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The *glmS* Ribozyme Tunes the Catalytically Critical pK_a of Its Coenzyme Glucosamine-6-phosphate

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Supporting Information

ABSTRACT: The *glmS* ribozyme riboswitch is the first known natural catalytic RNA that employs a small-molecule cofactor. Binding of glucosamine-6-phosphate (GlcN6P) uncovers the latent self-cleavage activity of the RNA, which adopts a catalytically competent conformation that is nonetheless inactive in the absence of GlcN6P. Structural and analogue studies suggest that the amine of GlcN6P functions as a general acid−base catalyst, while its phosphate is important for binding affinity. However, the solution pK_a of the amine is 8.06 ± 0.05, which is not optimal for proton transfer. Here we used Raman crystallography directly to determine the pK_a's of GlcN6P bound to the *glmS* ribozyme. Binding to the RNA lowers the pK_a of the amine of GlcN6P to 7.26 ± 0.09 and raises the pK_a of its phosphate to 6.35 ± 0.09. Remarkably, the pK_a's of these two functional groups are unchanged from their values for free GlcN6P (8.06 ± 0.05 and 5.98 ± 0.05, respectively) when GlcN6P binds to the catalytically inactive but structurally unperturbed G40A mutant of the ribozyme, thus implicating the ribozyme active site guanine in pK_a tuning. This is the first demonstration that a ribozyme can tune the pK_a of a small-molecule ligand. Moreover, the anionic *glmS* ribozyme in effect stabilizes the neutral amine of GlcN6P by lowering its pK_a. This is unprecedented and illustrates the chemical sophistication of ribozyme active sites.

In Gram-positive bacteria, the *glmS* ribozyme is part of the mRNA encoding the essential enzyme glucosamine-6-phosphate (GlcN6P) synthetase. Binding of GlcN6P to the ribozyme domain leads to self-cleavage, degradation of the mRNA, and negative-feedback regulation of GlcN6P synthesis.[1−3] Three lines of evidence indicate that GlcN6P functions as a catalytic cofactor or coenzyme rather than as an allosteric activator. First, crystallographic[4] and biochemical[5,6] analyses have demonstrated that the RNA is prefolded in the absence of GlcN6P and does not undergo conformational changes as it binds GlcN6P and catalyzes RNA cleavage through internal transesterification. Indeed, the *glmS* ribozyme from the bacterium *Thermoanarobacter tengcongensis* binds GlcN6P and catalyzes RNA cleavage in the crystalline state.[4] Second, GlcN6P binds in the active site of the ribozyme with its amine in van der Waals contact with the scissile phosphate and within hydrogen-bonding distance of the 5′-oxo leaving group of the reaction (Figure 1A).[7,8] Consistent with a chemical role for the amine, glucose-6-phosphate [Glc6P; Figure S1 in the Supporting Information (SI)], which differs from GlcN6P only in having a hydroxyl group in place of the amine, is not an activator.[9] Glc6P competes with GlcN6P for binding to the RNA in solution,[9] and crystal structures show that the two compounds bind in precisely the same manner.[8] Third, several compounds that have vicinal amino and hydroxyl groups, such as glucosamine (GlcN; Figure S1), serinol, and L-serine, can weakly activate the *glmS* ribozyme.[9] The stereochemical arrangement of the amine and hydroxyl groups in these compounds is analogous to that of the anomeric hydroxyl and amine of GlcN6P. Importantly, the apparent pK_a of the ribozyme-catalyzed reaction tracks the pK_a of amine of the analogue employed.[9] On the basis of the foregoing structural and biochemical observations, it has been proposed that GlcN6P functions as a coenzyme and carries out general acid−base catalysis (Figure 1B).[4]

General acid−base catalysis is a strategy employed by other ribozymes that, without a coenzyme, carry out sequence-specific RNA cleavage through internal transesterification (reviewed in ref 10). Proton transfer is most efficient by functional groups with pK_a's near neutrality.[11] Although RNA does not normally have such functional groups, the active sites of the hepatitis δ virus (HDV) and hairpin ribozymes have been demonstrated to perturb the ground-state pK_a's of nucleobases, bringing them closer to neutral pH. Raman microscopic measurements using ribozyme crystals have indicated that the functionally essential active-site C75 of the HDV ribozyme[12,13] has a pK_a of 6.4 (vs 4.1 for CMP)[14] and that the catalytically critical active-site A38 of the hairpin ribozyme[15−17] has a pK_a of 5.5 (vs 3.7 for AMP).[18,19] These results and previous solution experiments (reviewed in ref 20) demonstrate that ribozyme active sites can tune the pK_a's of RNA residues to improve their catalytic activity. Because the *glmS* ribozyme is catalytically inactive in the absence of GlcN6P, its active-site nucleobases are not expected to have perturbed ground-state pK_a's. However, it is possible that binding to the *glmS* ribozyme modulates the properties of the coenzyme GlcN6P, enhancing its catalytic capacity. The apparent pK_a of the *glmS* ribozyme-catalyzed reaction has been variously estimated to be 7.8 or 6.9 by fitting of the pH−rate profile[9,21]. McCarthy et al.[9] used potentiometric titrations to measure the pK_a of the amine of free GlcN6P and found it to be ∼8.2. Thus, if the reaction pK_a reflects acid−base catalysis by GlcN6P, the

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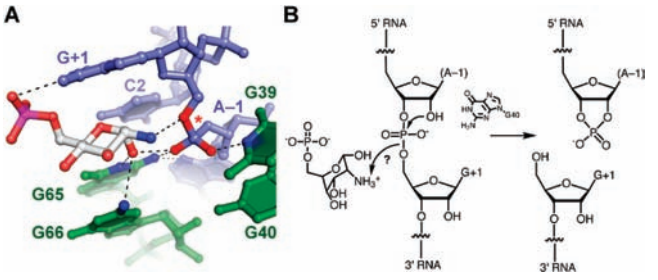

Figure 1. Active site and reaction catalyzed by the *glmS* ribozyme. (A) Active-site structure. The scissile phosphate (*) is flanked by residues A−1 and G+1 (purple; O atoms involved in hydrogen bonding are shown in red). The bound GlcN6P (in CPK coloring with gray carbons) stacks under G+1. Dashed lines denote hydrogen bonds. (B) Internal transesterification reaction. The putative general acid function of GlcN6P is indicated.

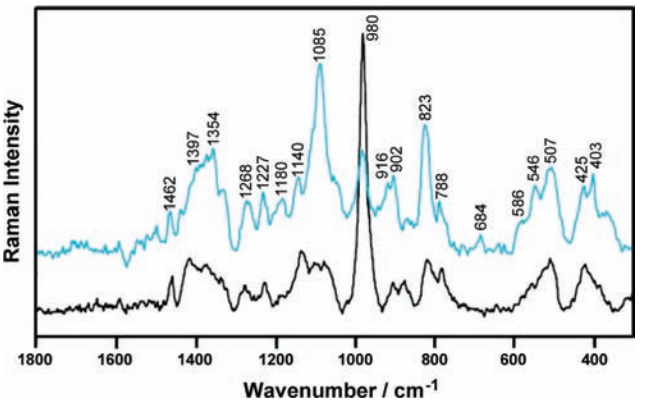

Figure 2. Raman difference spectra of [*glmS* ribozyme with GlcN6P] minus [*glmS* ribozyme] at pH 5.0 (blue trace) and pH 10.0 (black trace).

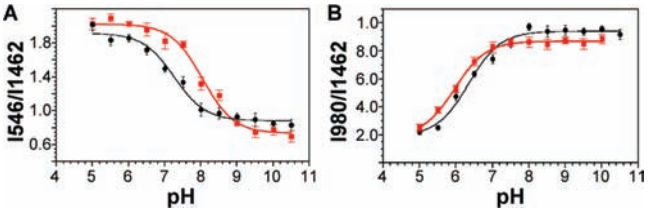

Figure 3. Response to changes in pH for the (A) amine and (B) phosphate groups of GlcN6P bound to the wild-type (black) and G40A mutant (red) *glmS* ribozymes. The pK_a's are 7.26 ± 0.09 and 8.04 ± 0.08 for the amine of GlcN6P bound to the wild-type and G40A mutant, respectively, and 6.35 ± 0.09 and 5.96 ± 0.04 for the phosphate of GlcN6P bound to the wild-type and G40A mutant, respectively. Error bars depict standard deviations based on three independent measurements.

RNA might be lowering the pK_a of the amine of the small molecule.[9] Two molecular dynamics studies on the *glmS* ribozyme reached opposite conclusions in this regard. Xin and Hamelberg[22] suggested that binding to the *glmS* ribozyme would decrease the pK_a of the amine of GlcN6P to 6.2, whereas the pK_a would increase to 8.4 when the small molecule binds to the catalytically inactive G40A mutant[23] of the ribozyme. On the other hand, Banáš et al.[24] concluded that the crystallographic structures of the ribozyme are not consistent with a significant shift in the pK_a of the amine of the bound GlcN6P.

To establish definitively whether the *glmS* ribozyme modulates the pK_a's of its small-molecule coenzyme, we employed Raman crystallography[25] and directly measured the ionization state of the amine and phosphate groups of GlcN6P bound to ribozyme single crystals. The crystals were composed of a *trans*-acting form of the *T. tengcongensis* ribozyme with a single 2′-deoxyribose substitution at the cleavage site, which traps the RNA in the precleavage state. Previously, these crystals yielded the most accurate structure of the RNA to date (1.7 Å resolution). That structure was indistinguishable from the structure determined using crystals of an all-ribose ribozyme construct that was catalytically active in the crystalline state.[4,8]

Difference spectra were measured as a function of pH by subtracting the Raman spectrum of the *glmS* crystal from that of the same crystal containing stoichiometric amounts of active-site bound GlcN6P, following "soak in" of the ligand. The resulting spectrum of bound GlcN6P contained marker bands reporting on the ionization states of the ligand (Figure 2).[26,27] The peak at 980 cm^{-1} is a phosphate stretching mode that is present only for the $-O-PO_3^{2-}$ form of the phosphate, and the feature near 546 cm^{-1} is a skeletal mode of the glucose ring that is present only for the $-NH_3^+$ form of the amine. The intensities of these bands relative to that of the mode at 1462 cm^{-1} were plotted against pH. The latter band is an internal intensity standard (due to C−H deformations) that does not vary with pH (see Experimental Procedures and Figures S2−S4 in the SI). GlcN6P exists in rapidly interconverting α and β anomeric forms in solution.[21] Crystal structures indicate that the ribozyme-bound GlcN6P is exclusively in the α conformation.[4,8] Consistent with this, we observed low-intensity peaks in the solution Raman spectra that were absent from the Raman spectra of the *glmS* ribozyme-bound GlcN6P. We used the 546 cm^{-1} peak in the Raman spectra of free and bound GlcN6P to arrive at the pK_a of the amino group. Since this is assigned to a vibrationally coupled mode involving the ring and the $-NH_3^+$ groups that would be sensitive to conformation of the ring, it is very likely that we were titrating the α anomer in both the solution and crystalline states.

We previously discovered that a G40A mutant *glmS* ribozyme is catalytically inactive even in the presence of saturating concentrations of GlcN6P. The 2.7 Å resolution crystal structure of the mutant shows that the RNA is in a conformation nearly identical to that of the wild-type *glmS* ribozyme. Remarkably, the experimental electron density maps indicate that GlcN6P binds to the G40A mutant in the same location as in the wild-type ribozyme. Thus, although GlcN6P is indispensable for the catalytic activity of the wild-type ribozyme, the presence of guanine at position 40 is also absolutely required.[23] To determine whether this catalytically essential guanine residue might exert its effect, at least in part, through GlcN6P, we also carried out Raman pH titrations of GlcN6P using crystals of the G40A mutant *glmS* ribozyme. These experiments showed that the pK_a of the amine of the ribozyme-bound GlcN6P differs substantially for the wild-type and G40A mutant ribozymes (Figure 3A). The pK_a of the phosphate of GlcN6P also differs for the wild-type and mutant ribozymes, but to a smaller extent (Figure 3B).

The pK_a values for the phosphate and amino groups were derived using a two-state model and eq 1:

$$I_{obs}/I_{IS} = I_R/I_{IS} + \frac{(I_{RH^+}/I_{IS}) - (I_R/I_{IS})}{1 + 10^{pH - pK_a}} \quad (1)$$

where I_{RH^+} and I_R represent the intensities of the Raman marker bands of the protonated and deprotonated amine or phosphate

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Table 1. Amine and Phosphate pK_a Values for Free GlcN6P and GlcN6P Bound to Wild-Type or G40A Mutant *glmS* Ribozyme

	free	bound to wild-type	bound to G40A
amine	8.06 ± 0.05	7.26 ± 0.09	8.04 ± 0.08
phosphate	5.98 ± 0.05	6.35 ± 0.09	5.96 ± 0.04

group, respectively, and I_{IS} is the intensity of the internal standard band, which is insensitive to pH change. The pK_a's for the amino and phosphate groups of GlcN6P bound to wild-type and G40A *glmS* ribozymes are compared in Table 1 to those for free aqueous GlcN6P (details of the measurements on free GlcN6P are given in Figures S2 and S5 and Table S1). The pK_a values for the GlcN6P amine and phosphate groups for the G40A mutant-bound ligand are very similar to those seen for the free ligand in aqueous solution. However, in the complex with the wild-type ribozyme, the pK_a of the amine of GlcN6P is lowered by 0.8 units to near neutrality while the pK_a of its phosphate is raised by 0.37 units.

Binding to the wild-type *glmS* ribozyme modulates the pK_a's of both the amine and the phosphate of GlcN6P, whereas binding to the G40A mutant does not. Moreover, both pK_a's shift toward neutrality, in principle improving the ability of each functional group to function as a general acid−base catalyst. Either could account for the experimentally determined[9,21] reaction pK_a of 6.9 or 7.8. From the structural standpoint, however, only the amine is likely to participate in catalysis. While the amine of the bound GlcN6P is 3.1 Å from the 5′-oxo leaving group of transesterification reaction and therefore well-placed to function as a general acid catalyst, the phosphate is ∼12 Å from the 2′-OH nucleophile and ∼9 Å from the 5′-oxo leaving group, making it unlikely to participate in catalytic proton transfer as either a general base or acid, respectively (Figure 1A).[4] This is consistent with the results of analogue studies, which imply that the amine is directly involved in catalysis while the phosphate probably only contributes to the binding energy, since GlcN, which has the amine but lacks the phosphate, is an activator (albeit weaker than GlcN6P), while Glc6P, which has the phosphate but lacks the amine, is not an activator.[9] In this regard, it is noteworthy that the pK_a shift of the amine of GlcN6P is twice as large as that of its phosphate (Table 1).

To our knowledge, this is the first report of the tuning of a catalytically relevant property of a small molecule by a ribozyme. This discovery extends the previously documented ability of ribozyme active sites to tune the pK_a's of RNA functional groups to improve their catalytic activity.[11] In addition, this is the first example of the lowering of the pK_a of an ionizable catalytic group by RNA. The active sites of the HDV and hairpin ribozymes raise the pK_a's of RNA functional groups.[14,18,19,28] In principle, it is easy to see how the strongly negatively charged environment of the interior of an intricately folded RNA could stabilize the cationic forms of nucleobases such as adenine and cytosine and thereby raise their pK_a's. How the *glmS* ribozyme preferentially stabilizes the neutral amine form of GlcN6P over its ammonium form is not immediately apparent, but our experiments indicate that the nucleobase at position 40 (which is at least 7 Å away from the amine nitrogen of GlcN6P) plays a critical role. (While this paper was under review, Davis et al.[21] independently reported NMR studies suggesting that the wild-type *glmS* ribozyme from *Bacillus anthracis* also perturbs the pK_a of its cofactor, but those authors did not examine the role of G40.) Although it is possible that ribozyme-bound metal ions could contribute to lowering the pK_a of GlcN6P, there are no crystallographically ordered cations directly bound to either the amine of GlcN6P or the scissile phosphate. Moreover, the structure of the G40A mutant, including the location of the tightly bound metal ions, is indistinguishable from that of the wild-type ribozyme. This points to the nucleobase of residue 40, rather than bound metal ions, as playing a key role in perturbing the pK_a of the bound GlcN6P.

Several catalytic RNAs, such as the hammerhead, hairpin, and VS ribozymes, have active-site guanine residues that could function as general acid−base catalysts (reviewed in ref 10). Because the unperturbed pK_a of GMP is ∼10, the mechanisms available to RNA to lower the pK_a's of such active-site functional groups are of considerable biological importance. The present work establishes the *glmS* ribozyme as a model system with which to dissect the mutual modulation of the chemical properties of an RNA and its small-molecule ligand.

■ ASSOCIATED CONTENT

Supporting Information. Structures of GlcN6P, GlcN, and Glc6P (Figure S1); Raman spectra of aqueous GlcN6P, GlcN, and Glc6P in the pH range from 5 to 10 (Figures S2−S4); plots of the intensities of the 546 cm^{-1} skeletal ring mode originating from the $-NH_3^+$ group and the band at 980 cm^{-1} due to the $-PO_3^{2-}$ form of the phosphate group relative to the internal intensity standard (the C−H deformation at 1462 cm^{-1}) for free GlcN6P as functions of pH (Figure S5); results of data analysis using an equation with a nonunity Hill coefficient (Table S1); and details of the Raman experimental protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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