Electronic Supplementary Material (ESI) for Molecular BioSystems

SUPPORTING INFORMATION

FOR

Selective binding of 2'-F-c-di-GMP to Ct-E88 and Cb-E43, new class I riboswitches from *Clostridium tetani* and *Clostridium botulinum* respectively

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Synthesis of c-di-GMP and analogs (1-4)

For the synthesis of c-di-GMP and analogs (**1-4**), see references 1-3. C-di-GMP and 2'-OMe-c-di-GMP are also commercially available from axxora (http://www.axxora.com).

Preparation of c-di-GMP class I Riboswitch Aptamer RNAs

The aptamer sequences of Vc2 RNA, Ct-E88 RNA, Cb-17B RNA, Cb-E43 RNA and Cd-630 RNA were obtained from the Rfam database.[4] For riboswitches that contained flexible loop on P3 longer than four nucleotides, this loop was changed to a UUCG tetraloop to facilitate synthesis of the RNA. A pUC57 plasmid containing each target sequence, including T7 promoter sequence, was constructed by GenScript [Piscataway, NJ].

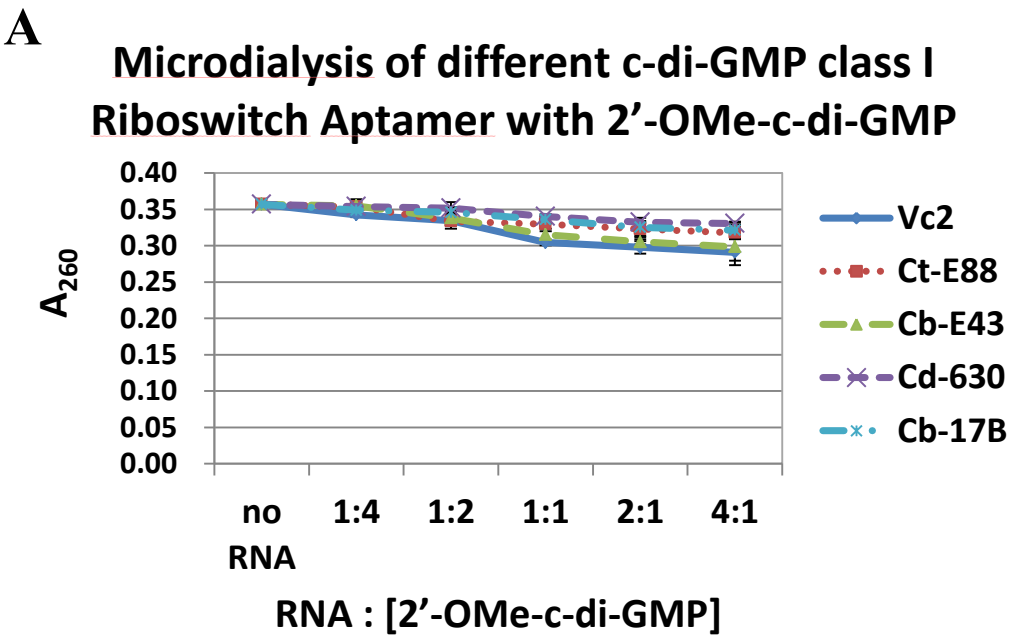
RNAs were synthesized by *in vitro* transcription with T7 RNAP from DNA template including T7 RNAP promoter sequence produced by PCR amplification (one of the PCR primers was 5′ - CTA ATA CGA CTC ACT ATA G, the T7 RNAP promoter sequence, and the other PCR primer was a 17 nt of the 3'-end of RNA sequence). The mutant T7 RNAP (P266L,[5] plasmid was obtained from Marc Dreyfus) was expressed

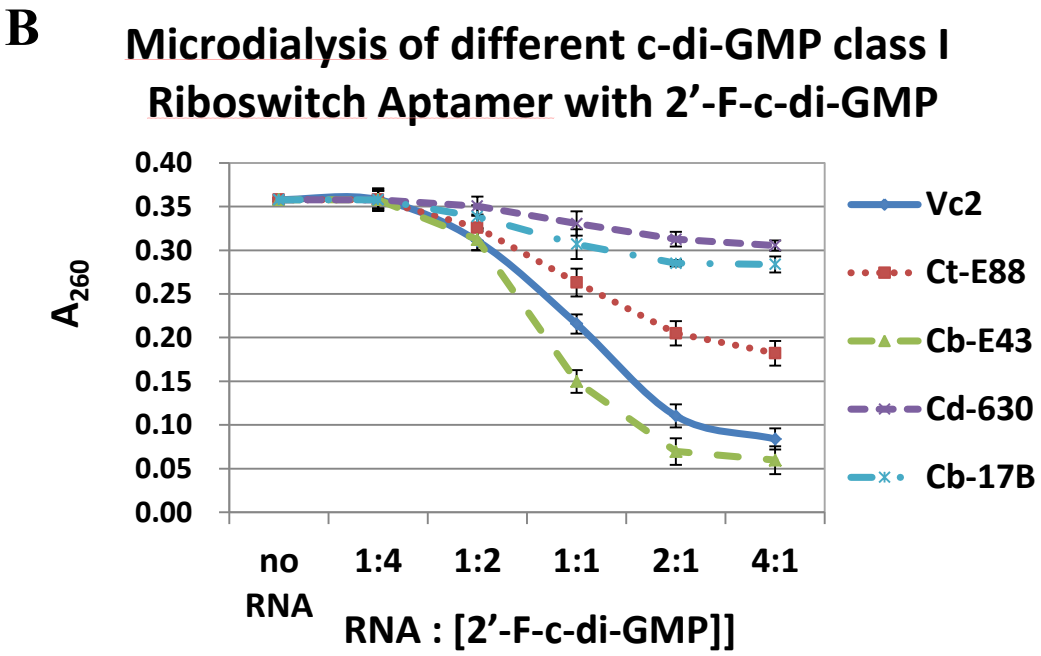
in *Escherichia coli* BL21 (DE3) and purified on a Ni-chelating Sepharose column (Pharmacia). The transcription for each RNA was done in transcription buffer (40 mM Tris-HCl (pH 8.1), containing 1 mM spermidine), 10 mM total NTPs and 15 mM total Mg^{2+}, 10 mM total dithiothreitol (DTT), 0.01% Triton X-100, 80 mg/mL PEG 8000, 2 units of RNase inhibitor (New England Biolabs), 2 units of inorganic pyrophosphatase (New England Biolabs)], 200 μL of each PCR DNA template, and 200 μL of 4 mg/ml T7 RNAP per 2 mL of transcription volume. The reactions were incubated for 3 h at 37˚C. After 3 h, 2 units of Turbo DNase (Ambion) were added and the samples were incubated for another 15 min. The RNA transcripts were purified by 10% denaturing PAGE/8M urea/1×TBE gel. The product band was detected by UV-shadowing, excised and electro-eluted in an Elutrap electro-separation system (Schleicher and Schuell). The purified RNA was precipitated with three volumes of absolute ethanol and 0.3 M sodium acetate pH 5.2. The RNA pellet was then dissolved in water and dialyzed in a Biodialyzer (Nestgroup) with a 5 kDa MWCO membrane (Nestgroup). The dialysis step was performed for 12 h against 10 mM EDTA and 12 h against double-distilled H_2O twice. Finally, the RNAs were lyophilized and re-dissolved into folding buffer as described below.

Microdialysis assay of RNA with ligand

Microdialysis was used to gain insights into the binding between the various c-di-GMP class I riboswitches and c-di-GMP or analogs. The microdialysis assay was

carried out in two 25 µL chambers (HARVARD APPARATUS) separated by a 10 kDa cut-off regenerated cellulosed membrane (HARVARD APPARATUS). 25 µL of ligand and 25 µL of RNA in the same buffer (20 mM KCl, 6 mM $MgCl_2$, and 10 mM sodium cacodylate, pH=6.8) were added to chamber A and B respectively. Equilibration was assumed to have occurred after 24 h, having left the microdialysis apparatus at room temperature (with slight shaking). UV absorbance readings at 260 nm (A_{260}) of c-di-GMP or analog in chamber A were taken after 24 h of equilibration, using JASCO V-630 spectrophotometer with 1 cm path length cuvette at 25°C.





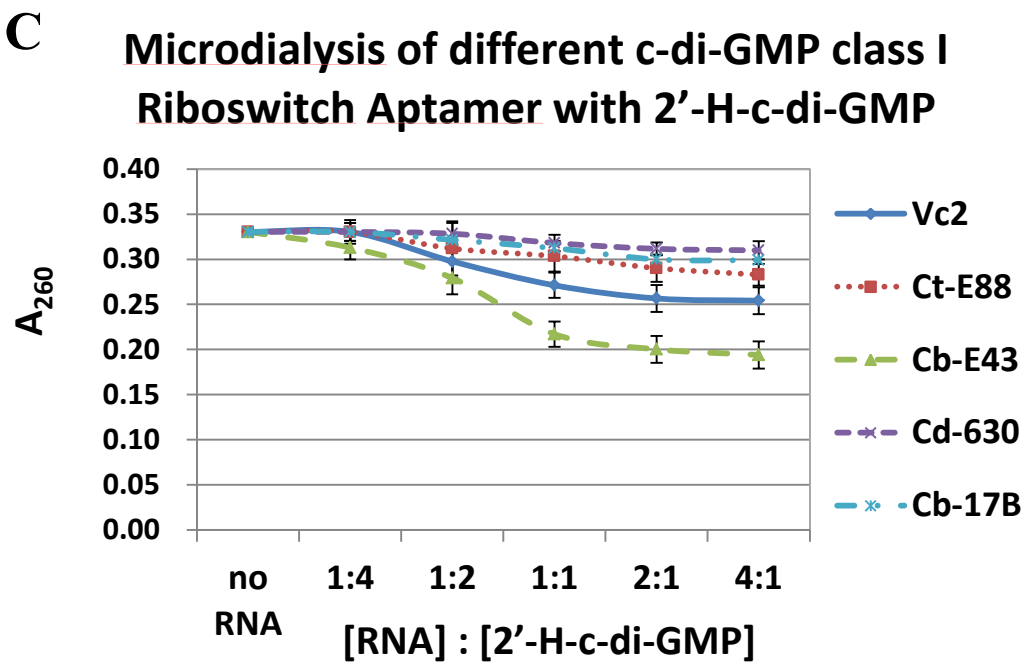


Figure S1: Equilibrium microdialysis of c-di-GMP analogs. (A) 2'-F-c-di-GMP, (B) 2'-H-c-di-GMP and (C) 2'-OMe-c-di-GMP with various class I riboswitch aptamers.

Preparation of labeled c-di-GMP

E. coli containing wild type WspR from pVL791 plasmid was obtained from Dr. Vincent Lee. The plasmid was isolated, using commercial plasmid isolation kit, sequenced and mutated into WspR (D70E) using QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies), using primers 5'- GGT GAT CCT CCA GGA **G**CT GGT GAT GCC -3' and 5'- CCG GGC ATC ACC AG**C** TCC TGG AGG ATC -3'. The sequence of the mutant WspR (D70E) was confirmed via sequencing and it was transformed into *E. coli* BL21 Star^{TM} (DE3) competent cell (One Shot BL21 Star^{TM} (DE3) chemically competent *E. coli*, Invitrogen). Then *E. Coli BL21 (DE3)* was used for production of WspR (D70E) protein. The bacteria were grown at 30°C in Luria-Bertani (LB) medium with shaking. IPTG was added at $OD_{600\ nm}$ = 0.6 and protein expression was induced for 6 h. The protein, which has a His-tag, was then purified on a nickel resin using 10 mM Tris-HCl pH 8.0/ 100 mM NaCl / 8 mM imidazole as washing buffer and 10 mM Tris-HCl pH 8.0/ 100 mM NaCl / 200 mM imidazole as elution buffer. Finally, WspR (D70E) protein was dialyzed into a 10 mM Tris/ 100 mM NaCl solution for the enzymatic reaction. α-^{32}P-c-di-GMP was generated from purified WspR (D70E) protein as described.[6] For 333 µL of enzymatic reaction, 8 µL of 3.33 µM α-^{32}P-GTP and 7 µL of 350 µM WspR (D70E) were mixed in the reaction buffer containing 10 mM Tris (pH 8.0), 100 mM NaCl and 5 mM $MgCl_2$ and incubated at 37^{o} C overnight. The protein was killed at 90^{o} C for 5 mins before being removed via filtration using a 3 kDa cut-off spin filter. The yield of the reaction was near quantitative (> 95%), confirmed via polyethyleneimine cellulose

TLC plate (mobile phase consisting of 1:1.5 of saturated $(NH_4)_2SO_4$ and 1.1 M KH_2PO_4).

K_d measurements of c-di-GMP and analogues by gel-shift and microdialysis assay

RNA was folded by heating to 70ºC and slow cooling in folding buffer (20 mM KCl, 6 mM $MgCl_2$, and 10 mM sodium cacodylate, pH 6.8) in the presence of 50 pM of radiolabeled c-di-GMP. Binding reactions were incubated at room temperature (25ºC) until equilibrium was assumed to have been reached (24 h for different class I riboswitches). The dissociation constant (K_d) of c-di-GMP for each class I riboswitch was then determined using a gel-shift or microdialysis assay. For gel-shift assay, unbound c-di-GMP was separated from RNA/c-di-GMP complex using 10% native PAGE (100 mM Tris/HEPES pH=7.5, 0.1 mM EDTA, 1 mM $MgCl_2$) at 4 ºC. Gels were dried and scanned using a STORM phosphorimager (Molecular Dynamics). For each ligand concentration, the bands corresponding to free and bound c-di-GMP were boxed and quantitated using ImageQuant software (Molecular Dynamics). The fraction of bound c-di-GMP was calculated using Equation (1).

$$FB = I_B / (I_F + I_B) \qquad \text{Equation (1)}$$

Where FB = fraction of c-di-GMP bound; I_B =intensity of bound c-di-GMP band; I_B = intensity of free c-di-GMP band;

For microdialysis assay, the pre-incubated RNA withα-^{32}P c-di-GMP sample was transferred to chamber A and 25 µL of binding buffer was added into chamber B. After 5 h equilibrium, 2 µL of solution from chamber A and B were spotted on a TLC

plate (Sorbtech). TLC plates were dried and scanned using a STORM phosphorimager (Molecular Dynamics). For each ligand concentration, the fraction of bound c-di-GMP was calculated using Equation (2).

$$FB = (I_A - I_B)/ (I_A + I_B) \quad \text{Equation (2)}$$

Where I_A is the intensity of the spot from chamber A and I_B is the intensity of the spot from chamber B.

Then all data was fit to the following Equation (3) to determine K_d values[7]:

$$FB = \frac{([RNA]_{tot}+[P32cdGMP]+Kd)-\sqrt{([RNA]_{tot}+[P32cdGMP]+Kd)^2-4\times[RNA]_{tot}\times[P32cdGMP]}}{2\times[P32cdGMP]}$$

Equation (3)

Where FB = fraction of c-di-GMP bound; [P32cdGMP] = concentration of α-^{32}P- c-di-GMP, which is at 50 pM; K_d= dissociation constant of c-di-GMP for the riboswitch RNA; $[RNA]_{tot}$ = total concentration of riboswitch RNA.

Competition experiments to determine the K_d values of analogues were performed similarly as described above. In this case, α-^{32}P c-di-GMP and unlabeled competitor analog were premixed in folding buffer before adding RNA to a final concentration of 100 nM of RNA. RNA was heated to 70 ºC for 3 min and slowly cooled in the presence of both labeled and unlabeled ligand and incubated at room temperature for 48-72 h before resolving free and bound c-di-GMP by 10% native PAGE or microdialysis equilibrium. Then the fraction bound (FB) of labeled c-di-GMP was quantified and the K_d value of the unlabeled competitor analogue was determined from quadratic solution of the Lin and Riggs equation determined by Weeks and Crothers[8], Equation (4):

FB =

$$FB_{\infty} + \frac{FB_0}{2\times[P32cdGMP]} \times \{K_d^{cdGMP} + \frac{K_d^{cdGMP}\times[analog]}{K_d^{analog}} + [RNA]_{tot} + [P32cdGMP] - [\left(K_d^{cdGMP} + \frac{K_d^{cdGMP}\times[analog]}{K_d^{analog}} + [RNA]_{tot} + [P32cdGMP]\right)^2 - 4\times[RNA]_{tot}\times[P32cdGMP]]^{\frac{1}{2}}\}$$

Equation (4)

Where FB = fraction of c-di-GMP bound; FB_0 = fraction bound in the absence of c-di-GMP competitor; FB_∞ = fraction bound in the presence of saturating c-di-GMP competitor; [P32cdGMP] = concentration of α-^{32}P-c-di-GMP, which is at 50 pM; K_d^{cdGMP}= dissociation constant of c-di-GMP for the riboswitch RNA; [analog] = concentration of unlabeled competitor analog; $[RNA]_{tot}$ = total concentration of riboswitch RNA; K_d^{analog}= dissociation constant of competitor analog.

The binding energy (ΔG_{bind}) of c-di-GMP or its analog was calculated from the following equation:

$$\Delta G_{bind} = RT\ln K_d$$ Equation (5)

Where R = universal gas constant (1.985×10^{-3} $kcalK^{-1}mol^{-1}$) and T = temperature (295K).

The change in binding energy ($\Delta\Delta G_{bind}$) was determined according to

$$\Delta\Delta G_{bind} = \Delta G_{bind}(analog) - \Delta G_{bind}\ (c\text{-}di\text{-}GMP)$$ Equation (6)

Reference:

1. B. L. Gaffney and R. A. Jones, *Curr. Protoc. Nucleic Acid Chem*., 2012, **14**, 14.8.1-7.

2. I. Kiburu, A. Shurer, L. Yan and H. O. Sintim, *Mol. Biosyst.*, 2008, **4**, 518-520.

3. C. A. Shanahan, B. L. Gaffney, R. A. Jones and S. A. Strobel, *J. Am. Chem. Soc*., 2011, **133**, 15578-15592.

4. P. P. Gardner, J. Daub, J. G. Tate, E. P. Nawrocki, D. L. Kolbe, S. Lindgreen, A. C. Wilkinson, R. D. Finn, S. Griffiths-Jones, S. R. Eddy and A. Bateman, *Nucleic Acids Res*., 2009, **37**, D136.

5. J. Guillerez, P. J. Lopez, F. Proux, H. Launay and M. Dreyfus. *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 5958.

6. V. T. Lee, J. M. Matewish, J. L. Kessler, M. Hyodo, Y. Hayakawa and S. Lory, *Mol. Microbiol*., 2007, **65**, 1474.

7. T. D. Pollard, *Mol. Biol. Cell*, 2010, **21**, 4061.

8. (a) K. M. Weeks and D. M. Crothers, *Biochemistry*, 1992, **31**, 10281; (b) S. Y. Lin and A. D. Riggs, *J. Mol. Biol*., 1972, **72**, 671.