A divalent cation-dependent variant of the \textit{glmS} ribozyme with stringent Ca\textsuperscript{2+} selectivity co-opts a preexisting nonspecific metal ion-binding site

MATTHEW W.L. LAU, ROBERT J. TRACHMAN III, and ADRIAN R. FERRÉ-D’AMARÉ
National Heart, Lung and Blood Institute, Bethesda, Maryland 20892-8012, USA

ABSTRACT
Ribozymes use divalent cations for structural stabilization, as catalytic cofactors, or both. Because of the prominent role of Ca\textsuperscript{2+} in intracellular signaling, engineered ribozymes with stringent Ca\textsuperscript{2+} selectivity would be important in biotechnology. The wild-type \textit{glmS} ribozyme (\textit{glmS}\textsubscript{WT}) requires glucosamine-6-phosphate (GlcN6P) as a catalytic cofactor. Previously, a \textit{glmS} ribozyme variant with three adenosine mutations (\textit{glmS}AAA) was identified, which dispenses with GlcN6P and instead uses, with little selectivity, divalent cations as cofactors for site-specific RNA cleavage. We now report a Ca\textsuperscript{2+}-specific ribozyme (\textit{glmS}Ca) evolved from \textit{glmS}AAA that is >10,000 times more active in Ca\textsuperscript{2+} than Mg\textsuperscript{2+}, is inactive in even 100 mM Mg\textsuperscript{2+}, and is not responsive to GlcN6P. This stringent selectivity, reminiscent of the protein nuclease from \textit{Staphylococcus}, allows rapid and selective ribozyme inactivation using a Ca\textsuperscript{2+} chelator such as EGTA. Because \textit{glmS}Ca functions in physiologically relevant Ca\textsuperscript{2+} concentrations, it can form the basis for intracellular sensors that couple Ca\textsuperscript{2+} levels to RNA cleavage. Biochemical analysis of \textit{glmS}Ca reveals that it has co-opted for selective Ca\textsuperscript{2+} binding a nonspecific cation-binding site responsible for structural stabilization in \textit{glmS}\textsubscript{WT} and \textit{glmS}AAA. Fine-tuning of the selectivity of the cation site allows repurposing of this preexisting molecular feature.

Keywords: catalytic RNA; in vitro selection; phosphorothioate interference; SAXS; molecular exaptation

INTRODUCTION
Divalent cations stabilize the 3D architecture of ribozymes and other structured RNAs (for review, see Draper 2004; Koculi et al. 2007). In addition, alkaline earth ions directly participate in the catalytic mechanism of several ribozymes (for review, see Fedor and Williamson 2003; Ferré-D’Amaré and Scott 2010; Aufinger et al. 2011). The specific identity of the divalent cation is generally less important for electrostatic structural stabilization than for catalytic cofactor function. For instance, the \textit{Tetrahymena} ribozyme can adopt a near-native fold in the presence of Ca\textsuperscript{2+}, Ba\textsuperscript{2+}, or Sr\textsuperscript{2+}, but is inactive unless its active site is bound to Mg\textsuperscript{2+} (Grosshans and Cech 1989; Celander and Cech 1991; Koculi et al. 2007). Structure-guided engineering and in vitro evolution have been used to alter the cation specificity of several natural ribozymes. In the case of the \textit{Tetrahymena} ribozyme, in vitro selection produced variants that have 300-fold increased activity in Ca\textsuperscript{2+} (Lehman and Joyce 1993; Riley and Lehman 2003). However, these also had increased activity in Mg\textsuperscript{2+}. Biochemical analyses suggested that their active sites did not acquire specificity for Ca\textsuperscript{2+}; instead they relaxed their cation selectivity (Cernak et al. 2008). Similarly, in vitro evolution of the ribonuclease P ribozyme for cleavage in Ca\textsuperscript{2+} produced a point mutant that acquired the ability to cleave in this cation. However, and despite a decrease of reactivity in Mg\textsuperscript{2+}, the cleavage rate of the mutant in Ca\textsuperscript{2+} was only 91-fold higher than in Mg\textsuperscript{2+} (Frank and Pace 1997). Consistent with its intracellular abundance, Mg\textsuperscript{2+} is preferentially used by most natural ribozymes that depend on a divalent cation as a catalytic cofactor. Ribozymes specific for Ca\textsuperscript{2+} would have biotechnological applications, given the importance of this metal ion in intracellular signaling (for review, see Clapham 2007). Altering the cation selectivity of natural ribozymes so that they are stringently dependent on Ca\textsuperscript{2+} has remained a challenging molecular engineering problem.

The \textit{glmS} ribozyme is a bacterial gene-regulatory RNA that controls the abundance of glucosamine-6-phosphate (GlcN6P), an essential precursor for cell-wall biosynthesis (for review, see Ferré-D’Amaré 2010). It is part of the mRNA for the enzyme GlcN6P synthetase. GlcN6P binds to the ribozyme and activates its self-cleavage (Winkler et al. This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see http://rnajournal.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.
activating Ca2+. Such RNAs could potentially be functionalized to high stability of this ribozyme fold, and demonstrating the coenzyme function of GlcN6P, the activity of this ribozyme is largely insensitive to the identity of divalent cations. Thus, and as expected for their role solely in structural stabilization, the glmS ribozyme exhibits comparable GlcN6P-catalyzed activity in the presence of Mg2+, Ca2+, Mn2+, or even the complex ion cobalt (III) hexammine (Roth et al. 2006; Klauwuhn et al. 2010). The amino ligands of the latter are kinetically inert, and therefore activity of the ribozyme in this compound is strong evidence that inner-sphere cation coordination is not required for folding or catalysis (Cowan 1993).

Previously, we examined whether RNAs closely related to the wild-type glmS ribozyme (glmSWT) could perform divalent cation-mediated catalysis without using the coenzyme GlcN6P (Lau and Ferré-D’Amaré 2013). The evolutionary ancestors of glmSWT could have been such unregulated ribozymes. Acquisition of GlcN6P-dependence by RNAs like these would have given rise to the gene-regulatory capability of the present-day glmS WT (Ferré-D’Amaré 2011). We discovered that three adenosine mutations in the ribozyme core produced a variant (termed glmSAAA) that is fully active (kobs ~ 2.5 min⁻¹, which is within 20-fold of glmSWT) in the presence of divalent cations alone. This mutant ribozyme was not stimulated by GlcN6P. As is the case for many engineered variants of natural ribozymes, glmSAAA has only modest cation specificity; its maximum activity in Ca2+ exceeds that in Mg2+ by less than a factor of 50 (Lau and Ferré-D’Amaré 2013). The crystal structure of glmSAAA revealed essentially the same 3D-architecture as that of glmSWT, underscoring the high stability of this ribozyme fold, and demonstrating that this RNA structural scaffold can be functionalized to support divergent biochemical activities (GlcN6P and cation-mediated catalysis by glmSWT and glmSAAA, respectively; Lau and Ferré-D’Amaré 2013). The functional plasticity of the structurally rigid glmS ribozyme fold we uncovered suggested that other variants that exhibit a higher degree of cation selectivity than glmSAAA might exist. To test this hypothesis, we performed further in vitro selection experiments to isolate variants of the glmS ribozyme that are selectively activated by Ca2+. Such RNAs could potentially be developed into tools that link intracellular signaling by Ca2+ with RNA stability and gene expression. Unlike in the glmSAAA selection (Lau and Ferré-D’Amaré 2016a), these new experiments explicitly selected and counterselected for catalysis based on the identity of the divalent cation (Ca2+ versus Mg2+) in solution. This work has now produced a mutant of the glmS ribozyme that does not use GlcN6P for catalysis and is exquisitely selective for Ca2+. Small-angle X-ray scattering (SAXS) demonstrates that the mutant can fold equally in Ca2+ or Mg2+, excluding a primarily structural role for the cation. Biochemical analysis indicates that rather than evolving a new Ca2+-binding site, the mutant RNA co-opted a nonspecific cation-binding site that had played a structural role in both the glmSWT and glmSAAA ribozymes.

RESULTS

In vitro selection yields a highly Ca2+-selective glmS ribozyme variant

To seek variants of the glmS ribozyme that function without GlcN6P and are highly selective for specific metal ions, we performed in vitro selection using a pool of RNA sequences based on glmSAAA in which nucleotides near the active site that do not participate in forming the characteristic, triply pseudoknotted secondary structure (Klein and Ferré-D’Amaré 2006) of the ribozyme were mutagenized (Fig. 1), and enriched for cleavage in either Mg2+ or Ca2+ (Materials and Methods). Ribozymes with varying degrees of selectivity for Ca2+ were isolated after eight selection rounds (Fig. 2).

A group of ribozymes that displayed strong discrimination against Mg2+ was chosen for further analyses. These RNAs all have in common 10 point mutations from the wild-type sequence (eight mutations relative to glmSAAA; Figs. 1B, 3), and reversion of any of these to the wild-type nucleotide reduced or eliminated Ca2+-dependent activity (not shown). A ribozyme containing just these mutations (hereafter glmS(Ca)) displayed high selectivity for Ca2+. Its cleavage rate was at least 10,000-fold faster in 2 mM Ca2+ than in 2 mM Mg2+. This is a lower bound because this ribozyme exhibited no detectable activity in Mg2+, and therefore the estimate is limited by the sensitivity of our cleavage assay. Even in the presence of 100 mM Mg2+ (Fig. 4A), glmS(Ca) was inactive. This Ca2+-selective ribozyme is also inactive in common monovalent and lanthanide ions and the soft divalent ion Cd2+, but exhibits residual activity in Sr2+ (Fig. 4A). Like glmSAAA (Lau and Ferré-D’Amaré 2013), glmS(Ca) is inactive in cobalt hexammine, indicating that it requires inner-sphere cation coordination for activity.

Comparison of the activity of glmS(Ca) in the presence and absence of GlcN6P shows no stimulation by the cofactor of glmSWT (Table 1). Indeed, glmS(Ca) is not stimulated by a number of other small molecule activators of glmSWT with vicinal amine and hydroxyl groups (McCarthy et al. 2005; Lim et al. 2006; Posakony and Ferré-D’Amaré 2013; Fei et al. 2014), nor a recently synthesized GlcN6P analog activator of glmSWT that bears a guanidinium group instead of the amine (Table 1). Thus, glmS(Ca), like glmSAAA from which it evolved, is a small molecule cofactor-independent ribozyme.

Mg2+ inhibits glmS(Ca) catalysis but not folding

Kinetic analysis shows that glmS(Ca) attains half-maximal activity (K1/2) at 14 mM Ca2+ (in the presence of 100 mM KCl) (Fig.
This is lower than for glmS\textsuperscript{WT} (in saturating GlcN\textsubscript{6}P and 100 mM KCl) and glmS\textsuperscript{AAA} (\(K_{1/2} = 31\) and 93 mM, respectively, Lau and Ferré-D’Amaré 2013), indicating a higher affinity for the divalent cation. Addition of 5 mM Mg\textsuperscript{2+} to the Ca\textsuperscript{2+} titration of glmS\textsuperscript{Ca} yielded a sigmoidal relationship, and increased \(K_{1/2}\) by approximately twofold (Fig. 4B). Lineweaver–Burk analysis shows a nonlinear relationship in the presence of Mg\textsuperscript{2+}, suggestive of mixed-type inhibition by this cation (Fig. 4C). Competition by Mg\textsuperscript{2+} with Ca\textsuperscript{2+} for inner-sphere binding to functionally important sites is further supported by analogous experiments in which a Ca\textsuperscript{2+} titration was supplemented with 5 mM Ba\textsuperscript{2+}. In these, the \(K_{1/2}\) was increased by \(\sim 1.5\) fold, but the kinetics remained Michaelis–Menten. Presumably, the larger ionic radius of Ba\textsuperscript{2+} (Table 2) precludes it from competing efficiently for functionally important inner-sphere Ca\textsuperscript{2+} binding sites, but it can displace some diffusely RNA-associated (Draper 2004) Ca\textsuperscript{2+} ions (Fig. 4B).

To examine whether glmS\textsuperscript{Ca} requires Ca\textsuperscript{2+} for folding, we compared its overall three-dimensional structure in solution in the presence of a near-physiological concentration of MgCl\textsubscript{2} (Romani and Scarpa 1992; Grubbs 2002), or the same concentration of CaCl\textsubscript{2} or SrCl\textsubscript{2} (2.5 mM of the alkaline earth salt in addition to 100 mM KCl in each case, Materials and Methods) by small-angle X-ray scattering (SAXS). In the presence of Mg\textsuperscript{2+}, glmS\textsuperscript{Ca} exhibits a radius of gyration (\(R_g\)) of 41.4 \(\pm 1.2\) Å. Unexpectedly, given the inactivity of the ribozyme in Mg\textsuperscript{2+}, the shapes of its pair-probability distribution function \(P(r)\) (Fig. 5A) and Kratky plots (Fig. 5B) are indicative of a folded RNA. The \(R_g\) of the RNA in the presence of either Ca\textsuperscript{2+} or Sr\textsuperscript{2+} is identical within experimental error to that in Mg\textsuperscript{2+}. Moreover, the \(P(r)\) and Kratky plots of the RNA in the three divalent cations tested are virtually identical (Fig. 5B). This analysis indicates that all three divalent cations can globally fold the RNA to a comparable extent at the same ionic strength. SAXS analysis, therefore, suggests that inactivity of glmS\textsuperscript{Ca} in Mg\textsuperscript{2+} reflects inhibition of the chemical step, rather than a deficit in folding. Under our experimental conditions, the \(R_g\) of the glmS\textsuperscript{Ca} ribozyme (in the equivalent cis-acting form) in 2.5 mM MgCl\textsubscript{2} is 38.5 \(\pm 2.3\) Å (not shown), suggesting that the parental ribozyme may be slightly more compact than glmS\textsuperscript{Ca}.

The glmS\textsuperscript{Ca} ribozyme cleaves one nucleotide 3’ to glmS\textsuperscript{WT}

Both glmS\textsuperscript{WT} and glmS\textsuperscript{AAA} cleave RNA by internal transesterification, yielding products with 2’,3’-cyclic phosphate and 5’-OH termini (Winkler et al. 2004; Lau and Ferré-D’Amaré 2013). To characterize the cleavage reaction of

![FIGURE 1. Mutagenized RNA pool based on glmS\textsuperscript{AAA} for in vitro selection. (A) Cartoon depiction of the crystal structure of glmS\textsuperscript{AAA} (Lau and Ferré-D’Amaré 2013). Nucleotides mutagenized for this study are in red. Blue residues denote the three adenosine mutations (relative to wild-type, A49, A51, G65) that characterize glmS\textsuperscript{AAA}. (*) Location of scissile phosphate. (B) Sequence and hypothetical secondary structure of glmS\textsuperscript{Ca}. Nucleotides in red lower case were mutagenized by 30% relative to glmS\textsuperscript{AAA}. Nucleotides in gray were constant. Two of the three adenosine mutations that characterize glmS\textsuperscript{AAA} (blue) were retained in glmS\textsuperscript{Ca}. Nucleotides in green denote the eight mutations in glmS\textsuperscript{Ca} relative to glmS\textsuperscript{AAA}.](www.rnajournal.org)}
glmS\textsubscript{Ca}, we compared its 5′ cleavage product with those of glm\textsubscript{S\textsuperscript{WT}} and glm\textsubscript{S\textsuperscript{AAA}}. Upon treatment of the 5′ cleavage products of the three ribozymes with acid or polynucleotide kinase, they exhibited parallel gel mobility shifts consistent with opening and removal, respectively, of a 2′,3′-cyclic phosphate (Fig. 6A). This suggests that glmS\textsubscript{Ca}-catalyzed RNA cleavage also proceeds through internal transesterification.

Unexpectedly, the 5′ cleavage product of glm\textsubscript{S\textsuperscript{Ca}} had an electrophoretic mobility consistent with being one nucleotide longer than the products of the two other RNAs. This hinted that the cleavage site of the Ca\textsuperscript{2+}-selective ribozyme shifted one nucleotide 3′ to the cleavage site of the wild type. To confirm this, we subjected oligonucleotides bearing a 2′-deoxyribose at positions −2, −1, or +1 (by convention, there is no zero position) to cleavage by the three ribozymes (Fig. 6B). Because the 2′-OH group is the nucleophile in the transesterification reaction, substitution of the reactive ribose with a deoxyribose is expected to abrogate cleavage. Consistent with previous experiments, glm\textsubscript{S\textsuperscript{WT}} and glm\textsubscript{S\textsuperscript{AAA}} cleavage was dependent on a 2′-OH at position −1 (Winkler et al. 2004; Lau and Ferré-D’Amaré 2013). In contrast, glm\textsubscript{S\textsuperscript{Ca}} could cleave a substrate with the deoxy substitution at position −1, but not at position +1. Overall, these experiments indicate that glm\textsubscript{S\textsuperscript{Ca}} cleaves its substrate between positions +1 and +2 by using the ribose 2′-OH of the former as the nucleophile.

Phosphorothioate interference suggests location of an essential Ca\textsuperscript{2+}

To locate Ca\textsuperscript{2+} ions important for glm\textsubscript{S\textsuperscript{Ca}} activity, we performed phosphorothioate interference mapping. This technique exploits the altered affinity for hard cations (e.g., Mg\textsuperscript{2+} or Ca\textsuperscript{2+}) resulting from substitution of nonbridging phosphate oxygen (NBPO) atoms of an RNA with sulfur (Schatz et al. 1991). We synthesized glm\textsubscript{S\textsuperscript{Ca}} analogs with random sulfur substitution of pro-Rp NBPOs by transcription, and separated active from inactive RNAs by electrophoresis following self-cleavage. Positions within glm\textsubscript{S\textsuperscript{Ca}} with deleterious substitutions were identified by comparing the relative abundance of active versus inactive RNA fragments after selective phosphorothioate cleavage with iodine. In our mapping analysis of glm\textsubscript{S\textsuperscript{Ca}}, which provided readout between residues G3 and G90, we observed strong interferences only at residues A40 and G41 (Fig. 7).
FIGURE 4. The glmS<sup>Ca</sup> ribozyme is highly Ca<sup>2+</sup>-selective. (A) glmS<sup>Ca</sup> activity in the presence of various metal ions. Autoradiogram of PAGE analysis of cleavage of a 5'<sup>32</sup>P-end-labeled deoxyribonucleotide by different ribozymes for 30 min. Reaction rates are indicated. (†) Cobalt hexamine (CoH); (‡) not determined (ND). (B) glmS<sup>Ca</sup> activity in different Ca<sup>2+</sup> concentrations (blue) and in the presence of an additional 5 mM Mg<sup>2+</sup> (red) or 5 mM Ba<sup>2+</sup> (brown). All reactions were in the presence of a background of 100 mM KCl. (C) Lineweaver–Burk representation of the reaction rates from B. Error bars represent standard errors of the mean of at least three independent experiments.

Previously, we identified functionally important divalent cations for glmS<sup>WT</sup> and glmS<sup>AAA</sup> by combining unbiased interference mapping and site-specific phosphorothioate substitution. We found that for both RNAs, the pro-R<sub>p</sub> NBPO of residue C2 is important in metal ion coordination. The best ordered structural metal ion discovered in multiple crystallographic analyses of the two ribozymes (Fig. 8A) is coordinated by this oxygen atom (Klein and Ferré-D’Amaré 2006; Cochrane et al. 2007, 2009; Klein et al. 2007a,b). This hexacoordinate cation, M<sub>A</sub>, is too far from the cleavage sites of either glmS<sup>WT</sup> or glmS<sup>AAA</sup> to participate directly in catalysis. Moreover, full activity of glmS<sup>WT</sup> in cobalt hexamine (Roth et al. 2006) strongly argues against any essential direct (i.e., inner-sphere) role for this, or any other divalent cation in folding or catalysis by the wild-type ribozyme (Cowan 1993).

Activity of glmS<sup>AAA</sup> was also dependent on metal ion coordination by the pro-R<sub>p</sub> NBPO of residue A38 and the pro-S<sub>p</sub> NBPO of residue C2 (Lau and Ferré-D’Amaré 2013). Crystallographic analysis confirmed the presence of a divalent cation bound at this position (M<sub>B</sub>). Multiple lines of evidence indicated that this cation participates directly in the catalytic mechanism of glmS<sup>AAA</sup> (but not glmS<sup>WT</sup>). In contrast, we did not observe any significant interference at A38 for glmS<sup>Ca</sup>, which implies that this ribozyme does not require M<sub>B</sub> for catalysis. The interference at residues A40 and G41 of glmS<sup>Ca</sup> is consistent with the presence of a metal ion (M<sub>C</sub>) observed in crystal structures of glmS<sup>WT</sup> and glmS<sup>AAA</sup> (Fig. 8B). M<sub>C</sub> is too far from the active site of glmS<sup>Ca</sup> to participate in catalysis, and thus probably plays a structural role in all three ribozymes.

If neither M<sub>B</sub> nor M<sub>C</sub> is involved in catalysis by glmS<sup>Ca</sup>, where is the essential metal ion cofactor? Examination of crystal structures suggests that by virtue of its novel cleavage site one nucleotide 3′ to the scissile phosphates of glmS<sup>WT</sup> and glmS<sup>AAA</sup>, glmS<sup>Ca</sup> may recruit M<sub>A</sub> for catalysis (Fig. 8B). We tested this by site-specific phosphorothioate substitution. Consistent with lack of involvement of M<sub>B</sub> in catalysis by glmS<sup>Ca</sup>, sulfur substitution of the pro-S<sub>p</sub> NBPO of C2 only reduced the maximal cleavage fraction to <~80% (Fig. 8C). In contrast, substitution of the pro-R<sub>p</sub> NBPO of C2, which coordinates M<sub>A</sub>, markedly impaired glmS<sup>Ca</sup> activity, with the maximum extent of cleavage reduced to <~9%. This impairment could not be relieved with Sr<sup>2+</sup>, which suggests a high degree of cation selectivity of the M<sub>A</sub> site in glmS<sup>Ca</sup> (it could also not be rescued with the thiophilic cation Cd<sup>2+</sup>, in which the ribozyme is inactive, not shown). Substitution of both NBPOs at G3 had no effect on activity, arguing against a nonlocal, nonspecific effect of sulfur substitution. Finally, we confirmed the importance of the structural cation M<sub>C</sub> suggested by our mapping results by synthesizing glmS<sup>Ca</sup> constructs containing a diastereomeric mixture of phosphorothioates at residues A40 and G41 (the internal position of the substitution precluded

| Small molecule | glmS<sup>WT</sup> in Mg<sup>2+</sup> (min<sup>−1</sup>) | glmS<sup>Ca</sup> in Mg<sup>2+</sup> (min<sup>−1</sup>) | glmS<sup>Ca</sup> in Ca<sup>2+</sup> (min<sup>−1</sup>) |
|----------------|---------------------------------|---------------------------------|---------------------------------|
| Glucosamine 6-phosphate | 34 ± 1.9<sup>a</sup> | Not detectable | 0.46 ± 0.01 |
| 3-azido-glucosamine | 0.08 ± 0.003 | Not detectable | 0.36 ± 0.05 |
| 6-azido-glucosamine | 0.4 ± 0.05 | Not detectable | 0.37 ± 0.01 |
| 2, 6-diaminoglucose | 0.5 ± 0.02 | Not detectable | 0.32 ± 0.04 |
| 2-deoxy-2-guanidinylglucose-6-phosphate | 0.6 ± 0.02 | Not detectable | 0.30 ± 0.02 |

<sup>a</sup>Rate reported previously (Lau and Ferré-D’Amaré 2013).
chromatographic resolution of the diastereomers). These had moderately impaired activity that could be partially relieved by Sr²⁺ (Fig. 8C). Overall, our analysis indicates that MA is essential for glmS³Ca activity, without precluding a role in facilitating overall ribozyme folding.

**DISCUSSION**

Ten point mutations distinguish glmS³Ca from the GlcN6P-dependent wild-type ribozyme (Figs. 1, 3). Two of these, U49A and G65A, are shared with glmS³AAA. Previous analyses of glmS³AAA demonstrated that while both adenosine mutations contribute to the coenzyme-independence of this ribozyme, the mutation at position 65 is the most important in abrogating the ability of the RNA to use GlcN6P as a general acid catalyst (Lau and Ferré-D’Amaré 2013). These mutations are therefore consistent with the GlcN6P-independence of glmS³Ca. Site-directed mutagenesis has previously demonstrated that the phylogenetically conserved guanine at position 40 is essential for the activity of both glmS³WT and glmS³AAA (Cochrane et al. 2007; Klein et al. 2007a; Lau and Ferré-D’Amaré 2013). Structural analysis implicated the purine base of this residue in orienting the nucleophile of the transesterification reaction (the 2’-OH of residue −1), and possibly in facilitating its deprotonation (Klein and Ferré-D’Amaré 2006; Cochrane et al. 2007; Klein et al. 2007a). Moreover, Raman crystallographic investigation of the ribozyme demonstrated that G40 contributes to lowering the pKₐ of the amine of GlcN6P, the general acid catalytic moiety (Gong et al. 2011). The presence of the G40A mutation in glmS³Ca thus explains why this ribozyme no longer cleaves its substrates between positions −1 and +1 (Fig. 6). Of the remaining seven mutations, A35U is particularly interesting, as this residue is located in close proximity to the MA site (Fig. 8), and therefore may be involved in endowing Ca²⁺ selectivity to glmS³Ca.

Since the ionic radii of Ca²⁺ and Mg²⁺ differ by only 28 pm (Table 2), definitive characterization of the exquisite cation selectivity glmS³Ca will require atomic-resolution structural analysis of the transition state of the ribozyme. However, none of the mutations that characterize glmS³Ca disrupt the secondary structure elements that define the unusually stable glmS ribozyme fold (Fig. 1B). Therefore, previously determined structures of the RNA can guide provisional interpretation of our results (Fig. 8A,B). Because glmS³Ca is itself overall structurally stable (indicated by the fact that it can adopt structures indistinguishable by SAXS in the presence of either Mg²⁺, Ca²⁺, or Sr²⁺) (Fig. 5), and because its structure is comparable in compactness to that of the wild-type and glmS³AAA, the cation selectivity of glmS³Ca likely results from a localized reorganization of the MA cation binding site. Our biochemical data are consistent with an enlargement of this site relative to that of the wild-type, such that it cannot achieve tight binding (at least in the transition state) of Mg²⁺ (ionic radius = 86 pm) while being optimal for Ca²⁺ (ionic radius = 114 pm), tolerant for the somewhat larger Sr²⁺ (ionic radius = 132 pm) and unable to accommodate the much larger Ba²⁺ (ionic radius = 149 pm; Table 2). This expansion of the MA site could be largely the result of the mutation of residue A35, which is immediately adjacent to this cation-binding site, to the smaller uracil (Fig. 8B). The inability of Sr²⁺ to activate a glmS³Ca ribozyme containing a sulfur substituent of the MA-coordinating pro-R,NBPO of C2, despite its ability to weakly support catalysis of the unmodified glmS³Ca (Fig. 4), could hint that replacement of the nonbridging phosphate oxygen with sulfur makes the MA site too small to accommodate the larger Sr²⁺ cation. In addition, the inability of Cd²⁺ to rescue the same phosphorothioate-containing RNA, despite an ionic radius (109 pm) comparable to

| Cation   | Ionic radius (pm) | Absolute hardness (η) | Coordination numbers |
|----------|-------------------|------------------------|---------------------|
| Mg²⁺    | 86                | 32.55                  | 4, 5, 6, 8          |
| Cd²⁺    | 109               | 10.29                  | 4, 5, 6, 7, 8, 12   |
| Ca²⁺    | 114               | 19.52                  | 6, 7, 8, 9, 10      |
| Sr²⁺    | 132               | 16.3                   | 6, 7, 8, 9, 10, 12  |
| Ba²⁺    | 149               | 12.8                   | 6, 7, 8, 9, 10, 11, 12 |

aRadii are for the hexacoordinate cations (Huheey 1983).
bAbsolute hardness values from Parr and Pearson (1983) and Pearson (1988).

**FIGURE 5.** SAXS analysis indicates that glmS³Ca adopts the wild-type fold in a variety of divalent cations. (A) Pair-probability functions of glmS³Ca in the presence of 2.5 mM of either Mg²⁺, Ca²⁺, or Sr²⁺ (red, blue, and black, respectively) in addition to 100 mM KCl. The radii of gyration of the RNA in the three divalent cations are indicated (mean ± standard error of the mean of thirty measurements). (B) Kratky plots of glmS³Ca color-coded as in A.
that of Ca\(^{2+}\) suggests that the remaining ligands of the MA site, or the substrate atoms with which MA interacts during catalysis, have a strong preference for hard cations (Table 2).

Compared to the glm\(_{\text{SAAA}}\) ribozyme from which it was selected, glm\(_{\text{CA}}\) has adopted a new catalytic strategy that relies on MA. In the wild-type ribozyme, this cation is structural, since it lies too far away from the cleavage site of that ribozyme (Klein and Ferré-D’Amaré 2006). Moreover, as glm\(_{\text{SWT}}\) is fully active in a variety of metal ions (including cobalt hexamine), its structural cation binding sites (e.g., MA) have limited selectivity (Roth et al. 2006; Klawuhn et al. 2010). Likewise, glm\(_{\text{AAA}}\) has only modest cation selectivity, and functions even in high concentrations of the monovalent cation Li\(^{+}\). Both crystallographic and phosphorothioate interference analyses indicate that MA retains a structural function in glm\(_{\text{CA}}\) (Lau and Ferré-D’Amaré 2013). The co-option of an erstwhile structural metal ion for catalysis (directly or indirectly) by glm\(_{\text{CA}}\) is an example of molecular exaptation, that is, the evolutionary repurposing of preexisting features for adaptation to new selective pressures (Gould and Vrba 1982). The evolution of glm\(_{\text{CA}}\) illustrates both the rugged fitness landscape of ribozymes, wherein a small number of mutations can have large effects on activity (Pitt and Ferré-D’Amaré 2010; Lau and Ferré-D’Amaré 2016b), and the exceptional structural stability of the triply pseudoknotted glm\(_{\text{S}}\) fold (Hampel and Tinsley 2006; Klein and Ferré-D’Amaré 2006; Tinsley et al. 2007; Lau and Ferré-D’Amaré 2013).

In addition to demonstrating that a self-cleaving ribozyme that requires a metal ion for activity can achieve remarkable specificity for Ca\(^{2+}\), glm\(_{\text{CA}}\) is a potentially useful biotechnological tool. Like in the case of the Ca\(^{2+}\)-dependent protein nuclease from Staphylococcus (Heins et al. 1967), RNA cleavage by glm\(_{\text{CA}}\) can be initiated in the presence of Ca\(^{2+}\), and then abruptly stopped by addition of the Ca\(^{2+}\)-specific chelator EGTA (Fig. 9). In addition, because this ribozyme is active in modest Ca\(^{2+}\) concentrations (∼1 mM), it may serve as the basis for new live-cell Ca\(^{2+}\) sensors whose output is site-specific RNA cleavage. Organelles such as the endoplasmic reticulum, mitochondria, and nucleus contain total Ca\(^{2+}\) levels of ∼0.01 to 1 mM. In stimulated tumor mast cells and hyperplastic prostate epithelial cells, this level can reach as high as 1.2 and 7 mM, respectively (Tvedt et al. 1987; Chandra et al. 1994). Such Ca\(^{2+}\) concentrations would provide strong and selective activation of glm\(_{\text{CA}}\) in vivo.

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**FIGURE 6.** Similar to glm\(_{\text{SWT}}\) and glm\(_{\text{SAAA}}\), glm\(_{\text{CA}}\) RNA cleavage proceeds by internal transesterification, but at a site 1 nt distal. (A) 5′ Cleavage product end-group analysis. 5′ 32P-radiolabeled substrate was cleaved in trans with the three ribozymes for 30 min and treated with acid or with phage T4 polynucleotide kinase prior to analysis by denaturing PAGE, as previously described (Winkler et al. 2004; Xiao et al. 2008; Lau and Ferré-D’Amaré 2013). (B) Mapping of glm\(_{\text{CA}}\) cleavage site. 5′ 32P-radiolabeled of RNA substrates of varying lengths with or without single-site 2′-deoxy substitutions were cleaved by the three ribozymes and analyzed by denaturing PAGE. (Hy.) Hydrolysis ladder.

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**FIGURE 7.** Strong phosphorothioate interferences in glm\(_{\text{CA}}\). Normalized interference values (Lau and Ferré-D’Amaré 2013) are shown. Only positions with interferences >1 are shown, and interferences >5 are plotted as having the value 5.0. Interferences from A\(\alpha\)S (adenosine phosphorothioate) and G\(\alpha\)S are in cyan and orange, respectively. Significant interferences were not observed from C\(\alpha\)S and U\(\alpha\)S. (Inset) Close-up of residues with strong interferences near the active site (res., residue numbers). Residues are colored green and gray in the plane of the secondary structure to represent the eight mutations in glm\(_{\text{CA}}\) relative to glm\(_{\text{SAAA}}\), and positions that were not addressed in the phosphorothioate mapping experiment, respectively.
MATERIALS AND METHODS

RNA pool synthesis and selection

A double-stranded mutagenized DNA pool was synthesized as described, with the exception of constant residues A49, A51, and A65 (Fig. 1; Lau and Ferré-D’Amaré 2013, 2016a). Approximately 100 nmol of DNA (∼6 × 10^15 unique sequences) were used to template transcription of 32P body-labeled RNA for the first selection round. Two parallel selections were performed with 15 nmol of RNA in incubation buffers containing 50 mM HEPES-KOH (pH 7.5), and either 10 mM MgCl2 (Pool A) or 10 mM CaCl2 (Pool B). Active ribozymes were reverse-transcribed as previously described (Lau and Ferré-D’Amaré 2013). In round 2, cDNAs from Pool B were further divided into two before the second PCR step, with one (Pool B) amplified using standard PCR and the other (Pool C) using mutagenic PCR (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 7 mM MgCl2, 0.5 mM MnCl2, 0.2 mM dATPs, 0.2 mM dGTPs, 1 mM dCTPs, 1 mM dTTPs) for 13 cycles (Cadwell and Joyce 1994). Starting with round 3, the divergent cation concentrations in the incubation buffers were reduced to 2 mM, and the 5′ primer was switched to 5′-TTCTAATACTCA CTCACTATAGGTGCACTTAAACTCTG ACCAATCTAAGGATGCCATCGTGACGCTGG ACTTTAACGGCCAGG-3′ (T7 promoter in italics) during the second PCR step to yield a different leader sequence. A more stringent selection scheme was used starting with round 5, in which RNA pools were 5′ labeled with 6-thioguanosine 5′-monophosphate (6SGMP) and selected using an [(N-acryloylaminophenyl)]mercuroic chloride (APM) gel mobility-shift assay (Igloi 1988; Lau and Ferré-D’Amaré 2013). A counter-selection step was also introduced starting with round 6 in which 6SGMP tagged RNAs were first incubated overnight in 50 mM HEPES, pH 7.5, with either 25 mM CaCl2, 25 mM MgCl2, or 25 mM MnCl2 for Pools A, B, and C, respectively. RNAs that did not self-cleave were recovered by electrophoresis through APM gels, and ribozymes specifically active in either Mg2+ or Ca2+ were selected using conditions described above.

Kinetics

Ribozyme rate measurements in different metal ion conditions were performed as previously described (Lau and Ferré-D’Amaré 2013). For determination of the inhibitory effects of Mg2+ and Ba2+ on glmS Ca activity, 5 mM MgCl2 or 5 mM BaCl2 was added to cleavage reactions in varying concentrations of CaCl2 (from 2 to 150 mM). Pseudo–first-order rates were determined as previously described (Lau and Ferré-D’Amaré 2013). All cleavage rate measurements were performed on a background of 100 mM KCl.

Small-angle X-ray scattering

SAXS experiments were performed at beamline 12-ID-C of The Advanced Photon Source at Argonne National Laboratories. Unimolecular glmS ribozyme constructs were used in this study, rather than the bimolecular RNAs used by Lau and Ferré-D’Amaré (2013). The constructs correspond to those in Figure 1A, except that their first nucleotide is residue +1. All cleavage rate measurements were performed on a background of 100 mM KCl.
Phosphorothioate interference mapping and NBPO substitution experiments

Phosphorothioate interference mapping was performed using 185-nt cis-cleaving glmS Ca⁺⁺ constructs, and interference values were calculated as previously described (Lau and Ferré-D’Amaré 2013). For individual NBPO substitution experiments, RNA oligos 5'-AGGCGCCU-3' and 5'-AGCCGUUAAGGCGAG-3' ([*] indicates position of diastereomeric phosphorothioate substitutions) were purchased from Dharmacon. Rp and Sp phosphorothioate isomers were resolved by reversed-phase HPLC (Frederiksen and Piccirilli 2009). Rates for NBPO substitutions at C2 or G3 were determined by incubation of HPLC-purified RNA oligos (Rp or Sp), respectively, with 126-nt glmS WT ribozyme truncated to start at position 42 (CAGGGU…CAGAA), with RNA oligo 5'-GGCCUUAGGUUGCAGG-AG-3' and 5'-GGCCUUAGGUUGCAGAAG-3', respectively. The ligated RNAs were purified by 8% denaturing PAGE. The ribozymes were incubated with 5'-32P-labeled RNA oligo 5'-AGGCGCCUGAGCUUAAGGCGAG-3' to determine the effects of A40 or G41 NBPO substitutions. The same RNA constructs and conditions were used for Sr2⁺ experiments, except 5 mM Ca2⁺ was replaced with 5 mM Sr2⁺.

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