Ground-state coordination of a catalytic metal to the scissile phosphate of a tertiary-stabilized Hammerhead ribozyme

W. LUKE WARD^{1,2} and VICTORIA J. DEROSE^{1,2,3}

¹Department of Chemistry, University of Oregon, Eugene, Oregon 97403-1253, USA

²Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403-1253, USA

ABSTRACT

Although the Hammerhead ribozyme (HHRz) has long been used as a model system in the field of ribozyme enzymology, several details of its mechanism are still not well understood. In particular, significant questions remain concerning the disposition and role of catalytic metals in the HHRz. Previous metal-rescue experiments using a “minimal” HHRz resulted in prediction of a catalytic metal that is bound in the A9/G10.1 site in the ground state of the reaction and that bridges to the scissile phosphate further along the reaction pathway. “Native” or extended HHRz constructs contain tertiary contacts that stabilize a more compact structure at moderate ionic strength. We performed Cd^{2+} rescue experiments on an extended HHRz from *Schistosoma mansoni* using stereo-pure scissile phosphorothioate-substituted substrates in order to determine whether a metal ion makes contact with the scissile phosphate in the ground state or further along the reaction coordinate. Inhibition in $\text{Ca}^{2+}/\text{Mg}^{2+}$ and rescue by thiophilic Cd^{2+} was specific for the $\text{R}_p\text{-S}$ stereoisomer of the scissile phosphate. The affinity of the rescuing Cd^{2+} , measured in two different ionic strength backgrounds, increased fourfold to 17-fold when the pro- R_p oxygen is replaced by sulfur. These data support a model in which the rescuing metal ion makes a ground-state interaction with the scissile phosphate in the native HHRz. The resulting model for Mg^{2+} activation in the HHRz places a metal ion in contact with the scissile phosphate, where it may provide ground-state electrostatic activation of the substrate.

Keywords: Hammerhead ribozyme; metal rescue; catalytic RNA; kinetics; mechanism; metals

INTRODUCTION

A continuing challenge in the field of ribozyme enzymology is the precise determination of the contributions of metal ions to RNA catalytic activity. RNA has a general electrostatic requirement for cations for proper folding, and metal ions can specifically bind RNA ligands via inner or outer sphere coordination to provide a deeper level of structural stabilization. Site-specifically bound divalent metal ions can have a direct role in the chemical mechanism of the ribozyme as well (DeRose 2003; Sigel and Pyle 2007). Using RNase P and the group I intron as model ribozymes, Steitz and Steitz (1993) proposed that ribozymes might rely on bound metal ions to perform general acid/base chemistry. However, insights into the mechanisms of small nucleolytic ribozymes have indicated that the nucleobase functional

groups of RNA can also play critical roles in catalysis, with the extreme example of the hairpin ribozyme that is proposed to catalyze phosphoryl transfer in the complete absence of metal ion participation in the chemical mechanism (Nesbitt et al. 1997; Bevilacqua 2003). A thorough understanding of the range of mechanisms used by ribozymes has not yet been realized, because the detailed mechanisms including the specific roles of metal ions in ribozyme active sites are difficult to ascertain.

The Hammerhead ribozyme (HHRz) has been a particularly challenging system with regard to determining the roles of metal ions in structure and catalysis. The HHRz is a small nucleolytic ribozyme that catalyzes the site-specific nucleophilic attack of a 2'-OH on its 3'-phosphodiester bond, resulting in strand cleavage. Hammerhead ribozymes were originally discovered as the genome processing component of RNA-based viroids and have since been found in genomes throughout the domains of life (Buzayan et al. 1986; de la Pena and Garcia-Robles 2010). In medium ionic strength, HHRz activity is extremely sensitive to the concentration and identity of divalent metals (Hunsicker

³Corresponding author.

E-mail derose@uoregon.edu.

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and DeRose 2000; Boots et al. 2008). Moderate rates of catalysis can also be achieved in molar concentrations of monovalent cations, an important property that helped to uncover the critical roles of nucleobases in the HHRz reaction mechanism (Murray et al. 1998; O'Rear et al. 2001; Bevilacqua et al. 2004). At physiological ionic strengths, the HHRz requires divalent ions for appreciable rates of catalysis; therefore, it is reasonable to assume that the divalent metal-dependent channel is the primary mode of catalysis in nature (Khvorova et al. 2003).

The HHRz was studied for years in its simplest active form, as three short helices meeting at a junction of conserved nucleotides that form the active site of the ribozyme (for review, see Blount and Uhlenbeck 2005). Studies using this “truncated” form of the HHRz (trHHRz) led to a model of catalysis in which a catalytic metal in the P9/G10.1 site coordinates the pro-R oxygen of the scissile phosphate, presumably to stabilize the negative charge of the phosphorane transition state (Peracchi et al. 1997; Wang et al. 1999). Based on detailed metal-rescue experiments, Wang et al. (1999) predicted that the metal ion coordinates to the P9/G10.1 site in the ground state and bridges to the scissile phosphate in the transition state of the trHHRz reaction. A ground state that is very different from the transition state is consistent with structural studies of the truncated HHRz, which in general did not show catalytically relevant atoms within appropriate distances of the active site (Blount and Uhlenbeck 2005). In these structures, the P9/G10.1 metal ion site is ~ 20 Å away from its predicted ligand during catalysis, the pro-R oxygen of the scissile phosphate (Pley et al. 1994; Scott et al. 1995). To corroborate these seemingly conflicting data, it was proposed that the trHHRz periodically sampled a thermodynamically unstable “active” structure in which the implicated functional nucleobases and metal ion would be in the correct position to stimulate catalysis (for review, see Blount and Uhlenbeck 2005).

Tertiary-stabilized or “extended” Hammerhead ribozymes contain nonconserved unpaired regions in helices I and II that interact to promote the formation of the active site in the helix junction. These tertiary interactions result in a higher population of functionally folded ribozymes, leading to higher observed rates at significantly lower divalent metal concentrations in vitro, as well as activity in vivo (Khvorova et al. 2003). New structures of constructs based on native HHRz's (2GOZ, 2OEU, 2QUS) largely satisfy predictions based on previous mutations and also provide new hypotheses concerning players in the HHRz mechanism (Martick and Scott 2006; Lee et al. 2007; Chi et al. 2008; Martick et al. 2008). The conformation of the native HHRz in crystal-forming conditions is significantly different from that of the truncated HHRz. The native HHRz structures show a well-organized active site, with functional groups from the G8 and G12 guanosines in place to participate in general acid/base catalysis (Fig. 1). One fascinating aspect of these

FIGURE 1. (A) Secondary structure of the modified *Schistosoma mansoni* HHRz (MSL1L2) (Osborne et al. 2005) used in these studies. (B) Crystallographic active site of the *S. mansoni* HHRz (2OEU) (Martick et al. 2008) showing (1) proximity of the G12 N1, proposed to act as general base for activation of the 2' nucleophile (2, a 2'-OMe in this structure) and the 2'-OH of G8 that has been proposed to act as a general acid for the 5'-O[−] leaving group (3). The Mn²⁺ ion positioned in the P9/G10.1 active site (4) would be proposed to coordinate the pro-R oxygen of the scissile phosphate based on these studies. (C) Results expected for Cd^{2+} rescue of phosphorothioate substitution at the HHRz scissile phosphate. If there is no ground-state interaction between the metal and the substituted site (left), the value of $K_{1/2}(\text{Cd}^{2+})$ for Cd^{2+} activation of the reaction should not change for the O and S substrates. For a ground-state interaction, $K_{1/2}(\text{Cd}^{2+})$ should decrease to reflect the higher affinity of the Cd^{2+} –S interaction in the substituted site.

structures, however, is that even in this proposed “active” conformation, the metal modeled at the P9/G10.1 site is still not making contact with functional groups directly involved in catalysis.

The extended HHRz from *Schistosoma mansoni* demonstrates Cd^{2+} rescue of P9 and scissile phosphorothioate substitutions, supporting the prediction that inner-sphere coordination of a metal ion to these phosphates is required for efficient catalysis in the stabilized HHRz (Osborne et al. 2005). These prior studies were done with mixtures of phosphorothioates and fixed Cd^{2+} concentrations and did not provide sufficient basis to determine ground-state affinities of the rescuing metal ions. Assuming that the metal

ion observed in the P9/G10.1 site of native HHRz's is the catalytically relevant ion implicated in coordinating the pro-R oxygen of the scissile phosphate, as is predicted by studies in the trHHRz, two simple models exist that might explain how this metal ion interacts with the active site during catalysis. In one model, named here the "dynamic model," the metal ion could be recruited to the scissile phosphate at some point during the reaction resulting in stabilization of the negative charge of the phosphorane transition state (Wang et al. 1999). This model, combined with native HHRz crystal structures, has been the basis for recent computational studies that calculated the position of the metal ion during different points along the HHRz reaction coordinate. These molecular simulations began with the crystallographically predicted P9/G10.1 metal site and predicted that a slight negative charge build-up resulting from nucleophile deprotonation is enough to rapidly and stably recruit the P9/G10.1 metal into a bridging coordination mode between the pro-R oxygen atoms of the P9 and scissile phosphates (Lee et al. 2008, 2009). Alternatively, the positioning of the metal ion may be specific to crystallography, in that the described structure is the most stable state under crystal-forming conditions. In solution, the most active form of the ribozyme may be slightly different or be in equilibrium with the conformation observed in X-ray structures. A pre-initiation coordination (or an "activated ground-state coordination") of the catalytically relevant metal ion to the scissile phosphate could theoretically provide a strong chemical advantage over the dynamic model via several methods. For instance, in a ground-state pro-R oxygen coordination position, the metal ion could lower the reaction barrier by stabilizing in-line attack geometry, activating the nucleophile via direct or indirect coordination, and/or withdrawing electron density from the phosphate, thereby increasing the electrophilicity of the scissile phosphorus. Proximity of the metal ion may also lower the pK_a of the putative general base and/or nucleophilic 2'-OH. In the predicted mechanisms of other metal-dependent ribozymes such as the Group I intron, one or more metal ions are coordinated in the active site of the ribozyme without the need for metal recruitment during the reaction (Frederiksen and Piccirilli 2009).

We have performed detailed cadmium rescue experiments on HPLC-separated stereoisomers of a scissile phosphorothioate-substituted HHRz derived from *S. mansoni* in order to test whether a catalytic metal coordinates the scissile phosphate in the ground state or is instead recruited to the scissile phosphate during the transition state of the HHRz mechanism. Our results are consistent with a mechanism in which the rescuing metal makes a ground-state coordination with the pro-R oxygen of the scissile phosphate. These results differ from prior studies of cadmium rescue using trHHRz constructs, which demonstrated transition-state and not ground-state coordination (Peracchi et al. 1997; Wang et al. 1999), indicating that the

tertiary interactions in native HHRz constructs stabilize a ground state that may be more advanced on the reaction coordinate. These results also imply that the current structural view of the HHRz, with a metal ion coordinated only in the P9/G10.1 site, may not represent the most active conformation of the HHRz active site. Instead, the crystal structure may represent just one of several conformations available to the HHRz core leading to catalytic activation, whereby the activating metal coordinates the pro-R oxygen of the scissile phosphate to stimulate catalysis.

RESULTS AND DISCUSSION

Cd^{2+} rescue in 100 mM Na^+ and 15 mM Ca^{2+}

The HHRz has a general dependence on divalent metal cations for folding. In a background of 100 mM Na^+ , the $K_{1/2}$ for folding is ~ 1 mM M^{2+} for a variety of metal cations (Kim et al. 2005; Boots et al. 2008; WL Ward and VJ DeRose, in prep.). Catalytic activation of the ribozyme occurs at much higher metal concentrations than does folding, however, and reaction rates vary greatly depending on cation identity (Kim et al. 2005; Boots et al. 2008; WL Ward and VJ DeRose, in prep.). For instance, Cd^{2+} is an especially stimulatory cation, while Ca^{2+} is a poor stimulator of activity (Boots et al. 2008). We used these relationships in thiophosphate substitution- Cd^{2+} rescue experiments to separate the effects of Cd^{2+} on catalytic rescue from its contributions to global folding. HHRz kinetic experiments were performed in a background of 15 mM Ca^{2+} and 100 mM Na^+ , which supports a folded ribozyme population with very low activity, and the ability of Cd^{2+} to stimulate wild-type (WT) and thiophosphate-substituted samples was compared.

Stereoisomers (R_p and S_p) of the scissile phosphorothioate-substituted HHRz substrate strand were separated by reverse-phase HPLC for use in Cd^{2+} rescue titrations alongside the wild-type scissile phosphate construct (WT). The rate for each construct in 15 mM Ca^{2+} and 100 mM Na^+ is very low, reaching only 0.04 min^{-1} for the WT construct (Fig. 2). The S_p construct has a rate reduction of one-half the WT rate ($k = 0.02 \text{ min}^{-1}$), indicating that the S_p construct is only slightly inhibited by the sulfur substitution. In contrast, the R_p construct cleaves 100-fold more slowly than the WT construct under these conditions ($k = 0.0004 \text{ min}^{-1}$). General catalytic inhibition by a phosphorothioate substitution is possible in ribozymes (Forconi and Herschlag 2009) and could be due to slight structural perturbation caused by the larger sulfur atom in place of an oxygen atom. Additionally, the less electronegative sulfur atom may withdraw less electron density from the phosphorus, causing the phosphorus to have a lower electrophilicity than the native phosphate, leading to a lower intrinsic reaction rate (Erat and Roland 2011). The 50-fold additional inhibition from the R_p thio substitution

FIGURE 2. Cadmium rescue of HHRz constructs in 100 mM Na⁺ and 15 mM Ca²⁺. The wild-type (WT) (triangle) and S_p phosphorothioate construct (square) show similar Cd²⁺ profiles compared with the R_p phosphorothioate construct (circle), which is significantly inhibited in the absence of Cd²⁺. The rates in 0 mM Cd²⁺ are 0.04 min⁻¹ for WT, 0.02 min⁻¹ for S_p construct, and 0.0004 min⁻¹ for the R_p construct. The data were fit to Equation 3 to give apparent binding affinities of the rescuing Cd²⁺ of WT $K_{1/2} = 4 \pm 1$ mM, S_p $K_{1/2} = 10 \pm 7$ mM, and R_p $K_{1/2} = 900 \pm 300$ μ M. (Inset) Presents the data using rates normalized to the predicted $k_{\max}$ in order to highlight the differences in $K_{1/2}$ values.

in comparison with the S_p isomer is consistent with further loss of activity that may be due to the very low affinity of the hard Ca²⁺ ion for the R_p sulfur.

The addition of Cd²⁺, which supports faster reaction rates in comparison with both Mg²⁺ and Ca²⁺, causes a rate increase for each HHRz construct. Upon titration with Cd²⁺, the activity of the S_p construct approximately mirrors that of the WT, with the S_p phosphorothioate causing a small and constant rate reduction (Fig. 2). Due to the limits of manual kinetics techniques, rates for the S_p and WT ribozyme were not collected above 10 mM Cd²⁺. The data were fit to Equation 3 (in Materials and Methods) and give a predicted $k_{\max}$ of ~ 25 min⁻¹ for both ribozymes, which likely represents a lower limit for the $k_{\max}$ values. The stimulation of WT activity over this range of Cd²⁺ concentrations is ~ 600 -fold. Over the same range of Cd²⁺ concentrations, the initial inhibition in the R_p construct is almost completely rescued, ending in a saturated activity curve with a $k_{\max}$ that is ~ 10 -fold lower than the WT ribozyme. Incomplete rescue of the R_p construct to the WT rates may be due to the properties of phosphorothioate substitutions mentioned above. The 5000-fold stimulation in R_p activity, however, is consistent with the rescue of an essential metal coordination with the pro-R position of the scissile phosphate.

The $K_{1/2}$ value of activity stimulation can be considered to reflect the apparent affinity of the activating metal to the HHRz in the ground state. Therefore, if the coordination environment of the metal ion in the activated complex is changed such that the affinity between a metal ion and the respective ligand is increased, this should be reflected as a decrease in the $K_{1/2}$ of stimulation (Fig. 1C). The WT ribozyme represents the standard state of the metal binding pocket, with the “rescuing” or “stimulating” metal ion coordinating a given set of ligands. If the metal ion coordinates a scissile phosphate oxygen in the ground state, then in the thio-substituted construct the increased affinity of a Cd²⁺—S bond over a Cd²⁺—O bond should be reflected in an increased affinity of the rescuing metal. Based on the data of Figure 2, the WT and S_p constructs have predicted stimulation $K_{1/2}$ values of 4 ± 1 mM and 10 ± 7 mM, respectively, for Cd²⁺ stimulation in a background of 15 mM Ca²⁺/100 mM Na⁺. Due to incomplete saturation of $k_{\max}$, these values likely represent lower limits for the apparent $K_{1/2}$ of the catalytically stimulating metal. For the R_p construct, a $K_{1/2} = 900 \pm 300$ μ M Cd²⁺ is measured, which is fourfold to 10-fold lower than the constructs with a pro-R oxygen. This increased affinity of the rescuing metal for the R_p thio-construct is consistent with coordination of the activating metal to the pro-R position of the scissile phosphate in the ground state of the HHRz.

In model nucleotide systems, the binding affinity of Cd²⁺ to a phosphorothioate is 20–100-fold tighter than the affinity to a phosphate oxygen (Pecoraro et al. 1984; Sigel et al. 1997). The affinity increase in these model systems calls into question whether a fourfold to 10-fold increase can correctly be considered to reflect a “ground-state” coordination. Consideration should be given to the fact that the rescuing metal ion in our experiment competes with 15 mM Ca²⁺ and 100 mM Na⁺ for positioning in the active site. Wang et al. (1999), using similar ionic conditions of 10 mM Ca²⁺/100 mM Na⁺, measured a fourfold increase in apparent affinity for Cd²⁺ stimulation of activity in a trHHRz with an A9 R_p—S substitution, leading to the conclusion that the rescuing metal ion made a ground-state interaction with the A9 pro-R oxygen. For the same P9 substitution but in a background of 10 mM Mg²⁺, Peracchi et al. (1997) found an 8.8-fold increased $K_{1/2}$ for stimulating Cd²⁺. Metal coordination to the A9/G10.1 site in activity-inhibited constructs has also been spectroscopically measured, providing further support for the description of the A9 interaction as a ground-state interaction (Horton et al. 1998; Hunsicker and DeRose 2000; Suzumura et al. 2002; Vogt et al. 2006). Additionally, in the present study, the $K_{1/2}$ values for Cd²⁺ activity stimulation in the WT and S_p constructs likely represent low estimates of the actual $K_{1/2}$ values in this ionic background. Therefore, it is likely that the $K_{1/2}$ difference between the R_p and the pro-R oxygen constructs is also a lower limit. The fourfold to 10-fold increase in the affinity of Cd²⁺ for the R_p scissile phos-

phorothioate in the native HHRz is, therefore, consistent with affinity increases of other characterized ground-state interactions in the HHRz.

Cd^{2+} rescue in 20 mM Na^+ and 5 mM Ca^{2+}

Metal coordination is subject to general cation competition, and therefore the presence of high concentrations of Na^+ and Ca^{2+} , while intended to deconvolute the role of Cd^{2+} in metal rescue, will also raise the level of Cd^{2+} required for achieving maximum activities. We have recently used 2-aminopurine fluorescence to measure global HHRz folding in a reduced ionic background of 20 mM Na^+ and found a $K_{1/2, \text{fold}}^{\text{Ca}}$ value of $230 \pm 20 \mu\text{M}$ (WL Ward and VJ DeRose, in prep.). The lowered Na^+ background also reduces the k_{max} and $K_{1/2}$ for Mg^{2+} activation. By reducing the background Na^+ and Ca^{2+} concentrations, competition for the rescuing Cd^{2+} ion should be reduced in the HHRz active site while still satisfying the general ionic requirements for global folding. We therefore repeated the Cd^{2+} activity titrations for the WT and R_p constructs in a background of 20 mM Na^+ and 5 mM Ca^{2+} (Fig. 3).

Under these lower background ionic conditions, the $k_{\text{max}}^{\text{app}}$ for stimulating Cd^{2+} is reduced in each construct, consistent with observations for Mg^{2+} activation in reduced

Na^+ (WL Ward and VJ DeRose, in prep.). With added Cd^{2+} the activity of the WT construct increases and then peaks at $\sim 3 \text{ mM}$ Cd^{2+} , followed by catalytic inhibition at higher concentrations of Cd^{2+} . Inhibition could be explained by the presence of a relatively low-affinity metal coordination site that stabilizes an inactive conformer of the HHRz at high concentrations of Cd^{2+} . Catalytic inhibition by soft metals is a known problem due to the propensity for non-native site binding (Forconi and Herschlag 2009), and Cd^{2+} inhibition of the trHHRz has been observed as well (Wang et al. 1999). The R_p construct is able to reach a saturated k_{max} at low micromolar Cd^{2+} concentrations, and it too begins to show catalytic inhibition at 3 mM Cd^{2+} , supporting this inhibition as a general effect of high Cd^{2+} on the HHRz in these background ionic conditions. Since this inhibitory effect was not observed in 100 mM Na^+ and 15 mM Ca^{2+} , it may indicate that the inhibitory metal is effectively outcompeted by higher background ionic conditions. Additionally, as it is also not observed for Mg^{2+} (Kim et al. 2005), this inhibitory effect may be specifically attributed to the binding of soft metal ions or transition metal ions.

The WT and R_p Cd^{2+} rate profiles, excluding the inhibition phase, were fit to Equation 3. The reduced cation competition clearly amplifies the differences in $K_{1/2}$ values for Cd^{2+} stimulation of activity in these constructs. In 5 mM Ca^{2+} /20 mM Na^+ , the $K_{1/2}$ for Cd^{2+} stimulation of WT activity is $170 \pm 10 \mu\text{M}$, while the $K_{1/2}$ for the R_p construct is now $10 \pm 3 \mu\text{M}$. This 17-fold increase of Cd^{2+} affinity for the R_p thio-construct over the WT phosphate strongly supports a ground-state metal coordination to the pro-R oxygen of the HHRz.

CONCLUSIONS/SUMMARY

The HHRz relies on divalent metal ions to achieve appreciable rates of catalysis in physiological ionic strength. We investigated the catalytically important coordination of a metal ion to the scissile phosphate using Cd^{2+} rescue titrations under ionic conditions in which the HHRz is globally folded with low background catalytic activity. Consistent with previous work on minimal HHRz constructs, we find that Cd^{2+} rescue is stereo-specific for the pro-R position of the scissile phosphate. However, we also find a fourfold to 17-fold increased affinity of the rescuing Cd^{2+} for the R_p phosphorothioate construct. This increased affinity of the rescuing metal supports a model in which a catalytic metal coordinates the pro-R oxygen of the scissile phosphate in the ground state of the HHRz mechanism, a result that differs from current mechanistic models derived from trHHRz data in combination with crystallographic structures of native HHRz's (Wang et al. 1999; Lee et al. 2009).

The “minimal” trHHRz that lacks tertiary interactions is proposed to predominantly occupy an “open” state that requires a conformational change to a “closed” or active

FIGURE 3. Cadmium rescue of the HHRz construct in 20 mM Na^+ and 5 mM Ca^{2+} . WT (triangle) and R_p phosphorothioate construct (circle) both experience catalytic inhibition phases at $[\text{Cd}^{2+}] > 3 \text{ mM}$. The rates in 0 mM Cd^{2+} are 0.02 min^{-1} for WT and $<0.0004 \text{ min}^{-1}$ for the R_p construct. The data, excluding inhibited points, were fit to Equation 3 to give apparent binding affinities of the rescuing Cd^{2+} of WT $K_{1/2} = 170 \pm 10 \mu\text{M}$ and R_p $K_{1/2} = 10 \pm 3 \mu\text{M}$. (Inset) Presents the data using rates normalized to the predicted k_{max} in order to highlight the differences in $K_{1/2}$ values.

state for catalysis. This closed form seems to be only transiently populated, meaning that “ground-state” pre-equilibration of metal occupancies in the trHHRz would mainly reflect the open conformation. Both structure (Scott et al. 1995) and spectroscopic measurements (Maderia et al. 2000; Vogt et al. 2006; Osborne et al. 2009) indicate higher-affinity population of the P9/G10.1 metal ion site in the trHHRz. Thus, metal-rescue studies of the trHHRz that predict metal coordination to the P9 site in the ground state, and the scissile phosphate at a later point on the reaction pathway, are consistent for that system.

Native HHRz constructs, however, stabilize a “closed” conformation through tertiary interactions. Given the higher stability of this closed conformation and the fact that P9 and scissile phosphate oxygens are much closer in native HHRz crystal structures, a bridging coordination mode for the catalytic metal ion seems to be achievable in the ground state of these constructs. Despite this, current crystallographic models locate a Mn^{2+} ion only in the nearby P9/G10.1 coordination site (Martick et al. 2008) or not strongly localized in the case of Mg^{2+} (Martick and Scott 2006; Chi et al. 2008). In contrast, the data presented here strongly support a ground-state metal coordination to the scissile phosphate. A caveat to thio-substitution and Cd^{2+} rescue experiments is the potential creation of a non-native coordination environment due to the increased affinity of Cd^{2+} for sulfur over oxygen. The potential drawbacks of metal-rescue methods for ribozyme mechanistic studies are well discussed in the literature (Forconi and Herschlag 2009; Frederiksen and Piccirilli 2009; Erat and Roland 2011). While caveats must be considered, such methods have been used successfully to identify important coordination sites for several ribozymes (Frederiksen and Piccirilli 2009). A quantitative analysis of the apparent affinity of the rescuing metal, such as performed here, expands the information gained to identify the point along the reaction coordinate at which the metal ion contacts the substituted ligand. The fourfold to 17-fold increase in affinity for Cd^{2+} for the substituted R_p scissile phosphorothioate observed for this native HHRz is very similar to results previously obtained for the P9/G10.1 site in the trHHRz, whose occupancy has been corroborated by crystallography and spectroscopic methods. The simplest interpretation of the current data is therefore that the HHRz has a catalytic metal ion bound to the scissile phosphate. Although current crystallographic results place a metal ion in the nearby P9/G10.1 position, simulations in which the metal has moved to bridge to the scissile phosphate show small structural changes, including an increase in the in-line fitness for nucleophilic attack (Lee et al. 2008). Structural changes along with beneficial electrostatic effects would enhance the catalytic potential of the ribozyme. We propose, therefore, that the most active form of the HHRz involves a ground-state coordination of an activating metal ion to the pro-R oxygen of the scissile

phosphate, which is then expected to aid catalysis throughout the reaction coordinate.

MATERIALS AND METHODS

Oligonucleotides

RNA oligomers were purchased from Dharmacon (CO), deprotected according to the manufacturer’s instructions, reconstituted in deionized and filtered water, and quantified by absorbance at 260 nm. The sequences for ribozyme and substrate strands are as follows: Enzyme Strand: 5′-GCAGGUACAUCAGCUGAUGA GUCCCAAUAGGACGAAACGCG. Substrate Strand: 5′-CGCG UC*CUGUAUCCACUGC. The asterisk denotes the scissile phosphorothioate substitution site.

Reagents

All reagents used were molecular biology grade or better. $CaCl_2 \cdot 2H_2O$ was obtained from Sigma-Aldrich and $Cd(NO_3)_2 \cdot 4H_2O$ from Alfa Aesar.

Separation of phosphorothioate stereoisomers

Phosphorothioate-substituted RNAs consisting of a mixture of the S_p and R_p diastereomers were separated using reverse-phase HPLC (Akta purifier; Amersham Biosciences) on a C18 column (Hypersil GOLD, 5 μm 4.6/250 mm; Thermo Scientific). A stationary phase of 10 mM triethylammonium acetate (TEAA) and a mobile phase of 80% acetonitrile/20% 10 mM TEAA were used. A multistep gradient was used that provided baseline separation of two major peaks.

The configurations of the separated stereoisomers were determined using stereospecific digestion by phosphodiesterase I (Slim and Gait 1991). This treatment leaves an undigested dinucleotide in the S_p fraction that is identified using HPLC separation, while the R_p fractions are completely digested into single nucleotides. Digestion mixtures contained 2.5 nmol RNA, 10 mM Tris (pH 8), 10 mM $MgCl_2$, 0.1 unit of snake venom phosphodiesterase I (Sigma-Aldrich), and 1 unit of calf intestine alkaline phosphatase (USB). Reactions were incubated for 1 h at 37°C. A dinucleotide peak was observed only in fractions from the second peak, identifying this peak as the S_p diastereomer fraction.

HHRz cleavage assays

Catalytic activities of the HHRz were determined by using the 43-nt enzyme strand (ES) and 5′- ^{32}P -labeled 20-nt substrate strand (S). Substrate strands were radioactively labeled (5′- ^{32}P) using [γ - ^{32}P]ATP and polynucleotide kinase (New England Biolabs). Labeled RNA was purified by PAGE, eluted in 100 mM acetic acid (pH 4.0) with TEMED, and desalted by EtOH precipitation and run through a G-25 column. The enzyme strand at 3 μM was mixed with trace-labeled substrate, in the presence of $2 \times Na^+$ and 100 mM Bis-tris propanediol buffer (pH 6.5). The complex was annealed by heating for 2 min to 90°C, cooling for 20 min to 4°C, and equilibrating for 10 min at 20°C. Reactions were initiated by the addition of an equal volume of $2 \times [Ca^{2+}]$ and $2 \times [Cd^{2+}]$ to the annealed RNA. The final conditions were 1.5 μM ES, trace S*, $1 \times Na$, 50 mM buffer, $1 \times Ca^{2+}$, and $1 \times Cd^{2+}$. Time points were quenched by the addition of 166 mM EDTA in 66% formamide

(SCHEME 1)

with trace amounts of xylenecyanol and Bromophenol Blue. Cleavage products were separated by dPAGE, then quantified using storage phosphor screens (Kodak) and imaging by Imagequant. A zero-time-point correction was used to account for background cleavage during the annealing steps.

We assume a model in which the rate in Ca^{2+} represents the folded, but nonactivated HHRz, and the addition of Cd^{2+} activates the ribozyme (Scheme 1).

Rate constants were determined by fitting the corrected fraction cleaved versus time to either a monophasic (Eq. 1) or biphasic (Eq. 2) model in which A and B represent the extents of cleavage and k_{obs1} and k_{obs2} are observed rate constants (Kim et al. 2005; Osborne et al. 2005).

$$y(t) = A \left(1 - e^{-k_{\text{obs1}}^t} \right) \quad (1)$$

$$y(t) = A \left(1 - e^{-k_{\text{obs1}}^t} \right) + B \left(1 - e^{-k_{\text{obs2}}^t} \right) \quad (2)$$

Most fits of “native” HHRz kinetic data are best described by a model of two noninteracting populations, with the faster phase corresponding to the majority population and a slow phase of $<0.01 \text{ min}^{-1}$. In the case of the $\text{R}_p\text{-S}$ substitution, Equation 1 fit the very slow rates in 0 to $\sim 10^{-6} \text{ M}$ $[\text{Cd}^{2+}]$, and Equation 2 was used when the fitting parameters indicated $\sim 50\%$ for the second population and significantly improved values of R^2 . The Cd^{2+} dependence of the rate constant for the fastest population was fit to a binding equation (3):

$$k_{\text{activated}} = k_0 + \frac{k_{\text{max}} x^n}{k_{1/2}^n + x^n} \quad (3)$$

where k_0 is set as the rate in only Na^+ and Ca^{2+} , k_{max} is the predicted maximum rate of cleavage at saturating Cd^{2+} , and n is the cooperativity coefficient. Nonlinear regression fits were performed using SigmaPlot 11 (Systat Software).

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W. Luke Ward and Victoria J. DeRose

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