Supporting Information

Differential analog binding by two classes of c-di-GMP riboswitches

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Characterization of c-di-GMP and analogs. The identity of all analogs was confirmed by ESI-MS in negative ion mode (Table S1) and purity was analyzed by high performance liquid chromatography (HPLC) (Figure S1). All analogs were purified to >95% purity. The [M-H]/1 and [M-2H]/2 peaks were observed for all analogs. These observed masses correspond to the mass of the compound after the loss of one or both protons on the phosphate oxygens, respectively.

k_{on} and k_{off} measurements for the class I riboswitch using 2-aminopurine (2AP) fluorescence. On-rates (k_{on}) were measured under pseudo-first order conditions with ligand in a 10-fold excess over RNA. 200 nM G94(2AP) RNA in buffer containing 10 mM NaCl, 10 mM $MgCl_2$, and 10 mM sodium cacodylate, pH 6.8 was prepared in a quartz cuvette. The fluorescence intensity at 360 nm was measured prior to the addition of c-di-GMP to a final concentration of 2 µM and recorded as time= 0 min. After ligand addition, the fluorescence intensity at 360 nm was monitored over 5 minutes. Data was fit to a single exponential equation as follows:

$$FI = A\left(1 - e^{-kt}\right) + FI_0 \quad \text{(S1)}$$

where A= amplitude, k= rate, t= time and FI_0= fluorescence intensity at time 0. We attempted to fit the data for c-di-GMP to a double exponential but the vast majority of the ligand (92%) reacted in the single exponential with a faster on-rate, while only a very small fraction reacted at a slower rate. The faster on-rate determined from the double exponential fit did not significantly differ from the k_{on} determined from fitting to a single exponential. Because the additional two analogs tested fit best to a single exponential, the k_{on} values reported in Table S2 were determined from the single exponential fits.

To measure the off-rate (k_{off}) of ligand binding, a 200 nM sample of RNA in buffer was prepared as described above with ligand added to a final concentration of 200 nM. The mixture was equilibrated for 45 minutes before a 10-fold excess of the wild-type, non-fluorescently labeled class I riboswitch was

added as a quench and the increase in fluorescence intensity at 360 nm was monitored over time until the signal leveled off. Data fit to the following equation provided the k_{off}:

$$FI = Ae^{-kt} + FI_{\infty} \quad \text{(S2)}$$

where A= amplitude, k= rate, t= time, and FI_{∞}= fluorescence intensity at time= ∞.

Affinity measurements of c-di-GMP and analogs for the class I riboswitch using fluorescence.

Fluorescence measurements have been previously used to characterize RNA-small molecule interactions[1, 2] as well as to study RNA structural transitions and folding pathways induced by ligand binding[3-5]. Using fluorescence techniques, binding can be monitored in real-time allowing for facile determination of when equilibrium is achieved, a particularly desirable goal with the class I c-di-GMP-binding riboswitch. In-line probing analysis showed that nucleotides in the P1 helix undergo reduced scission in the presence of c-di-GMP, suggesting that these residues are unstructured in the absence of ligand and become more structured after c-di-GMP binding[6]. Therefore, we hypothesized that ligand binding could be detected by monitoring the fluorescence of a 2-aminopurine (2AP) inserted within this region of the RNA. We selected several purine residues in the P1 helix (G94, A95, G98) as candidates for 2AP replacement (Figure S2) because these nucleotides do not make any direct contacts with c-di-GMP, but are hypothesized to undergo conformational changes in response to c-di-GMP binding. We also tested replacement of A91 with 2AP because this nucleotide is located in close proximity to the binding pocket and would likely undergo a substantial reorganization upon ligand binding and therefore exhibit a change in fluorescence. Of these four positions tested, only the G94(2AP) and A95(2AP) variants showed a difference in fluorescence intensity between the ligand bound and unbound states. Because the largest change in fluorescence upon ligand addition was observed for the G94(2AP) RNA construct, we chose to use this RNA for binding studies.

Replacement of G94 with 2AP yielded the fluorescently labeled RNA, G94(2AP). A minimum RNA concentration of 200 nM was necessary for sufficient fluorescence signal and in measuring the K_d of c-di-GMP by equilibrium titration, we found that the K_d was too low to accurately measure using this concentration of RNA. As a second approach, we measured the on (k_{on}) and off (k_{off}) rates and used these values to estimate the K_d using $K_d = k_{off}/k_{on}$ (Figure S3). The k_{on} was measured as 7.5 x10^5 $M^{-1}min^1$, which was within two-fold of the previous value reported for the wild-type class I RNA[7, 8], whereas the k_{off} observed was 1.1 x10^{-2} min^{-1}, giving a complex half life ($t_{1/2}$) of 58 min which is considerably faster than that for the wild-type RNA (44 days)[7, 8] (Table S2). From the ratio of the off-rate and on-rate, the K_d of c-di-GMP was estimated to be 16 nM (Table S2). To confirm the accuracy of this K_d determination, we measured the K_d of two weaker binding analogs, c-(R_p)-G_{ps}-GMP and c-2'F-G-GMP, using the equilibrium titration method and by calculating the K_d from the measured rate constants (k_{on} and k_{off}). Comparison of the K_d values from the two methods for both analogs found that they were within experimental error of one another (Table S2), verifying that this is a valid approach for determining the affinity of c-di-GMP for this system.

In measuring the affinities of analogs for the class I riboswitch using this fluorescence assay, we found that the range of K_d values we were able to measure was limited and for weaker binding ligands ($K_d \geq 15\mu M$) a complete binding curve could not be obtained. At higher analog concentrations (approximately > 15 µM) background fluorescence in the absence of G94(2AP) RNA was observed from exciting the ligand alone, impeding us from obtaining a full binding curve when concentrations greater than this threshold were required to complete the titration. This observed background signal was both concentration and volume dependent, suggesting that aggregation or intermolecular interactions of the ligand may be the cause. Given the propensity of c-di-GMP to exist in multiple oligomeric states in solution[9, 10], this observation was not surprising. Furthermore, this phenomenon has been observed

previously for fluorescence quenching experiments with small molecules, particularly with ligands containing aromatic moieties[11].

Effects of the 2-aminopurine mutation on ligand binding. To investigate whether or not the introduction of 2AP into the P1 helix affected the $\Delta\Delta G_{bind}$ measured for analogs and to determine if these values are reflective of what would be expected in the background of the wild-type RNA, we performed additional binding studies with two other class I riboswitch mutants. Using the competition gel-shift assay, we measured the affinity of c-(R_pR_p)-di-G_{ps} for a class I RNA containing a mutation affecting the tetraloop/tetraloop receptor interaction distal to the site of ligand binding, termed GUAA RNA, as well as for the C92U RNA, which contains a mutation in the binding pocket and is also a naturally occurring variant. We previously characterized both the GUAA and C92U RNAs[7, 8], as well as reported binding of the 2′-OMethyl c-di-GMP analog to the GUAA RNA[12], for which we observed the same relative loss in energy as we did with the G94(2AP) RNA (~6 kcal/mol).

We measured the K_d of c-(R_pR_p)-di-G_{ps} for the GUAA RNA and found that the fold loss in binding affinity relative to c-di-GMP differed by approximately 2-fold as compared to that measured in the background of the G94(2AP) RNA (Table S3). This amounts to a difference in the $\Delta\Delta G_{bind}$ of approximately 0.6 kcal/mol and indicates that the 2AP mutation in the P1 helix does not affect the relative loss in binding energy determined for each c-di-GMP analog.

Although, the G94(2AP) and GUAA class I variants contain mutations that are not in the binding pocket, we sought to further confirm that measurements made in the background of the G94(2AP) RNA are reflective of what is expected in the wild-type RNA. To do so, we measured the affinity of c-(R_pR_p)-di-G_{ps} for the C92U binding pocket mutant. Because C92U is important for recognizing one of the guanine bases (G_β) and not the phosphates, this nucleotide change should not perturb any specific contacts made to the ligand. However, a perturbation in the binding pocket itself could have additional effects on binding that cannot be solely attributed to the specific ligand mutations. We noted a 80-fold

loss for c-(R_pR_p)-di-G_{ps} binding to C92U, which is also within 2-fold of that measured in the G94(2AP) RNA (Table S3). Between these three class I variants (G94(2AP), GUAA, and C92U), the $\Delta\Delta G_{bind}$ for c-(R_pR_p)-di-G_{ps} differ by ≤ 0.6 kcal/mol, despite the fact that one of these RNAs contains a mutation directly in the ligand binding pocket. Taken together, this data suggests that mutations made in RNA structural elements outside of the ligand binding pocket, specifically the P1 helix and the tetraloop/tetraloop receptor, do not affect the relative free energies of the c-di-GMP analogs. Furthermore, while the existence of the P1 helix is highly conserved in class I riboswitches, the nucleotide identity and length of this stem is not. This suggests that this structural region of the RNA is resilient to mutation and that nucleotide changes in this helix should not affect contacts made to the ligand.

TABLES

Table S1. Characterization of c-di-GMP analogs by ESI-MS (negative ion mode).

		Observed Mass	
Analog	**Exact Mass**	**[M-H]/1**	**[M-2H]/2**
c-di-GMP	690.09	688.95	343.98
c-GMP-IMP	675.08	674.08	336.55
c-di-IMP	660.07	659.06	329.03
c-c^{7}G-GMP	689.1	688.09	343.53
c-di-c^{7}GMP	688.1	687.12	343.05
c-GMP-AMP	674.1	673.12	336.06
c-di-AMP	658.11	657.05	328.02
c-dG-GMP	674.1	673.04	336.03
c-di-dGMP	658.11	657.08	328.05
c-2′F-G-GMP	692.09	691.08	345.03
c-di-2′F-GMP	694.09	693.01	346.02
c-2′OMe-G-GMP	704.11	703.12	351.06
c-di-2′OMe-GMP	718.13	716.87	357.94
c-dG-AMP	658.11	657.11	328.05
c-G-dAMP	658.11	657.11	328.05
c-N^{1}mG-GMP	704.11	703.11	361.06

Table S2. Rates of ligand binding for the class I riboswitch.

Analog	**k_{off} (min^{-1})**	**$t_{1/2}$ (min)**	**k_{on} ($M^{-1}min^{-1}$)**	**K_d, measured[a] (nM)**	**K_d, calculated[b] (nM)**
c-di-GMP	1.2 ± 0.07 x10^{-2}	58	7.5 ± 0.5 x10^{5}	-[c]	16
c-2′F-G-GMP	1.3 ± 0.2 x10^{-2}	53	4.9 ± 0.2 x10^{5}	25 ± 3.7	27
c-(R_p)-G_{ps}-GMP	4.9 ± 0.08 x10^{-2}	14	7.5 ± 0.6 x10^{5}	67 ± 11	65

[a] K_d measured by equilibrium binding using fluorescence.

[b] K_d calculated by taking the ratio of k_{off}/k_{on}.

[c] K_d could not be accurately measured using equilibrium binding methods.

Table S3. Binding affinity of c-(R_pR_p)-di-G_{ps} for the GUAA and C92U class I variants compared to that measured for the G94(2AP) RNA.

	G94(2AP)			**GUAA**			**C92U**		
Analog	**K_d (nM)**	**Fold Loss**	**$\Delta\Delta G_{bind}$ (kcal/mol)**	**K_d (nM)**	**Fold Loss**	**$\Delta\Delta G_{bind}$ (kcal/mol)**	**K_d (nM)**	**Fold Loss**	**ΔΔ (kca**
c-di-GMP	1.4	-	-	2.2 ± 0.8[a]	-	-	15[a]	-	
c-(R_pR_p)-di-G_{ps}	150 ± 53	100	2.8	95 ± 21	43	2.2	1200 ± 88	80	

[a] Values reported previously[7, 8].

FIGURES

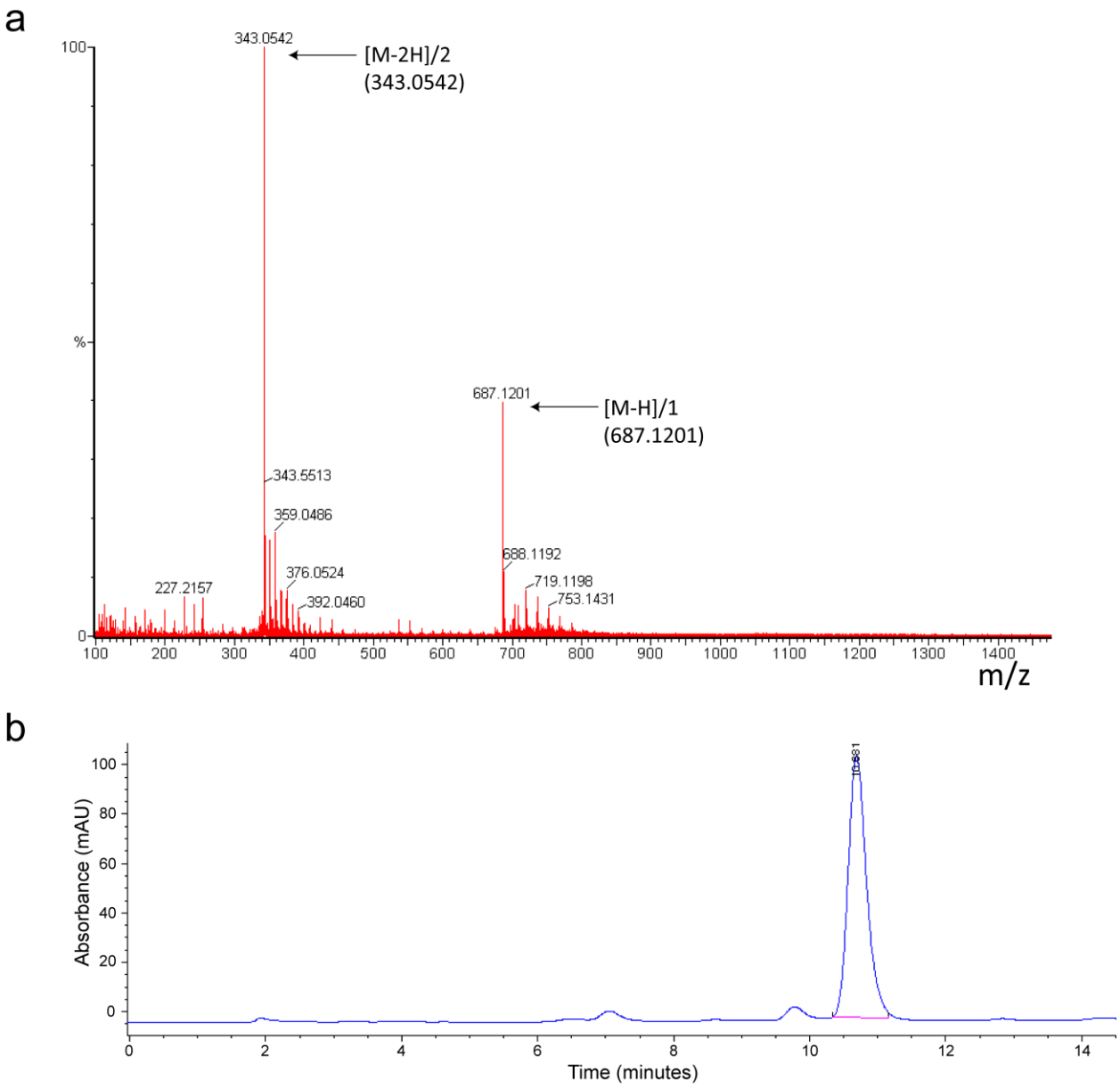

Figure S1. Characterization of c-di-GMP and analogs by ESI-MS (negative ion mode) and HPLC. (a) Sample spectrum from ESI-MS analysis of synthesized di-nucleotide analogs. The example shown here is for c-di-c^{7}GMP (exact mass= 688.1). Both the [M-H]/1 (687.1201) and [M-2H]/2 (343.0542) peaks are observed. (b) Sample HPLC trace for purity analysis of synthesized di-nucleotide analogs by HPLC (example shown again for c-di-c^{7}GMP). All compounds ran as a single peak and were >95% pure.

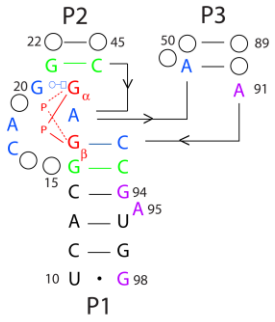

Figure S2. Secondary structure of the class I riboswitch. c-di-GMP is colored in red, nucleotides making direct contacts with the ligand are shown in blue and those that stack directly above and below the ligand are shown in green. Purine residues shown in purple (A91, G94, A95, and G98), which do not make any direct contacts with c-di-GMP, were replaced with 2-aminopurine. G94(2AP) and A95(2AP) variants displayed a large fluorescence signal in the absence of c-di-GMP that was quenched upon ligand binding. A91(2AP) and G98(2AP) variants showed little to no change in fluorescence upon addition of c-di-GMP.

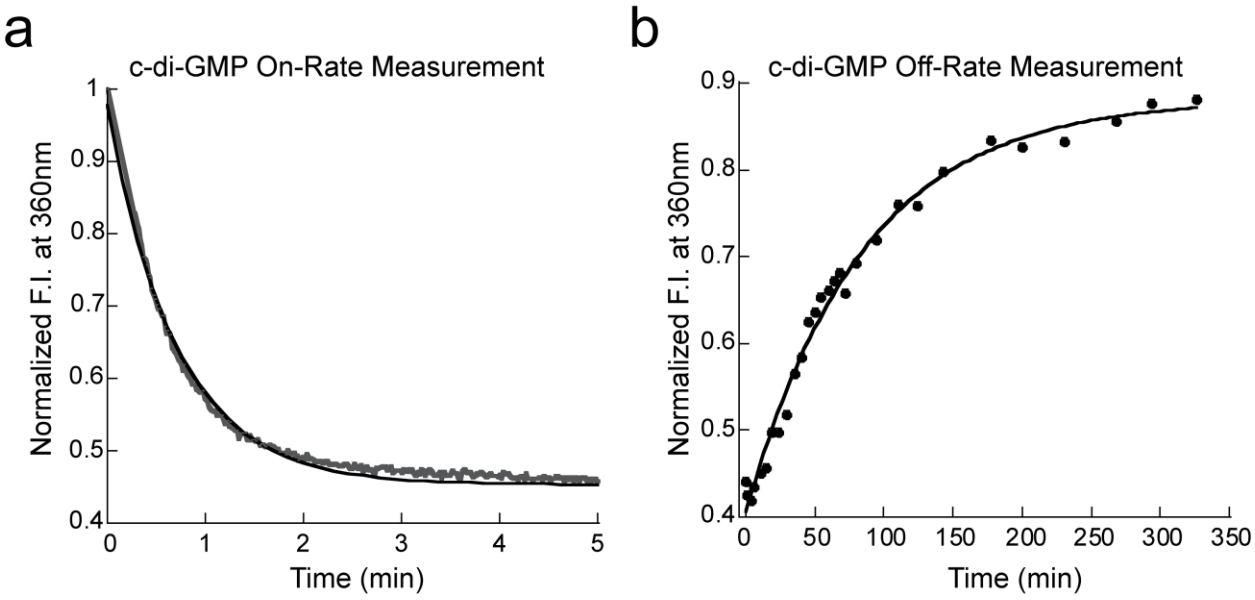

Figure S3. Measurement of rate constants using 2AP fluorescence for the class I riboswitch. (a) On-rate measurement of c-di-GMP. Data was fit to a single exponential (black line) according to equation S1. (b) Off-rate measurement of c-di-GMP. Data was fit to equation S2. The K_d of c-di-GMP was estimated by taking the ratio of k_{off}/k_{on}.

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