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Analysis of Metal Ion-Dependence in *glmS* Ribozyme Self-Cleavage and Coenzyme Binding

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Abstract

The bacterial *glmS* ribozyme is a mechanistically unique functional RNA among both riboswitches and RNA catalysts. Its self-cleavage activity is the basis of riboswitch regulation of glucosamine-6-phosphate (GlcN6P) production, and catalysis requires GlcN6P as a coenzyme. Previous work has shown that the ligand amine of GlcN6P is essential for *glmS* ribozyme self-cleavage as is its protonation state. Metal ions are also essential within the *glmS* ribozyme core for both structure and function of the ribozyme. Although metal ions do not directly promote catalysis, we show that metal ion identity and the varying physicochemical properties of metal ions impact the rate of *glmS* ribozyme self-cleavage. Specifically, these studies demonstrate that metal ion identity impacts the overall apparent pK_a of ribozyme self-cleavage, and metal ion binding largely reflects phosphate oxygen affinity. Results suggest that metal ions serve alternative roles supporting the mechanism of catalysis.

Keywords

ribozymes; RNA; metalloenzymes; structure-activity relationships; molecular recognition

INTRODUCTION

The most recent catalytic RNA (ribozyme) discovered is the bacterial *glmS* riboswitch.[1] Bacterial riboswitches commonly provide a feedback mechanism of genetic regulation, where they are generally situated in non-coding regions of mRNAs that encode proteins

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required to produce the metabolite with which the riboswitch directly interacts.[2] While most riboswitch-metabolite interactions modulate transcription termination or translation initiation of the associated mRNA[2], the *glmS* riboswitch uniquely utilizes metabolite as a coenzyme to perform self-cleavage [1,3] and promote mRNA degradation.[4]

The cognate metabolite and coenzyme for the *glmS* ribozyme is glucosamine-6-phosphate (GlcN6P), the direct product of the GlmS enzyme required for bacterial cell wall biosynthesis. Prior studies using GlcN6P and various analogs have shown that the ligand amine is required for catalysis and that the apparent pK_a for the overall self-cleavage reaction largely reflects that of the ligand amine.[3] Additionally, the ligand phosphate affects the pK_a of the amine and is an important determinant for ligand recognition and affinity. Both X-ray crystallographic analyses [5-9] and biochemical analyses [1,10-12] of the *glmS* ribozyme agree that coenzyme binding does not alter the conformation of the ribozyme, but positions the ligand amine adjacent to the scissile phosphodiester linkage (Figure 1). Furthermore, crystallographic data suggest there are as many as four divalent metal ion-binding sites within the core of the RNA. Two metal ions appear to mediate ligand phosphate interaction with one helical segment adjacent to the ribozyme active site, and two additional metal ions are associated with another helical segment adjacent the active site. Both X-ray crystallographic and biochemical analyses [5-9,13] agree that metal ions do not directly interact with the scissile phosphodiester to promote catalysis. Rather, the coenzyme amine does so to initiate self-cleavage by a presumed mechanism of acid-base catalysis.[3,5,8,12,14]

Nevertheless, metal ions are indeed required for the *glmS* ribozyme to perform coenzyme-dependent self-cleavage.[1,13] In general, metal ion binding to RNA can involve a network of inner- and outer-sphere contacts involving multiple coordination sites and hydrogen bond donor and acceptor sites. Nucleotide Analog Interference Mapping (NAIM) and Suppression (NAIS) experiments have revealed important functional groups within the core ribozyme that lie in the vicinity of metal ion binding sites evident in the crystal structures of the *glmS* ribozyme.[5,8,12] While such data support the positions and interactions of metal ions in the *glmS* ribozyme core, it is difficult to distinguish whether metal ion binding serves only to coordinate the catalytically competent structural conformation or additionally promote catalytic mechanism. In regard to the latter, pK_a values of RNA functional groups can be altered by metal ions bound at a distance from the active site.[15] For example, direct coordination of a transition state metal ion to the N7 of guanine can lower the N1 pK_a by ~2 pH units from ~10 to a physiologically relevant pK_a of ~8.[16] In the VS ribozyme it has been shown that metal ions are largely responsible for the observed alteration of catalytic nucleobase pK_a. [17] Such alternative means for metal ions to augment catalysis in the *glmS* ribozyme have not been considered previously.

RESULTS & DISCUSSION

Divalent metal ions and *glmS* ribozyme self-cleavage

Prior work established only whether self-cleavage is observed in the presence of various divalent metal ions.[1] This study showed that self-cleavage is supported in the presence of 5 mM Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, or Sr^{2+}, but that Zn^{2+} or Cd^{2+} do not promote catalysis. We extended the time course of such reactions and show that both Cd^{2+} and Zn^{2+} do support *glmS* self-cleavage albeit at substantially reduced rates compared to Mg^{2+} (see Figures 2 and 3). However, to further investigate the roles that metal ions play in *glmS* ribozyme self-cleavage, we examined the pH-dependence of reaction rate in the presence of various divalent metal ions including Mg^{2+}, Mn^{2+}, Ca^{2+}, Cd^{2+}, or Ba^{2+}. With GlcN6P concentration at 10 μM (near apparent K_D)[12] and divalent metal ion concentration at 10 mM, the *glmS* ribozyme exhibits greatest self-cleavage activity in the presence of Mg^{2+} followed by Ca^{2+},

Mn^{2+} and Ba^{2+} (Figure 2a). At sub-saturating concentration of GlcN6P, Cd^{2+} minimally supports self-cleavage but substantially effects RNA degradation, which can be alleviated somewhat using saturating concentrations of ligand (data not shown). For divalent metal ions that strongly support *glmS* ribozyme self-cleavage, the rate constant increases with pH and reaches a maximum approaching pH 8. Apparent pK_a values determined for the self-cleavage reaction are 7.8 for Mg^{2+}, 7.3 for Ca^{2+}, and 7.1 for Mn^{2+}. The apparent pK_a for the reaction with Mg^{2+} is consistent with prior work in which the value is presumed to reflect the pK_a of the ligand amine. [3] Nevertheless, the overall pK_a of the reaction varies by 0.7 pH units and the rate constant at physiological pH varies by ~1.5 log units dependent upon divalent metal ion. These data strongly suggest that the ligand amine is not the only important functional group involved in catalysis and that metal ions factor into the detailed mechanism of RNA catalysis.

In consideration of the physicochemical properties of divalent metal ions (Table 1), it becomes possible to identify correlations with ribozyme reactivity. For over half of the metal ions tested there is a linear relationship between increasing log(k_{obs}) and pK_a (Figure 3a) for metal ions with pK_a values in the range from 9 to 12. Conversely, for metal ions with pK_a values >12 the rate constant decreases as pK_a value increases. Although this relationship alone cannot fully explain the role of hydrated metal ions in *glmS* self-cleavage it suggests that outer-sphere contacts to RNA functional groups play a role in catalysis.

Ionic radius and absolute hardness of a metal ion have been observed to correlate with log(k_{obs}) for some small self-cleaving RNAs.[18] The Schistosoma hammerhead ribozyme showed such correlation with the exception of Mg^{2+}. It was proposed that Mg^{2+} exhibits a greater k_{obs} value than other ions with similar ionic radii due to its greater absolute hardness. Absolute hardness is a measure of the affinity of metal ions for hard ligands such as phosphate oxygens. Therefore the catalytic utility of a metal ion is dictated by steric restriction of the binding site and the presence of hard or soft ligands such as phosphate oxygens or N7 of guanine, respectively. Analysis of our data shows no correlation between log(k_{obs}) and ionic radii (Figure 3b). However there exists some relationship between rate and absolute hardness (Figure 3c), suggesting that metal ion-phosphate interactions are important. This relationship makes sense based on previous biochemical and biophysical analyses indicating metal ion interactions with ligand phosphate.[5-9,12]

Lastly, we analyzed our data in relationship to metal ion-phosphate affinities in the order of the Irving-Williams series, which corresponds to complex stabilities between metal ions and various biological ligands. The k_{obs} values for the alkaline earth M^{2+} ions (Ba^{2+}, Sr^{2+}, Ca^{2+}, Mg^{2+}) follow the phosphate affinities of the Irving-Williams series, within which rate increases with affinity of the metal ions for phosphate monoester (Figure 3d).[19-21] Conversely, the cleavage rates observed for transition state M^{2+} ions of the series do not follow the trend of phosphate affinities, where k_{obs} decreases from left to right across the Irving-Williams series. Overall these analyses suggest that phosphate binding dominates the metal ion effect in the *glmS* ribozyme, although metal ion effects on the overall pK_a of the reaction remain to be determined.

Monovalent metal ions and *glmS* ribozyme self-cleavage

With regard to *glmS* ribozyme self-cleavage in the presence of monovalent metal ions, prior work only established that a relatively low rate of self-cleavage is observed in the presence of high concentrations (up to 3 M) of K^+.[13] We similarly examined the pH-dependence of reaction in the presence of various monovalent metal ions including Li^+, NH_4^+, Na^+, K^+, Rb^+ or Cs^+, which required a higher concentration of ligand to observe substantial self-cleavage. With GlcN6P concentration at 10 mM and monovalent metal ion concentration at 1 M, the *glmS* ribozyme exhibits greatest self-cleavage activity in the presence of Li^+

followed by NH_4^+, and Na^+ (Figure 2b). Under these conditions, nominal self-cleavage activity was observed in the presence of K^+, Rb^+, and Cs^+. For monovalent metal ions that strongly support *glmS* ribozyme self-cleavage, the rate constant again increases with pH and reaches a maximum approaching pH 8. However, apparent pK_a values determined for the self-cleavage reaction are 7.5 independent of monovalent metal ion identity. These data demonstrate that monovalent metal ions can support *glmS* ribozyme activity, but they do not differently affect the overall apparent pK_a for the reaction as do divalent metal ions.

In consideration of the physicochemical properties of monovalent metal ions (Table 1), correlations with ribozyme reactivity are apparent with ionic radius and Pearson hardness. Thus, it is pertinent to highlight the near identical properties of Li^+ and Mg^{2+}, which provide for maximal performance of the *glmS* ribozyme. Otherwise, ribozyme reactivity with metal ions appears to correlates with Pearson hardness to the point that ionic radius exceed approximately 1.3 to 1.5 Å, where larger metal ions such as Rb^+ and Cs^+ do not support self-cleavage.

To further investigate monovalent metal ion binding and the steric and chemical restrictions of metal ion binding sites, *glmS* ribozyme activity was examined in the presence of Na^{2+} with GlcN6P or glucosamine (GlcN). The rate constant for ribozyme self-cleavage with 10 mM GlcN6P exhibits a log-linear relationship with Na^{2+} concentration up to 2 M (Figure 4a), and reactivity approaches the maximum observed for saturating concentrations of 2 M Li^+ (data not shown) or 10 mM Mg^{2+}.[12] These data suggest that metal ion binding sites are just nearing saturation at the highest achievable concentrations of monovalent metal ions, which is consistent with prior work using K^+.[13] We are therefore able to examine effects of metal ion interaction with ligand phosphate under reasonably comparable conditions at pH 7.5. In the presence of 10 mM Mg^{2+}, self-cleavage activity is substantially decreased in the presence of 10 mM GlcN versus GlcN6P, whereas in the presence of 2 M Na^+, both ligands support self-cleavage activity (Figure 4b). Based on observed rate constants, there is a relative 3-fold enhancement in self-cleavage activity for GlcN, with Na^+ versus Mg^{2+}.

The apparent effect that ligand phosphate is discriminated less by Na^+ might be attributable to a number of phenomena that highlight the roles that divalent versus monovalent ions can play in RNA structure and function. First, the absence of ligand phosphate as a hard ligand could soften the preference for hard metal ions and change the possible coordination sites available for monovalent versus divalent metal ions. MD simulations of the Hepatitis Delta Virus (HDV) ribozyme show that two Na^+ ions could physically replace a divalent metal ion without utilizing the same coordination sites.[22] Indeed crystal structures of the HDV ribozyme in monovalent versus divalent metal ions indicate that the coordination sites of these different ions do not overlap completely.[23] Second, the absence of ligand phosphate could reduce steric hindrance and enable the accommodation of larger metal ions. Accordingly, we observe a similar relative enhancement in *glmS* ribozyme self-cleavage activity with GlcN and Ba^{2+} (data not shown). These results suggest that aside from an obvious difference in divalent and monovalent metal ion charge density and binding affinity, mediation of ligand phosphate recognition and binding contribute to the *glmS* ribozyme's preference for divalent metal ions.

These studies provide evidence for divalent metal ion preference in association with the ligand phosphate, but demonstrate that other metal ion binding sites can utilize monovalent or divalent metal ions that conform to appropriate physicochemical properties. While there is a strong correlation between ribozyme self-cleavage and the hardness of associated metal ions, there are nuances to metal ion coordination that are less intuitive. For example, NH_4^+ supports catalysis although it is not a hard metal ion, and its proposed ionic radius is equivalent to or exceeds that of K^+.[24] Nevertheless, these studies further demonstrate that

metal ion identity can impact the overall apparent pK_a of ribozyme self-cleavage as well as phosphate oxygen affinity, thus suggesting that metal ions can serve alternative roles supporting the mechanism of catalysis. We have previously proposed an acid-base mechanism of catalysis that invokes a proton relay within the catalytic core of the ribozyme.[14] The identity of metal ions binding in the vicinity of such a proton relay could differently affect functional groups engaged in proton transfer and thus influence the efficiency of catalysis. In support of this hypothesis, the *glmS* ribozyme exhibits 7-deazaguanine interference at the active site G33 in the presence of Mn^{2+} (Soukup and Soukup, unpublished observations) that is not observed in the presence of Mg^{2+}.[12] G33 is important in the proposed proton relay[14], and has been identified by others as being somehow important to catalysis.[5-6,8-9]

Although determination of the precise role(s) of metal ions in the structure and detailed mechanism of *glmS* ribozyme self-cleavage requires further analysis, the impact of metal ion interaction on coenzyme binding and catalysis is relatively clear. Mg^{2+} interaction with ligand phosphate in the context of the ribozyme core is essential to achieving rates of self-cleavage biologically relevant to regulation of *glmS* expression. In this regard, our work underscores the importance of preserving the phosphate group or another hard ligand for Mg^{2+} in coenzyme analogs designed to function as artificial agonist that elicit *glmS* ribozyme activity. Such artificial agonists might be used as novel antibiotics to inhibit *glmS* expression, GlcN6P biosynthesis, and bacterial cell growth for a number of human pathogens that possess the *glmS* riboswitch.

EXPERIMENTAL SECTION

Templates for transcription were prepared by primer extension and PCR amplification using synthetic DNA corresponding to *B. cereus glmS* ribozyme sequence as previously described.[3] Ribozymes were prepared by in vitro transcription with T7 RNA polymerase and ^{32}P-labeled by incorporation of [α-^{32}P]-UTP. Transcription products were purified by denaturing 10% polyacrylamide gel electrophoresis (PAGE), eluted in solution containing HEPES (50 mM at pH 7.5 at 23°C) and NaCl (200 mM), precipitated with ethanol, redissolved in water, and quantitated by scintillation counting.

Ribozyme reactions were initiated by the addition of divalent or monovalent metal ion to yield solutions containing ribozyme (250 nM), buffering agent (50 mM), ligand at indicated concentration, and metal ion at indicated concentration. Sulfonic acid (non-amine) buffering agents used to achieve various reaction pH values at 23°C were HEPES, MES, PIPES, TAPS, or CHES. Reactions containing monovalent metal ion additionally included EDTA (10 mM) to chelate contaminating divalent metal ions. Aliquots from reactions incubated at 23°C were removed and terminated at various time points by addition of an equal volume of gel loading dye containing urea (10 M) and EDTA (20 mM). Products were separated by denaturing 10% PAGE and analyzed using a PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

k_{obs} values for self-cleavage were derived by plotting the natural logarithm of the fraction of uncleaved ribozyme versus time and establishing the negative slope of the resulting line. Stated values represent the average of at least two replicate assays. Apparent pK_a values were determined by fitting average data from at least two replicate assays to the Hill equation: $k_{obs} = (k_{max} \times pH^n)/(pH^n + \text{apparent } pK_a{}^n)$, where n represents the Hill coefficient.

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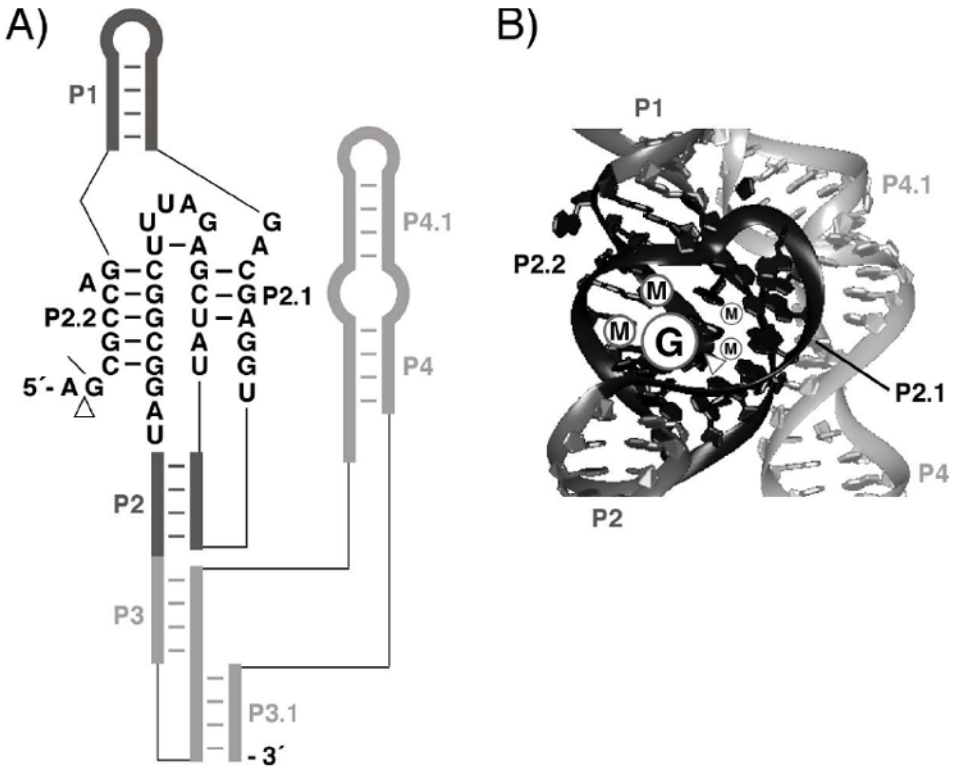

Figure 1. Coenzyme and metal ion binding within the structure of the *glmS* ribozyme

a) Secondary structure. The highly conserved core sequence of the *glmS* ribozyme is shown in black (P2.1 and P2.2), while requisite structural elements (P1 and P3) and peripheral structural elements (P3-P4) are schematically shown in dark gray and light gray, respectively. The arrowhead denotes the site of self-cleavage. b) Tertiary structure. The ribbon model depicts the core structure of the *glmS* ribozyme.[8] Indicated are the sites of self-cleavage (arrowhead), GlcN6P binding (encircled G), and metal ion binding on the "front" side of the ribozyme (large encircled M) and on the "back" side of the ribozyme (small encircled M).

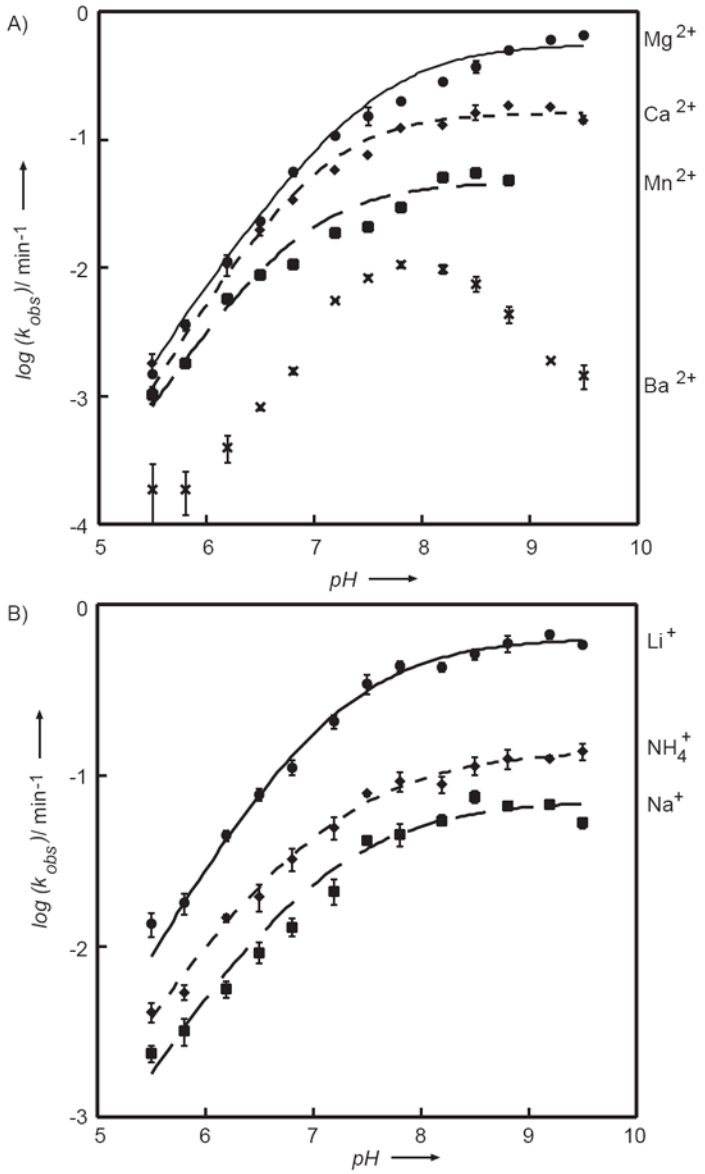

Figure 2. Effect of metal ion identity on pH reactivity profiles of *glmS* ribozyme self-cleavage

a) pH reactivity profiles in the presence of indicated divalent metal ions. Reactions were performed using 10 μM GlcN6P and 10 mM divalent metal ion. b) pH reactivity profiles in the presence of indicated monovalent metal ions. Reactions were performed using 10 mM GlcN6P and 1 M monovalent metal ion with 10 mM EDTA. Data points represent the average of three replicate assays, and error bars indicate standard deviation.

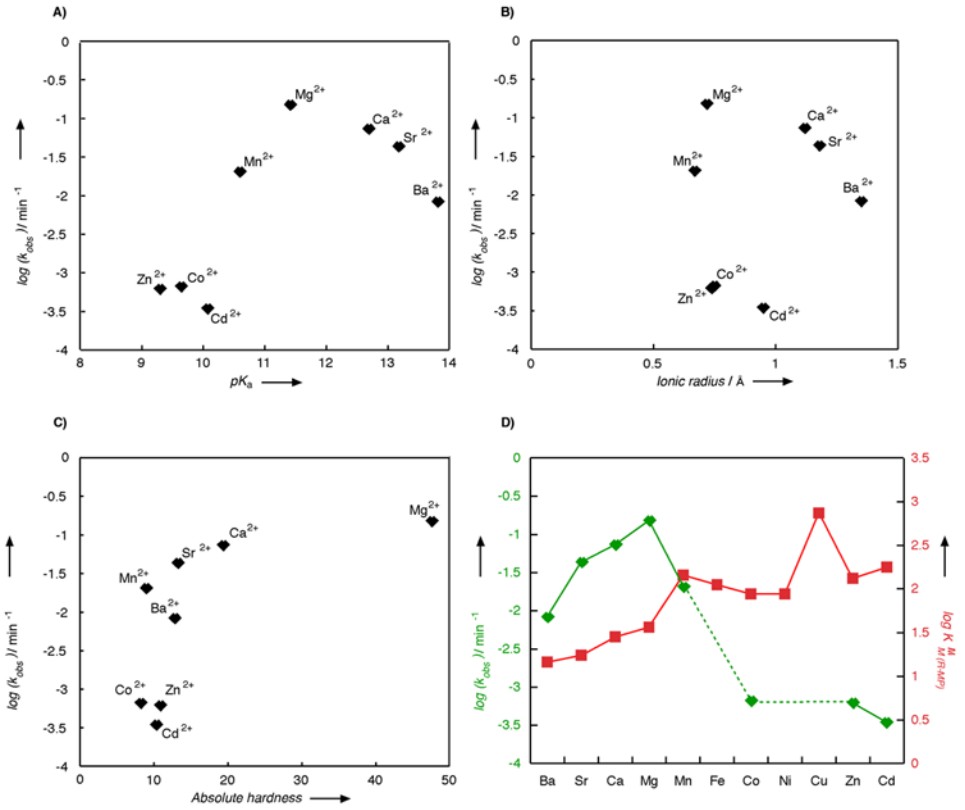

Figure 3. Effect of metal ion physicochemical properties on *glmS* ribozyme self-cleavage

a) pK_a of the hydrated metal ion versus rate. b) Ionic radius of the metal ion versus rate. c) Absolute hardness of the metal ion versus rate. d) Classical Irving-Williams series as observed for metal ion binding to phosphate monoester (red).[19-21] *glmS* ribozyme self-cleavage rates (green) follow the phosphate affinities of the Irving-Williams series for the alkaline earth metal ions. Values for pK_a, ionic radius, and absolute hardness are referenced in Table 1.

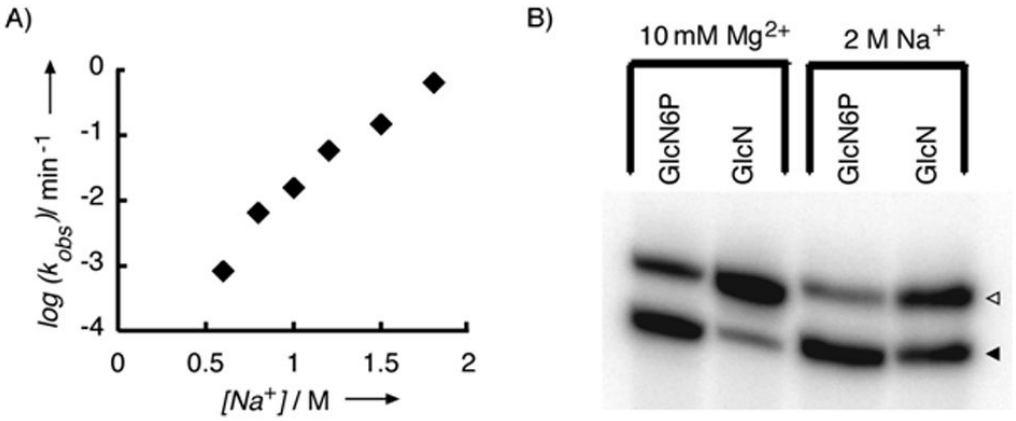

Figure 4. Alleviation of divalent metal ion requirement in the absence of ligand phosphate

a) Log-linear relationship between the rate constants for *glmS* ribozyme self-cleavage and Na^+ concentration at pH 7.5. Reactions were performed using 10 mM GlcN6P. Self-cleavage activity is nearly maximal at the highest achievable Na^+ concentration indicating near-saturation. Each data point represents the average of two or three measurements. b) Effect of metal ion interaction with ligand phosphate on *glmS* self-cleavage activity at pH 7.5. Depicted are the reaction products separated by denaturing PAGE for 30 s reactions in the presence of monovalent or divalent metal ions with GlcN6P or GlcN as indicated. Open arrowheads denote the full-length (uncleaved) ribozyme, and filled arrowhead denote the 3′ cleavage product. Observed rate constants are 0.04 s^{-1} (Mg^{2+}, GlcN6P), 0.006 s^{-1} (Mg^{2+}, GlcN), 0.05 s^{-1} (Na^+, GlcN6P), and 0.02 s^{-1} (Na^+, GlcN).

Table 1

Physicochemical Properties of Divalent and Monovalent Metal Ions.[a]

Ion	Ionic radius, Å	First pK_a of [$M(H_2O)_n$]	Absolute hardness (η)
Mg^{2+}	0.72	11.42	47.59
Ca^{2+}	1.12	12.7	19.52
Mn^{2+}	0.67	10.6	9.02
Cd^{2+}	0.95	10.08, 11.7	10.29
Ba^{2+}	1.35	13.82	12.8
Li^{+}	0.59	13.8	35.12
Na^{+}	1.02	14.48	26.21
K^{+}	1.38	–	17.99

[a] Listed values are compiled from various sources: ionic radii in angstroms for +2 or +1 oxidation state[25-28], first pK_a of hydrated metal ion[28-29], absolute hardness.[30-31]