Metal ion specificities for folding and cleavage activity in the *Schistosoma* hammerhead ribozyme

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ABSTRACT

The effects of various metal ions on cleavage activity and global folding have been studied in the extended *Schistosoma* hammerhead ribozyme. Fluorescence resonance energy transfer was used to probe global folding as a function of various monovalent and divalent metal ions in this ribozyme. The divalent metals ions Ca^{2+} , Mg^{2+} , Mn^{2+} , and Sr^{2+} have a relatively small variation (less than sixfold) in their ability to globally fold the hammerhead ribozyme, which contrasts with the very large difference (>10,000-fold) in apparent rate constants for cleavage for these divalent metal ions in single-turnover kinetic experiments. There is still a very large range (>4600-fold) in the apparent rate constants for cleavage for these divalent metal ions measured in high salt (2 M NaCl) conditions where the ribozyme is globally folded. These results demonstrate that the identity of the divalent metal ion has little effect on global folding of the *Schistosoma* hammerhead ribozyme, whereas it has a very large effect on the cleavage kinetics. Mechanisms by which the identity of the divalent metal ion can have such a large effect on cleavage activity in the *Schistosoma* hammerhead ribozyme are discussed.

Keywords: ribozyme; metal ions; RNA catalysis; hammerhead; fluorescence resonance energy transfer; RNA folding

INTRODUCTION

The hammerhead ribozyme (HHRz) is one of the best-studied small ribozymes. It was first discovered in the satellite RNA of tobacco ringspot virus (Buzayan et al. 1986) and avocado sunblotch viroid (Hutchins et al. 1986) and has been implicated in rolling-circle replication of plant satellite viruses (Forster and Symons 1987; Haseloff and Gerlach 1988), where it performs both self-cleavage and self-ligation reactions. The HHRz is also found in repetitive satellite DNA in *Schistosoma mansoni*, newts, salamanders, and crickets (Zhang and Epstein 1996; Ferbeyre et al. 1998; Rojas et al. 2000) and more recently in the genome of *Arabidopsis thaliana*, where it is expressed in several tissues (Przybilski et al. 2005).

The HHRz consists of three helical regions surrounding a 13-nucleotide (nt) catalytic core, which performs a *trans*-esterification reaction where site-specific RNA cleavage produces a 2',3'-cyclic phosphate and a 5'-hydroxyl group.

This “minimal” ribozyme requires relatively high concentrations of divalent metal ions for efficient cleavage in vitro (i.e., ≥ 10 mM Mg^{2+}) (Hertel et al. 1994). Inclusion of the naturally occurring tertiary interaction between loop motifs in stems I and II (Fig. 1) leads to much faster cleavage rates, and this interaction has been shown to be essential for efficient catalysis in vivo (De la Pena et al. 2003; Khvorova et al. 2003). In vitro kinetic studies on these “extended” HHRzs show that the loop-loop interaction can increase the observed cleavage rates under physiological conditions by over a factor of 100 (De la Pena et al. 2003; Khvorova et al. 2003; Canny et al. 2004).

Comparison of the X-ray structures of the minimal HHRz (Pley et al. 1994; Scott et al. 1995) with the recent structure of an extended HHRz (Martick and Scott 2006) shows that inclusion of the loop-loop tertiary interaction leads to a substantial conformational rearrangement in the catalytic core. The conformation of the catalytic core in the extended hammerhead rationalizes much of the extensive biochemical data that were in serious disagreement with the structure of the minimal HHRz (McKay 1996; Blount and Uhlenbeck 2005; Nelson and Uhlenbeck 2008). Biophysical studies, such as FRET and EPR, have also been used to probe the mechanism for increased catalytic activity in the extended HHRz. These studies showed that the inclusion of the tertiary interaction leads to a lower metal ion requirement

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.1010808>.

FIGURE 1. (A) The wild-type *Schistosoma* hammerhead construct used for the kinetics studies. (Black) The ribozyme strand; (gray) the substrate strand. The cleavable substrate strand has either a ^{32}P on the 5'-end or a fluorescein label on the 3'-end and is cleaved at C₁₇, as indicated by the arrow. (B) The noncleavable wild-type *Schistosoma* hammerhead ribozyme construct used for FRET studies. Cy3 was coupled to a modified U in loop II. Cy5 was coupled to the modified 3'-end of the substrate strand. The substrate strand has a 2'-deoxyribose on C₁₇ to prevent cleavage. (C) The UUCG control construct used for FRET studies. Cy3 was coupled to a modified U in loop II. The same noncleavable substrate strand was used for the UUCG control and the wild-type complex shown in B.

for folding of the extended than the minimal HHRz (Penedo et al. 2004; Heckman et al. 2005; Kim et al. 2005; Kisseleva et al. 2005). Together, the biochemical, biophysical, and structural data support a model where the loop-loop tertiary interaction increases the population of catalytically active species of the HHRz (Khvorova et al. 2003; Martick and Scott 2006; Nelson and Uhlenbeck 2006, 2007) and where divalent metal ions help to stabilize formation of the loop-loop tertiary interaction.

Previous studies on the minimal HHRz indicate that divalent metal ions are primarily involved in stabilizing the catalytically active structure and play only a small role in the chemistry of the cleavage/ligation reaction of the HHRz (Murray et al. 1998; Curtis and Bartel 2001; O'Rear et al. 2001). It was earlier hypothesized that all catalytic RNAs are metalloenzymes (Pyle 1993; Yarus 1993), where metal ions are required to perform chemistry in the catalytic reaction. The *Tetrahymena* group I intron is thought to be such a metalloenzyme since it has no activity in monovalent cations alone and phosphothioate rescue experiments have shown that there are multiple divalent metal ions within the core that are required for catalytic activity (for reviews, see Cech and Golden 1999; Hougland et al. 2006). Recent structural data support this model where the X-ray structure of the *Azoarcus* group I ribozyme (Stahley and Strobel 2005) has two Mg^{2+} ions in the active site, which are poised to carry out the classical two-metal ion catalytic mechanism for a phosphoryl transfer reaction (Steitz and Steitz 1993). Other ribozymes such as the hairpin ribozyme, the HDV ribozyme, and the ribosome have been shown to use RNA

functional groups to act as general acids or general bases in catalysis (for recent reviews, see Fedor and Williamson 2005; Bevilacqua and Yajima 2006; Hoogstraten and Sumita 2007; Scott 2007; Walter 2007). Based on biochemical and structural studies, RNA functional groups have also been proposed to act in the catalytic mechanism of the HHRz (Han and Burke 2005; Martick and Scott 2006).

FRET (Penedo et al. 2004) and EPR (Kim et al. 2005) studies have shown that global folding occurs at much lower Mg^{2+} concentrations in the extended HHRz compared to the minimal HHRz. Previous biochemical studies have also shown that the identity of the divalent metal ion has a significant effect on the cleavage rate for the minimal HHRz (Dahm and Uhlenbeck 1991; Dahm et al. 1993) and a SELEX-derived extended HHRz (Roychowdhury-Saha and Burke 2006). To try to better understand the roles of metal ions in the extended

HHRz, the observed rate constants for cleavage (k_{obs}) and global folding of the *Schistosoma* HHRz were measured for various divalent and monovalent metal ions. These results show that the identity of the divalent metal has a very large effect on the cleavage kinetics, whereas it has only a small effect on global folding of this HHRz.

RESULTS

Cleavage activity of the *Schistosoma* HHRz with different metal ions

Previous studies have shown that the inclusion of the loop-loop interaction in the HHRz leads to higher catalytic activity at lower Mg^{2+} concentrations (De la Pena et al. 2003; Khvorova et al. 2003; Canny et al. 2004) and that divalent metals are much more efficient at promoting catalysis than monovalent metal ions (Nelson et al. 2005). To test the effect of metal ion identity on catalysis, single turnover cleavage experiments were performed on the *Schistosoma* HHRz construct shown in Figure 1A in reaction buffer containing 1.0 mM of one of the following divalent ions: Ca^{2+} , Cd^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} , Sr^{2+} , or Zn^{2+} . The fraction of cleaved substrate was monitored as a function of time and fit to Equation 1 yielding k_{obs} for cleavage under these conditions (0.1 M NaCl, at pH 7.0, 50 mM Tris, 25°C) (Table 1; Supplemental Fig. S1). These k_{obs} values vary by more than 10,000-fold (from 0.018 min^{-1} for Sr^{2+} to $\sim 200 \text{ min}^{-1}$ for Mn^{2+}) with the following trend: $\text{Mn}^{2+} > \text{Co}^{2+} > \text{Zn}^{2+} \approx \text{Cd}^{2+} > \text{Mg}^{2+} > \text{Ca}^{2+} > \text{Sr}^{2+}$. Single turnover

TABLE 1. Metal dependence of k_{obs} for cleavage in the *Schistosoma* HHRz

Cation	k_{obs} (min^{-1}) ^a in 0.1 M NaCl	Fraction substrate cleaved in 0.1 M NaCl ^b	k_{obs} (min^{-1}) ^a in 2 M NaCl	Fraction substrate cleaved in 2 M NaCl ^b
Mn ²⁺	200 ± 10	0.41 ± 0.03	230 ± 25 ^c	0.43 ± 0.06
Co ²⁺	140 ± 20	0.36 ± 0.04	ND	ND
Zn ²⁺	82 ± 9	0.44 ± 0.05	ND	ND
Cd ²⁺	72 ± 5	0.41 ± 0.05	ND	ND
Mg ²⁺	2.6 ± 0.3	0.69 ± 0.02	1.0 ± 0.1	0.70 ± 0.06
Ca ²⁺	0.14 ± 0.04	0.53 ± 0.05	≤0.50	>0.40
Sr ²⁺	0.018 ± 0.006	0.46 ± 0.07	ND	ND
Na ⁺	<0.02	>0.50	ND	ND
NH ₄ ⁺	<0.02	>0.40	ND	ND

ND, not determined.

^a k_{obs} measured in 50 mM Tris-HCl (pH 7.0) and 25°C in the presence of 1.0 mM divalent cation or 600 mM monovalent cation, unless otherwise indicated. Values represent the average of at least three experiments, with errors of one standard deviation.^bThe fraction cleaved was determined from the plateau of the rapidly cleaving population using Equation 1.^cThis k_{obs} at 1.0 mM MnCl₂ (pH 7.0) was calculated using the measured k_{obs} of 3.5 min⁻¹ at 0.15 mM MnCl₂ (pH 6.0) and assuming a linear relation for k_{obs} on [Mn²⁺] and log-linear relation of k_{obs} with pH (see the text).

cleavage kinetics were also performed on the *Schistosoma* HHRz in the presence of 600 mM of the monovalent ion Na⁺ or NH₄⁺ and yielded upper limits of $k_{\text{obs}} < 0.02 \text{ min}^{-1}$ (Table 1).

As seen in Table 1, the fraction of substrate cleaved varies for the different metal ions from a plateau of 0.36 for Co²⁺ to 0.69 for Mg²⁺. The interpretation of the plateau is complicated because this plateau is not simply determined by the internal equilibrium for cleavage and ligation ($k_{\text{cleave}}/k_{\text{ligate}}$) but is also affected by the *Schistosoma* HHRz having a significant population of catalytically inactive species (Canny et al. 2007). These different metal ions may be differentially affecting the population of active ribozymes, which would lead to changes in the cleavage plateau. However, as previously discussed (Canny et al. 2007), the k_{obs} here is determined from the rapidly cleaving population, which is a minimum of 36% of the HHRz molecules for all metals (Table 1).

Global folding of the noncleavable *Schistosoma* HHRz with different metal ions

In addition to accelerating the rate of cleavage at a given Mg²⁺ concentration, the extended HHRz also globally folds at lower concentrations of Mg²⁺ when compared to a ribozyme lacking the tertiary interaction (Penedo et al. 2004; Kim et al. 2005). To examine if the large variation in k_{obs} for different divalent metal ions results from each metal differentially inducing global folding, FRET was used to monitor folding of the noncleavable wild-type

hammerhead construct shown in Figure 1B. Figure 2A shows the FRET efficiency (E_{FRET}) for the *Schistosoma* HHRz (filled symbols) as a function of concentration of the following divalent metals: Ca²⁺, Mg²⁺, Mn²⁺, or Sr²⁺. The FRET results clearly show that these four divalent ions have similar efficiencies for globally folding the *Schistosoma* ribozyme, which is in contrast to the very large variation in k_{obs} for cleavage for these same metal ions (Table 1). FRET experiments were not performed with Co²⁺, Zn²⁺, and Cd²⁺, for which k_{obs} values were determined, due to interference with the fluorescence measurements from the colored Co²⁺ and limited solubility at higher concentrations of Zn²⁺ and Cd²⁺.

FRET was also used to monitor global folding of the noncleavable UUCG control HHRz construct (Fig. 1C),

FIGURE 2. (A) FRET efficiency (E_{FRET}) versus divalent metal ion concentration for folding of the noncleavable *Schistosoma* HHRz (filled symbols) wild-type and (open symbols) UUCG control constructs in (circles) Mn²⁺, (diamonds) Mg²⁺, (squares) Ca²⁺, or (triangles) Sr²⁺. (B) FRET efficiency versus monovalent metal ion concentration for folding of the noncleavable wild-type construct in (squares) Na⁺ or (circles) NH₄⁺. Error bars for replicate measurements are within the symbols.

which cannot form the loop–loop tertiary interaction. Like the wild-type ribozyme, an increase in E_{FRET} was observed with increasing metal ion concentration for Ca^{2+} , Mg^{2+} , Mn^{2+} , and Sr^{2+} (open symbols in Fig. 2A), and all of these divalents again show comparable efficiencies for folding. However, higher concentrations of these metals are required to globally fold the control ribozyme compared to the wild-type ribozyme.

To more quantitatively compare folding with different metal ions, the FRET data were fit to a two-state folding equilibrium assuming fully cooperative metal ion binding (Equation 2 in Materials and Methods). As seen in Table 2, all the divalent metal ions have very similar values for both $M_{1/2}$, the apparent dissociation constant for the metal ion, and n , the Hill coefficient for metal-binding induced folding of the wild-type HHRz ($M_{1/2}$ ranges from 0.87 to 2.0 mM, and n ranges from 0.41 to 0.65). Furthermore, the divalent ions show a similar change in FRET efficiency (ΔE_{FRET} ranging from 0.22 to 0.25) upon folding, indicating that the global fold of the HHRz is comparable in all of these metals (Supplemental Table S1). Together these results show that the identity of the divalent metal ion has little influence on global folding of the HHRz.

The FRET-based folding data for the UUCG control ribozyme construct as a function of Ca^{2+} , Mg^{2+} , Mn^{2+} , or Sr^{2+} (Fig. 2A) were also fit to the two-state folding equilibrium (Eq. 2) and again showed small ranges of $M_{1/2}$ (5.2–12 mM) (Table 2), n (0.7–1.0) (Table 2), and ΔE_{FRET} (0.13–0.17) (Supplemental Table S1). The $M_{1/2}$ values for the UUCG control are fivefold to sixfold higher and the Hill coefficients are larger for the UUCG than the wild-type HHRz. The average ΔE_{FRET} for the UUCG control of 0.16 is lower than for the wild-type construct ($\Delta E_{\text{FRET}} \sim 0.25$), which is not unexpected given that the position of the donor fluorophore, Cy3, is altered in the UUCG loop (Fig. 1C).

To assess how the loop–loop interaction affects global folding in the presence of only monovalent cations, FRET studies were performed for both the wild-type (Fig. 2B) and UUCG control (data not shown) noncleavable HHRz

constructs as a function of Na^+ or NH_4^+ concentration. The FRET data for monovalent ions were fit to Equation 2, yielding $M_{1/2}$ values for the wild-type HHRz in Na^+ and NH_4^+ of ~ 170 mM and ~ 245 mM with Hill coefficients of 1.3 and 1.1, respectively (Table 2). The UUCG control HHRz showed higher $M_{1/2}$ values for both Na^+ and NH_4^+ of >500 mM and >800 mM and Hill coefficients of 1.0 and 1.1, respectively (Table 2). Thus, as observed for the divalent metals, the HHRz containing the loop–loop interaction requires considerably lower concentrations of monovalent metal ions to achieve its global fold.

Inactive populations in the *Schistosoma* HHRz

In addition to the very large changes in k_{obs} for the wild-type HHRz, the divalent metal ions show a variation in the fraction of substrate cleaved (ranging from 0.36 to 0.69) (Table 1). As previously reported, the *Schistosoma* HHRz has an inactive population that slowly converts to an active population (Canny et al. 2007). To determine if interconversion of inactive and active hammerhead species involves a change in global folding, the FRET of the noncleavable *Schistosoma* HHRz system was monitored over time. As seen in Figure 3A, the E_{FRET} values were essentially unchanged over 8 h in 1 mM Mg^{2+} ($<3\%$ change). On the other hand, kinetic experiments with a cleavable substrate show a substantial increase in the fraction of substrate cleaved (from 0.30 to 0.65), yielding an apparent $k_{\text{obs}} = 0.003 \text{ min}^{-1}$ over the same 8-h time period (Fig. 3B). The FRET data here only monitor global folding of the HHRz, i.e., stems I and II coming together, and may not be sensitive to local conformational changes. Thus, an inactive-to-active transition involving a local conformational change in the loop–loop tertiary interaction or in the catalytic core would not generally be detected by this assay.

To rule out multiple turnover ribozyme reactions being responsible for the apparent inactive-to-active conversion in the single-turnover kinetics experiments, pulse-chase nondenaturing gel experiments were performed to monitor the rate of substrate dissociation (Fedor and Uhlenbeck

TABLE 2. Metal dependence of global folding of the *Schistosoma* hammerhead ribozyme

Cation	Wild type		UUCG Control	
	$M_{1/2}$ (mM)	n	$M_{1/2}$ (mM)	n
Mn^{2+}	0.87 ± 0.5	0.41 ± 0.1	5.2 ± 1.3	0.7 ± 0.1
Mg^{2+}	1.2 ± 0.2	0.56 ± 0.02	6.2 ± 0.9	1.0 ± 0.1
Ca^{2+}	1.4 ± 0.2	0.57 ± 0.2	9.0 ± 0.7	0.9 ± 0.1
Sr^{2+}	2.0 ± 0.2	0.65 ± 0.02	12 ± 4	0.8 ± 0.1
Na^+	170 ± 15	1.3 ± 0.1	>500	1.0 ± 0.1
NH_4^+	245 ± 15	1.1 ± 0.1	>800	1.1 ± 0.1

$M_{1/2}$ represents the apparent metal ion dissociation equilibrium constant, n corresponds to the Hill coefficient, and the values were determined from the average of three or more experiments with errors of one standard deviation.

FIGURE 3. (A) Plot of FRET efficiency of the noncleavable wild-type *Schistosoma* HHRz FRET construct as a function of time in 1 mM Mg^{2+} . (B) Plot of fraction cleaved substrate as a function of time for the wild-type *Schistosoma* HHRz (chemically synthesized Cy3-labeled ribozyme strand and transcribed substrate strand) in 1 mM Mg^{2+} . The line represents the fit to Equation 1 and gives a k_{obs} of 0.003 min^{-1} .

1992). These experiments showed no evidence for dissociation of the noncleavable substrate strand from the transcribed or chemically synthesized ribozymes (see Supplemental Fig. S2). Thus, the slow increase in the fraction of substrate cleaved in Figure 3B is not due to substrate dissociating from an inactive ribozyme and binding to an active ribozyme in these single-turnover kinetic experiments. In addition, the ribozyme-noncleavable substrate complexes for both transcribed and chemically synthesized ribozymes show a single band in the nondenaturing gel, so there is no evidence for conformational heterogeneity in these complexes (Supplemental Fig. S2).

Cleavage activity with different divalent metal ions in a globally folded HHRz

Although divalent metal ions are normally required for efficient folding of RNAs, RNAs sometimes fold in high concentrations of monovalent ions (Draper 2004). To try to separate the effects of the identity of the divalent metal ions on global folding from the effects of the identity of the metal ion on cleavage activity, single-turnover cleavage experiments were performed in a reaction buffer containing 2 M NaCl and 1.0 mM Ca^{2+} , Mg^{2+} , or Mn^{2+} (Table 1). The FRET data in Figure 2B show that the wild-type *Schistosoma* HHRz is essentially fully folded in the presence of 2 M NaCl, so any variation in k_{obs} is not due to global folding. The k_{obs} in 2 M NaCl, 1.0 mM Mn^{2+} was too fast to measure manually at pH 7.0, so kinetic experiments were repeated with 0.15 mM Mn^{2+} at pH 6.0 yielding a k_{obs} of 3.5 min^{-1} . A linear dependence of k_{obs} on Mn^{2+} concentration and log-linear dependence with pH was observed over these ranges (data not shown), yielding a calculated k_{obs} of 230 min^{-1} for Mn^{2+} at 1 mM (pH 7.0). Thus, the

k_{obs} for Ca^{2+} , Mg^{2+} , and Mn^{2+} still vary by >4600-fold even in the globally folded HHRz (2 M NaCl) (Table 1). Furthermore, the fraction of substrate cleaved in a given metal is unchanged in both the 0.1 M and 2 M NaCl backgrounds (Table 1), indicating that higher concentrations of monovalent ions do not affect the populations of rapidly cleaving ribozymes.

DISCUSSION

Metal ions are important for catalytic activity of the HHRz (Dahm and Uhlenbeck 1991; Murray et al. 1998). Previous studies on a minimal hammerhead system required higher than physiological divalent metal ion concentrations for efficient catalysis (Hertel et al. 1994).

More recently, it was shown that formation of a tertiary interaction distant from the catalytic core of the HHRz allows for efficient cleavage and ligation at physiological Mg^{2+} concentrations (De la Pena et al. 2003; Khvorova et al. 2003). Although the size and specific sequence of this tertiary interaction varies, all of the natural hammerhead systems have the potential to form a tertiary interaction between loop motifs on stems I and II (Khvorova et al. 2003; Przybiski et al. 2005). One model for how inclusion of these additional motifs generates faster kinetics is that there is a rapid equilibrium between highly active and low or inactive species in the HHRz and that formation of the tertiary interaction leads to a higher population of catalytically active molecules (Khvorova et al. 2003; Martick and Scott 2006; Nelson and Uhlenbeck 2006). The effects of the identity of the metal ions on both the cleavage kinetics and the global folding of the extended *Schistosoma* HHRz are studied here.

The identity of the metal ion has a very large (>10,000-fold) effect on the k_{obs} for cleavage in the *Schistosoma* HHRz, where the divalent ions follow the trend of $Mn^{2+} > Co^{2+} > Zn^{2+} \approx Cd^{2+} > Mg^{2+} > Ca^{2+} > Sr^{2+}$ (at 1 mM metal ion, 50 mM Tris, 0.1 M NaCl, pH 7, and 25°C) (see Table 1). Previous studies on the *Schistosoma* HHRz showed an ~100-fold difference in k_{obs} for Mg^{2+} and Mn^{2+} (Osborne et al. 2005), similar to the results here. Except for Cd^{2+} , this trend is the same as what was previously observed for a SELEX-derived rapidly cleaving HHRz with a stable hairpin loop-internal bulge tertiary interaction, which had k_{obs} of $Mn^{2+} > Cd^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+} > Sr^{2+}$ (at 1 mM metal ion, 50 mM Tris, pH 7.4, and 37°C) (Roychowdhury-Saha and Burke 2006). A somewhat different trend for metal ions was previously observed for a minimal HHRz with k_{obs} of $Mn^{2+} \approx Cd^{2+} > Mg^{2+} > Co^{2+} \approx Zn^{2+} > Ca^{2+} > Sr^{2+}$ (at 2 mM metal ion,

50 mM Tris, 0.5 mM spermine, pH 7.5, and 25°C) (Dahm et al. 1993). Interestingly, the identity of the divalent metal ion has a much larger effect on the cleavage rates in the extended HHRz compared with the minimal HHRz. Only a relatively small effect on k_{obs} (~ 25 -fold change) was observed for the various divalent metals in the minimal HHRz (Dahm et al. 1993), whereas a much larger effect in k_{obs} was observed for the same divalent metals in the *Schistosoma* HHRz ($>10,000$ -fold change) and the tertiary-stabilized SELEX-derived HHRz (>5000 -fold change) (Roychowdhury-Saha and Burke 2006). Thus, although these three hammerhead systems all show the fastest k_{obs} with Mn^{2+} , intermediate k_{obs} with Mg^{2+} , and slower cleavage with Ca^{2+} and Sr^{2+} , inclusion of the loop-loop tertiary interaction leads to a much higher sensitivity of k_{obs} to the identity of the divalent metal ion.

FRET was used here to monitor global folding of the HHRz to examine if the large variations in k_{obs} with different divalent ions arise from different efficiencies of global folding. In contrast to the cleavage kinetics, the FRET data in Figure 2A unambiguously show that the identity of the divalent ion has little effect on the global folding of the *Schistosoma* HHRz. This is confirmed by fitting the data to Equation 2, which shows that there are small ranges for both the apparent dissociation constants for metal binding, $M_{1/2}$, and the Hill coefficients for the different divalent ions (Table 2). As previously discussed (Misra and Draper 2002), the two-state model used to derive Equation 2 involves multiple assumptions for metal-induced folding of RNA, and these assumptions may not be valid for folding of the HHRz. Therefore, although the data in Table 2 can be used to compare the overall effects that different metal ions have on folding, they cannot be used to probe the mechanism of folding this RNA. Independent of whether the folding of the HHRz is well described by the two-state model (or follows a more complex pathway), the raw FRET data in Figure 2A clearly demonstrate that global folding of the *Schistosoma* HHRz is independent of the identity of the divalent ion. It is important to note that this FRET assay only probes the proximity of stems I and II and does not give information on formation of the specific tertiary interaction observed in the X-ray structure of the *Schistosoma* HHRz (Martick and Scott 2006). Thus, the divalent metals could be differentially stabilizing a local conformational change required for efficient cleavage or could be stabilizing slightly different local structures, which then result in the large variation in k_{obs} . The UUCG control HHRz, which lacks the tertiary interaction, requires higher concentrations of divalent metal ions to globally fold than the wild-type HHRz (approximately sixfold higher) (Table 2), but folding is also independent of the identity of the divalent metal ion (Fig. 2A).

Previous studies showed that the minimal and extended constructs of the sTRSV HHRz have similar k_{obs} in 4 M Li^+ , from which it was proposed that the loop-loop

interaction may not be able to form in the presence of only monovalent cations (Nelson et al. 2005). Cleavage was measured here for the *Schistosoma* HHRz in the presence of 600 mM Na^+ or NH_4^+ and yielded an upper limit on k_{obs} of $<0.02 \text{ min}^{-1}$. The FRET studies show evidence for global folding of the *Schistosoma* HHRz with monovalent ions, consistent with previous folding studies on this ribozyme (Penedo et al. 2004). As seen in Table 2, the wild-type *Schistosoma* HHRz globally folds at threefold lower metal ion concentration than the UUCG control for both Na^+ and NH_4^+ . However, the FRET experiments cannot determine whether the molecules are forming the correct tertiary interaction required for cleavage or whether the monovalent ions simply reduce electrostatic repulsion between stems I and II.

Various biochemical and structural studies have provided strong evidence for metal ion-binding site(s) within the core of the hammerhead ribozyme (for reviews, see Feig and Uhlenbeck 1999; Hoogstraten and Sumita 2007). Cd^{2+} rescue experiments led to the prediction that in the transition state a single metal ion coordinates the phosphate of A9 and N7 of G10.1 and the phosphate at the cleavage site in the transition state (Wang et al. 1999). This prediction was essentially validated in the X-ray structure of the extended HHRz (Martick and Scott 2006), where the phosphate oxygens at A9 and the cleavage site C1.1 are separated by 4.3 Å and thus are poised to bind a single metal (Wang et al. 1999). A subsequent X-ray structure of the *Schistosoma* HHRz (Martick et al. 2008) showed a Mn^{2+} ion with inner sphere coordination to both the phosphate oxygen of A9 and the N7 of G10.1. Although no bridging metal ion was observed between the A9 and C1.1 phosphates, these phosphates are still separated by only 4.3 Å. EPR studies also showed evidence for a single Mn^{2+} ion-binding site with $\sim 10 \mu\text{M}$ affinity in the minimal HHRz at 1 M NaCl (Horton et al. 1998). A follow-up study identified this as the Mn^{2+} coordinated to the A9 phosphate and the N7 of G10.1 (Vogt et al. 2006). Furthermore, an EPR study on an extended HHRz (Kisseleva et al. 2005) showed a single Mn^{2+} binding site with even higher affinity, where the binding affinity for the Mn^{2+} has increased by >100 -fold compared to the minimal HHRz ($K_d < 10 \text{ nM}$ for Mn^{2+} binding in 0.1 M NaCl). These results demonstrate that the loop-loop interaction of the extended HHRz dramatically increases the affinity for binding of a Mn^{2+} ion. Achieving such high affinity ($K_d < 10 \text{ nM}$) likely requires a highly optimized metal-binding site in the RNA, and such a site could also show high metal ion selectivity, potentially resulting in the large variation in k_{obs} measured here for different metal ions.

To try to understand what property of the divalent metal ion gives rise to the changes in the k_{obs} for the *Schistosoma* HHRz, Figure 4 shows plots of $\log(k_{\text{obs}})$ versus $\text{p}K_a$ of the hydrated metal ion, ionic radius, or absolute hardness of the metal ion. The $\text{p}K_a$ plot shows the best correlation for

FIGURE 4. (A) Plot of the pK_a of the hydrated metal ion as a function of the $\log(k_{\text{obs}})$ for the *Schistosoma* HHRz in 1 mM metal ion, 50 mM Tris, 0.1 M NaCl (pH 7.0). The line represents the best fit between $\log(k_{\text{obs}})$ and pK_a for the *Schistosoma* HHRz (slope = -0.96 , $R = 0.93$). (B) Plot of the ionic radius of the metal ion as a function of $\log(k_{\text{obs}})$ for the *Schistosoma* HHRz. (C) Plot of the absolute hardness of the metal ion as a function of $\log(k_{\text{obs}})$ for the *Schistosoma* HHRz. The values for pK_a , ionic radius, and absolute hardness are from Feig and Uhlenbeck (1999).

all the metals. There is a good linear correlation for pK_a versus $\log(k_{\text{obs}})$ for the *Schistosoma* HHRz ($R = 0.93$) (Fig. 4A) with a slope of approximately -1 . A similar linear correlation of $\log(k_{\text{obs}})$ and pK_a was previously observed for the SELEX-derived tertiary-stabilized HHRz (Roychowdhury-Saha and Burke 2006). Although this trend could arise from a metal hydroxide acting directly as the general base for the 2'-OH at the cleavage site for the HHRz (Dahm et al. 1993), there are various lines of evidence that this is not the mechanism of action of metal ions in the HHRz. Multiple studies have shown that the minimal HHRz efficiently cleaves in high concentrations of monovalent metal ions (Murray et al. 1998; Curtis and Bartel 2001; O'Rear et al. 2001; Takagi et al. 2004). The monovalent ions have very high pK_a values, strongly suggesting that the metal hydroxide does not directly act as the general base for the 2'-OH in the cleavage reaction (Murray et al. 1998; Curtis and Bartel 2001). One possibility for how the pK_a of the divalent metal (or related parameter such as ionic radius) could effect the k_{obs} is if there is direct inner-sphere metal binding (Pontius et al. 1997) to the 2'-O of G8, where, as previously proposed, this hydroxyl then acts as a general acid in the catalytic mechanism of the HHRz (Martick and Scott 2006).

There are several examples where the ionic radius of a metal ion has been shown to correlate with the binding affinity of metals to an RNA (Bukhman and Draper 1997; Feig et al. 1999; Curtis and Bartel 2001), so the $\log(k_{\text{obs}})$ for the *Schistosoma* HHRz was plotted as function of ionic radius (Fig. 4B). Except for Mg^{2+} , there is a good linear correlation of $\log(k_{\text{obs}})$ with ionic radii ($R = 0.92$). Mg^{2+} fits very poorly in this trend, where it has much slower kinetics than predicted from its ionic radius. A log-linear relationship between k_{obs} and the ionic radius of Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+ was observed in the minimal HHRz

(Curtis and Bartel 2001) from which it was interpreted that these metals differentially stabilize the catalytically active structure. Furthermore, a specific metal-binding site on G5 in the minimal HHRz also showed a log-linear correlation between binding affinity and the ionic radius for a series of divalent metals (Feig et al. 1999). One explanation for why Mg^{2+} fits so poorly in Figure 4B is that although it has an ionic radius similar to some of the transition metals, Mg^{2+} is very "hard" compared to these metals. This is illustrated in Figure 4C, which shows that for the *Schistosoma* HHRz there is a good linear correlation (again except for Mg^{2+}) between $\log(k_{\text{obs}})$ and the absolute hardness (Feig and Uhlenbeck 1999) of the divalent metal ($R = 0.98$). The N7 of purine preferentially coordinates soft versus hard metals, so the data in Figure 4, B and C, can be qualitatively rationalized if there is a critical metal-binding site that coordinates to a purine N7 in the extended HHRz. Therefore, the various divalent metal ions could differentially stabilize an active (or inactive) conformation within the core, leading to the large differences in the k_{obs} .

To test whether the large differences in k_{obs} in the *Schistosoma* HHRz could be eliminated by using monovalent ions to replace a structurally important metal-binding site, the k_{obs} was measured in 2 M NaCl and 1 mM Ca^{2+} , Mg^{2+} , or Mn^{2+} ions. As seen in Figure 2B, 2M NaCl results in global folding of the HHRz, and this high concentration of monovalent ions can also compete with binding of other divalent metals ions (Draper 2004). Paralleling what was observed in 0.1 M NaCl, there is still a very large variation in k_{obs} for the divalent metals (>4600 -fold) (Table 2). Such large differences for these divalent metals contrast with what was previously observed for two structurally important divalent metal-binding sites in the P4-P6 domain of the *Tetrahymena* group I intron (Travers et al. 2007). In that study, the metals Ca^{2+} , Mg^{2+} , and Mn^{2+}

showed only a low level of specificity for two well-defined divalent metal ion-binding sites in 2M NaCl (less than sixfold variation in $M_{1/2}$ for these metals). Thus the catalytic activity of the extended HHRz is much more sensitive to a specific property of the divalent metal ion (e.g., pK_a , ionic radius or coordination geometry) than metal binding in this P4–P6 domain.

There are several examples of metal-binding sites that also show high selectivity for the identity of the divalent metal ion in a pre-folded RNA. For example, a metal-binding site at G5 in the minimal HHRz shows moderate selectivity (~ 250 -fold) for different divalent metal ions in a background of 10 mM Mg^{2+} (Feig et al. 1999). Another example is a 58-nt fragment of the rRNA, where tertiary folding was measured as a function of Mg^{2+} , Ca^{2+} , Sr^{2+} , and Ba^{2+} in 1.6 M NH_4Cl (Bukhman and Draper 1997). These metals showed a 60-fold variation in their apparent binding affinities, and there was a log-linear relationship between binding affinities and ionic radius of the metal. There are also examples of larger ribozymes where certain divalent ions are required for catalytic activity. The *Tetrahymena* group I ribozyme has a strict requirement for the divalents Mn^{2+} or Mg^{2+} (Grosshans and Cech 1989). The *Escherichia coli* RNase P RNA is active with Mg^{2+} and Mn^{2+} in 1 M NaCl and still shows substantial activity with Ca^{2+} (Smith et al. 1992). However, with the exception of the *Tetrahymena* group I ribozyme, the *Schistosoma* HHRz has a higher level of divalent metal ion selectivity (>4600 -fold for Ca^{2+} compared to Mn^{2+} in 2 M NaCl) than exhibited by other catalytic RNAs.

The results here demonstrate that the changes in k_{obs} in the *Schistosoma* HHRz with different metal ions do not arise from differential effects on global folding, defined here as stems I and II coming together. Potential explanations for the variation in k_{obs} with metal ion identity include: (1) there is a structurally important metal-binding site required for catalysis that is very sensitive to the identity (i.e., properties) of the divalent ion; (2) there is a rapid equilibrium between a catalytically active and inactive species for the HHRz (Khvorova et al. 2003; Nelson and Uhlenbeck 2007) and the various divalent metal ions differentially affect the populations and/or conformations of these species; or (3) there is a specific, selective metal-binding site that helps to stabilize the negative charge in the transition state. Previous studies on the minimal hammerhead comparing kinetics of mutants/modifications in 10 mM Mg^{2+} and 4 M Li^+ concluded that divalent metal ions played only a small direct chemical role in catalysis (O'Rear et al. 2001). However, divalent metal ions may play a larger direct chemical role for divalents in the extended than the minimal HHRz. One candidate for a structural (or catalytic) metal is the Mn^{2+} ion that coordinates to the nonbridging R_p oxygen of A9 and the N7 of G10.1 in the X-ray structures of both the minimal and extended HHRzs (Pley et al. 1994; Martick et al. 2008). This metal is close to

the active site in the X-ray structure of the *Schistosoma* HHRz (Martick et al. 2008) and could help to stabilize the negative charge in the transition state, so differential affinities, or a slightly different coordination/conformation, of the metal ions in this site could alter the catalytic activity.

MATERIALS AND METHODS

Sample preparation

The ribozyme and substrate RNA oligonucleotides used in the cleavage studies were prepared by in vitro transcription with T7 RNA polymerase from synthetic DNA templates (Integrated DNA Technologies) and purified using 20% denaturing polyacrylamide gel electrophoresis as described (Milligan and Uhlenbeck 1989). To remove trace amounts of divalent metal ions, the RNAs were heated to 90°C in 50 mM Tris, 100 mM NaCl, and 10 mM EDTA (pH 7) and cooled to room temperature. The concentration of EDTA was then reduced by multiple rounds of buffer exchange using a 3K Microcon centrifugal device (Millipore) into reaction buffer (50 mM Tris, 100 mM NaCl, 0.1 mM EDTA at pH 7.0). Prior to use, the reaction buffer was run over a chelating resin (Chelex; Sigma) to remove any trace divalent metal ions. The substrate strand (Fig. 1A) was labeled with either fluorescein at the 3'-end (Qin and Pyle 1999) or with a ^{32}P phosphate group at the 5'-end using T4 polynucleotide kinase and buffer exchanged as described above. To prevent oxidation, the metal ion solutions were prepared by diluting stock solutions into the reaction buffer, where the stock solutions were kept at $-20^\circ C$ and low pH until ready for use. The stock solutions of metal ions were examined for contaminating metals by inductively coupled plasma optical emission spectroscopy. The RNAs used in FRET studies (Fig. 1B,C) were chemically synthesized (Dharmacon) with a 5-amino-allyl modification on the C5 of an internal U in the ribozyme strand and a 2'-deoxyribose at the cleavage site and a six-atom amino linker on the 3'-end of the substrate strand. The Cy3 (donor) or Cy5 (acceptor) N-succinimidyl ester fluorophores (Amersham Biosciences) were coupled to the RNA and purified as previously described (Downey et al. 2006) and then exchanged into reaction buffer as described above.

Single-turnover cleavage experiments on the *Schistosoma* HHRz

The observed rate constants for cleavage, k_{obs} , for the *Schistosoma* HHRz were measured under single turnover conditions in the presence of various metal ions, using the cleavable construct in Figure 1A. A 10:1 ratio of ribozyme strand to either fluorescein-labeled or ^{32}P -labeled substrate strand was annealed in the reaction buffer by heating for 2 min to 70°C and slowly cooling to 25°C in a thermocycler (Eppendorf). The final concentration of ribozyme strand was 0.5–1 μM . All kinetic experiments were performed at 25°C.

In manual experiments, the cleavage reaction was initiated by adding 90 μL of the annealed ribozyme-substrate complex to 10 μL of the appropriate concentration of metal ion in reaction buffer. Ten-microliter aliquots were removed at various time points and quenched by addition of 10 μL of 80% formamide and

25 mM EDTA. For metals that had too rapid cleavage kinetics to be measured by manual methods (Mn^{2+} , Co^{2+} , Zn^{2+} , Cd^{2+} , and Mg^{2+}), a Kin-Tek quench-flow model RQF-3 instrument was used. In these experiments, 15 μL of annealed hammerhead complex was rapidly mixed (2 msec dead-time) with 15 μL of two times the appropriate concentration of metal ion in reaction buffer followed by a rapid quench with 90 μL of solution containing 80% formamide and 25 mM EDTA. To separate cleaved and uncleaved substrate strands, the quenched samples were boiled for 1 min at 90°C and loaded on a 20% denaturing polyacrylamide gel. The gels were scanned for either fluorescence or ^{32}P exposure using a Typhoon Imager (Amersham Biosciences), and the fraction of cleaved substrate was quantified using ImageQuant software (Molecular Dynamics). These data were then fit to Equation 1 using the programs Excel (Microsoft) or KaleidaGraph (Synergy):

$$F_{\text{cl}}(t) = f_i + \Delta f(1 - e^{-k_{\text{obs}}t}) \quad (1)$$

where $F_{\text{cl}}(t)$ is the fraction cleaved at time t , f_i is the initial fraction cleaved at $t = 0$, Δf is the change in the fraction cleaved between $t = 0$ and $t = \infty$, and k_{obs} is the observed rate constant for cleavage. The k_{obs} represents an approach to equilibrium and therefore is the sum of the cleavage and ligation rate constants. The parameters f_i , Δf , and k_{obs} were varied to give the best fit to the experimental data.

Substrate dissociation assay

Pulse-chase experiments were used to test for substrate dissociation from the transcribed and chemically synthesized ribozyme strands (Fedor and Uhlenbeck 1992). The 0.5 μM ribozyme strand and trace ($<0.05 \mu\text{M}$) ^{32}P -labeled noncleavable substrate strand were annealed as described above. The annealed hammerheads were incubated in reaction buffer containing 1 mM Mg^{2+} for 30 min at room temperature. Then a 75 μM (2 μL) unlabeled noncleavable substrate strand was added to 2- μL aliquots of annealed HHRz complex to initiate the chase reactions, which were then incubated for various times before loading on the gel. One microliter of 30% glucose was added to each sample as loading buffer, including 2- μL aliquots of chase-free complex and substrate-only solutions, and the samples were loaded on a 12% nondenaturing gel containing 1 mM Mg^{2+} . The radioactive bands were identified using PhosphorImager analysis as described above.

Design of *Schistosoma* HHRz constructs for probing global folding by FRET

FRET was used here to monitor global folding and formation of the loop-loop tertiary interaction in the *Schistosoma* hammerhead ribozyme-substrate complex. Various constructs with a fluorophore-modified U residue in stem II or in loop II were tested for cleavage activity. Single-turnover cleavage kinetics in 1 mM Mg^{2+} were measured on these modified ribozymes, and only the construct with the Cy3 fluorophore on the U in loop II (Fig. 1B) showed the same k_{obs} for cleavage as the unmodified, unlabeled construct (data not shown). Modeling of this complex using the X-ray structure of the *Schistosoma* HHRz (Martick and Scott 2006) indicates that the donor Cy3-fluorophore in loop II

and the acceptor Cy5-fluorophore on the 3'-end of the substrate (stem I) are separated by $\sim 30 \text{ \AA}$ in the docked complex, which is well below the Förster radius for this FRET pair (Sabanayagam et al. 2005). To separate folding from the effects of catalytic activity, the FRET experiments were performed using a noncleavable substrate that has a 2'-deoxyribose at the cleavage site (see Fig. 1B).

A control construct where loop II was substituted with a UUCG tetraloop was used to test the importance of the loop-loop interaction on the global folding of the extended HHRz. For the FRET studies, a Cy3 fluorophore was coupled to a C5-modified U residue in the second position in the UUCG tetraloop (Fig. 1C). This control ribozyme strand was annealed to the same noncleavable substrate that was used with the wild-type ribozyme strand above.

Although the measured k_{obs} for cleavage was the same for the chemically synthesized Cy3-modified ribozyme and the in vitro transcribed ribozyme, these two ribozymes showed differences in the fraction of cleaved substrate in single-turnover cleavage experiments. In 1 mM Mg^{2+} , the constructs have plateaus of 0.40 and 0.70 fraction of cleaved substrate after 10 min for the chemically synthesized and transcribed ribozyme strands, respectively; and plateaus of 0.60 and 0.85 fraction of cleaved substrate after 24 h (data not shown). A chemically synthesized ribozyme strand without the C5-modified U also showed the lower fraction cleaved, thus the chemically synthesized ribozymes have a higher inactive (or slowly cleaving) population than their transcribed counterpart. RNAs often form inactive populations, and previous studies of the *Schistosoma* HHRz showed an $\sim 30\%$ "inactive" population for transcribed RNAs, where this "inactive" population converts slowly to an active species (Canny et al. 2007). Thus, the hammerhead constructs used here for the folding experiments have a somewhat higher population of catalytically "inactive" species than the constructs employed in the kinetic studies; however, both constructs show the same k_{obs} for cleavage for the rapidly cleaving population.

Metal-induced folding of the *Schistosoma* HHRz as probed by FRET

Stock solutions of *Schistosoma* HHRz for ensemble FRET measurements were prepared by annealing equal volumes of 1 μM Cy3-labeled ribozyme strand with 1.1 μM Cy5-labeled substrate strand (Fig. 1B,C) in reaction buffer by heating for 2 min to 70°C and slowly cooling to 25°C in a thermocycler. These stock solutions were diluted to $\sim 25 \text{ nM}$ in 0.5 mL, and fluorescence measurements were performed on a Photon Technology International spectrofluorometer at 25°C. The donor fluorophore was excited at 500 nm, and emission was measured at both 560 nm (donor emission) and 660 nm (acceptor emission) with slit widths for the emission and excitation monochromators of 4 mm and 8 mm, respectively. A total of 20 pts/sec for 20 sec at each emission wavelength was recorded and averaged for each metal ion concentration. The FRET efficiency, E_{FRET} , was calculated as $I_A / (I_A + I_D)$, where I_A is the average acceptor intensity and I_D is the average donor intensity.

Metal ion titrations were performed by adding 2–10 μL of a concentrated (25 or 250 mM) metal ion solution in reaction buffer to a 0.5 mL sample of the hammerhead complex. The E_{FRET} values were determined for each metal ion concentration after correcting for dilution effects and were fit to Equation 2, which

assumes a two-state metal-induced folding model consisting of an equilibrium between unfolded and folded states:

$$E_{\text{FRET}} = E_i + \Delta E_{\text{FRET}} \left(\frac{[M^{z+}]^n}{((M_{1/2})^n + [M^{z+}]^n)} \right) \quad (2)$$

where E_i is the FRET efficiency in the reaction buffer (no added metals ions), ΔE_{FRET} is the overall change in FRET efficiency induced between no metal ion added and infinite metal ion concentration, $M_{1/2}$ is the apparent dissociation constant for the metal ion, n is the Hill coefficient, $[M^{z+}]$ is the metal ion concentration, and z is the charge of the metal ion used in the titration. The parameters E_i , ΔE_{FRET} , $M_{1/2}$, and n were varied to give the best fit to the experimental data.

To determine if metal ions lead to the quenching of the fluorophores independent of FRET, experiments were performed where only the Cy3 (donor) or Cy5 (acceptor) dyes were incorporated into the hammerhead complex, and the fluorescence was monitored as a function of metal ion concentration (data not shown). For Mg^{2+} , Ca^{2+} , and Sr^{2+} , there was no significant change in the fluorescence intensity of the donor-only or acceptor-only constructs as a function of metal ion concentration. However, at higher concentrations of Mn^{2+} (>10 mM), for both the donor-only and acceptor-only constructs, there was a linear reduction in the fluorescence intensity with Mn^{2+} concentration, with 10% quenching of original fluorescence at the highest concentration (~60 mM). Therefore, when measuring the fluorescence of the HHRz in the presence of Mn^{2+} , a correction was applied based on the linear relationship between fluorophore quenching and Mn^{2+} concentration.

SUPPLEMENTAL DATA

Supplemental material can be found at <http://www.rnajournal.org>.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute of Health (AI 30726) and the W.M. Keck Foundation initiative in RNA science at the University of Colorado, Boulder. We thank Drs. Robert Batey, Chris Downey, Dan Herschlag, Robert Kuchta, Michael Latham, Marcelo Sousa, and Olke Uhlenbeck for valuable discussions.

Received January 23, 2008; accepted July 2, 2008.

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RNA 2008 14: 2212-2222 originally published online August 28, 2008

Access the most recent version at doi:[10.1261/rna.1010808](https://doi.org/10.1261/rna.1010808)

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