Riboswitches

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Characteristics of Ligand Recognition by a *glmS* Self-Cleaving Ribozyme**

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The *glmS* ribozyme[1–7] from *Bacillus cereus* is a representative of a unique riboswitch class[8,9] whose members undergo self-cleavage with accelerated rate constants when bound to glucosamine-6-phosphate (GlcN6P). These metabolite-sensing ribozymes are found in numerous Gram-positive bacteria, where they control expression of the *glmS* gene. The *glmS* gene product (glutamine:fructose-6-phosphate amidotransferase) generates GlcN6P,[10,11] which binds to the ribozyme and triggers self-cleavage by internal phosphoester transfer.[1] The ribozyme is embedded within the 5′ untranslated region (UTR) of the *glmS* messenger RNA and self-cleavage prevents production of GlmS protein, thereby decreasing the concentration of GlcN6P. The combination of molecular

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sensing, self-cleavage, and gene control functions allows this small RNA to operate both as a ribozyme and as a riboswitch.

Previous studies have shown that the *glmS* ribozyme from *B. cereus* and the homologous ribozyme from *Bacillus subtilis* respond to GlcN6P with an apparent dissociation constant (K_D) of approximately 200 μM.[1,3,6] Although this K_D value is greater than those determined for most other natural riboswitches, *glmS* ribozymes exhibit a high level of molecular recognition specificity and are able to reject even close chemical analogues of GlcN6P. For example, glucosamine-6-sulphate can induce ribozyme activation to the same extent as GlcN6P, albeit when present at concentrations that are approximately 100-fold greater. In contrast, glucose-6-phosphate, wherein the 2-amine group of GlcN6P is replaced with a hydroxy group, completely fails to trigger ribozyme action.[1,3]

Riboswitches must be capable of discriminating against compounds related to their natural ligands to prevent undesirable regulation of metabolic genes. However, it is possible to generate analogues that trigger riboswitch function and inhibit bacterial growth, as has been demonstrated for riboswitches that normally respond to lysine[12] and thiamine pyrophosphate.[13] Proper expression of the GlmS protein is critical for bacterial viability,[10,11] and analogues of GlcN6P that could interfere with normal gene expression by triggering *glmS* ribozyme activity might serve as new types of antimicrobial agents. Therefore, we sought an increased understanding of the molecular recognition characteristics of *glmS* ribozymes.

We evaluated the molecular recognition characteristics of the *glmS* ribozyme by determining the effects of GlcN6P and various GlcN6P analogues on the self-cleavage activity of a 200-nucleotide *glmS* ribozyme construct from *B. cereus* (Figure 1). K_D values for each ligand were determined by plotting ribozyme rate constants versus ligand concentrations (see Experimental Section). Previous studies using similar methods revealed that the phosphate moiety of GlcN6P (Figure 1b; **1a**) is necessary for maximal affinity between ligand and *glmS* ribozyme.[1,3] The amine group of the ligand is also known to be essential for ribozyme function.[1,3] However, linear amine-containing compounds can induce modest ribozyme activity,[3] suggesting that acyclic (**1b**) or alternative anomeric forms (**1c**) of GlcN6P might be active. Therefore, we tested a series of analogues (Figure 2) to probe the importance of structural conformation of GlcN6P and of individual functional groups on the pyranose ring.[14]

Under physiological conditions, GlcN6P equilibrates between an acyclic form (**1b**) and two cyclic β-anomer (**1a**) and α-anomer (**1c**) forms (Figure 1b).[15] The relative ratio of **1a** and **1c** in solution is 60:40 at 25 °C as determined by ^{1}H NMR spectroscopy in D_2O (data not shown), with less than 1% in the acyclic form.[15] Each conformer could exhibit differences in RNA binding affinity and ribozyme activity similar to that observed for the GlmS protein.[16]

Small molecules such as serinol and ethanolamine promote ribozyme activity, although they are orders of magnitude less effective than GlcN6P.[3] Similarly, the acyclic analogue **3** has no detectable activity under the assay conditions used in the current study (see Figure 2b and

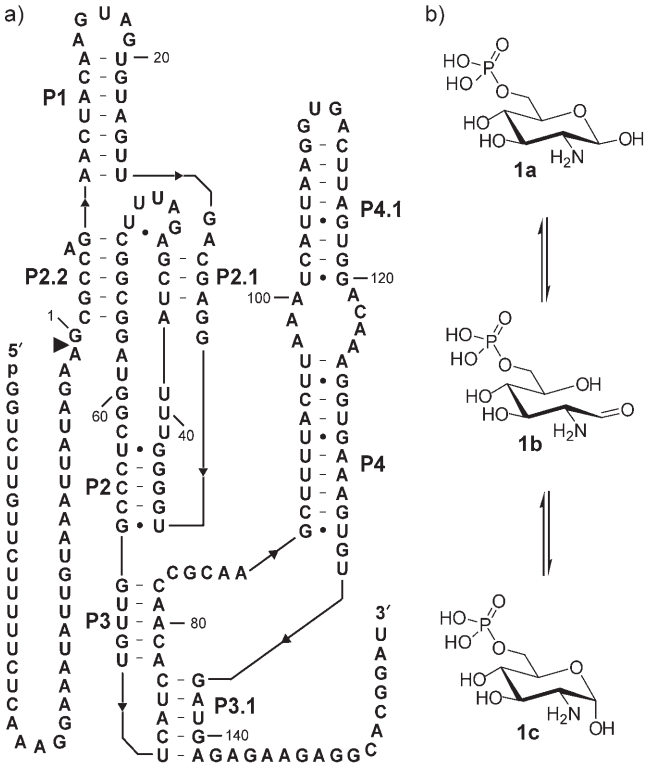
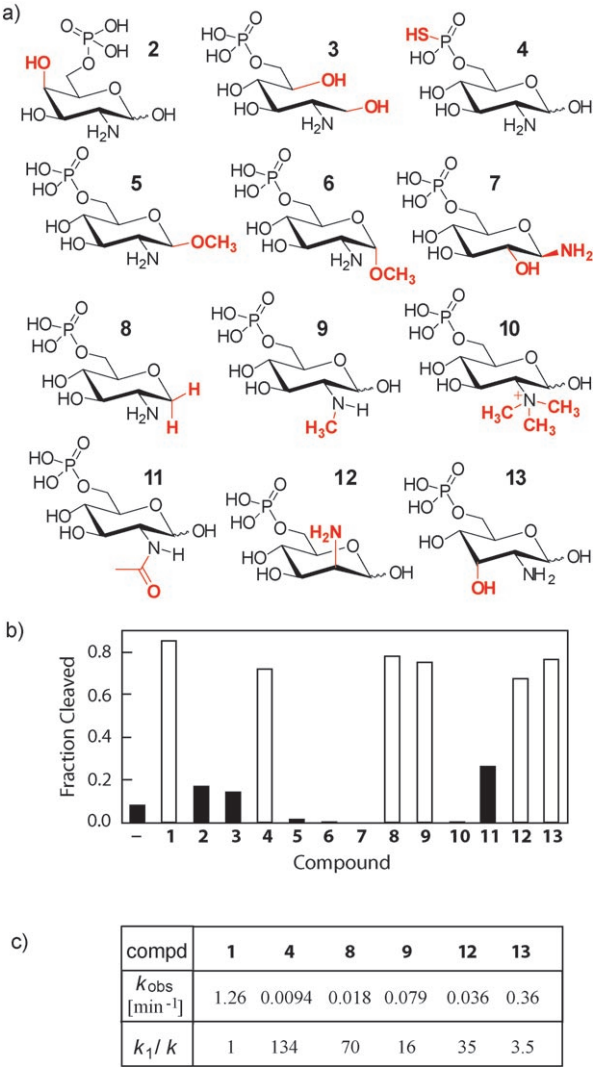


Figure 1. a) Secondary structure model of the *glmS* ribozyme from *B. cereus*. The model was adapted using data from a model for the *glmS* ribozyme from *Thermoanaerobacter tengcongensis* based on X-ray crystal structural data.[23] b) Equilibrium of the β-anomer (**1a**), acyclic form (**1b**), and α-anomer (**1c**) of GlcN6P, the ligand of *glmS* ribozyme.

Experimental Section). In contrast, the cyclic analogue **8**, which lacks the hydroxy group at the 1-position, activates ribozyme self-cleavage to approximately 1/70th of the activity exhibited by GlcN6P (Figure 2c, Figure 3). These results demonstrate that alteration of the chemical structure at the 1-position of the pyranose ring has only a modest effect on ribozyme activity, but opening of the ring at this position (as in **3** and most likely in **1b**) is far more deleterious.

Because **1b** is unlikely to be relevant for normal function of the ribozyme, one or both of the anomers of GlcN6P must serve as the activator. We tested analogues of **1a** (**5**) and **1c** (**6**), in which the stereochemistry of the analogues are maintained by methylation of the oxygen atoms at the 1-position. Unfortunately, neither **5** nor **6** induced ribozyme cleavage, and this result prevents us from establishing the activities of the two anomers by using these analogues. Perhaps a future study might exploit similar analogues in which the methoxy groups are replaced with fluorine. However, the current results suggest the ribozyme forms a tight binding pocket near the 1-position that precludes analogue binding. As **8** can activate ribozyme cleavage substantially, the modification at the 1-position might only modestly disrupt a molecular interaction between the ligand and ribozyme. Alternatively, the influence of the 1-hydroxy group on the pK_a value of the 2-amine group also could be the cause of the reduction in activity of **8**. For example, ethyl-



c)

compd	1	4	8	9	12	13
k_{obs} [min^{-1}]	1.26	0.0094	0.018	0.079	0.036	0.36
k_1/k	1	134	70	16	35	3.5

Figure 2. GlcN6P analogues and their influence on *glmS* ribozyme self-cleavage. a) Chemical structures of GlcN6P analogues.[14] Red portions designate differences from GlcN6P. b) Yields of ribozyme cleavage actions using a 200-nucleotide *glmS* RNA construct incubated with GlcN6P (**1**) or with various compounds as indicated. 5′-^{32}P-labeled precursor (Pre)-RNAs were incubated for 30 minutes in the presence of 1 mM effector as noted for each lane. The bar labeled with "–" reveals the extent of RNA cleavage when a reaction containing 1 mM GlcN6P was terminated with loading buffer[6] at time zero. Data for all assays were corrected for the amount of cleaved RNA present in the reaction generating the lowest amount of cleavage (reaction containing **7**). Unshaded bars designate active compounds that were further examined to establish ribozyme rate constants. c) Observed rate constants (k_{obs}) for ribozyme cleavage and the ratio of rate constants (k_1/k) measured using GlcN6P (**1**) versus the most active GlcN6P analogues, each at 100 μM. Rate constants for the remaining compounds are estimated to be less than 0.001 min^{-1}.

amine ($pK_a = 10.7$) has a higher pK_a value than ethanolamine ($pK_a = 9.50$),[17] indicating that an adjacent hydroxy group can reduce the basicity of an amine by more than one unit. Therefore the change in the pK_a value caused by the absence of the 1-hydroxy group in **8** could disrupt either ligand binding or ribozyme catalysis.

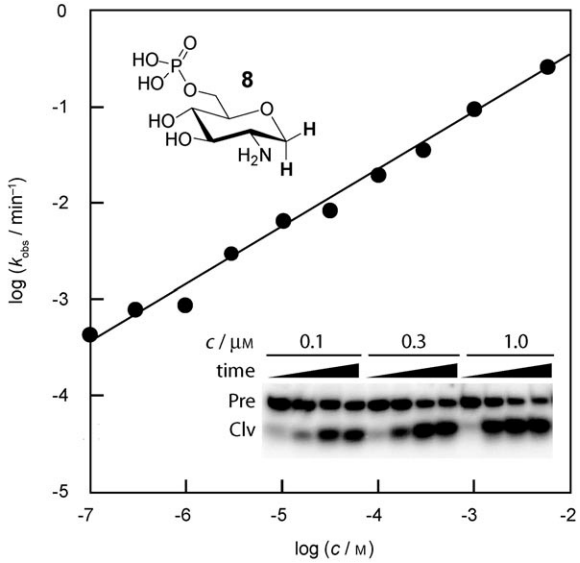


Figure 3. Observed rate constants for ribozyme self-cleavage at different concentrations of **8**.[14] The inset depicts three representative assays in which the radiolabeled precursor (Pre) was incubated for various times with different concentrations (c) of **8** as indicated. Cleaved (Clv) RNAs were separated by polyacrylamide gel electrophoresis (PAGE). Aliquots of ribozyme reactions were removed and terminated at $t = 0$, 4, 15.3, and 19.5 h.

Compounds **2** and **13** were examined to determine the importance of the 3- and 4-hydroxy groups for ribozyme activation. While **13** exhibits activity that is similar to that of GlcN6P, **2** does not induce activity under the assay conditions used (Figure 2b and c). These results imply that the 4-hydroxy group is critical for binding. In contrast, the 3-hydroxy group might have only a modest impact on binding or otherwise might influence reactivity by means of an inductive effect on the 2-amine group.

Replacement of the phosphate group with sulfate reduces affinity for the ligand by about 100-fold.[1] However, removal of the phosphate group (glucosamine) causes an even greater loss of ligand affinity. To further assess the importance of phosphate oxygen atoms, we generated the phosphorothiolate analogue **4**. This change also reduces the rate constant of ribozyme cleavage by approximately two orders of magnitude relative to **1**. However, GlcN6P is bound by the ribozyme about 1000-fold more tightly than is glucosamine.[1,3,18,19] Although the phosphate modifications tested might only disrupt a single interaction between ligand and ribozyme, it is likely that more than one binding interaction is made to this part of the ligand.

The 2-amine group, or an analogous amine, is present in all compounds that induce ribozyme activity.[1,3] Therefore, we tested a series of structural and stereochemical isomers of **1** in which this functional group was altered. The interchange of 1-hydroxy and 2-amine groups in **7** does not support ribozyme cleavage, which suggests that the location of the 2-amine group is critical for activity. The ribozyme is activated by **9** with only a modest reduction in efficiency compared to GlcN6P, despite the steric hindrance that might be caused by the methyl group. In contrast, **10** and **11** are inactive,

suggesting that the ability of the amine to accept or donate protons for bonding or catalysis is essential.

Compound **12** carries an amine group at the 2-position with opposing stereochemical configuration and surprisingly induces cleavage to 1/35th of that of the natural ligand. It seems possible that **12** could be bound using the same contacts that are used to bind GlcN6P, but the relocation of the amine group in this pocket only slightly detracts from its ability to bind or to participate in proton-transfer-mediated catalysis.

On the basis of our findings, we generated a series of predicted molecular recognition determinants for GlcN6P binding (Figure 4). The 6-phosphate and 4-hydroxy groups

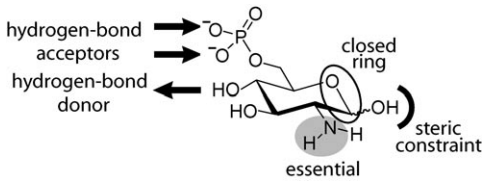


Figure 4. Predicted molecular recognition determinants of *glmS* ribozymes. Confirmation of the precise type or number of molecular contacts for some functional groups requires testing of additional analogues.

are likely to serve as hydrogen-bond donor and acceptor sites. Although the nonbridging oxygen atoms of a phosphate group can interact with metal ions by inner-sphere coordination, this is unlikely for this RNA because the ribozyme can attain full activity when Mg^{2+} ions are replaced with cobalt hexamine.[6] Cobalt hexamine simulates fully hydrated Mg^{2+} ions and can only form hydrogen bonds with an adjacent phosphate.

The exact role of the essential 2-amine group remains unclear. The reduced activity for **8** could be a result of the expected increase in the pK_a value of the amine, which would influence its ability to function in proton-transfer reactions. However, it is not clear whether the loss of activity observed with **8** is due entirely to a shift in pK_a value of the amine or due at least in part to disruption of a molecular recognition contact. We have suggested that GlcN6P could function as a cofactor for RNA cleavage,[1] and nucleic acid enzymes that use small molecules presumably to assist in proton transfer have been identified previously.[20] Both the absence of ligand-induced shape change in the RNA[21] and pH profile changes brought about by the use of various ligand analogues[3] are consistent with the hypothesis that GlcN6P directly participates in the chemical step of the reaction.

If the amine group of GlcN6P is a key moiety in the ribozyme active site, then the simplest explanation for the data is that the ligand serves as a general base catalyst. The logarithm of k_{obs} for ribozyme activity with increasing pH value increases linearly with a slope of 1.[1,3,6] Furthermore, GlcN6P analogues that exhibit higher pK_a values for the amine group are less effective inducers of ribozyme activity (i.e. **8**) or exhibit an increase in the pH required to reach half-maximal ribozyme activity.[3] Although other more complex mechanisms are possible, the ribozyme might use GlcN6P to assist in deprotonation of the 2-hydroxy group at the labile internucleotide linkage.[6]

Previous studies of the molecular recognition characteristics of other riboswitch classes revealed that a high level of molecular discrimination can be achieved by natural ligand-binding RNAs.[22] Although the *glmS* ribozyme also strongly discriminates against many closely related GlcN6P analogues, there appears to be considerable opportunity to design novel analogues that efficiently and selectively trigger *glmS* ribozyme cleavage. Such compounds possibly could be used to reduce the expression of GlmS metabolic enzymes in pathogenic bacteria, which is expected to disrupt their normal cellular function.

Experimental Section

The *glmS* ribozyme from *B. cereus* (Figure 1a) was generated by in vitro transcription as described previously,[6] 5′-^{32}P-radiolabeled,[22] and purified by PAGE. Rate constants were established by using methods and reaction conditions similar to those described previously,[6] with the exception that reaction mixtures contained 50 mM HEPES buffer (pH 7.5 at 23 °C) in place of Tris-HCl buffer. Ligand concentrations and incubation times used are defined for each assay. Ribozyme activity was established by quantifying the amounts of cleaved and uncleaved RNAs using a Typhoon imager (Amersham Biosciences).[14]

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