contained 2 mM dNTPs, 1x ThermoPol Reaction Buffer (New England Biolabs, Inc.), and 1 unit of Taq DNA polymerase. In vitro transcription reactions contained 32 mM MgCl₂, 10 mM DTT, 4 mM each NTP, 0.25 mg/ml T7 RNA polymerase, 50 nM DNA template, and 1x Transcription Buffer (10x contains 300 mM Tris-HCl, pH 8.0, 100 mM DTT, 0.1% Triton-X 100, 5 mg/ml spermidine). Reactions were incubated at 37 °C for 4 hrs, and the RNA precipitated in 70% EtOH. RNA was resuspended in 5 ml of 4 M Urea, 125 mM Na-EDTA, pH 8.0, 1x Formamide Load Buffer (90% Formamide, 0.2 mg/ml Bromophenyl Blue, 0.2 mg/ml xylene cyanol). RNA was purified by 12 % denaturing polyacrylamide gel electrophoresis, and electroeluted from the gel into sample buffer. RNA was subsequently dialysed into a suitable buffer, 10 mM K⁺- HEPES and 2 mM 2,4,6-triaminopyrimidine for X-ray crystallography, or 10 mM K⁺- HEPES, 100mM KCl, and 10 mM MgCl₂ for ITC.

Crystallization, Data Collection and Processing, Structure Refinement. Crystals were grown by hanging drop vapor diffusion method by mixing in a 1:1 ration, GR(C74U)-3AY complex with mother liquor containing 20% PEG 3000-, 480 mM

ammonium acetate, 12 mM cobalt hexaammine, and 10 mM K⁺- HEPES, pH 7.5. Crystals grew to maximum size within one week at 25 °C, and were subsequently cryoprotected in mother liquor plus 30% 2-methyl-2,4-pentanediol for 5-10 minutes, mounted on a cryoloop (Hampton Research), and flash frozen in nitrogen. Diffraction data was collected on a ALS synchrotron beamline 8.2.1.

Data was indexed, integrated and scaled using D*TREK in the Crystal Clear package (Rigaku MSC). Crystals diffracting to 1.6 Å were analyzed according to a C2 space group. Molecular replacement in CNS was used to calculate an electron density map of GR(C74U):3AY complex using the previously solved guanine riboswitch bound to hypoxanthine (PDB ID 1U8D) stripped of solvent and ligand molecules as the search model. Clear electron density corresponding to the 2,4,6-triaminopyrimidine ligand, coordinated cobalt hexaammines, and an acetate adjacent to G32 was readily observable in the initial 2F_o-F_c map. To minimize model bias, successive rounds of simulated annealing slow cooling from 5000 K in 25 K steps was performed. Ligands and structure associated ions were added to the model using the O graphical interface and solvent placed by the automated water picking function in CNS. Model refinement was carried out by energy minimization and B-factor refinement in CNS, with a final R-factor of 19.5 % and an R_{free} of 24.2 %.

Isothermal Titration Calorimetry. Sample were prepared as described above. Purine and pyrimidine analogs were dissolved at a concentration 10-fold higher in identical buffer to the RNA. All ITC experiments were carried out at 30 °C. Titrations were performed on an VP-ITC microcalorimeter (Microcal, Inc.) by titrating ligand into a sample cell containing RNA in thirty 10 ml injections with a reference power of 5 mcal/sec. Data analysis was performed using the Origin 5.0 (Origin Laboratories) by the single site binding model:

$$q = \nu \times \Delta H \times \left(\frac{K_a [L]_i^n}{1 + K_a [L]_i^n} - \frac{K_a [L]_{i-1}^n}{1 + K_a [L]_{i-1}^n} \right)$$

where q is the measure amount of heat released, ν is the known value of the reaction, and L_i is the concentration of ligand at the i^{th} injection. The relationship:

$$\Delta G = RT \ln K_d$$

was used to calculate ΔG , the change in free energy, from the fitted parameters.

References

- (1) Gilbert, S. D.; Stoddard, C. D.; Wise, S. J.; Batey, R. T. *J. Mol. Biol.* **2006**, 359, 754-768.

Table S1. Additional thermodynamic parameters obtained from ITC results for the binding of various nucleobases to the *xpt-pbuXGR*(C74U) and the addAR (*add*) purine riboswitches.

Ligand	ΔH (kcal mol ⁻¹)	ΔS (cal mol ⁻¹ K ⁻¹)	N*
2,6-diaminopurine	-40 ± 3	-100 ± 4.0	1 ± 0.04
adenine (GR(C74U))	-30 ± 3	-70 ± 10	0.9 ± 0.1
2,4,5,6-tetraaminopyrimidine	-40 ± 2	-100 ± 6	0.8 ± 0.04
2,4,6-triaminopyrimidine	-40 ± 4	-100 ± 10	0.9 ± 0.1
2,4,-diaminopyrimidine	n.d.	n.d.	n.d.
2,4,6-triamino-1,3,5-triazine	-30 ± 6	-50 ± 4	0.5 ± 0.2
2,6-diaminopurine (<i>add</i>)	-30 ± 1	-70 ± 2	0.7 ± 0.05
adenine (<i>add</i>)	-20 ± 2	-30 ± 7	0.9 ± 0.1
2,4,6-triamino-pyrimidine (<i>add</i>)	-20 ± 1	-30 ± 3	0.9 ± 0.1

*N refers to the experimental molar ratio of nucleobase to riboswitch; for the purine riboswitches, the theoretical value is 1.

Table S2. Crystallographic StatisticsData Collection

Spacegroup:	C2
a, b, c (Å)	131.99, 35.10, 41.80
β (°)	90.43°
Resolution ^a :	20-1.58 Å (1.64-1.58 Å)
Wavelength:	0.9169 Å
% Completeness:	99.3 % (98.7 %)
Measured reflections:	92,995
Unique reflections:	26,388
Average redundancy:	3.52 (3.57)
$\langle I \rangle / \langle \sigma(I) \rangle$:	12.4 (3.3)
R_{sym}^b :	4.7 % (26.1 %)

Refinement

Resolution (Å):	20 – 1.70 (1.76 – 1.70)
Number of reflections:	
Working:	19,673 (92.0 %)
Test set:	1530 (7.2 %)
R_{xtal}^c :	19.5 (23.5)
R_{free}^c :	24.2 (29.6)
r.m.s.d. bonds:	0.017 Å
r.m.s.d. angles:	1.90°
Cross-validated Luzzati coordinate error:	0.26 Å
Cross-validated Sigma-a coordinate error:	0.19 Å
Average B-factor:	27.8 Å ²
RNA	25.1 Å ²
Ligand	20.1 Å ²
Water (353 total)	36.0 Å ²

- a. Numbers in parenthesis correspond to the highest resolution shell.
- b. $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the average intensity of multiple measurements of symmetry related reflections.
- c. $R_{\text{xtal}} = \sum |F_o - F_c| / \sum |F_o|$, R_{xtal} from the working set and R_{free} from the test set.

Figure S1. Alignment of GR(C74U):3AY (green) with GR:hypoxanthine (magenta). The six-membered ring of the purine ligand aligns identically to the the pyrimidine ring, leaving a cavity where the five-membered ring would be.