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Structural and Biochemical Characterization of Linear Dinucleotide Analogues Bound to the c-di-GMP-I Aptamer

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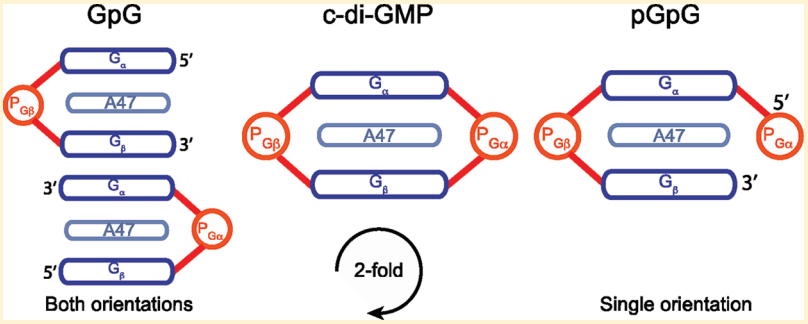
Supporting Information



ABSTRACT: The cyclic dinucleotide c-di-GMP regulates lifestyle transitions in many bacteria, such as the change from a free motile state to a biofilm-forming community. Riboswitches that bind this second messenger are important downstream targets in this bacterial signaling pathway. The breakdown of c-di-GMP in the cell is accomplished enzymatically and results in the linear dinucleotide pGpG. The c-di-GMP-binding riboswitches must be able to discriminate between their cognate cyclic ligand and linear dinucleotides in order to be selective biological switches. It has been reported that the c-di-GMP-I riboswitch binds c-di-GMP 5 orders of magnitude better than the linear pGpG, but the cause of this large energetic difference in binding is unknown. Here we report binding data and crystal structures of several linear c-di-GMP analogues in complex with the c-di-GMP-I riboswitch. These data reveal the parameters for phosphate recognition and the structural basis of linear dinucleotide binding to the riboswitch. Additionally, the pH dependence of binding shows that exclusion of pGpG is not due to the additional negative charge on the ligand. These data reveal principles that, along with published work, will contribute to the design of c-di-GMP analogues with properties desirable for use as chemical tools and potential therapeutics.

The cyclic dinucleotide bis(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a second messenger signaling molecule that regulates many processes in bacterial species that allow these organisms to adapt to their environment. These processes include motility, pathogenesis, and biofilm formation (for recent reviews see refs 1−3). In addition to receptor proteins, c-di-GMP responsive riboswitches have been discovered that bind the second messenger with high affinity and regulate diverse downstream genes, underscoring the importance of RNA receptors in this pathway.[4,5] These RNAs reside in the 5′ untranslated region (UTR) of genes in many bacterial species and regulate gene expression primarily at the transcriptional or translational level.[6−10]

Two previously reported structures of the c-di-GMP-I riboswitch show that the RNA primarily uses three conserved nucleotides to bind this small molecule (Figure 1a).[11−13] Riboswitch residue G20 forms a Hoogsteen pair with G_α, one of the purine bases of c-di-GMP. G_β, the other ligand guanine base, forms a Watson−Crick pair with C92. A47 of the riboswitch inserts between G_α and G_β, forming a stacking network that extends into two helical stems of the RNA. These interactions result in a very tight complex with a binding affinity of 10 pM.[11] We have also reported structures of several mutant riboswitches bound to c-di-GMP or the related molecule, c-di-AMP.[12] These structures, along with biochemical analysis, reveal the interactions that are important for binding and recognition of c-di-GMP and establish the molecular basis for how the RNA is able to distinguish between its cognate ligand and related cyclic molecules, such as c-di-AMP.

In addition to other cyclic dinucleotides, the riboswitch must also be able to exclude linear dinucleotides, including the cellular breakdown product of c-di-GMP, pGpG. This linear form of diguanosine monophosphate is produced from the enzymatic hydrolysis of the second messenger by c-di-GMP specific phosphodiesterases (PDEs).[1,2] Additionally, pppGpG is another linear diguanosine nucleotide found in cells as an intermediate produced in the synthesis of c-di-GMP by diguanylate cyclase (DGC) enzymes.[14] pppGpG, along with other dinucleotides, is also formed as a product of abortive transcription by RNA polymerase.[15−19] Biochemical evidence

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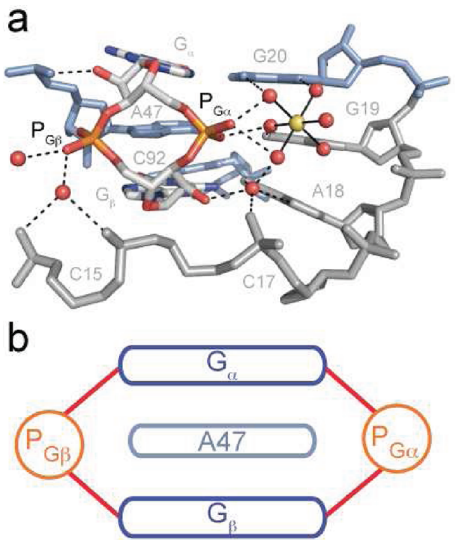

Figure 1. Differential recognition of $P_{G\alpha}$ and $P_{G\beta}$. (a) c-di-GMP is colored by atom with carbon shown as white, oxygen shown as red, nitrogen shown as dark blue, and phosphorus shown as orange. Nucleotides that directly contact the ligand are shown in light blue. Magnesium is shown as a yellow sphere and waters are shown as red spheres. Hydrogen bonds are shown as black dashed lines. (b) Schematic of c-di-GMP shown from the same perspective as in (a). The two guanine bases of the ligand are shown in dark blue, A47 is shown in light blue, the phosphates are shown in orange, and the phosphodiester bonds are shown in red.

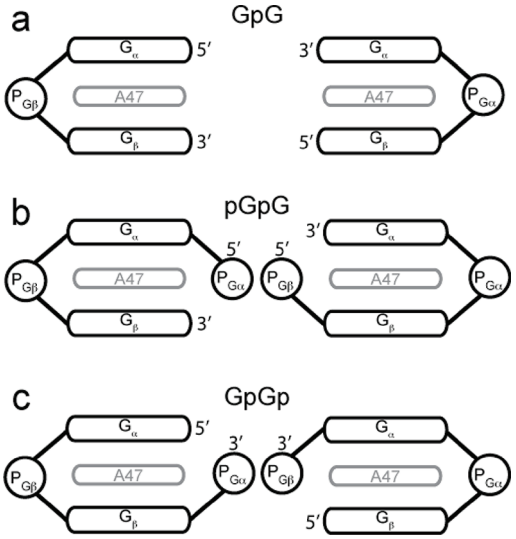

Figure 2. Possible binding orientations for linear dinucleotides: (a) GpG, (b) pGpG, and (c) GpGp.

shows that the riboswitch has evolved a strong preference for the cyclic form of diguanosine monophosphate relative to the linear form.[4]

When the c-di-GMP-I riboswitch was first reported, several linear and cyclic analogues of c-di-GMP were tested for binding to the RNA.[4] These results show that there is an ~100,000-fold decrease in binding affinity for the linear form of the dinucleotide relative to the cyclic form. Additionally, these data establish that the riboswitch is specific for molecules that contain guanine exclusively. None of the compounds that contained both a guanine and adenine base showed any binding. They also indicate that while two guanine bases are necessary for binding, a single phosphate is sufficient in the case of linear analogues.[4]

The structure of c-di-GMP bound to the riboswitch revealed that although the ligand has a 2-fold rotational axis of symmetry, the two phosphates are recognized asymmetrically (Figure 1).[11,12] The phosphate 5′ of G_α, termed $P_{G\alpha}$, is coordinated by a metal ion as well as the exocyclic amine of A47, the base that intercalates between the two guanines of the ligand. The second phosphate, termed $P_{G\beta}$, is not contacted by either the RNA or metal ions.[12] Linear molecules have distinct 5′ and 3′ ends and therefore do not have rotational symmetry. As a result, these linear dinucleotides have two distinct ways in which they could bind to the riboswitch. In all cases, either the 5′ or 3′ base of the ligand could occupy the position of G_α (Figure 2). Whether the 5′ or 3′ end of the molecule is in the α position has consequences for the placement of the phosphate(s) (Figure 2).

We set out to determine the binding orientation of these ligands and to answer questions about the nature of the phosphate interactions within the binding site. Does the riboswitch preferentially bind molecules like pGpG in a single orientation or in a mixture of the two possibilities? Does the riboswitch have a preference for whether the terminal phosphate is on the 5′ or 3′ end? It is possible that some positions of the terminal phosphate may create steric clashes with residues of the riboswitch, leading to the exclusion of certain orientations or of some molecules entirely. Can three phosphates be accommodated? If only a single phosphate is present, as in the case of GpG, is there a preference for placing the phosphate in the position of either $P_{G\alpha}$ or $P_{G\beta}$? And what is the source of the large energetic difference in binding affinity between the cyclic and linear diguanosine monophosphate molecules?

Here we report a biochemical and structural analysis of linear dinucleotide binding to the class I riboswitch aptamer. These data, along with pH-dependence experiments, reveal the parameters of phosphate recognition by the riboswitch and suggest a molecular basis for the 5 orders of magnitude discrimination between c-di-GMP and its breakdown product, pGpG.

MATERIALS AND METHODS

Materials. *RNAs.* Riboswitch RNAs were produced by in vitro transcription using T7 RNA polymerase and purified by denaturing polyacrylamide gel electrophoresis (PAGE) as previously described.[11,12] Riboswitch RNAs used for biochemistry in this study were in the background of the 110 Vc2 RNA[4,11] while those for crystallographic analysis were in the background of the V2U construct.[11]

Linear Analogues. Non-radiolabeled pGpG, pGpA, GpG, GpA, and ApG were purchased from Dharmacon. Each molecule was deprotected and then desalted using a Waters Sep-Pak Plus C18 column. The dinucleotides were bound to the column in 50 mM triethylammonium acetate (TEAA), pH 6.0, and eluted with 30% acetonitrile (ACN). GpGp was synthesized on solid-phase using an *O*-methyl phosphoramidite purchased from ChemGenes using standard conditions. It was purified using HPLC with a gradient from 50 mM TEAA, pH 6.0, to 10% ACN.

To produce radiolabeled pGpG, pGpA, and pApG, the dinucleotides GpG, GpA, and ApG were phosphorylated using polynucleotide kinase (PNK) and [γ-^{32}P]ATP and purified by PAGE. Radiolabeled pGpGp was produced by labeling GpGp using a 3′ phosphatase minus PNK as described above.

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For molecules that did not contain a 5′ phosphate, an enzymatic synthesis was developed that allows for label incorporation at the central linking phosphate. First, 3′ GMP was synthesized from an *O*-methyl-protected phosphoramidite purchased from ChemGenes. This phosphoramidite was coupled to solid support, oxidized, cleaved from the bead, and deprotected. The product was purified by HPLC using the conditions described above for GpGp. Gp and Ap (commercially available) were then labeled with PNK and [γ-^{32}P]ATP to produce *pGp and *pAp (the asterisk indicates the position of the radioactive phosphate). Using RNA ligase, these molecules were then ligated onto the 3′ end of oligonucleotides ending in either A or G where the second to last base was a pyrimidine. This ligation produces the products NNNNNYR*pRp, where R represents an adenine or guanine and Y represents a pyrimidine. These internally labeled oligonucleotides were then digested with RNase A, which cleaves after all pyrimidine residues, producing molecules of the form R*pRp. The reactions were monitored, and the final products were purified by PAGE. To produce labeled GpG, GpA, and ApG, the products from the last reaction were incubated with calf intestine phosphatase (CIP) in order to remove the 3′ phosphate, and the reaction was purified by PAGE.

Methods. *Measurement of Equilibrium Constants by Gel-Shift.* Binding constants for linear analogues were measured by native gel-shift as described previously,[11,12] where each radiolabeled ligand was added in trace to the RNA. Briefly, riboswitch, ligand, and folding buffer (10 mM sodium cacodylate, pH 6.8, 25 °C, 10 mM $MgCl_2$, and 10 mM KCl) were heated to 70 °C for 3 min and allowed to slow cool. Each binding reaction was allowed to reach equilibrium, and then free and bound ligand were separated by native PAGE.

Measurement of pH-Dependent Equilibrium Constants by Equilibrium Dialysis. Binding constants across a pH range of 5.5−8.8 were measured using equilibrium dialysis. A three-component buffer system was used for these experiments: 20 mM MES, 20 mM MOPS, 20 mM Bicine, 10 mM $MgCl_2$, and 10 mM KCl. Riboswitch RNA and trace radiolabeled pGpG or c-di-GMP were mixed in the presence of buffer and folded by heating to 70 °C for 3 min and allowed to slow cool. This complex was loaded onto one side of a Dispo Equilibrium Dialyzer (Harvard Apparatus), and buffer was loaded on the opposite side of the membrane. The dialyzers were allowed to equilibrate overnight at room temperature (~23 °C) with gentle shaking. The length of time required to reach equilibrium was determined by monitoring how long it took for radiolabeled c-di-GMP loaded on one side of the membrane to become equally distributed on both sides of the membrane. The amount of radiolabeled ligand on each side of the membrane was determined by scintillation counting. The counts from the buffer side were subtracted from the counts on the RNA side in order to obtain the counts that correspond to ligand molecules bound to the riboswitch. This value divided by the total counts on the RNA side gives the fraction of ligand bound. The K_d can be obtained from the following equation:

$$F = F_\infty C/(C + K_d) \quad (1)$$

where F is the fraction bound, F_∞ the fraction bound at saturation, and C the riboswitch concentration. The reactions with pGpG were done with wild-type RNA while those with c-di-GMP were done with the C44U/G83A mutant, an RNA we have previously used to successfully measure binding constants on a reasonable time scale.[12]

For pGpG, the K_d values were inverted and these values were plotted versus pH. These data were fit to the following equation:

$$L_H = \frac{L_{HA}[H^+] + L_{-A}K_a}{K_a + [H^+]} \quad (2)$$

where $L_H = 1/K_d$ (observed), $L_{HA} = 1/K_d$ of the protonated ligand, $[H^+]$ is the concentration of H_3O^+ at each pH, $L_{A-} = 1/K_d$ of the deprotonated ligand, and K_a is the acid dissociation constant of the ligand.

Crystallization and Structure Determination. Crystals were grown as previously described[11] with the following modifications. Crystals of pGpG and GpG in complex with the wild-type RNA were grown with either 1 mM pGpG or 500 μM GpG with 24% PEG 550 MME in the well solution. Crystals of pGpA or GpA bound to the C92U RNA were grown with 500 μM ligand and a precipitant concentration of 26% or 24% PEG 550 MME, respectively. All crystals were stabilized and frozen as described for the wild-type crystals,[11] where the stabilization solution was supplemented with 500 μM of the appropriate ligand. Data were collected at beamline X25 at the NSLS at Brookhaven National Laboratory and processed using HKL2000.[20] All structures were solved using molecular replacement with the program Phaser.[21] The search model included the U1A protein and the riboswitch but no ligand. Clear density was observed for all of the ligands in the initial maps from molecular replacement. Rebuilding was done in Coot[22] and refinement was performed in Refmac.[23] Pymol[24] was used to make the structural figures.

RESULTS

Analysis of Linear Guanine-Only Analogues. The dinucleotide GpG has a 5′ and 3′ guanine base connected by a single phosphate group. In complex with the riboswitch, either residue could theoretically occupy the position of G_α, leading to two possible binding orientations (Figure 2a). c-di-GMP has two guanine bases and two phosphates that are equivalent due to the symmetry of the molecule. When one of the phosphodiester bonds is hydrolyzed, either the 3′-O can act as the leaving group, resulting in a linear molecule with a 5′ phosphate (pGpG), or the 5′-O can be the leaving group, leading to a dinucleotide with a 3′ phosphate group (GpGp). Phosphodiesterases specific for c-di-GMP exclusively catalyze the former reaction.[1,2] Like GpG, either guanine base in these linear molecules could occupy the position of G_α (Figure 2b,c).

Several linear analogues of c-di-GMP have previously been reported to bind to the c-di-GMP-I riboswitch with dissociation constants of ~1 μM: pGpG, GpG, and GpGpG.[4] To determine the number of phosphates that can be accommodated by the riboswitch and if the riboswitch has a preference for the position of the terminal phosphate, we determined the binding affinities for an expanded series of linear c-di-GMP analogues. These included molecules with phosphates on either the 5′ or 3′ end as well as molecules with only the single internal phosphate or with three phosphates. We then solved the cocrystal structures of those that bound the RNA to define the orientation of these linear molecules. All of the compounds used in affinity measurements were radiolabeled with ^{32}P either at the 5′ terminus or at the central linking phosphate, and K_ds were determined using a previously reported gel-shift assay.[11]

We sought to determine if GpG, with only a single linking phosphate, binds to the riboswitch in a single conformation or as a mixture of the two possible orientations. In agreement with

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Table 1. Binding Affinities of Linear Analogues of c-di-GMP[a]

RNA	K_d (nM) c-di-GMP	K_d (nM) GpG	K_d (nM) pGpG	K_d (nM) GpGp	K_d (nM) pGpGp
wild-type[b]	0.01[c]	210 ± 24	490 ± 150	22,000 ± 14,000	–
C92U	15 ± 1.1	>100 μM[c]	–	–	n.d.

[a]All values are the average of three independent trials ± the standard deviation. A dash indicates that no binding was observed at 100 μM RNA. n.d. means not determined. [b]The wild-type RNA used here is the 110 Vc2 RNA. The C92U mutation was made in this RNA background. [c]Both values for c-di-GMP binding were previously reported.[11] [d]A partial shift was observed at 100 μM RNA.

the previous report,[4] GpG binds to the c-di-GMP-I riboswitch with a K_d of 200 nM (Table 1). This corresponds to a 20,000-fold loss in binding affinity relative to the cyclic molecule. We determined the crystal structure of GpG in complex with the riboswitch and found that the ligand binds in both possible orientations (Figure 3a) (Table S1). The initial map (2.8 Å resolution) obtained by molecular replacement showed clear density for the two guanine bases as well as peaks in the position of what would be $P_{G\alpha}$ and $P_{G\beta}$. Modeling each orientation at 50% occupancy fit the data well (Supporting Information), suggesting that GpG binds the RNA with either guanine base occupying the α position with approximately equal affinity. Additionally, weak density ($F_o - F_c$ peak of 3.3σ) is observed in the position of the hydrated metal in the c-di-GMP structure (Figure 3a), suggesting that this metal is present but has a lower occupancy, presumably due to the mixed population.

The addition of a phosphate on the 5′ terminus of GpG results in pGpG, which is the enzymatic breakdown product of c-di-GMP. We found that this molecule binds to the riboswitch with a K_d of ~500 nM (Table 1), in good agreement with previous work.[4] These data indicate that if the molecule is not cyclic, a phosphate at the 5′ end does not improve the affinity relative to a molecule with a single linking phosphate. In fact, accommodation of this terminal phosphate results in a 2-fold loss in affinity. To investigate whether the riboswitch has a preference for the placement of this additional phosphate relative to GpG, we determined the structure of pGpG bound to the riboswitch and found that the addition of this terminal phosphate causes the molecule to bind in only one of the two possible orientations (Table S1). pGpG binds to the riboswitch with the terminal phosphate positioned 5′ of G_α and near G20 (Figure 3b). The structure (2.8 Å) reveals that while all contacts to the guanine bases of the ligand are the same as was observed for c-di-GMP (with the exception of the A18 contact to G_β), the free phosphate 5′ of G_α moves ~4 Å relative to its position in the c-di-GMP complex. This shift eliminates the hydrogen bond between A47 and $P_{G\alpha}$. The phosphate is solvent-exposed, and the nearest nucleotide is G20, the residue involved in a Hoogsteen pair with G_α. Because of rotational flexibility in the terminal phosphoester bond of pGpG, the phosphate can be modeled with slightly different positions of the phosphate oxygens. As a result, the nearest phosphate oxygen is located between 3.4 and 4.3 Å from the O6 of G20. A small (~3σ) peak observed in the $F_o - F_c$ density may correspond to either a water molecule or a magnesium ion bridging between the phosphate and the O6 of G20 (Figure 3b). If there is a water or metal involved in coordinating the 5′ phosphate, it may not be highly occupied at the pH used for crystallization (pH 6.0), since a large percentage of the pGpG may be in the protonated form at this pH. No density is observed in the location of the hydrated magnesium found in the c-di-GMP structure. This metal normally contacts two binding pocket residues, G19 and G20, interactions that are therefore lost in the pGpG complex. Loss of contacts to this metal may contribute a small amount to the differential affinity observed between c-di-GMP and pGpG.

To investigate if the riboswitch has a preference for whether the terminal phosphate is located on the 5′ or 3′ end, we next tested molecules that had 3′ terminal phosphates. In contrast to pGpG, which binds with submicromolar affinity, the related molecule, GpGp, binds very poorly (22 μM), with more than a 2 million-fold loss in affinity relative to c-di-GMP and a 45-fold loss relative to pGpG (Table 1). The difference in affinity between pGpG and GpGp suggests that they do not bind with the terminal phosphate in equivalent orientations and that the accommodation and position of the phosphate in the complex with pGpG is much more favorable than that for GpGp. We also tested pGpGp and did not detect any binding to the riboswitch even at RNA concentrations as high as 100 μM. If the effects of adding a phosphate to both the 5′ and 3′ ends of GpG were additive, we should be able to detect some binding of pGpGp under these conditions. This result shows that two terminal phosphates cannot be accommodated by the riboswitch within the constraints of the binding site. Because RNA is a polyanion and every phosphate on the ligand adds additional negative charge, a greater number of phosphates may be expected to result in weaker binding. This set of analogues reveals that the c-di-GMP-I riboswitch can bind linear molecules with a 5′ phosphate group but that addition of a phosphate on the 3′ terminus essentially eliminates binding.

Binding Constants of Chimeric G/A Analogues. All of the analogues described above contain two guanine bases and therefore could in principle bind in either the 5′ to 3′ or 3′ to 5′ orientation. In order to more fully understand the relationship between the phosphate and base orientation, we biochemically and structurally characterized a series of linear analogues that contain a mixture of guanine and adenine bases that enabled us to control the orientation of the ligand. We used the C92U mutant riboswitch[12] that eliminates the symmetry of the binding site and is expected to orient the dinucleotide by formation of an A_β-U92 base pair. Our group has previously used this strategy to investigate the effect of a single deoxy sugar in the context of a cyclic dinucleotide.[25] We confirmed that the molecule binds in the expected orientation through formation of the A–U pair by determining the crystal structure of two analogues (GpA and pGpA) bound to the riboswitch (see below).

When the mixed base analogues with a single phosphate were tested, only the molecule with a 5′ guanine bound. GpA bound with an affinity of 8 μM, 38-fold weaker than the GpG/wild-type complex, while ApG showed no binding (Table 2) (Supporting Information). The observation that GpA binds, but ApG does not, implies either that the riboswitch has a preference for the linking phosphate to occupy the position of $P_{G\beta}$ or that it cannot bind molecules with an A on the 5′ terminus. The fact that GpG binds in both orientations suggests that the 5′ A is what eliminates binding (see below). The weaker affinity of GpA for the C92U riboswitch compared to

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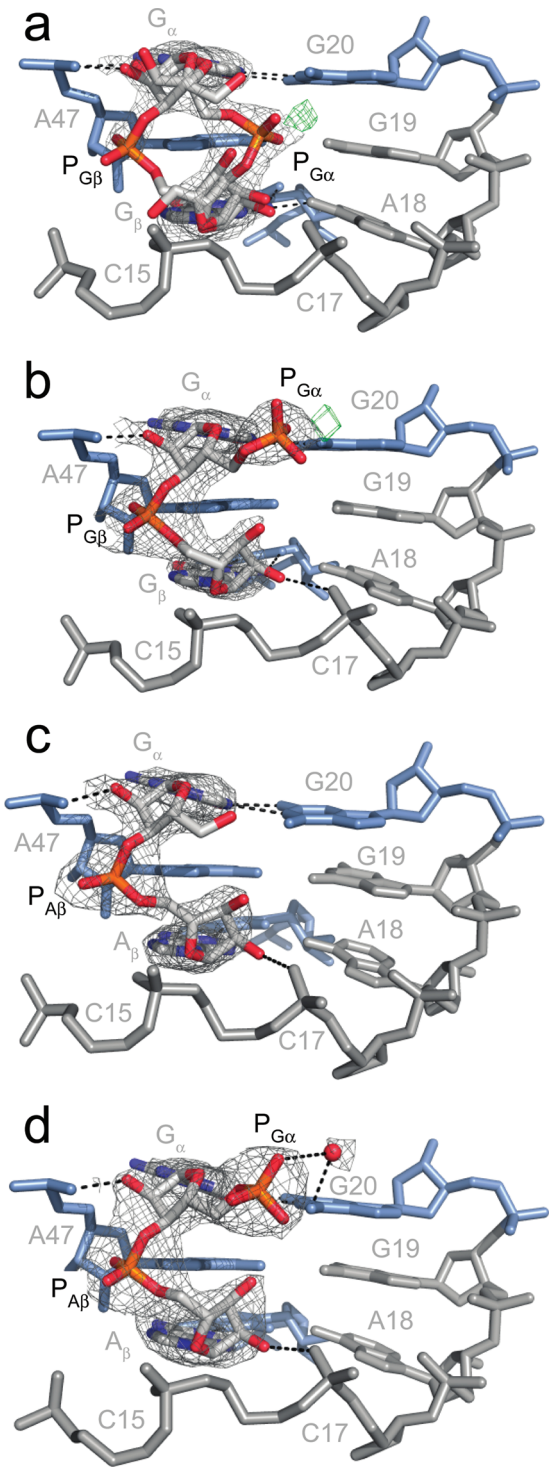

Figure 3. Structures of linear analogues bound to the c-di-GMP-I riboswitch. Coloring is the same as in Figure 1. $2F_o - F_c$ density is shown in gray, contoured to 1σ. $F_o - F_c$ density is shown in green. (a) Complex with GpG. Both orientations of the ligand are shown. $F_o - F_c$ density is contoured to 3.0σ. (b) Complex with pGpG. $F_o - F_c$ density is contoured to 2.4σ. (c) Complex of GpA with the C92 mutant riboswitch. (d) Complex of pGpA with the C92U mutant riboswitch. The water molecule is shown as a red sphere.

GpG for the wild-type RNA most likely reflects the decreased stability and poorer stacking interactions of an AU pair relative to a GC pair. We determined the structure of GpA (2.7 Å) bound to the C92U riboswitch and, as expected for this base combination, found that the ligand binds in a single orientation (Figure 3c) (Table S1). An AU pair is formed between the adenosine of GpA and U92. The phosphate of GpA is in the $P_{G\beta}$ position, and as expected for this orientation, no density is observed for a metal ion in the binding pocket. This result confirms that by using a chimeric G/A ligand and the C92U mutant riboswitch, the small molecule can be oriented by the AU pair.

We tested two different chimeric ligands that had one terminal phosphate on the 5′ end. One molecule, pGpA, has a 5′ guanine while the second, pApG, has a 5′ adenine. Two additional molecules with the phosphate on the 3′ end, GpAp and ApGp were also assayed for binding. Of the four molecules tested, only pGpA bound to the C92U riboswitch, suggesting that the free phosphate can only be accommodated 5′ of G_α. The binding constant of this complex (16 μM) is 33-fold weaker than that of the pGpG/wild-type complex, similar to the difference observed between GpA and GpG (Table 2). However, the affinity of pGpA is 2-fold weaker than that of GpA (Table 2). This is equivalent to the difference between pGpG and GpG binding to the wild-type riboswitch, an observation that implies that the modest 2-fold decrease in affinity due to the presence of the terminal phosphate is a reproducible effect. The other chimeric molecules, pApG, GpAp, and ApGp, showed no binding even at 100 μM. These data suggest that pGpA is oriented in the same way as pGpG, with the 5′ phosphate near G_α. This is confirmed by the structure (3.1 Å resolution) of pGpA bound to the C92U riboswitch (Figure 3d) (Table S1). As observed in the GpA structure, the adenine base forms a Watson−Crick base pair with U92 while the guanine forms a Hoogsteen interaction with G20. The terminal phosphate is positioned similarly to the 5′ phosphate of pGpG. A water molecule is observed that bridges between the terminal phosphate and the O6 of G20 (Figure 3d). This suggests that the small peak observed in this position in the pGpG complex may indeed be a water molecule.

These structures do not explain the biochemical observation that while GpA binds the C92U mutant RNA, ApG does not. If it did bind, the phosphate of ApG would be placed in the position of $P_{G\alpha}$ which appears to be equally favorable to $P_{G\beta}$ based on the GpG structure. Additionally, placing the phosphate in this position would retain the hydrogen bond with A47, a contact that is lost in the GpA complex. The lack of binding must therefore result from the 5′ adenosine. Interestingly, none of the analogues with a 5′ A bound to the riboswitch, although the other dinucleotides also had terminal phosphates that may interfere with binding.

pH Dependence of Binding. In addition to determining the nature of the interactions between the riboswitch and linear molecules, we also sought to investigate the molecular basis for the nearly 5 orders of magnitude difference in the dissociation constants of the cyclic and linear diguanosine monophosphates. One potential contribution to the significant difference in binding affinities may be a loss of metal interactions in the case of the linear molecule, as observed in the pGpG complex (Figure 3b). Another difference between the two molecules is that c-di-GMP has a net −2 charge while pGpG has a net −3 charge. It is possible that the RNA has difficulty accommodating this additional negative charge on the ligand.

In order to determine if the charge on the free phosphate contributes to the differential binding of pGpG versus c-di-GMP, we measured the binding affinity for both of these ligands across the pH range of 5.5−8.8 using equilibrium dialysis. The pK_a of the 5′ phosphate of pGpG is expected to be

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Table 2. Binding Affinities for G/A Linear Analogues of c-di-GMP[a]

RNA	K_d (nM) GpA	K_d (nM) ApG	K_d (nM) pGpA	K_d (nM) pApG	K_d (nM) GpAp	K_d (nM) ApGp
wild-type[b]	–	–[c]	–	–	–	–
C92U	8000 ± 1500	–	16000 ± 5200	–	–	–

[a]All values are the average of three independent trials ± the standard deviation. A dash indicates that no binding was observed at 100 μM RNA. [b]The wild-type RNA used here is the 110 Vc2 RNA. The C92U mutation was made in this RNA background. [c]A small amount of smearing was observed at 100 μM RNA in the native gel shift, indicating that some small amount of binding may be occurring.

between 6 and 7,[26] so at pH 5.5 a large fraction of the molecules will be in the protonated form while at pH 8.8 the majority will be deprotonated. If the negative charge reduces binding, the affinity is expected to decrease as the pH is increased. Atoms in the riboswitch are not expected to be titrated in this pH range, so any changes we observe in binding affinity should be primarily due to titration of the terminal phosphate of pGpG.

We first tested the pH-dependent binding of c-di-GMP and found that the binding affinity is independent of pH. It should be noted that for measurements with c-di-GMP we used the C44U/G83A mutant RNA because the wild-type riboswitch binds c-di-GMP too tightly to measure using equilibrium methods.[11,12] This mutant alters a conserved Watson−Crick base pair that is involved in stabilizing the packing of helical stems P2 and P3 in the riboswitch. Mutation of this interaction from a CG pair to a UA pair reduces the affinity presumably by weakening this helical interface (see ref 12 for a greater description). This base pair is located ∼10 Å from the binding pocket and is not expected to interfere with ligand binding. The dissociation constants measured for c-di-GMP are all within 5-fold of each other from pH 5.5−8.8 (Figure 4a). This result is

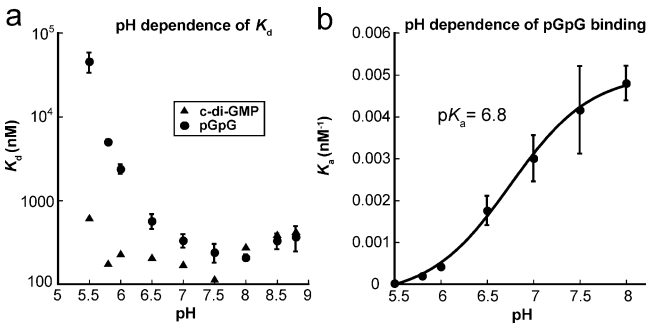

Figure 4. pH dependence of binding. (a) pH dependence of K_d for c-di-GMP (triangles) and pGpG (circles). Error bars for the pGpG values represent the standard deviation from at least three trials. The values for c-di-GMP are from a single measurement. (b) The association constants ($K_a = 1/K_d$) for pGpG as a function of pH. Error bars for the pGpG values represent the standard deviation from at least three trials.

consistent with the hypothesis that no atoms on either the c-di-GMP ligand or the RNA are being titrated in this pH range, and therefore any observed pH dependence for pGpG binding is not due to titration of the RNA.

In contrast to c-di-GMP, we observed that the riboswitch binds pGpG more tightly at high pH than low pH. Contrary to the expectation that the greater the negative charge on the ligand the weaker the binding, we observed a greater than 120-fold increase in affinity for the riboswitch binding to pGpG as the pH was increased from low to high (Figure 4a). At pH 5.5, binding was so weak that the entire curve could not be measured. When the data were forced to fit to an end point equivalent to that found for other pHs, the K_d is ∼45 μM, while at pH 8.8, the K_d is ∼370 nM. This pH dependence for pGpG reveals that deprotonation of the terminal phosphate positively affects binding. The affinity data for pHs up to 8.0 fit well to a single proton model with a pK_a of 6.8 (Figure 4b). This correlates with the expected pK_a of a 5′ phosphate. At pH 8.5 and 8.8, the K_d increases relative to pH 8.0. This may suggest that at higher pHs another functional group(s) on either the RNA or the ligand may undergo a partial protonation change. RNA degradation is also a possibility at high pH, and a small amount (∼5%) of cleavage was observed over the time of the experiment at pH 8.8.

These data imply that as the terminal phosphate of pGpG becomes more negatively charged, the binding affinity is improved. One hypothesis to explain this result is that as the phosphate becomes more anionic, a water molecule or metal ion is recruited to coordinate this functional group. Data from the structures of pGpG and pGpA are consistent with this hypothesis.

DISCUSSION

Using a series of linear c-di-GMP analogues, we have defined the phosphate specificity of the c-di-GMP-I riboswitch and determined the interactions made between this RNA and linear dinucleotides. The results establish that only a single terminal phosphate can be accommodated and binding is significantly tighter when that phosphate is located on the 5′ end of the molecule. Structural analysis revealed that GpG binds as a nearly equal mixture of the two possible orientations while pGpG binds in a single position. Attempts to model the other potential binding orientations for both pGpG and GpGp suggest that steric and/or electrostatic clashes may be created with RNA atoms. The structures of the complexes of the c-di-GMP-I riboswitch with linear dinucleotides show that the contacts between the RNA and the guanine bases of the ligand are essentially identical to those with the cyclic ligand.

If the interactions between the riboswitch and these cyclic and linear dinucleotides are largely identical, what then is the source of the ≥50 000-fold difference in binding affinity between c-di-GMP and pGpG? Our pH-dependence data contradict a potential hypothesis that the additional negative charge present on pGpG relative to c-di-GMP is detrimental to binding. Structural data reveal that metal and RNA contacts to $P_{G\alpha}$ are lost in the pGpG complex compared with c-di-GMP. While this loss of interaction probably contributes to the decrease in binding affinity of pGpG, it is unlikely to account for the majority of the >6 kcal/mol effect. Two additional parameters seem likely to play a role: base stacking in the free versus bound form of the ligand and the entropic cost associated with binding a flexible ligand (pGpG) instead of a more constrained molecule (c-di-GMP).

Consistent with the potential role of base stacking in contributing to the differences in binding between c-di-GMP and pGpG, the bases of single-stranded oligonucleotides are known to form stacks, even in dinucleotides such as dpTpT.[27]

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This suggests that in the unbound form the bases of pGpG may also stack. It is possible that in order for A47 of the riboswitch to intercalate between G_α and G_β upon binding, stacking interactions present in the free pGpG must first be disrupted. Crystal structures of c-di-GMP alone show that it can form dimers of two intercalated c-di-GMPs.[36,37] These structures show that the bases of free dimeric c-di-GMP are spaced identically to their positions in the riboswitch bound state, which means it is preorganized for RNA binding. However, it is likely that breaking these dimers (if they are present under these experimental conditions) requires an input of free energy. Even so, the degree to which c-di-GMP is preorganized likely contributes to the large negative free energy of binding.

In addition, constraining small molecules into a form close to their bound conformation has been shown to dramatically increase binding affinity. One example is from a screen of small peptides that bind to the protein streptavidin. Several linear peptides were identified that bound to streptavidin with weak affinity. In an effort to improve binding, several libraries of cyclic peptides were screened, and cyclic peptides with binding affinities 2−3 orders of magnitude better than those of the linear molecules were found.[28] When the crystal structures of the cyclic and linear peptides bound to streptavidin were solved, it was observed that the critical three amino acids, HPQ, were bound in the same orientation in each case, strongly suggesting that the increased affinity of the cyclic peptide was due to preorganization of these residues.[29] Additionally, there are instances of peptide-based antibiotics as well as other small molecules for which the cyclic form of the molecule has greater activity than the linear version (for some examples see refs 30−32).

In analogy to these examples, we hypothesize that a large portion of the difference in binding affinity between c-di-GMP and pGpG is due to the preorganization of the two guanine residues of the ligand. The adenosine that stacks between these two bases is universally conserved, not only in the c-di-GMP-I riboswitch but also in a second class of c-di-GMP-binding RNAs, the c-di-GMP-II riboswitch.[5] If this base is mutated, large decreases in binding affinity are observed in both classes.[11,33] The stacking networks formed with the ligand in both class I and class II riboswitches are the only conserved feature of ligand binding between these two structurally distinct RNAs,[33] further underscoring the importance of the spacing of the bases of the ligand that allows it to intercalate into the RNA structure. The 6 kcal/mol difference in binding affinity between c-di-GMP and pGpG most likely results from a combination of several factors where preorganization contributes a substantial amount while loss of interactions with the metal and A47 contribute in smaller ways to the energy difference.

Understanding the origin of the large difference in binding affinity between c-di-GMP and pGpG is not only of biochemical interest but is also important for the design of small molecule inhibitors of this riboswitch class. Because of the widespread use of c-di-GMP signaling and its role in biofilm formation and virulence, there is great interest in having small molecules that can manipulate pathways under the control of this second messenger, either as novel antibiotics or as biochemical tools.[3,34,35] Understanding the role of the phosphates in ligand binding, one of the groups most desirable for modification, is critical in this effort. This functional group is cleaved enzymatically in the native ligand, and so modifications here may result in nuclease-resistant compounds. Additionally, the negative charge on the phosphates may inhibit compounds from entering cells. Our lab has previously reported that modifications to the bases of c-di-GMP are more detrimental to binding than backbone alterations such as substituting the phosphates with phosphorothioates.[25] The results of this current study show that, at least in the case of linear analogues, a single linking phosphate is preferable to two phosphate groups. Additionally, in the case of GpA (and one of the orientations of GpG), this phosphate makes no contacts to the RNA or metals and may be able to be replaced with a neutral group. If this functional group could hold the two guanine bases at the appropriate distance apart, a tight binding, neutral analogue may result that could be used as a biochemical tool or a potential therapeutic.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental results and one table as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 3UCZ, 3UCU, 3UD4, and 3UD3.

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■ ABBREVIATIONS

c-di-GMP, bis(3′-5′)-cyclic dimeric guanosine monophosphate; PEG, poly(ethylene glycol); MES, 2-(*N*-morpholino)-ethanesulfonic acid.

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