Characteristics of the *glmS* ribozyme suggest only structural roles for divalent metal ions

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ABSTRACT

The *glmS* ribozyme is a riboswitch class that occurs in certain Gram-positive bacteria, where it resides within mRNAs encoding glucosamine 6-phosphate synthase. Members of this self-cleaving ribozyme class rapidly catalyze RNA transesterification upon binding GlcN6P, and genetic evidence suggests that this cleavage event is important for down-regulating GlmS protein expression. In this report, we present a refined secondary structure model of the *glmS* ribozyme and determine the importance of a conserved pseudoknot structure for optimal ribozyme function. Analyses of deletion constructs demonstrate that the pseudoknot, together with other structural elements, permits the ribozyme to achieve maximum rate constants for RNA cleavage at physiologically relevant Mg²⁺ concentrations. In addition, we show that substantial rate enhancements are supported by an exchange-inert cobalt (III) complex and by molar concentrations of monovalent ions. Our findings indicate that the *glmS* ribozyme forms a complex structure to employ catalytic strategies that do not require the direct participation of divalent metal ions.

Keywords: ribozyme; riboswitch; *glmS*; glucosamine 6-phosphate; metal ions; pseudoknot

INTRODUCTION

Riboswitches are structured RNA elements that most commonly reside within 5' untranslated regions (UTRs) of bacterial mRNAs, where they control gene expression through direct binding interactions with small molecule ligands. A number of these RNA-based regulatory motifs have been identified that respond to compounds with fundamental roles in metabolism, including the amino acids lysine and glycine, the nucleobases guanine and adenine, and the coenzymes adenosylcobalamin, thiamine pyrophosphate, flavin mononucleotide, and S-adenosylmethionine (Mandal and Breaker 2004; Winkler 2005). Recently, we reported the discovery of a novel riboswitch class (Winkler et al. 2004) that represses gene expression in response to rising concentrations of GlcN6P, a sugar-phosphate compound that is a fundamental metabolic intermediate in the biosynthesis of bacterial cell walls.

Riboswitches typically make use of two distinct functional domains to modulate gene expression in response to changing concentrations of their cognate metabolites. An aptamer domain mediates selective binding of the target compound, and structural changes brought about by binding induce an adjoining expression platform to exert control over gene expression (Soukup and Soukup 2004). The aptamer domain of a riboswitch is most easily identified because its role as a metabolite receptor demands a precise structural organization that is well conserved phylogenetically. In contrast, an individual aptamer class can be associated with different expression platforms that can vary widely in sequence and structure, but still control gene expression by one of several different mechanisms. Among bacteria, riboswitches most commonly exert gene control at the level of transcription termination or translation initiation, although the modular construction of riboswitches allows them to be adapted for participation in a variety of control mechanisms. The majority of riboswitches appear to function as allosteric RNA switches, wherein ligand binding to the aptamer domain causes structural and functional changes to occur distally within the expression platform.

The *glmS* riboswitch is unique because it controls gene expression by undergoing ligand-induced self-cleavage. Examples of self-cleaving *glmS* ribozymes are present in the 5' UTRs of *glmS* mRNAs in *Bacillus subtilis* and numerous other Gram-

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positive bacteria (Winkler et al. 2004). At high concentrations of GlcN6P (the metabolite product of the *glmS* protein), the rate constant for self-cleavage by the ribozyme is accelerated at least 10-million-fold over the uncatalyzed rate constant for RNA transesterification (Li and Breaker 1999). Experiments demonstrating a negative correlation between the levels of *glmS* RNA-driven reporter gene expression in vivo and ribozyme activity in vitro have established the *glmS* ribozyme as a type of riboswitch whose mechanism of gene regulation has an autocatalytic component (Winkler et al. 2004).

Despite its unique metabolite dependence, the *glmS* ribozyme has several characteristics in common with four other natural classes of self-cleaving RNAs. The hammerhead, hairpin, hepatitis delta virus (HDV), and *Neurospora* Varkud satellite (VS) ribozymes each employ an internal phosphoester transfer mechanism for RNA scission, in which a 2'-hydroxyl group at the cleavage site is activated for nucleophilic attack on the adjacent phosphorus center (Fedor and Williamson 2005). The *glmS* ribozyme uses an identical phosphoester transfer mechanism to yield products with 5' hydroxyl and 2',3'-cyclic phosphate termini (Winkler et al. 2004). Another feature the *glmS* ribozyme shares with some of the previously identified self-cleaving RNAs is relaxed metal ion specificity (Dahm and Uhlenbeck 1991; Chowrira et al. 1993; Collins and Olive 1993; Suh et al. 1993), as a variety of divalent metal ions can substitute for Mg^{2+} in promoting GlcN6P-dependent self-cleavage (Winkler et al. 2004). Interestingly, several of the small RNA enzymes initially reported to exhibit relaxed metal ion specificities have subsequently been shown to employ catalytic strategies that do not require the direct participation of divalent metal ions (Hampel and Cowan 1997; Nesbitt et al. 1997; Young et al. 1997; Murray et al. 1998), raising the prospect that the *glmS* ribozyme might be similarly devoid of an absolute requirement for divalent metals.

Approximately one-half of the *glmS* ribozyme secondary structure, composed of several phylogenetically conserved stems at the 3' end (the P3/P4 domain), may be deleted without abrogating GlcN6P-responsive cleavage activity in vitro (Winkler et al. 2004). Similarly, certain small nucleolytic ribozymes have been reported to contain functionally or structurally conserved domains that are formally dispensable for catalysis, but that nonetheless are required for stabilization of the respective active structures under physiological conditions (Lilley 2003). These parallel observations prompt the question of whether the P3/P4 domain of *glmS* RNA might be serving an analogous purpose.

In this study, we investigate in greater detail the metal ion requirements of the *glmS* ribozyme. In addition, we demonstrate the existence of a phylogenetically conserved pseudoknot and evaluate whether the P3/P4 subdomain in which it resides may be functionally analogous to the stabilizing domains identified in other nucleolytic ribozymes.

RESULTS

Identification of an additional representative of the *glmS* motif

Based on a phylogeny containing 19 sequences from mostly Gram-positive bacteria, a revised secondary structure model was assigned for the *glmS* ribozyme (Fig. 1). The modified consensus structure of this self-cleaving RNA contains a newly identified pairing element (P3a) in addition to the four stem-loop structures proposed in Barrick et al. (2004) (Fig. 2). Two of the stem-loop structures (P2 and P4) carry conserved sequences within internal loops, while most of the remaining primary sequence conservation occurs in the region between the cleavage site and P1, in the P1–P2 joining sequence (J1/2), and in loop 4 (L4). The 18 original members of the *glmS* ribozyme phylogeny were identified using both the BLISS database (Barrick et al. 2004), which assesses sequence similarity among intergenic regions from a collection of microbial genomes, and the SEQUENCESNIFFER program (J. Barrick and R.R. Breaker), which is similar to RNAMotif (Macke et al. 2001) and searches for specific motifs according to defined sequence and secondary structure parameters.

In the course of examining an expanded version of the BLISS database (Corbino et al. 2005), we encountered an additional *glmS* RNA motif not noted in the earlier report. Interestingly, this sequence was identified not in a species of the Firmicutes (Bacillus/Clostridium group), where the majority of *glmS* motifs have been identified, but rather in association with the *glmS* gene from *Deinococcus radiodurans*, a species of the Deinococcus/Thermus group (Fig. 1). As a result, this newly identified representative expands the occurrence of the *glmS* motif to three bacterial lineages, including the previous report of one such motif in Fusobacteria (*Fusobacterium nucleatum*) (Barrick et al. 2004).

While the *glmS* element from *D. radiodurans* largely conforms to the proposed secondary structure model, there are some subtle departures from the consensus. Among the predicted stems of the *glmS* structural model, the strands depicted as comprising the P2a stem (blue highlights [interior pairing] in Fig. 1) are highly conserved in primary sequence and exhibit by far the least amount of covariation. The sequence of the *D. radiodurans* element in this region, although it has been threaded as shown to conform to this base-pairing arrangement, could also be slightly adjusted in register to align more closely with the conserved primary sequence in this region, but this would come at a significant cost to the potential for the depicted Watson-Crick pairing arrangement. Thus, while the current rendering depicts sequences in this local area as a stem, there is the possibility that the proposed P2a stem sequences adopt an alternative structure, perhaps even one devoid of canonical base-pairing interactions.

Another deviation from the consensus exhibited by the *D. radiodurans* *glmS* motif is the loss of an otherwise absolutely

FIGURE 2. Consensus secondary structure for *glmS* ribozymes based on the revised sequence alignments in Figure 1. Nucleotides in red and in black are conserved in at least ~95% (18/19) and ~80% (15/19) of the representatives, respectively. Lines represent nonconserved sequences of variable length.

conserved adenosine triplet in the P4 stem, together with the apparent redistribution of unpaired sequences within this stem, resulting in a bulge and asymmetric internal loop. In addition, the nucleotides corresponding to L4 in the *D. radiodurans* *glmS* RNA fail to conform to the putative GNRA tetraloop sequence conserved in all other members of the phylogeny. Despite these deviations from the consensus, RNAs corresponding to the *glmS* element from *D. radiodurans* exhibit accelerated rate constants for site-specific self-cleavage in response to GlcN6P (data not shown). Moreover, the ability of this newly identified *glmS* ribozyme species to discriminate against compounds related to GlcN6P is similar to that of representatives adhering more closely to the consensus.

Expanding the conserved structure of the *glmS* ribozyme

Upon further inspection of the sequence representatives, we have identified a phylogenetically conserved base-pairing arrangement (P3a) that forms a pseudoknot between nucleotides within L3 and nucleotides 3' to the P4 stem (Figs. 1, 2). The pseudoknot ranges from 3 to 7 bp, and almost all of the nucleotides contained within L3 contribute to formation of the pseudoknot in most of the representatives examined. The number of L3 residues lacking a base-pairing partner appears typically to be limited to one or two, although sequences from two organisms (*B. subtilis* and *D. radiodurans*) suggest that additional nonduplex nucleotides may be accommodated within this loop. The length of the linker sequence between the P4 and the P3a stems (J4/3a) appears to be narrowly constrained to between 1 and 4 nt.

The *glmS* ribozymes from *B. subtilis* and *B. cereus* display similar kinetic profiles

We expect that each *glmS* ribozyme representative will exhibit similar biochemical and kinetic characteristics in order to

ensure comparable responses to changing intracellular concentrations of GlcN6P. To examine this assumption, we chose to establish the performance characteristics of the *glmS* ribozyme from *Bacillus cereus*, in part because this representative appears to have the lowest number of nonessential nucleotides residing within L1, L3, and the P4 internal bulge (Fig. 1). This reduced length might be advantageous for those who pursue future structural studies on *glmS* ribozymes.

A 5' 32 P-labeled *B. cereus* 201 *glmS* RNA (corresponding to nucleotides –159 to +41 relative to the translation start site), whose predicted secondary structure is shown in Figure 3A, undergoes rapid self-cleavage in the presence of elevated concentrations of GlcN6P. The response to GlcN6P over a wide range of concentrations is similar to the profile observed previously with the *glmS* ribozyme from *B. subtilis* (Fig. 3B). Ribozymes from both organisms exhibit a nearly log-linear dependence on effector concentration over ~2 orders of magnitude and attain half-maximum rate enhancements of ~1 min^{–1} in the presence of 200 μ M GlcN6P.

Under standard assay conditions, the *B. subtilis* and *B. cereus* *glmS* ribozymes undergo self-cleavage in the absence of effector with rates of between 0.001 min^{–1} and 0.01 min^{–1} (corresponding to the minimum k_{obs} values in Fig. 3B). While these rates are 2–3 orders of magnitude slower than those measured in the presence of saturating concentrations of GlcN6P, they nevertheless represent enhancements of as much as 10⁵-fold over the uncatalyzed rate of RNA strand cleavage. During the course of this study, we noticed (particularly when GlcN6P was absent) that *glmS* ribozyme incubations in solutions buffered with Tris-HCl (the chloride salt of protonated tris-(hydroxymethyl)-aminomethane) resulted in significantly more cleavage activity than in similar solutions containing other buffers. A series of reactions containing identical concentrations of HEPES (N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid) and varying concentrations of Tris-HCl demonstrates this stimulatory effect (Fig. 3B, inset). The effect of Tris-HCl appears to be exerted through structural mimicry of the amine-containing portion of GlcN6P (McCarthy et al. 2005). Since the *glmS* ribozyme recognizes GlcN6P in a highly specific manner, its affinity for the chemically dissimilar Tris compound is likely to be quite poor. However, the presence of the Tris-HCl at millimolar concentrations presumably permits substantive binding of the buffer in the binding site for GlcN6P, where it acts as a functional substitute for the natural ligand.

Because assays monitoring the effect of GlcN6P concentration on cleavage activity were buffered with Tris-HCl, rate measurements taken in the lower range of effector concentrations are likely to be artificially elevated. In order to learn the actual extent of effector-induced ribozyme activation, similar assays were repeated in solutions buffered with HEPES. A plot obtained with *B. cereus* 201 *glmS* under these modified conditions displays a similar maximum k_{obs} . As expected, the minimum k_{obs} value dropped to as low as 10^{–5} min^{–1}, indicating that saturating

FIGURE 3. Biochemical analyses of *glmS* ribozymes from *B. subtilis* and *B. cereus*. (A) Secondary structure model of *B. cereus* 201 *glmS* RNA. The G residue marked with an asterisk was introduced to improve the yield of transcription in vitro. (B) Effect of GlcN6P concentration on the rate of self-cleavage of the *glmS* ribozyme from *B. cereus* (filled circles). Data corresponding to the 246 *glmS* ribozyme from *B. subtilis* are shown for comparison (open circles). (Inset) Effect of Tris-HCl buffer on the rate of self-cleavage in the absence of GlcN6P. 5' ³²P-labeled *B. cereus* 201 *glmS* RNA was incubated for 4 h in reactions buffered to pH 7.5 with various concentrations of HEPES or Tris as indicated in mM. NR designates no reaction. Precursor and 5' cleavage product bands are indicated. (C,D) Effects of MgCl₂ concentration (c) and pH on rate constants under otherwise standard assay conditions. Reactions from pH 7.0–9.0 were buffered with Tris, and those below pH 7.0 were buffered with 2-[N-morpholino]ethanesulfonic acid (MES). Data corresponding to *B. cereus* and *B. subtilis* *glmS* ribozymes are represented as in B.

concentrations of GlcN6P stimulate the activity of this ribozyme by ~100,000-fold (data not shown).

Although the actual dynamic range of the *glmS* ribozyme in vitro is much greater than that suggested previously (Winkler et al. 2004), it is unlikely that the full range of this effector-dependent response would be exploited in vivo. It seems doubtful whether the intracellular concentration of a critical metabolite like GlcN6P would fluctuate to such an extent, as it is presumably required to be maintained at relatively high concentrations due to its role in cell wall biosynthesis and remodeling. Moreover, because of the rapid turnover of bacterial mRNAs (Kushner 2002), the extended half-lives in vitro of uncleaved RNAs at low GlcN6P concentrations would not be biologically relevant.

We also compared the effects of additional biochemical parameters on cleavage kinetics. Similarly to the *B. subtilis* variant, the *B. cereus* 201 *glmS* ribozyme exhibits a strong dependence on the concentration of Mg²⁺ under standard

assay conditions, approaching a rate maximum near physiological concentrations of this metal ion (Fig. 3C). The *B. cereus* ribozyme also behaves similarly in its response to pH, displaying a log-linear rate enhancement with increasing basicity that becomes maximal near neutral pH (Fig. 3D). Collectively, these data demonstrate that the *glmS* ribozyme from *B. cereus* behaves similarly to the ribozyme from *B. subtilis*, as might be expected for genetic switches that perform identical roles in different organisms. Furthermore, these findings suggest that the fundamental characteristics of one representative might apply equally to other members of this ribozyme class.

Metal dependence of the *glmS* ribozyme from *B. cereus*

It was determined previously that a number of different divalent metal ions could support self-cleavage of the *glmS* ribozyme (Winkler et al. 2004). To establish quantitatively the degree to which certain other divalent cations could substitute for Mg²⁺, we examined the cleavage rates of *B. cereus* 201 *glmS* over a range of Mn²⁺ and Ca²⁺ concentrations. The effects on the rate of increasing concentrations of each of these metal ions are quite similar to those observed with a series of Mg²⁺ concentrations, resulting in a sharply ascending profile that approaches a plateau in the low millimolar range (Fig. 4A). The ability of

other divalent metal ions functionally to replace Mg²⁺ indicates a certain amount of promiscuity in the specificity of the corresponding binding sites. This is a characteristic that is shared by several of the other small self-cleaving ribozymes (Dahm and Uhlenbeck 1991; Chowrira et al. 1993; Collins and Olive 1993; Suh et al. 1993).

The relaxed divalent metal ion specificity observed in other catalytic RNAs may be readily explained in light of several recent reports that certain small catalytic RNAs, including the hairpin, the hammerhead, and the VS ribozymes, do not depend strictly on direct interactions with divalent metals but instead require only the presence of dense positive charge in the form of transition metal complexes, monovalent ions, or even organic polycations (Fedor 2002). To begin assessing whether a mechanism independent of divalent metals might also apply in the case of the *glmS* ribozyme, we tested the ability of Co(NH₃)₆³⁺, a structural mimic of hexahydrated Mg²⁺, to

FIGURE 4. Cation requirements for self-cleavage of the *B. cereus* 201 *glmS* ribozyme. (A) Effect on the rate constant of substituting Mg^{2+} with varying concentrations of Mn^{2+} or Ca^{2+} . (B) Effect on the rate constant of varying concentrations of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$. (C) Cleavage activity in high concentrations of KCl. Reactions in B and in C were performed in the absence of added divalent metal ions and supplemented with EDTA at concentrations of 2.5 mM and 25 mM, respectively.

functionally replace divalent metal ions. Because Co^{3+} is inert, exchange of the inner sphere amine ligands is slow relative to the timescale of the cleavage reaction, resulting in a mode of association of $\text{Co}(\text{NH}_3)_6^{3+}$ with RNA that can be mediated only through electrostatic and outer sphere interactions (Jou and Cowan 1991; Cowan 1993). In *B. cereus* 201 *glmS* cleavage reactions supplemented with 2.5 mM EDTA, the presence of $\text{Co}(\text{NH}_3)_6^{3+}$ at a minimum concentration of ~ 1 mM supports rate accelerations that are only modestly diminished relative to the enhancements obtained in the presence of saturating Mg^{2+} levels (Fig. 4B). The observation that this kinetically stable transition metal complex permits elevated rates of self-cleavage suggests that the *glmS* ribozyme lacks a major catalytic or structural requirement for inner sphere divalent metal ion coordination. The relatively low concentrations of $\text{Co}(\text{NH}_3)_6^{3+}$ required for robust activity, combined with the superstoichiometric amounts of EDTA in this range, argue against the participation of contaminating divalent metal ions.

We examined the effects of high monovalent ion concentrations to determine whether cationic species other than hydrated divalent metal ions or their structural mimics are capable of sustaining *glmS* ribozyme activity. To minimize the potential influence of contaminating levels of divalent metal ions, we included EDTA in these assays at a concentration of 25 mM. In the presence of molar concentrations of KCl (Fig. 4C), *B. cereus* 201 *glmS* approaches enhancements of $\sim 10^5$ -fold over the uncatalyzed rate of

RNA transesterification (Li and Breaker 1999), although these values are lower by ~ 2 orders of magnitude compared with the maximum k_{obs} in the presence of saturating divalent metal ions. The rate accelerations supported by molar concentrations of monovalent ions were unaffected when EDTA concentrations were increased to 100 mM (data not shown), arguing against the possibility of divalent metal effects resulting from reduced chelation efficiency under high ionic strength conditions. The ability of monovalent ions at high concentrations to support substantial rate accelerations provides additional evidence that inner sphere metal ion coordination is not essential. Furthermore, our observations suggest the lack of a critical role for a divalent metal ion in any capacity in the active site, whether fully hydrated or chelated directly to the RNA.

The self-cleavage rates supported by monovalent ions alone are not accelerated optimally, however, and in this respect the *glmS* ribozyme differs slightly from the hairpin and VS ribozymes, whose rate enhancements in molar concentrations of monovalent ions equal or exceed those achieved in the presence of divalent metal ions (Murray et al. 1998). Rather, the *glmS* RNA behaves more similarly to the hammerhead ribozyme in this regard, which performs at rates reduced by ~ 30 -fold when high concentrations of monovalent ions are provided as a substitute for Mg^{2+} (Murray et al. 1998; Curtis and Bartel 2001; O'Rear et al. 2001). The relatively modest effect of Mg^{2+} on *glmS* ribozyme rate enhancement also invites a comparison with the HDV ribozyme, in which the divalent metal ion contribution to the catalytic step is ~ 25 -fold (Nakano et al. 2001).

These observations, together with the suboptimal rate accelerations supported by $\text{Co}(\text{NH}_3)_6^{3+}$, suggest that not all roles of divalent metal ions in the *glmS* ribozyme cleavage mechanism may be performed equally by other cationic species. Perhaps the lower effectiveness of high concentrations of KCl compared with saturating levels of Mg^{2+} or $\text{Co}(\text{NH}_3)_6^{3+}$ could result in part from less efficient structure stabilization due to the lower charge density of monovalent ions (Woodson 2005). Regardless, although divalent metal ions are critically important under conditions of physiological ionic strength, they appear not to be essential for *glmS* ribozyme catalysis. Instead, they are likely to fulfill roles such as electrostatic screening that may be compensated in large part by other cationic species. In summary, these data indicate that the *glmS* ribozyme is mechanistically similar to the majority of other self-cleaving ribozymes, at least with regard to the absence of an indispensable catalytic or structural role for a divalent metal ion.

Cleavage of phosphorothioate-modified substrate RNA by the *glmS* ribozyme

The promotion of substantial *glmS* ribozyme function by an exchange-inert metal complex as well as by high monovalent ion concentrations suggested that any catalytic strategy involv-

ing direct coordination of a divalent metal ion to the RNA is likely to be minor. Accordingly, catalytic strategies that utilize divalent metal ions as Lewis acids to facilitate deprotonation or to stabilize negative charge are not expected to be used by *glmS* ribozymes. To begin to assess this hypothesis, we examined the effect of thiophosphate substitutions at nonbridging phosphate oxygen atoms (NBPOs) of the cleavage site. Any effects on the rate constant for RNA cleavage resulting from these substitutions could be interpreted to reflect the reduced affinity for sulfur of hard metal ions (e.g., Mg^{2+}), especially if the observed effects are rescued in the presence of a soft metal ion (e.g., Mn^{2+}), which coordinates oxygen and sulfur ligands with similar affinities (Jaffe and Cohn 1979; Pecoraro et al. 1984).

Given our previous findings suggesting the absence of a major role for divalent metal ions in *glmS* ribozyme catalysis, we anticipated that any neutralization of developing negative charge that might occur at NBPOs would not involve direct metal ion coordination. Accordingly, we expected sulfur substitutions at these positions to have negligible effects on ribozyme function. To test this prediction, we first reconfigured the *glmS* ribozyme to form a bimolecular complex by splitting the *B. cereus* construct at L1, removing unpaired nucleotides in this region, and extending the P1 stem by 2 bp to strengthen the association of the resulting RNA fragments (Fig. 5A). The ability of the *B. subtilis glmS* ribozyme to function in this format has been demonstrated and is consistent with the lack of phylogenetic conservation among L1 sequences (Barrick et al. 2004).

Using chemical synthesis, two RNA “substrate” fragments were prepared that were identical in sequence but that contained at the cleavage site either a standard phosphate or a phosphate substituted with sulfur at one of the nonbridging positions. The modified substrate was synthesized as a diastereomeric mixture, with *Rp* and *Sp* sulfur substitutions expected to be present in approximately equal proportions (Burgers and Eckstein 1979). Such diastereomeric mixtures of phosphorothioate linkages at ribozyme cleavage sites have been used previously to establish whether various ribozymes and deoxyribozymes are sensitive to sulfur substitution at either NBPO (Breaker et al. 2003). For example, the hammerhead ribozyme is strongly inhibited by the *Rp* sulfur substitution and expectedly was found to process a diastereomeric substrate to ~50% completion in the presence of Mg^{2+} , but to process nearly 100% of the mixed substrate when incubated with Mn^{2+} . In contrast, other ribozymes and deoxyribozymes that do not exhibit a thio effect process mixed substrates nearly to completion with either Mg^{2+} or Mn^{2+} as the cofactor.

Under single-turnover conditions in reactions containing 10 mM Mg^{2+} , the rate constant of *glmS* ribozyme activity with the mixed thiophosphate substrate was approximately one-third of the rate constant with the unmodified substrate (Fig. 5B, left). Reactions performed in the same concentration of the more thiophilic Mn^{2+} ion displayed inhibition of mixed thiophosphate RNA cleavage to the same relative extent (Fig. 5B, right). The failure

FIGURE 5. Absence of phosphorothioate effects at cleavage site non-bridging phosphate oxygens (NBPOs). (A) Secondary structure model of the bimolecular *glmS* ribozyme construct (based on *B. cereus*) used to assess effects of phosphorothioate modification at the cleavage site. (B) Time courses of ribozyme cleavage of unmodified (P-O, filled circles) and phosphorothioate-modified (P-S, open circles) substrates in the presence of 10 mM Mg^{2+} or 10 mM Mn^{2+} , respectively. Assays were performed in 2 mM GlcN6P under single turnover conditions. Values were corrected for the fraction of substrate remaining uncleaved after exhaustive incubation. Approximate rate constants for initial phases of reactions in Mg^{2+} are 0.68 min^{-1} (P-O) and 0.20 min^{-1} (P-S); the corresponding values in Mn^{2+} are 0.40 min^{-1} (P-O) and 0.15 min^{-1} (P-S). (C) Extended time courses of ribozyme cleavage of phosphorothioate-modified substrate molecules in the presence of Mg^{2+} or Mn^{2+} as indicated. Experimental conditions were as described in B, except that values were not corrected for the fraction of substrate remaining uncleaved after exhaustive incubation.

of Mn^{2+} to rescue the observed inhibitory effect of the sulfur substitutions suggests the limited importance of divalent metal ion coordination to a single cleavage-site NBPO in *glmS* ribozyme catalysis. Alterations in the geometry or charge distribution of the sulfur-substituted linkage might contribute to the modest rate reduction through

adverse effects on active site conformation (Derrick et al. 2000). Alternatively, a specific interaction with an NBPO might be mediated by a functional group other than a metal ion.

Importantly, the yield of products resulting from thiophosphate-containing precursors substantially exceeds 50% at later time points in buffers containing Mg^{2+} as well as in ones containing Mn^{2+} (Fig. 5C). This argues against a profound impairment of catalysis resulting from the loss of a divalent metal contact with a specific thiophosphate isomer, since a catalytic requirement for such an interaction would result in the processing of only $\sim 50\%$ of the mixed thiophosphate substrate in buffer containing Mg^{2+} . This finding is consistent with our hypothesis that divalent metal ion coordination to a cleavage-site NBPO is not important for this ribozyme class.

The results obtained with phosphorothioate-modified substrates, together with the substantial rate enhancements observed in the absence of divalent metal ions, imply limited direct contributions of divalent metal ions to the chemical mechanism of the *glmS* ribozyme. The apparent lack of stringent divalent metal ion requirements may also be explained, however, by invoking a slow conformational rearrangement as the rate-limiting step, so that any large detrimental effects on chemical mechanism resulting from the absence of divalent metals would be masked. This scenario would require that the ribozyme catalyze the chemical step with a rate constant greatly exceeding the maximum k_{obs} value determined in our experiments. It is notable that the maximum rate constant for *glmS* ribozyme cleavage under optimized buffer conditions precisely matches that proposed for enzymes that fully employ two catalytic strategies for RNA cleavage: deprotonation of the nucleophilic 2'-hydroxyl group and establishment of a geometry for the linkage that is optimal for "in-line" nucleophilic attack (Breaker et al. 2003; Emilsson et al. 2003). Both the maximum rate constants and the kinetic characteristics of *glmS* ribozymes from *B. subtilis* and *B. cereus* suggest that this ribozyme class has reached a speed limit that is common for RNA-cleaving ribozymes and deoxyribozymes that have been created by using in vitro evolution.

Function of the P3/P4 domain

It was previously determined that a truncated *glmS* ribozyme construct delimited at the 3' end by the P2 element retains GlcN6P-dependent self-processing activity, albeit with some reduction in speed relative to the full length ribozyme (Winkler et al. 2004). The negative impact on ribozyme performance upon removal of

the P3/P4 domain was not unexpected, given the strong phylogenetic conservation of this substructure.

To define more precisely the contribution of the P3/P4 domain to *glmS* ribozyme catalysis, we examined the kinetic characteristics of a series of 3'-end deletion constructs based on the ribozyme sequence from *B. subtilis* (Fig. 6A). We reasoned that two aspects of ribozyme function in which the P3/P4 domain might be likely to participate are stabilization of the active conformation and recognition of the GlcN6P effector. To explore the former possibility, we monitored the rates of effector-induced self-cleavage of the *glmS* RNA deletion series in varying concentrations of Mg^{2+} (Fig. 6B). The truncated RNA (P2 term), whose 3' terminus immediately follows the P2 secondary structure element, exhibits a marked difference in Mg^{2+} -dependent activity when compared with a longer RNA (P3a term) that contains the entire P3/P4 domain including the P3a pseudoknot. Specifically, P2 term exhibits a rate constant for self-cleavage in 1 mM Mg^{2+} that is ~ 4 orders of magnitude lower than that of P3a term under identical conditions. The rate constants for these variant RNAs converge at higher Mg^{2+} concentrations, however, such that the rate of the P2 term ribozyme variant is within 10-fold of the rate of the full-length RNA in reactions containing 100 mM Mg^{2+} . These findings indicate that the P3/P4 domain permits optimal function of the *glmS* ribozyme at Mg^{2+} concentrations in the physiological range. Moreover, it appears that the deleterious effects resulting from the absence of this domain can be largely overcome by including high concentrations of Mg^{2+} in the reaction.

We explored the specific contribution of the P3a pseudoknot to ribozyme function by examining the activity of a

FIGURE 6. Altered Mg^{2+} dependence of *glmS* ribozyme constructs with 3'-end truncations. (A) Secondary structure model of *B. subtilis* 246 *glmS* RNA. Circled nucleotides correspond to the 3' termini of individual truncated RNAs, whose names (P2 term, P4 term, P3a term) refer to the 3'-most stem element completely contained within the respective construct. Two additional engineered *glmS* ribozyme variants (each terminating at C183) individually disrupt and restore base-pairing in the P3a pseudoknot (boxed). Bracketed symbols are used on the plot in B to represent the Mg^{2+} dependence of the corresponding variants. Arrowhead indicates the cleavage site. The G residues marked by asterisks were introduced to facilitate transcription in vitro. (B) Effect of Mg^{2+} concentration on the cleavage rates of the *glmS* ribozyme variants described in A.

deletion variant that lacks only this element (P4 term) and by examining the activities of two variants of P3a term that carry mutations in the pseudoknot (P3a disrupt and P3a restore) (Fig. 6A). The plot of the observed rate constants for P4 term largely overlaps that for P3a term over much of the Mg^{2+} concentration range (Fig. 6B). The effects of this truncation are manifested only in lower Mg^{2+} concentrations, with P4 term RNA exhibiting a rate constant that is slightly >1 order of magnitude lower than that of the full-length ribozyme in 1 mM Mg^{2+} . To confirm that this rate decrease in lower divalent metal ion concentrations results from the loss of the P3a base-pairing interaction, we constructed a mutant (P3a disrupt) in which the potential for the pseudoknot interaction is significantly attenuated. As expected, the P3a disrupt RNA exhibits a Mg^{2+} -dependence profile similar to the one observed for P4 term. Moreover, a mutant containing compensatory changes that restore the proposed pseudoknot interaction responds similarly to the wild-type ribozyme (P3a term) over the full range of Mg^{2+} concentrations. These findings support the existence of the P3a pseudoknot and, furthermore, demonstrate that the integrity of this element is necessary to achieve optimal rate enhancement at Mg^{2+} levels in the physiological range. It is apparent, however, that function of the P3/P4 domain is not entirely contingent upon pseudoknot formation, since considerable rate accelerations are conferred by the P3/P4 domain even when the P3a pseudoknot interaction is disallowed. This analysis of pseudoknot function is consistent with a previous report on the contribution of this element to *glmS* ribozyme self-cleavage (Wilkinson and Been 2005).

We also considered the possibility that the P3/P4 domain might contribute simultaneously to GlcN6P recognition. It was anticipated that any such effects mediated by the P3/P4 domain would likely be minor, since a truncated RNA lacking this domain was capable of attaining nearly wild-type rate enhancements without requiring an increase in the GlcN6P concentration beyond 200 μM (Fig. 6B). Two different ribozyme constructs lacking the P3/P4 domain, based on sequences from different organisms, were assayed over a range of GlcN6P concentrations. To facilitate rate constant determinations, the reaction kinetics of these truncated ribozymes were measured in buffers containing 100 mM Mg^{2+} .

Both the P2 term *glmS* RNA derived from *B. subtilis* (Fig. 6A) and an analogous construct derived from *B. cereus* exhibited kinetic profiles that are nearly identical, with both attaining half-maximum rate enhancements in $\sim 200 \mu\text{M}$ GlcN6P (Fig. 7A). The plots corresponding to the truncated ribozymes are highly similar to those of their full-length counterparts, which also achieve half-maximum rate acceleration in $\sim 200 \mu\text{M}$ GlcN6P. As observed previously, the presence of 100 mM Mg^{2+} was not sufficient to compensate completely for the missing P3/P4 domain, resulting in rate constants for the truncated RNAs that were uniformly reduced by approximately fivefold com-

FIGURE 7. Effector-dependent activation of truncated *glmS* ribozymes. (A) GlcN6P concentration-dependent responses of minimal *glmS* ribozymes (terminating after the P2 stem) are similar to that of a full-length ribozyme. Data were obtained using the *B. subtilis* P2 term (Fig. 6A) (C76 3' terminus, open circles) and a *B. cereus* 201 *glmS*-derived variant terminating at G68 (filled circles). Data corresponding to the full-length *B. subtilis* 246 *glmS* ribozyme are shown for comparison (filled squares). Cleavage reactions included 100 mM MgCl_2 . (B) Effector selectivity of a minimal *glmS* ribozyme. An internally ^{32}P -labeled P2 term ribozyme from *B. subtilis* was incubated in the absence (—) or the presence of 200 μM GlcN6P or related compound, as indicated. Reactions contained 100 mM MgCl_2 and were halted after 5 min at room temperature. Precursor (Pre) and the 5' (5' Clv) and 3' (3' Clv) cleavage products are indicated. NR represents no reaction. Compounds are abbreviated as follows: GlcN, glucosamine; GlcN6S, glucosamine 6-sulfate; Glc1P, glucose 1-phosphate; Glc6P, glucose 6-phosphate; Glc, glucose.

pared with full-length ribozyme rate constants under standard conditions.

The similarities in the responses to varying GlcN6P concentrations of the truncated and full-length RNAs suggest that the P3/P4 domain does not participate directly in ligand-RNA complex formation. Moreover, the P2 term RNA from *B. subtilis* displays no obvious differences in the specificity of ligand recognition. The P2 term ribozyme is activated selectively by GlcN6P and, to a lesser extent, by glucosamine 6-sulfate, but not by other related compounds (Fig. 7B). This pattern of discrimination matches that reported for a full-length *glmS* RNA from *B. subtilis* (Winkler et al. 2004). Collectively, these data suggest that all of the molecular determinants employed by the *glmS* ribozyme in GlcN6P recognition are contained entirely within the P1/P2 core structure.

Taken together, the requirement of the P3/P4 domain for maximum rate enhancement under low ionic strength conditions and its apparent lack of impact on effector recognition suggest that the primary role of this domain is to permit optimum catalytic function in physiological concentrations of Mg^{2+} . The P3/P4 structure thus seems to act as an auxiliary domain, probably by stabilizing the active conformation of the *glmS* ribozyme through tertiary interactions with the P1/P2 core element.

DISCUSSION

RNA enzymes generally rely on metal ions to adopt their catalytically active conformations. These cations participate in folding the RNA into a relatively compact globular structure by mediating packing of negatively charged phosphate groups through delocalized electrostatic shielding, and by stabilizing tertiary structures through more specific contacts with RNA functional groups (Hanna and Doudna 2000; Pyle 2002; Draper 2004). Furthermore, due to the limited intrinsic chemical functionality available to RNA enzymes, divalent cations provide a means by which the catalytic properties of RNA could be enhanced. For example, divalent metal ions could participate in chemical reactions as aqua complexes that promote general acid or general base catalysis (Fedor 2002; DeRose 2003). Indeed, ribonuclease P and the group I and II self-splicing introns display stringent divalent metal ion requirements (Pyle 2002). Similarly, the small self-cleaving ribozymes (hammerhead, hairpin, HDV, and *Neurospora* VS) were initially regarded as obligate metalloenzymes after early studies revealed strong dependencies on Mg^{2+} or certain other divalent cations (Dahm and Uhlenbeck 1991; Chowrira et al. 1993; Collins and Olive 1993; Suh et al. 1993).

In recent years, however, the degree to which divalent metal ions contribute to ribozyme function has been re-evaluated, particularly in the cases of the small catalytic RNAs. A series of studies probing the metal ion requirements of the small self-cleaving ribozymes has revealed that divalent cations are not indispensable for catalysis, as they may be substituted quite effectively by high concentrations of monovalent ions, or in certain cases by exchange-inert metal hydrate mimics or organic polyamines (Hampel and Cowan 1997; Nesbitt et al. 1997; Young et al. 1997; Murray et al. 1998; Nakano et al. 2000; Curtis and Bartel 2001; O'Rear et al. 2001). One of the implications of these findings is that functional groups intrinsic to the RNA make significant and direct contributions to catalysis. Indeed, evidence to support direct nucleobase participation in catalysis has accrued from both biochemical and X-ray crystallographic analyses of several of the small, self-cleaving RNAs (Ferre-D'Amare et al. 1998; Perrotta et al. 1999; Nakano et al. 2000; Rupert and Ferre-D'Amare 2001; Lafontaine et al. 2002; Jones and Strobel 2003; Das and Piccirilli 2005; Kuzmin et al. 2005).

Representatives of the *glmS* class of self-cleaving ribozymes appear not to require direct involvement of divalent metal ions in the catalytic step of phosphoester transfer. Robust acceleration of self-cleavage in the presence of the exchange-inert metal hydrate mimic $Co(NH_3)_6^{3+}$ supports the hypothesis that catalytic strategies employing inner sphere divalent metal ion coordination are not used by this ribozyme class. This conclusion is consistent with the absence of a substantial thio effect when a substrate containing cleavage site phosphorothioate substitutions is

incubated with the ribozyme in the presence of Mg^{2+} . Similarly to several other self-cleaving ribozymes, the *glmS* motif exhibits substantial rate enhancements in elevated concentrations of monovalent ions alone, supporting the absence of a requirement even for specific outer sphere divalent metal contacts. Thus, while it is likely that divalent metal ions fulfill important structural roles under conditions of physiological ionic strength, it appears that they do not make significant, direct contributions to *glmS* ribozyme-mediated catalysis.

The apparent absence of catalytic roles for divalent metal ions in the *glmS* ribozyme cleavage mechanism suggests the involvement of functional groups of the ribozyme itself or, perhaps, of the metabolite serving as the ribozyme activator. In the latter case, GlcN6P could serve as a coenzyme, with its amine group participating in a cleavage mechanism that involves proton transfer. Rate enhancements measured in the absence of GlcN6P are substantially elevated by Tris buffer, which is an imperfect structural mimic of a portion of GlcN6P. Additional studies with other analogs of GlcN6P (Winkler et al. 2004) or Tris (McCarthy et al. 2005) suggest that the amine group is essential for ligand-mediated rate enhancement of the *glmS* ribozyme.

The presence of GlcN6P stimulates the activity of the *glmS* ribozyme by $\sim 100,000$ -fold compared with the non-activated rate constant (measured using buffering compounds other than Tris). If GlcN6P serves as an allosteric effector and not as a coenzyme, this high level of ribozyme activation would demand remarkably tight control of *glmS* ribozyme conformation by the metabolite. Such an exquisite degree of allosteric modulation of ribozyme function by a small molecule would be unprecedented among metabolite-responsive allosteric ribozymes that have been engineered or selected in vitro (Silverman 2003), and thus would argue against an allosteric effector function for GlcN6P.

In this study we also demonstrate that *glmS* RNA constructs from *B. subtilis* and *B. cereus* that lack a complete P3/P4 domain retain GlcN6P-dependent ribozyme activity, although these constructs require higher Mg^{2+} concentrations for full activity. These results, together with the apparent lack of involvement of the P3/P4 domain in GlcN6P recognition, indicate that the P3/P4 domain serves primarily to permit optimum rate enhancement of the *glmS* ribozyme at physiological Mg^{2+} concentrations. In fulfilling this role, the P3/P4 substructure may act as an auxiliary domain, peripheral to the catalytic core, which stabilizes the active ribozyme conformation through tertiary interactions with the P1/P2 domain.

It is worth noting that the internal loop within the P4 stem of the *glmS* ribozyme contains several highly conserved, consecutive adenosine residues, which potentially could mediate helix packing through minor groove tertiary interactions in the manner described previously for the P4–P6 domain of the *Tetrahymena* group I intron and other

ribozymes (Doherty et al. 2001). Interestingly, the deviation from the consensus secondary structure model observed in the P3/P4 domain of the *glmS* ribozyme from *D. radiodurans* (Fig. 1) is consistent with the proposed involvement of this domain in stabilization of the catalytic core rather than in the reaction mechanism itself or in GlcN6P recognition. Presumably, there is a diversity of structural approaches by which an auxiliary domain may stabilize the active conformation of the *glmS* ribozyme catalytic core. The P1/P2 core domain itself, however, because it contains the bulk of both the catalytic and ligand recognition determinants, is probably subject to more stringent sequence constraints. Although the *glmS* ribozyme from *D. radiodurans* is the only example of this motif yet uncovered in which significant changes exist in the auxiliary P3/P4 domain, analogous observations have been made with other RNA enzymes. In the *Tetrahymena* group I intron, for example, the P5abc peripheral element, which is significantly less conserved than the catalytic core (Michel and Westhof 1990), participates in stabilizing the active structure of the ribozyme (Doherty et al. 1999; Engelhardt et al. 2000).

The use of auxiliary stabilizing domains appears to be widespread among natural ribozymes, with several examples of these elements having recently been described among the small self-cleaving RNAs. The hammerhead ribozyme is constructed essentially as a three-way helical junction, and in its minimized form appears to fold in a stepwise manner (Bassi et al. 1997; Hammann et al. 2001). Dissection of this folding pathway *in vitro* has revealed that the first structural transition occurs at $\sim 500 \mu\text{M}$ Mg^{2+} , when helices II and III are observed to stack coaxially. A subsequent reorganization, in which the catalytic core adopts its active conformation concomitantly with the rotation of helix I into apposition with helix II, requires a concentration of Mg^{2+} in excess of the physiological range. Recently, it has been demonstrated that hammerhead ribozymes in their natural context contain terminal or internal loops on helices I and II that drastically reduce the functional requirement for Mg^{2+} (De la Pena et al. 2003; Khvorova et al. 2003), thereby resolving the discrepancy between the high divalent metal ion concentrations required for cleavage activity of the minimal motif *in vitro* and the considerably lower estimated concentrations of free Mg^{2+} in the cellular environment (Romani and Scarpa 1992). These auxiliary loops, which exhibit a lesser degree of phylogenetic conservation than the catalytic core, appear to associate with one another through tertiary contacts to facilitate folding of the active structure (Penedo et al. 2004). Ostensibly, these enhancing effects on folding are elicited by restricting the conformational freedom of the relevant helices, thereby channeling the folding pathway toward intermediates that approximate the active geometry of the hammerhead ribozyme (De la Pena et al. 2003; Khvorova et al. 2003; Lilley 2003; Penedo et al. 2004). Moreover, by

obviating the requirement for high Mg^{2+} concentrations in the structural transition to the active fold, the auxiliary domains in helices I and II permit efficient function of the hammerhead ribozyme *in vivo*.

Another example of a nucleolytic RNA benefiting from auxiliary structural elements is provided by the hairpin ribozyme. This ribozyme occurs naturally in the context of a four-way helical junction, although the minimum catalytically competent motif requires only two of these helices, each containing conserved loop domains that interact to form the active center (Ferre-D'Amare 2004). In the minimized version, the two helices are hinged at the junction point to allow association of the loop domains, and folding of this form requires significantly higher Mg^{2+} concentrations as compared with the native form (Walter et al. 1999; Zhao et al. 2000). Thus, the interdomain junction of the hairpin ribozyme, which is remote from the catalytic core, nevertheless exerts a profound influence on the folding of this motif. Single-molecule fluorescence studies have offered a mechanism for this effect by demonstrating that the structural dynamics of the natural, four-way junction form predispose hairpin RNAs toward the population of a discrete structural intermediate in which the loop domains are poised to interact (Tan et al. 2003).

It is evident that, apart from its regulation by GlcN6P, the *glmS* ribozyme appears to be similar in many respects to some of the other small phosphoryl transfer ribozymes. In addition to exhibiting no stringent requirement for divalent metal ion participation in catalysis, the *glmS* RNA relies on a supplemental structural domain that, while it is not essential to the reaction mechanism, nonetheless is required for efficient function under physiological conditions. Accordingly, this self-cleaving RNA motif underscores the critical contribution to ribozyme function of structural elements peripheral to the catalytic core.

MATERIALS AND METHODS

Chemicals and oligonucleotides

D-glucosamine 6-phosphate, D-glucosamine, D-glucosamine 6-sulfate, β -D-glucose 1-phosphate, D-glucose 6-phosphate, and D-glucose were purchased from Sigma. Synthetic DNA oligonucleotides prepared using standard solid-phase methods were provided by the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory and were purified by denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE). Synthetic RNA oligonucleotides with chemical modifications at the cleavage site were purchased from Dharmacon RNA Technologies.

Construction of ribozyme templates

Templates encoding full-length *glmS* ribozymes and variants with 5' or 3'-end truncations were amplified from plasmid clones by PCR using appropriate primers. Plasmids were created with a TOPO TA

Cloning Kit (Invitrogen) according to the manufacturer's instructions, using PCR-amplified fragments corresponding to the *glmS* ribozyme-containing IGRs from *B. cereus* (ATCC 10987) and *B. subtilis* (strain 1A40). The template for the version of the *B. subtilis glmS* ribozyme containing a disrupted P3a stem (Fig. 5A) was generated by PCR as described above using a reverse primer bearing the appropriate point mutations. The DNA encoding the double mutant ribozyme, in which compensatory mutations restored base-pairing in the P3a stem, was prepared in the same manner except that the template for the PCR was not a plasmid but rather double-stranded DNA prepared by extending the two partially complementary oligonucleotides, 5'-CTTATTTTCTCAATAGGAAAAGAAGACGGGAT TATTGCTTTACCTATAATTATAGCGCCCGAACTAAGCGCCCG GAAAAAGGCTTAGTTGACGAGGATGGAGGTTATCGA and 5'-TGTGATTTCTCTTTGTTCAAGGAGTCACCCCTTGGTTTGAA GAAATCCTTACGGCTGTCTAGTGCACACTCAGCCGGGAGGC ATCCGCCGAAAATTCGATAACCTCCATCCT, with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

RNA preparations

Transcriptions in vitro were performed either with a RiboMAX Large Scale RNA Production System-T7 (Promega) according to the manufacturer's instructions or in a 50- μ L volume, incubated 2 h at 37°C, containing 50 mM Tris-HCl (pH 7.5 at 23°C), 15 mM MgCl₂, 5 mM dithiothreitol (DTT), 2 mM spermidine, 2.5 mM each of the four ribonucleoside 5' triphosphates (NTPs), 1 μ M double-stranded template DNA, and 5 U/ μ L T7 RNA polymerase (T7 RNAP). Transcriptions in vitro of internally radiolabeled ribozymes also contained 10 μ Ci [α -³²P] UTP. Note that, due to the apparent stimulatory effect of Tris on *glmS* ribozyme catalysis (Fig. 3B, inset), lower yields of unreacted RNA should be expected from transcriptions utilizing this buffer. RNAs were purified using denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE) and eluted from gel slices in 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA. Recovered material was concentrated by precipitation with ethanol. Enzymatically synthesized RNA molecules that were to be 5' ³²P-labeled were first dephosphorylated with alkaline phosphatase (Roche Diagnostics) and then radiolabeled using γ -³²P [ATP] and T4 polynucleotide kinase (New England Biolabs) according to the manufacturers' instructions. Subsequent purification by denaturing PAGE was performed as described above.

Ribozyme assays

Rate constants for ribozyme-mediated strand cleavage were measured by incubating trace amounts of radiolabeled RNA at 23°C under standard conditions (50 mM Tris-HCl [pH 7.5 at 23°C], 200 mM KCl, 10 mM MgCl₂, 200 μ M GlcN6P) or as otherwise indicated. Assays of cleavage *in trans* of the phosphorothioate-modified RNA included ~5 nM 5' ³²P-labeled "substrate" and 200 nM "ribozyme" (Fig. 5). Reactions were terminated at appropriate times with either an equal volume of urea-containing gel loading buffer (87 mM Tris base, 89 mM boric acid, 20% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol, 0.1% sodium dodecyl sulfate, 7.3 M urea, 100 mM EDTA) or three volumes of a formamide-based loading buffer (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). Reaction products

were separated by denaturing PAGE, and radiolabeled species were analyzed using a PhosphorImager and ImageQuant software (Molecular Dynamics). Rate constants (k_{obs}) were determined assuming first order reaction kinetics using data points from the first 10%–30% of the reaction and were derived from the slope of the line obtained by plotting the natural logarithm of the fraction of precursor RNA remaining versus time. Final extents of fast self-cleavage reactions typically ranged from 70%–90%, and were not observed to change significantly as a function of cation concentrations or pH conditions. For cleavage reactions *in trans*, significant deviations from linearity were observed even after correcting for the uncleaved fraction, suggesting that the use of chemically synthesized substrates may result in diverse cleavage efficiencies.

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