Published in final edited form as:
J Am Chem Soc. 2005 October 19; 127(41): 14134–14135. doi:10.1021/ja0541027.

Separate Metal Requirements for Loop Interactions and Catalysis in the Extended Hammerhead Ribozyme

Nak-Kyoon Kim, **Ayaluru Murali**, and **Victoria J. DeRose***
Department of Chemistry, Texas A&M University, College Station, Texas 77843

RNA function, including catalysis, depends strongly on ionic conditions.[1,2] In many cases, both structure and catalysis are most sensitive to divalent cations, where the physiologically relevant ion is assumed to be Mg^{2+}. A challenge in understanding Mg^{2+}-dependent RNA catalysis has been to deconvolute metal-induced structural transitions from the direct contributions of metals to catalysis. Here, we are able to separate one such contribution in the hammerhead ribozyme by using site-directed spin labeling (SDSL) in combination with fast-quench kinetics.

The hammerhead ribozyme (HHRz) is a small self-cleaving RNA motif which catalyzes a site-specific transesterification reaction.[3] This ribozyme is found in plant virus satellite RNAs, viroids, and other higher organisms, and is implicated in replication of circular RNA genomes.[4] HHRzs have long been studied as truncated, minimal, trans-cleaving motifs that include a catalytically essential conserved core of nucleotides at a junction of three short helices (Figure 1A).[3] X-ray crystal structures of these truncated motifs showed a Y-shaped conformation with stem I in proximity to stem II.[5] Many biophysical and biochemical studies have proposed similar global structures, but in solution, HHRz folding is highly dependent on Mg^{2+} concentrations.[6] Catalysis is also very sensitive to the concentration and type of cation present.[2,3]

For the truncated HHRz motifs, millimolar concentrations of Mg^{2+} are required for activity.[2,3] Recently, it has been found that extended HHRz motifs with the addition of two loops that interact between stems I and II have enhanced cleavage activity at lower concentrations of Mg^{2+}.[7–11] FRET measurements have predicted that a loop-carrying HHRz from the *Schistosome* performs onestep folding with half-maximum $[Mg^{2+}]_{1/2}$ = 160 μM for the Mg^{2+}-dependent step.[9] Canny and co-workers have measured Mg^{2+}-dependent cleavage activity in an extended HHRz using rapid-quench techniques and report a much weaker apparent affinity ($[Mg^{2+}]_{1/2}$ = 40 mM, 0.1 M NaCl) corresponding to the cleavage reaction.[9] In the latter study, maximum HHRz rates above 800 min^{-1} were predicted (pH 8.5, 25 °C), which is a breakthrough in HHRz research since this ribozyme has exhibited relatively slow kinetics in its much-studied truncated form. Combining these two independent experiments, a working hypothesis is that the loop-containing HHRz folds into the active form with the aid of inter-loop interactions at low Mg^{2+} concentrations and further rearranges to a more active structure with increasing metal concentration.[9]

In this study, a HHRz derived from *Schistosoma mansoni*, including conserved loops in stems I and II (Figure 1A),[10] has been examined to directly monitor the relationship between

derose@mail.chem.tamu.edu.

Supporting Information Available: Detailed materials, methods, kinetic data, and EPR simulation parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

docking of loops and its activity using SDSL and EPR spectroscopy as a probe of local conformational changes. SDSL has recently been applied to RNA structural biology through both dynamics and distance measurements, where these studies have shown that SDSL can provide a novel type of RNA structural probe.[11] Here, nitroxide spin labels were attached to 2′-NH_2 substitutions in the HHRz substrate strands (Figure 1A (red), Supporting Information) and the dynamics of the spin labels monitored as a function of added Mg^{2+}. The cleavage site at C_{17} is protected by a 2′-OMe modification for the EPR measurements. Three lines are observed in the EPR spectra of the singly labeled substrates, originating from the hyperfine coupling between the nitroxide radical and the I = 1 ^{13}N nucleus, with lineshapes that reflect the local environment of the spin label (Figure 1C). As the Mg^{2+} concentration is increased in a background of 0.1 M NaCl, the spectral lines broaden, and even additional features are observed at higher and lower magnetic field, which is typical when the motion of the spin label is restricted due to steric inhibition (Figure 1C(c)). Addition of Mg^{2+} causes broadening of the spectra obtained for HHRz labels at positions $U_{1.12}$ and $C_{1.9}$. For a spin label at position $U_{1.6}$, however, added Mg^{2+} induces a distinctive splitting of the spectra. Importantly, these stem I labels have little effect on ribozyme activity (see below).

The spectral changes observed for the $U_{1.6}$ spin label are assigned to docking of stems I and II through several lines of evidence. A HHRz construct with global loop II substitutions (the U-loop containing enzyme, Figure 1A) has distinctively reduced activity that is presumably due to only nonspecific interactions between two loops (Figure 2B inset).[10] With increasing Mg^{2+}, the $U_{1.6}$ label in the U-loop HHRz shows mild spectral line broadening, but there is no evidence for a second spin label site having restricted motion (Figure 1C(d)). To further confirm that the spectral changes at $U_{1.6}$ originate only from the folding of the HHRz, and not from Mg^{2+}-induced changes in the isolated loop I, a spin-labeled duplex mimicking stem I has been tested (Figure 1B). As shown in Figure 1C(e), almost no spectral changes occur with added Mg^{2+} for the spin label attached to loop I in the absence of the rest of the HHRz. These data indicate that only intramolecular tertiary interactions in the ribozyme influence the mobility of the spin labels. Finally, Heckman and co-workers observed a photo-cross-linking interaction between $U_{1.6}$ and $U_{L2.5}$ at 20 mM Mg^{2+} (desalted, pH 8) in a *Schistosoma* hammerhead with identical loop sequences,[12] indicating that $U_{1.6}$ may participate in the docking interaction and consistent with the EPR spectra.

The EPR spectral changes from the HHRz $U_{1.6}$ label, assigned to docking of stems I and II, are saturated at low [Mg^{2+}] (Figure 1C(c)). These spectra can be fit to a simple model consisting of two different sites for the $U_{1.6}$ label that differ in mobility (Supporting Information).[13] Fitting the Mg^{2+}-dependent spectra to populations of these two sites, the half-maximum concentration of Mg^{2+} for complete docking of the loops is obtained (Figure 2A, Supporting Information). The fit shows that Mg^{2+}-dependent docking occurs at low concentrations of Mg^{2+} with a $[Mg^{2+}]_{1/2,dock}$ = 0.7 mM (0.1 M NaCl).

To compare this structural transition with the activity of this ribozyme, fast cleavage kinetics have also been measured for the $U_{1.6}$ spin-labeled ribozyme using rapid-quench flow methods (Figure 2B, Supporting Information). The effect of the spin-labeling modification on the activity of HHRz is not significant (e.g., $k_{obs,slHHRz}$ = 1.5 min^{-1}, $k_{obs,wtHHRz}$ = 5.5 min^{-1},[10] at 1 mM Mg^{2+}, 0.1 M NaCl, and pH 7). The single turnover rate constant increases dramatically with added Mg^{2+} to a value of 28 min^{-1} at the highest concentration measured of 100 mM Mg^{2+} and a predicted maximum value of 54 min^{-1} (pH 7.0, 0.1 M NaCl) at saturating Mg^{2+}.[15] As recently reported,[9] these fast rates are unprecedented for “loopless” HHRzs, which generally have maximum rate constants of <5 min^{-1} at saturating Mg^{2+}. A rough fit of the kinetic data for the extended HHRz results in an apparent $[Mg^{2+}]_{1/2,cat}$ ~ 90 mM (0.1 M NaCl), indicating the influence of a Mg^{2+} interaction on activity that is 130

times weaker than that required for docking of the two loops. Thus, the fast rates in the HHRz require loop docking and also an additional cation-dependent event.

The $[Mg^{2+}]_{1/2}$ that is correlated here with loop docking may correspond to population of a metal site that was previously characterized in truncated HHRzs and consists of ligands from the A_9 phosphate oxygen and $G_{10.1}$ N_7. Phosphorothioate substitutions[10] and EPR measurements[15] support maintenance of this site in the loop-carrying HHRz construct of Figure 1, but further work is required to link it to HHRz docking. The nature of the much weaker Mg^{2+} interactions that support activity remains unknown, but this influence may be monitored in future experiments with site-specific structural probes located closer to the HHRz catalytic core.

In summary, the combination of SDSL and fast-quench kinetic measurements on the newly extended HHRz motif has shown that the docking of stems I and II occurs at low Mg^{2+} concentrations ($[Mg^{2+}]_{1/2,dock}$ = 0.7 mM, 0.1 M NaCl), but a much weaker Mg^{2+} interaction ($[Mg^{2+}]_{1/2,cat}$ ~ 90 mM) increases activity to very high maximum rates of ~1 s^{-1}. This situation is reminiscent of that found in the ribonucleoprotein ribozyme, RNase P, where RNA catalysis in the absence of protein requires very high cation concentrations. In the case of RNase P, addition of the protein component significantly reduces the Mg^{2+} concentration needed for chemistry.[16] At physiological Mg^{2+} concentrations of ~1 mM, catalysis in the HHRz motif of Figure 1 exceeds 1 min^{-1}, but is far below its capacity. While perhaps this rate is sufficient for biological purposes, the possibility remains of an additional and as-yet undiscovered structural element, either RNA or protein, that provides electrostatic support to the HHRz core.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (GM 58096) and NSF (CHE-0111696). Texas A&M University EPR facilities are supported by the NSF (CHE-0092010). We thank Seongho Oh for synthesis of the 4-isocyanato TEMPO, and the NIH ACERT RR (Cornell) for simulation programs.

References

1. (a) Draper DE. RNA. 2004; 10:335–343. [PubMed: 14970378] (b) Fedor MJ. Curr Opin Struct Biol. 2002; 12:289–295. [PubMed: 12127446] (c) Pyle AM. J Biol Inog Chem. 2002; 7:679–690.
2. DeRose VJ. Curr Opin Struct Biol. 2003; 13:317–324. [PubMed: 12831882]
3. Blount KF, Uhlenbeck OC. Annu Rev Biophys Biomol Struct. 2005; 34:415–440. [PubMed: 15869397]
4. (a) Symons RH. Annu Rev Biochem. 1992; 61:641–671. [PubMed: 1497321] (b) Ferbeyre G, Smith JM, Cedergren R. Mol Cell Biol. 1998; 18:3880–3888. [PubMed: 9632772]
5. (a) Pley HW, Flaherty KM, McKay DB. Nature. 1994; 372:68–74. [PubMed: 7969422] (b) Scott WG, Finh JT, Klug A. Cell. 1995; 81:991–1002. [PubMed: 7541315]
6. (a) Amiri KM, Hagerman PJ. J Mol Biol. 1996; 261:125–134. [PubMed: 8757281] (b) Bassi GS, Murchie AI, Walter F, Clegg RM, Lilley DM. EMBO J. 1997; 16:7481–7489. [PubMed: 9405376] (c) Hampel KJ, Burke JM. Biochemistry. 2003; 42:4421–4429. [PubMed: 12693938]
7. Khvorova A, Lescoute A, Westhof E, Jayasena SD. Nat Struct Biol. 2003; 10:708–712. [PubMed: 12881719]
8. De la Pena M, Gago S, Flores R. EMBO J. 2003; 22:5561–5570. [PubMed: 14532128]
9. Penedo JC, Wilson TJ, Jayasena SD, Khvorova A, Lilley DM. RNA. 2004; 10:880–888. [PubMed: 15100442]

10. Canny MD, Jucker FM, Kellogg E, Khvorova A, Jayasena SD, Pardi A. J Am Chem Soc. 2004; 126:10848–10849. [PubMed: 15339162]
11. Osborne EM, Schaak JE, DeRose VJ. RNA. 2005; 11:187–196. [PubMed: 15659358]
12. (a) Edwards TE, Okonogi TM, Robinson BH, Sigurdsson S. J Am Chem Soc. 2001; 123:1527–1528. [PubMed: 11456739] (b) Qin PZ, Butcher SE, Feigon J, Hubbell WL. Biochemistry. 2001; 40:6929–6936. [PubMed: 11389608] (c) Kim NK, Murali A, DeRose VJ. Chem Biol. 2004; 11:939–948. [PubMed: 15271352]
13. Heckman JE, Lambert D, Burke JM. Biochemistry. 2005; 44:4148–4156. [PubMed: 15766242]
14. Budil DE, Lee S, Saxena S, Freed JH. J Magn Reson Ser A. 1996; 120:155–189.
15. The maximum rates reported here are somewhat slower than those recently reported by Canny et al.[10] for a similar HHRz construct with identical loops. Canny et al. found that k_{max} = 220 ± 20 min^{-1} at pH 7 and 25 °C, which is ~4 times that for slS($U_{1.6}$)/wtE in Figure 2. The difference can be ascribed to lower temperature in these measurements (20 vs 25 °C) 800 min^{-1} and possible complications from the $U_{1.6}$ spin label. The k_{max} > was calculated by Canny et al. as a theoretical maximum (pH 8.5, 25 °C, and 200 mM Mg^{2+}).
16. Kisselva N, Khvorova A, Westhof E, Schiemann O. RNA. 2005; 11:1–6. [PubMed: 15611296]
17. Hsieh J, Andrews AJ, Fierke CA. Biopolymers. 2004; 73:79–84. [PubMed: 14691942]

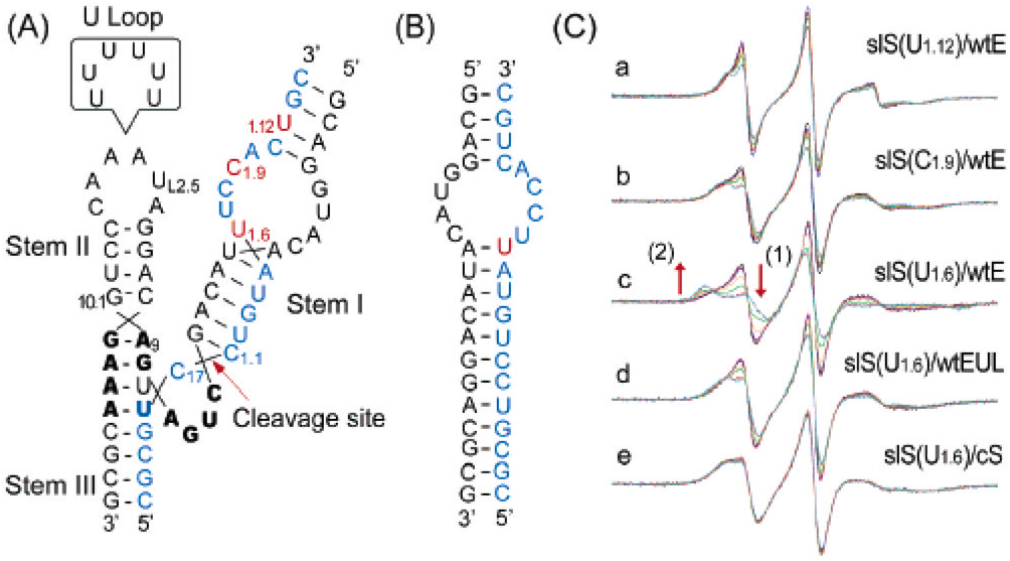

Figure 1.

Secondary structures of RNAs and SDSL EPR spectra. (A) *Schistosoma* hammerhead ribozyme. Enzyme strand is in black, substrate in blue, conserved core in bold, spin-labeled nucleotides in red, cleavage site at red arrow, and U-loop control sequence is shown in the box. (B) Spin-labeled duplex that mimics stem I. The spin-labeled uridine is in red. (C) EPR spectra for the metal dependence of the spin-labeled wild type (a–c), U-loop modified hammerheads (d), and duplex control (e). With increasing Mg^{2+} concentration, the spin label at $U_{1.6}$ changes from a more mobile (site1) to less mobile (site2) state only in the context of the active HHRz (c), which reflects docking of the two loops.

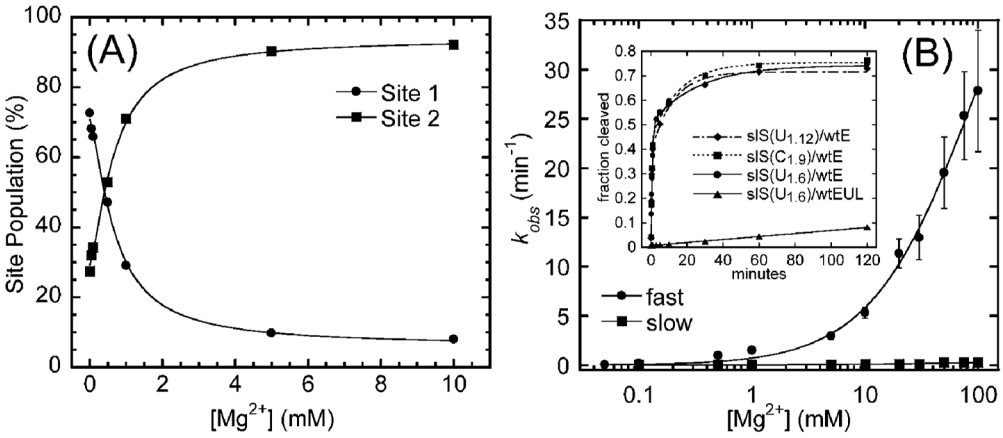

Figure 2.

Magnesium dependence of the folding and cleavage of the hammerhead in 5 mM HEPES, 0.1 M NaCl, pH 7.0, 20 °C. (A) Site population, calculated using the microscopic ordered macroscopic disordered (MOMD) model[13] (Supporting Information), predicts the half-maximum $[Mg^{2+}]_{1/2,dock}$ for complete docking as 0.69 ± 0.05 mM. (B) Biphasic catalysis of the hammerhead ribozyme. The single turnover rate constant of the fast phase (●) increases dramatically with high Mg^{2+} concentrations, with half-maximum $[Mg^{2+}]_{1/2,cat}$ = 91.4 ± 45.8 mM (n = 0.96, k_{max} = 54.2 ± 13.4 min^{-1}) for HHRz catalysis (Supporting Information). Inset compares activities between slS($U_{1.6}$)/wtE (●), slS($U_{1.9}$)/wtE (■), and slS($U_{1.12}$)/wtE (◆) with k_{obs} = 1.5 ± 0.2, 2.7 ± 0.5, and 3.8 ± 0.7 min^{-1}, respectively, and slS($U_{1.6}$)/wtEUL control (▲) at 1 mM Mg^{2+}.