

## Coordination Environment of a Site-Bound Metal Ion in the Hammerhead Ribozyme Determined by $^{15}\text{N}$ and $^2\text{H}$ ESEEM Spectroscopy

Matthew Vogt,<sup>†,||</sup> Simanti Lahiri,<sup>‡</sup> Charles G. Hoogstraten,<sup>‡,§</sup> R. David Britt,<sup>\*,‡</sup> and Victoria J. DeRose<sup>\*,†,⊥</sup>

Contribution from the Department of Chemistry, Texas A&M University, College Station, Texas 77842, and Department of Chemistry, University of California, Davis, California 95616

Received October 16, 2005; E-mail: rdbritt@ucdavis.edu; derose@uoregon.edu

**Abstract:** Although site-bound  $\text{Mg}^{2+}$  ions have been proposed to influence RNA structure and function, establishing the molecular properties of such sites has been challenging due largely to the unique electrostatic properties of the RNA biopolymer. We have previously determined that, in solution, the hammerhead ribozyme (a self-cleaving RNA) has a high-affinity metal ion binding site characterized by a  $K_{d,\text{app}} < 10 \mu\text{M}$  for  $\text{Mn}^{2+}$  in 1 M NaCl and speculated that this site has functional importance in the ribozyme cleavage reaction. Here we determine both the precise location and the hydration level of  $\text{Mn}^{2+}$  in this site using ESEEM (electron spin–echo envelope modulation) spectroscopy. Definitive assignment of the high-affinity site to the activity-sensitive A9/G10.1 region is achieved by site-specific labeling of G10.1 with  $^{15}\text{N}$  guanine. The coordinated metal ion retains four water ligands as measured by  $^2\text{H}$  ESEEM spectroscopy. The results presented here show that a functionally important, specific metal binding site is uniquely populated in the hammerhead ribozyme even in a background of high ionic strength. Although it has a relatively high thermodynamic affinity, this ion remains partially hydrated and is chelated to the RNA by just two ligands.

### Introduction

RNA structure and function depend strongly on ionic conditions. As a negatively charged polyelectrolyte, RNA attracts a diffuse counterion atmosphere.<sup>1,2</sup> In addition to this diffuse counterion atmosphere, site-bound  $\text{Mg}^{2+}$  and also monovalent cations have been invoked as specific cofactors that stabilize unique RNA folds and influence reactions catalyzed by ribozymes.<sup>1–6</sup> It has remained challenging to develop a molecular basis for the influence of specific cations on RNA function, in large part because it is difficult to determine properties of the specific sites. Intuition derived from the properties of metalloproteins would suggest that metal ion cofactors that are critical to biological function would have defined binding sites. In the context of the unique electrostatic properties of folded RNA, however, the properties of specific metal sites may differ

substantially from expectations based on metalloproteins.<sup>1,2,7,10</sup> Although many monovalent and divalent cations have been modeled as bound to specific RNA ligands in X-ray crystal structures of RNA molecules,<sup>1–9</sup> X-ray crystallographic models do not provide the direct link between site population and RNA function under solution conditions that is required to understand the influence of these ions on RNA activity.

Here we report properties of a specific metal ion site in the self-cleaving hammerhead ribozyme, including identification of the RNA ligands and hydration level of the ion. This information links thermodynamic and coordination properties of a defined, functional metal ion site in this ribozyme. The methods used in this investigation include functional metal substitution by paramagnetic  $\text{Mn}^{2+}$ , isotopic substitution of ligands, and ligand identification by electron-spin–echo envelope modulation (ESEEM) spectroscopy.

In metalloproteins, spectroscopic methods have been used extensively to explore the properties of metal coordination sites. These methods have recently been applied to metal interactions with the hammerhead ribozyme (HHRz, Figure 1), a small self-cleaving RNA whose activity in moderate ionic strength is

<sup>†</sup> Texas A&M University.

<sup>‡</sup> University of California.

<sup>§</sup> Current address: Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI.

<sup>||</sup> Current address: Laboratory of Pathology, National Cancer Institute, Bethesda, MD.

<sup>⊥</sup> Current address: Department of Chemistry, University of Oregon, Eugene, OR.

(1) Draper, D. E. *RNA* **2004**, *10*, 335–343.

(2) (a) Misra, V. K.; Shiman, R.; Draper, D. E. *Biopolymers* **2003**, *69*, 118–136. (b) Draper, D. E.; Grilley, D.; Soto, A. M. *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 221–243.

(3) Woodson, S. A. *Curr. Opin. Chem. Biol.* **2005**, *9*, 104–109.

(4) DeRose, V. J. *Curr. Opin. Struct. Biol.* **2003**, *13*, 317–324.

(5) Pyle, A. M. *J. Biol. Inorg. Chem.* **2002**, *7*, 679–690.

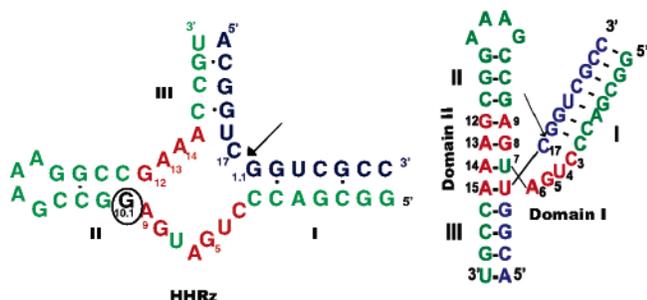
(6) Fedor, M. J.; Williamson, J. R. *Nat. Rev. Mol. Cell. Biol.* **2005**, *6*, 399–412.

(7) DeRose, V. J.; Burns, S.; Kim, N.-K.; Vogt, M. In *Comprehensive Coordination Chemistry II*; McCleverty, J., Meyer, T. J., Eds.; Elsevier: Oxford, 2003; pp 787–813.

(8) Stefan, L. R.; Zhang, R.; Levitan, A. G.; Hendrix, D. K.; Brenner, S. E.; Holbrook, S. R. *Nuc. Acids Res.* **2006**, *34*, Database issue D131–141.

(9) Klein, D. J.; Moore, P. B.; Steitz, T. A. *RNA* **2004**, *10*, 1366–1379.

(10) Das, R.; Travers, K. J.; Bai, Y.; Herschlag, D. *J. Am. Chem. Soc.* **2005**, *127*, 8272–8273.



The dG10.1 HHRz has a  $k_{\text{obs}}$  of approximately 4/min at a concentration of 300  $\mu\text{M}$   $\text{Mn}^{2+}$ , which is the same as the value reported for the WT HHRz,<sup>20</sup> as expected based on previous studies of this modification.<sup>15</sup>

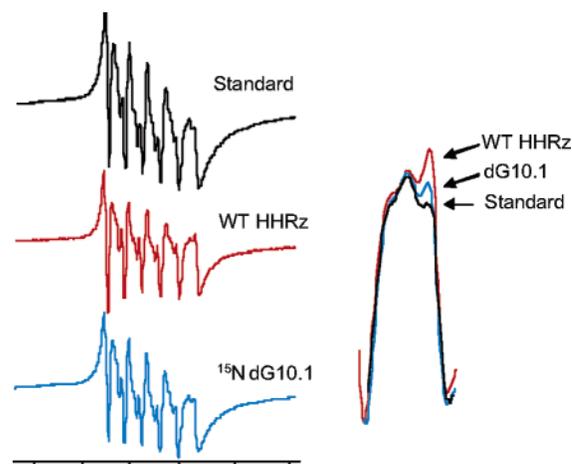
**EPR Sample Preparation.** Equal concentrations of 34nt enzyme strand and 13nt OMe substrate strand were combined, annealed for 90 s at 90 °C, and then cooled on ice for 30 min.  $\text{MnCl}_2$  was added from a stock solution freshly prepared from a 1 M  $\text{MnCl}_2$  source (Sigma). Typical sample volumes were 90 or 100  $\mu\text{L}$  and included 20% (vol/vol) ethylene glycol acting as a cryoprotectant. The samples were transferred to quartz EPR tubes (Wilmad) and frozen in liquid  $\text{N}_2$ . The dG10.1  $^{14}\text{N}$  sample contained 360  $\mu\text{M}$   $\text{Mn}^{2+}$  and 360  $\mu\text{M}$  HHRz. The wild-type HHRz natural abundance  $^{14}\text{N}$  sample contained 400  $\mu\text{M}$   $\text{Mn}^{2+}$  and 400  $\mu\text{M}$  RNA HHRz. The dG10.1  $^{15}\text{N}$  sample was prepared with 195  $\mu\text{M}$   $\text{Mn}^{2+}$  and 244  $\mu\text{M}$  RNA HHRz.

**Preparation of Deuterated Samples.** The ESEEM  $\text{H}_2\text{O}$  counting experiments require two samples, one prepared in  $\text{H}_2\text{O}$  and the other prepared in  $^2\text{H}_2\text{O}$ . For these samples, following ethanol precipitation, both the enzyme and substrate RNA strands were dried by speed vacuum, resuspended in  $^2\text{H}_2\text{O}$  or  $\text{H}_2\text{O}$ , and lyophilized. The pellet was redissolved in  $^2\text{H}_2\text{O}$  or  $\text{H}_2\text{O}$  and lyophilized again. This procedure was repeated 3 times. The final RNA pellets were then brought up in a buffer of 1 M NaCl, 5 mM TEA, pH = 7.53 (pD = 7.93) in  $^2\text{H}_2\text{O}$ . The EPR samples were prepared as described above, with 350  $\mu\text{M}$  HHRz and 250  $\mu\text{M}$  of  $\text{Mn}^{2+}$ , with 0.4 M sucrose as the cryoprotectant in either  $\text{H}_2\text{O}$  or  $^2\text{H}_2\text{O}$ . Samples were then transferred to quartz EPR tubes and frozen in liquid  $\text{N}_2$ .

**X-Band EPR Spectroscopy.** EPR spectra were obtained at a temperature of 10 K on a Bruker EMX EPR spectrometer operating at 9.4 GHz. Typical EPR parameters were 100 kHz modulation frequency, 15 G modulation amplitude, and a microwave power of 0.2 mW. EPR spectra were manipulated using the WinEPR program software suite from Bruker.

**ESEEM Spectroscopy.** Pulsed EPR spectroscopy was performed on a laboratory built 8–18 GHz spectrometer described previously.<sup>26</sup> Three-pulse ESEEM experiments<sup>27,28</sup> measure the  $T$  dependence of the stimulated echo amplitude from the  $\pi/2-\tau-\pi/2-T-\pi/2-\tau$ -echo pulse sequence and were analyzed as previously described.<sup>22–24</sup> Data were obtained at 4.2 K with a microwave frequency of 9.361 GHz, a repetition rate of 8 ms, and with tau values provided in the figure legends.

To determine the number of water ligands bound to  $\text{Mn}^{2+}$  in the HHRz high-affinity metal site, a previously reported “water-counting” procedure was employed.<sup>24,29</sup> ESEEM spectra were obtained for samples in both natural-abundance  $\text{H}_2\text{O}$  and in  $^2\text{H}_2\text{O}$ -exchanged media. The ESE (electron spin-echo) modulation pattern can be approximated as a product of the magnetic interactions from each set of relevant nuclei, where each interaction is related to the number and distance of the nuclei from the paramagnetic center. Thus, ratios of signals from HHRz samples in  $^2\text{H}_2\text{O}$  and  $\text{H}_2\text{O}$  provide the ESEEM contribution solely from exchangeable deuterium, removing contributions from other nuclei such as  $^{14}\text{N}$  and nonexchangeable protons.<sup>29</sup> The three-pulse ESEEM experiment allows selection of tau values that diminish background modulation from protons and so has been used extensively for these types of deuterium-quantitation studies.<sup>29</sup> Although the product rule applies formally only to two-pulse ESEEM, it is a good approximation for three-pulse ESEEM in the case of small hyperfine and dipolar contributions as expected for deuterium couplings measured in this experiment.<sup>29c</sup> To remove contributions from solvent molecules not



**Figure 3.** X-band EPR spectra of  $\text{Mn}^{2+}$  bound to hammerhead ribozymes. Inset shows an overlay of the sixth (highest-field) line of the EPR spectra of  $\text{Mn}^{2+}$  bound to WT HHRz, HHRz with a dG10.1 substitution, and  $\text{Mn}^{2+}$  in a buffer solution (Standard). Spectra were obtained at 10 K, with 250–350  $\mu\text{M}$  HHRz samples in 1 M NaCl, 5 mM TEA, pH = 7.8, and 20% ethylene glycol. EPR instrument conditions were X-band (9.4 GHz), 0.2 mW microwave power, 100 kHz magnetic field modulation at an amplitude of 15 G.

directly coordinated to  $\text{Mn}^{2+}$ , the ratioed  $^2\text{H}_2\text{O}/\text{H}_2\text{O}$  data are subsequently divided by the ESE modulation for  $\text{Mn}^{2+}$ –diethylenetriamine-pentaacetic acid (DTPA) in  $^2\text{H}_2\text{O}$ , which has no inner-sphere aqua ligands. The resulting HHRz ESEEM data consist solely of  $^2\text{H}$  modulation from inner-sphere  $^2\text{H}_2\text{O}$  ligands. The depth of the deuterium modulation is then compared to data sets that are predicted for  $N$  ( $=1-6$ ) aqua ligands. The predicted data for  $N = 1-6$  aqua ligands are created by raising the  $^2\text{H}$  ESE data from  $\text{Mn}(\text{H}_2\text{O})_6^{2+}$  (divided by the  $^2\text{H}$  Mn–DTPA ESE signal) to the  $N/6$  power.<sup>24</sup> To further control for possible effects of background modulation, ESEEM experiments were repeated with the magnetic field set to three different maxima of the  $\text{Mn}^{2+}$  ESE-EPR line shape, typically 3110, 3350, and 3551 G.

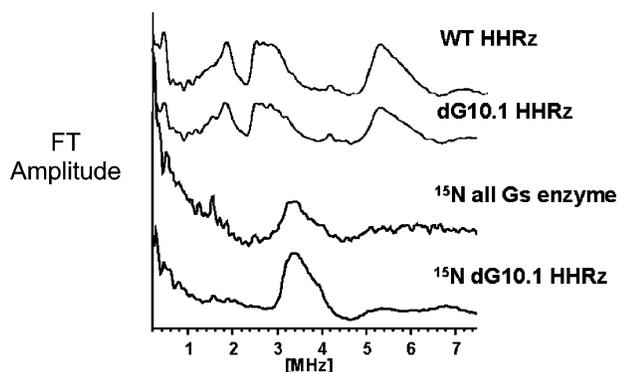
**Molecular Modeling of the A9/G10.1 Site.** A simple molecular mechanics approach was used to model the  $\text{Mn}^{2+}$  ion bound at the A9/G10.1 site in the hammerhead. The model was constructed from the HHRz crystal structure PDB 1HMH<sup>14</sup> by deleting all bases except G8, A9, and G10.1. The  $\text{Mn}^{2+}$  ion was modeled coordinated to the *pro*- $\text{R}_p$  oxygen of the A9 phosphate as predicted by functional,<sup>15</sup> X-ray structure,<sup>12,13</sup> and spectroscopic<sup>21</sup> studies, and the N7 position of G10.1 with four water molecules filling out the coordination sphere as determined from this work. The energy of the truncated model was minimized using the program Cerius2.

## Results

Previous activity studies on the HHRz predict a functional  $\text{Mg}^{2+}$  site involving A9/G10.1, and spectroscopic studies provide evidence for a high-affinity  $\text{Mg}^{2+}/\text{Mn}^{2+}$  site in the ribozyme. To definitively link these two observations and describe the properties of this cofactor, HHRz samples were examined by  $\text{Mn}^{2+}$  EPR and ESEEM spectroscopies in high salt (1M NaCl) to satisfy nonspecific cation interactions.  $^{15}\text{N}$  isotopic substitution of the proposed guanine ligand required the incorporation of 2'-deoxyguanosine at position G10.1, which has minimal effect on activity (see Materials and Methods).

**EPR Spectra.** The low-temperature EPR spectrum of  $\text{Mn}^{2+}$  in the wild-type HHRz sample shows the typical six-line spectrum, centered at  $g = 2$ , that results from hyperfine coupling between the unpaired electron spins and the  $I = 5/2$   $^{55}\text{Mn}$  nucleus (Figure 3). Compared to  $\text{Mn}^{2+}$  in a buffer sample, the WT HHRz sample has a distinct line shape, due to the slightly distorted

(26) Sturgeon, B. E.; Britt, R. D. *Rev. Sci. Instrum.* **1992**, *63*, 2189–2192.  
 (27) Kevan, L. In *Time Domain Electron Spin Resonance*; Kevan, L., Schwartz, R. N., Eds.; John Wiley & Sons: New York, 1979; pp 279–341.  
 (28) Mims, W. B.; Peisach, J. *Biol. Magn. Reson.* **1981**, *3*, 213–263.  
 (29) (a) McCracken, J.; Peisach, J.; Dooley, D. M. *J. Am. Chem. Soc.* **1987**, *109*, 4064–4072. (b) Peisach, J.; Davis, J. L. *J. Biol. Chem.* **1984**, *259*, 2704–2706. (c) Mims, W. B.; Davis, J. L.; Peisach, J. *J. Magn. Reson.* **1990**, *86*, 273–292.



**Figure 4.**  $^{14/15}\text{N}$  three-pulse ESEEM spectra of  $\text{Mn}^{2+}$  bound to the hammerhead ribozyme. Wild type and dG10.1 HHRz show similar  $^{14}\text{N}$  ( $I = 1$ ) ESEEM Fourier transform traces, consistent with  $\text{Mn}$ –guanine coordination. The “ $^{15}\text{N}$  all G’s enzyme” HHRz, in which all guanines on the enzyme strand are labeled with  $^{15}\text{N}$ , shows an ESEEM pattern consistent with  $\text{Mn}$ – $^{15}\text{N}$  ( $I = 1/2$ ) coordination.<sup>22</sup> A nearly identical spectrum is observed with specific  $^{15}\text{N}$  labeling of G10.1, identifying G10.1 as the  $\text{Mn}^{2+}$  ligand. Experiment conditions: magnetic field strength 3600 G,  $\tau = 192$  ns,  $\eta/2$  pulse length 15 ns, microwave power 3.2 W.

metal environment, that is most noticeable on the sixth line of the EPR signal. This feature has been previously observed for  $\text{Mn}^{2+}$  bound to the WT HHRz and attributed to population of the high affinity metal ion binding site.<sup>21</sup> The EPR spectrum for the dG10.1 HHRz shows a feature that is similar, although weaker in amplitude, to that observed for the WT HHRz sample (Figure 3 inset). The change in line shape observed in both  $\text{Mn}^{2+}$  HHRz samples relative to the buffer sample can be modeled as being due to a small difference in the zero field splitting ( $zfs$ ) of the  $M_s$  sublevels, which alters the positions of the forbidden transitions and results in more pronounced allowed lines.<sup>30</sup>

**Nucleobase Ligand Identification by  $^{14}\text{N}/^{15}\text{N}$  ESEEM Spectroscopy.** ESEEM spectroscopy is useful in observing weak hyperfine couplings that are not detected in conventional EPR spectra and has been previously used to detect  $^{14}\text{N}$ ,<sup>22,23,25</sup>  $^{15}\text{N}$ ,<sup>22,23</sup> and  $^2\text{H}$ <sup>24</sup> coupled to  $\text{Mn}^{2+}$  ions bound to RNA or model nucleotides. The three-pulse ESEEM spectrum of  $\text{Mn}^{2+}$  in the WT HHRz shown in Figure 4 displays a pattern that has been previously observed for  $\text{Mn}^{2+}$  coordinated to the  $^{14}\text{N}$  ( $I = 1$ ) of a guanine ligand.<sup>22,23</sup> The set of frequencies observed in these spectra result from the hyperfine and quadrupolar couplings of the  $^{14}\text{N}$   $I = 1$  nucleus.<sup>31</sup> This pattern has been observed previously for  $\text{Mn}$ –GMP complexes, for which  $\text{Mn}$  coordination is expected through the guanine N7 imino nitrogen. Isotopic substitution in  $\text{Mn}$ – $^{15}\text{N}$ –GMP results in replacement of this pattern with one consistent with the change to an  $^{15}\text{N}$  nucleus, with  $I = 1/2$  and no nuclear quadrupole moment.<sup>22,23</sup> Nearly identical spectral changes are observed in the HHRz when the enzyme strand is transcribed using  $^{15}\text{N}$ -GTP, resulting in globally labeled  $^{15}\text{N}$ -labeled guanines (Figure 4, third trace).<sup>22</sup> Thus, for the HHRz these ESEEM data represent coordination of  $\text{Mn}^{2+}$  to one or more guanine ligands. Identification of the exact ligand was achieved with site-specific  $^{15}\text{N}$  substitution at G10.1.

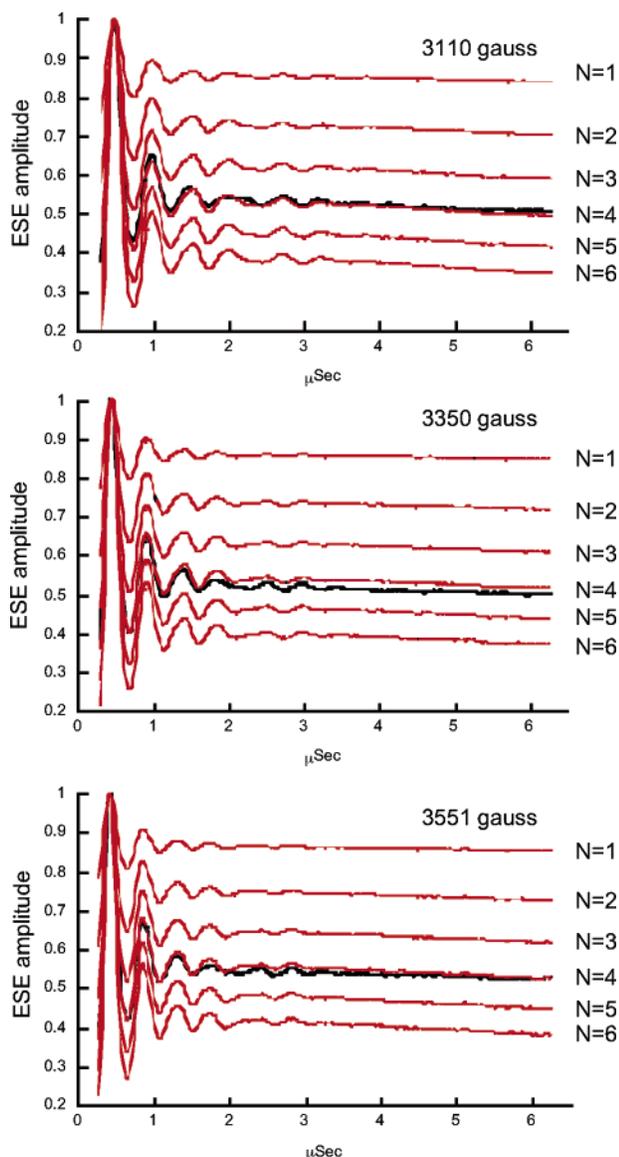
$\text{Mn}^{2+}$  bound to the natural-abundance HHRz dG10.1 sample gives rise to an  $^{14}\text{N}$  ESEEM pattern that is nearly identical to that of the unsubstituted WT HHRz (Figure 4, second trace),

indicating that the 2'-deoxyguanosine modification has little effect on the metal binding site. The intensity of the dG10.1 sample ESEEM signal is  $\sim 70\%$  that of the WT sample indicating a lowered concentration of bound  $\text{Mn}^{2+}$  ions, which is consistent with the slightly lower population of bound  $\text{Mn}^{2+}$  suggested by the EPR signal for this sample. With  $^{15}\text{N}$  substitution, the ESEEM spectrum from the  $^{15}\text{N}$  dG10.1 HHRz sample exhibits a single broad feature at 3.5 MHz (Figure 4, bottom trace), as observed for the globally  $^{15}\text{N}$ -labeled HHRz and for  $\text{Mn}$ – $^{15}\text{N}$ –GMP.<sup>22</sup> The ESEEM spectrum of the  $^{15}\text{N}$  dG10.1 HHRz sample does not show any additional features from  $^{14}\text{N}$  coordination, ruling out multiple high-affinity  $\text{Mn}^{2+}$  binding sites. The ESEEM spectrum for the HHRz sample in which the enzyme strand contains  $^{15}\text{N}$  in all guanine sites shows an additional peak at 1.5 MHz that arises from coupling to distant  $^{15}\text{N}$  nuclei and which is absent in the dG10.1 labeled spectrum. Taken together, these data provide conclusive evidence that  $\text{Mn}^{2+}$  binds tightly, and homogeneously, to the hammerhead G10.1 site.

**Hydration Level of the High-Affinity  $\text{Mn}^{2+}$  Ion.** Previous ENDOR studies of  $\text{Mn}^{2+}$ –HHRz samples demonstrated the presence of coordinated  $\text{H}_2\text{O}$  molecules.<sup>21</sup> However, such CW ENDOR methods lack the ability to quantify the precise number of aqua ligands bound to the  $\text{Mn}$  ion. To determine the exact number of  $\text{H}_2\text{O}$  molecules coordinated to  $\text{Mn}^{2+}$  in the high affinity ribozyme site,  $\text{Mn}^{2+}$ –HHRz samples were prepared in  $^2\text{H}_2\text{O}$ , and  $^2\text{H}$  ESEEM, which is sensitive to the number of coupled nuclei, was used to determine the number of  $^2\text{H}_2\text{O}$  ligands. As described under Materials and Methods, this technique isolates modulation from solvent molecules that are directly coordinated to the  $\text{Mn}^{2+}$  ion by a ratio method, using samples prepared in either  $\text{H}_2\text{O}$  or  $^2\text{H}_2\text{O}$ .<sup>24,29</sup> Figure 5 shows  $^2\text{H}$  ESEEM time-domain traces obtained from  $\text{Mn}^{2+}$  bound to  $^2\text{H}_2\text{O}$ -exchanged HHRz, appropriately ratioed (black), in comparison with data calculated for different hydration levels that are based on amplitudes obtained using a known standard (red). Data obtained at three different magnetic fields show excellent agreement in deuterium modulation depth. The average of fits to the three data sets results in a value of  $4.08 \pm 0.12$   $^2\text{H}_2\text{O}$  ligands, indicating that four aqua ligands are coordinated to the high affinity  $\text{Mn}^{2+}$  ion in HHRz.

(31) Although the assignment of these data to a directly coordinated  $\text{Mn}$ –guanine ligand is based solidly on comparison with model compounds and isotopic substitution, the interpretation of the  $^{14}\text{N}$  ESEEM data is complicated by the high-spin nature of the  $\text{Mn}(\text{II})$  spin system. Previous publications (refs 22, 23) have described this  $^{14}\text{N}$  ESEEM pattern, observed for a purine ligand coordinated to  $\text{Mn}^{2+}$ , as being near to the “exact cancellation” limit for  $^{14}\text{N}$ . In this limit,  $|A(^{14}\text{N})| \approx 2\nu_{\text{N}}(^{14}\text{N})$  and the lowest three FT peaks in the three-pulse ESEEM experiment represent quadrupolar frequencies  $\nu_{0,+,-}$ , with the property that  $\nu_0 + \nu_- = \nu_+$ . A higher frequency “double quantum” peak occurring at  $\nu_{\text{dq}} = 2[(\nu_{\text{N}} + A/2)^2 + K^2(3 + \eta^2)]^{1/2}$  is also expected, where  $K$  is related to the quadrupolar coupling constant  $e^2qQ$  as  $K = e^2qQ/4$ . At the observing magnetic field of 3600 G,  $\nu_{\text{N}}(^{14}\text{N}) = 1.1$  MHz. As previously described, assuming exact cancellation conditions and that the signal arises from the  $M_s = +1/2, -1/2$  sublevels of the  $\text{Mn}^{2+} S = 5/2$  spin manifold, and assigning features at 0.6, 1.9, and 2.5 MHz to  $\nu_0, \nu_-,$  and  $\nu_+$ , respectively, and the feature at 5.2 MHz to  $\nu_{\text{dq}}$ , gives estimated values of  $A_{\text{iso}}(^{14}\text{N}) \sim 2.3$  MHz and  $e^2qQ \sim 2.9$  MHz. Hoogstraten and co-workers used ESE-ENDOR spectroscopy to directly measure  $A(^{15}\text{N})$  in a  $\text{Mn}^{2+}$ –GMP model complex, obtaining predicted  $^{14}\text{N}$  values of  $A_{\text{iso}}(^{14}\text{N}) = 3.0$  MHz ( $A_{\text{dip}} = 0.6$  MHz). Attempts at simulating the  $^{14}\text{N}$  sample three-pulse ESEEM data using these values, however, did not fully reproduce features observed in the  $^{14}\text{N}$  ESEEM data (ref 23). Recently, Astashkin (ref 32a) and Dismukes (ref 32b) and co-workers have proposed that zero-field splitting may influence ESEEM frequencies, which would apply to the case of high-spin  $S = 5/2$   $\text{Mn}(\text{II})$ . Since the features of the EPR spectrum of  $\text{Mn}^{2+}$  site-bound to the HHRz show effects of increased zero-field splitting, this may indeed influence the predicted ESEEM frequencies.

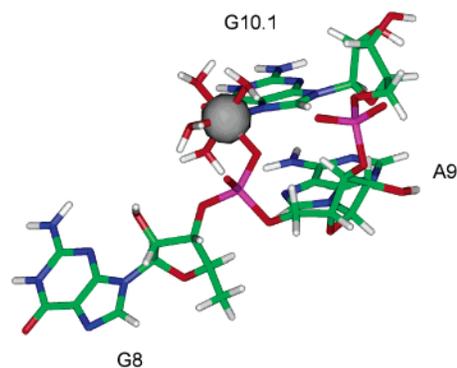
(30) Vogt, M. Ph.D. Dissertation, Texas A&M University, 2004.



**Figure 5.** Hydration level of the high-affinity  $\text{Mn}^{2+}$  site in the hammerhead ribozyme determined by  $^2\text{H}$  ESEEM spectroscopy.  $^2\text{H}$  three-pulse ESE time-domain traces from deuterium-exchanged HHRz samples, ratioed by data obtained on natural-abundance HHRz samples (see Materials and Methods), are compared with data calculated for hydration levels of  $N = 1-6$   $^2\text{H}_2\text{O}$   $\text{Mn}^{2+}$  ligands. Data are plotted as a function of  $T$  ( $\mu\text{s}$ ) (see Materials and Methods) and were obtained at three different magnetic field values with  $\eta$  values (in parentheses) selected for suppression of proton modulation: 3110 G (228 ns); 3350 G (210 ns); 3551 G (198 ns). The average fit to these data gives a value of  $4.08 \pm 0.12$   $\text{H}_2\text{O}$  molecules coordinated to the HHRz-bound  $\text{Mn}^{2+}$  ion.

**Model of  $\text{Mn}^{2+}$  Coordination in the High-Affinity Metal Site.** Figure 6 shows a model of the high affinity  $\text{Mn}^{2+}$  site in the hammerhead ribozyme that is based on the existing X-ray crystal structure of this construct and the results from EPR/ESEEM spectroscopy.

The ligands coordinated to the  $\text{Mn}^{2+}$  ion are the *pro*- $\text{R}_p$  oxygen from the phosphate of A9, the N7 from G10.1, and four  $\text{H}_2\text{O}$  molecules as determined by the ESEEM  $\text{H}_2\text{O}$  counting experiments. In the initial model,  $\text{Mn}^{2+}$  was placed into the coordinates of the A9/G10.1 site as excised from the crystal structure of metal-free HHRz (1HMH, ref 14). When  $\text{Mn}^{2+}$  is placed coordinated to G10.1 with a reasonable Mn–N distance of 2.2 Å, the starting distance between the  $\text{Mn}^{2+}$  ion and the



**Figure 6.** Model of the high affinity A9/G10.1  $\text{Mn}^{2+}$  site in the hammerhead ribozyme, based on energy minimization of  $\text{Mn}^{2+}$  complexed to G8, A9, and G10.1 excised from the HHRz X-ray structure 1HMH.<sup>14</sup> Specific  $^{15}\text{N}$  labeling of the G10.1 site and ESEEM spectroscopy identify the guanine ligand (Figure 4). Previous ENDOR studies predict phosphate coordination.<sup>21</sup> This site is proposed to be six-coordinate based on the ESEEM water-counting experiments (Figure 5).

phosphorus atom is approximately 3.9 Å. After energy minimization of the model, the Mn–P distance reduced to 3.2 Å, which is consistent with the 3.3 Å value observed in the crystal structure of Mn–ATP.<sup>33</sup> The remaining RNA structure rearranged only slightly following energy minimization, which suggests that this site has a geometry that favors chelation. The symmetry around the  $\text{Mn}^{2+}$  ion in this model deviates from pure octahedral symmetry, which is consistent with the unique line shape of the EPR spectrum observed for Mn–HHRz.

## Discussion

A major factor in determining if a metal ion binds to RNA in a diffuse or chelated manner is a balance between energetic terms that are unique to nucleic acids. Draper and co-workers have argued that electrostatic interactions dominate the energetics of RNA–cation association.<sup>1,2</sup> They point out that a “site-bound” ion pays an energetic cost by displacing other associated cations and also must be dehydrated in order to bind directly to the RNA. These energetic costs of ion displacement and dehydration must be balanced with large favorable energetic terms from electrostatic attraction to the RNA site and, presumably, formation of cation–RNA ligand interactions.<sup>1</sup> They have also shown that, for thermodynamic measurements (and therefore other techniques that measure the stability of folded RNA structures) of tRNA, properties attributed to localized, specific ion interactions may in fact arise from a class of ions that are weakly and nonspecifically bound.<sup>1,2</sup> This situation complicates the prospect of linking the occupation of cation sites to RNA function and even provokes the question as to whether such well-defined sites exist in solution.

The structural studies presented here confirm that the HHRz A9/G10.1 metal ion site is indeed a well-defined site that is occupied under solution conditions even in a competing background of high  $\text{Na}^+$  concentration. To compensate for energetic penalties, sites in RNA that favor chelated metal ions might be expected to have a high local negative electrostatic potential as well as well-placed ligands. Both properties are present in this HHRz metal site. These results suggest that the

- (32) (a) Astashkin, A. V.; Raitsimring, A. M. *J. Chem. Phys.* **2002**, *117*, 6121–6132. (b) Dasgupta, J.; Tyryshkin, A. M.; Kozlov, Y. N.; Klimov, V. V.; Dismukes, G. C. *J. Phys. Chem. B* **2006**, *110*, 5099–5111.  
 (33) Sabat, M.; Cini, R.; Haromy, T.; Sundaralingam, M. *Biochemistry* **1985**, *24*, 7827–7833.

N7 of G10.1 and the pro-R phosphate oxygen of A9 are positioned in a specific orientation to form a binding pocket for a metal ion to coordinate at this site. An electrostatic surface plot based on the hammerhead crystal structure also shows an area of high negative potential at the A9/G10.1 site.<sup>34</sup> The ESEEM “water-counting” experiments confirm that the high-affinity  $Mn^{2+}$  ion remains quite hydrated, having four water molecules while coordinated in this pocket. The fact that the  $Mn^{2+}$  ion retains a high level of hydration yet has a fairly high affinity ( $K_a > 10^5$  M in 1 M NaCl)<sup>20,21,25</sup> is consistent with the general picture of a special energetic balance for ions localized in structured RNAs.

The role of the metal ion at the A9/G10.1 site of the hammerhead ribozyme on the catalytic activity is remarkable considering the nearly 20 Å distance to the cleavage site predicted by X-ray crystal structures. Modifications of this metal ion-binding site at either the guanine base or the phosphate ligand can have large effects on activity.<sup>11,15–17</sup> A linkage between these two ligands was in fact observed in functional studies performed by Herschlag and co-workers, who found that removal of the N7 at G10.1 severely weakens the observed binding affinity for the metal ion that rescues activity with a phosphorothioate modification at A9, results that are consistent with the proposed metal binding site.<sup>15</sup>

Two main proposals have been made regarding the influence of this site on catalysis.<sup>4,15,35</sup> The most general proposal is that metal population of this site stabilizes the correct HHRz tertiary structure. HHRz crystal structures obtained under several different ionic conditions, including solely monovalent ions, all show the ribozyme folded into a compact gamma shape. Analyses of crystals soaked in  $Mg^{2+}$ ,  $Mn^{2+}$ , and  $Cd^{2+}$  ions predict several populated metal ion-binding sites including positions at A9/G10.1, at G5, and near the scissile phosphate.<sup>12,13</sup> Although in the crystal structures the hammerhead is fully folded, regardless of the presence of divalent ions, in solution the tertiary form of the ribozyme depends on the ionic conditions. Pardi and co-workers, using analysis of residual dipolar couplings, have demonstrated that, in a solution containing 0.1 M  $Na^+$ , the hammerhead is in an extended conformation with the stems fully separated.<sup>36</sup> Lilley and co-workers, based on FRET and <sup>19</sup>F NMR experiments, also predict that in the absence of  $Mg^{2+}$  the hammerhead ribozyme is in an extended conformation and that it undergoes a two-step folding transition to a more compact structure upon the addition of  $Mg^{2+}$  ions.<sup>37–39</sup> The first folding transition occurs within domain II upon the addition of  $\leq 500$   $\mu M$   $Mg^{2+}$ , reflecting the influence of an apparently high-affinity  $Mg^{2+}$  interaction, whereas the second folding transition of domain I occurs with the addition of 1–25 mM  $Mg^{2+}$  ions.<sup>38</sup>

The metal interaction responsible for the first stage of HHRz folding might be associated with the A9/G10.1 site based on this and other spectroscopic work. Pardi and co-workers observed a large  $Mg^{2+}$ -dependent <sup>31</sup>P chemical shift change that

predicted a high affinity  $Mg^{2+}$  site with an apparent  $K_d$  of  $\sim 100$   $\mu M$ . Using specific <sup>17</sup>O labeling, they were able to identify the shifted residue as the G13 phosphate.<sup>40</sup> Since G13 is in domain II, this  $Mg^{2+}$  site is consistent with the one predicted by FRET and <sup>19</sup>F NMR spectroscopy.<sup>37–39</sup> Here, the highest affinity site for  $Mn^{2+}$  is definitively shown to include coordination to G10.1, with a  $K_d$  of  $\sim < 10$   $\mu M$ . A 10-fold higher affinity for  $Mn^{2+}$  over  $Mg^{2+}$  is consistent with measurements on nucleotide model compounds<sup>41</sup> and also follows a trend observed in hammerhead activity studies.<sup>42</sup> Thus, it is possible that the site identified here is the same as the  $Mg^{2+}$  site identified by <sup>31</sup>P NMR. The coordinated A9 phosphate resonance was not observed in those studies, but the large upfield <sup>31</sup>P chemical shift at the G13 phosphate upon metal ion population of A9/G10.1 may be consistent with stabilization of a locally rearranged structure, as also detected by <sup>19</sup>F NMR studies.<sup>39</sup>

In addition to an apparent link to folding, it has also been hypothesized that the A9/G10.1 metal ion is directly involved in catalyzing the cleavage of the phosphodiester bond at the ribozyme active site. Herschlag and co-workers have proposed that only a single  $Cd^{2+}$  ion is needed to rescue catalytic activity in HHRz samples containing simultaneous  $R_p$  phosphorothioate substitutions of the A9 and the cleavage site phosphates.<sup>15</sup> In their model, this metal ion bridges both sulfur atoms, which would be distant according to X-ray crystallography of the native ribozyme. To accommodate their proposal, an “active state” conformer is proposed to be transiently formed, in which Stems I and II effectively dock, bringing the A9/G10.1 metal ion near enough to the cleavage site to directly coordinate to the scissile phosphate group. This bridged form of the ribozyme is proposed to be an infrequently populated, active state, and the gamma shape of the HHRz observed in the crystal structures is proposed to be a “ground state” conformation of the HHRz.

In the context of this model, the coordination of the A9/G10.1 metal ion as determined here is consistent with the ion coordinated in the “ground state” conformation of the HHRz.<sup>13,18</sup> The  $Mn^{2+}$  ion at this site remains highly hydrated with four water molecules and two ligands from the RNA. In order for the  $Mn^{2+}$  ion at the A9/G10.1 site to directly interact with a ligand from the cleavage site, the following would have to occur: the rearrangement of the hammerhead core to bring the A9 and C17 phosphates into close proximity,<sup>18</sup> including the displacement of metal ions in the core and near the cleavage site from electrostatic repulsion,<sup>1</sup> and the dehydration of the metal ion to open a coordination site for the scissile phosphate. The final coordination environment of the A9/G10.1 metal ion would have a more distorted geometry than that observed for the ion in the ground state, with three RNA ligands and three water molecules. Thus, adopting the “docked” conformation would have significant energetic penalties, consistent with an energetic barrier and with a lower population of the conformer in solution, as predicted by Herschlag and co-workers. Recent cross-linking studies also predict that the HHRz visits a conformation with a highly rearranged core structure.<sup>43</sup>

In line with this picture, it has recently been shown that extension of the HHRz to include interacting loops on Stems I

(34) Maderia, M.; Hunsicker, L. M.; DeRose, V. J. *Biochemistry* **2000**, *39*, 12113–12120.

(35) Butcher, S. E. *Curr. Opin. Struct. Biol.* **2001**, *11*, 315–320.

(36) Bondensgaard, K.; Mollava, E. T.; Pardi, A. *Biochemistry* **2002**, *41*, 11532–11542.

(37) Bassi, G. S.; Murchie, A. I. H.; Lilley, D. M. J. *RNA* **1996**, *2*, 756–768.

(38) Bassi, G. S.; Murchie, A. I. H.; Walter, F.; Clegg, R. M.; Lilley, D. M. J. *EMBO J.* **1997**, *16*, 7481–7489.

(39) Hammann, C.; Norman, D. G.; Lilley, D. M. J. *Proc. Nat. Acad. Sci. U.S.A.* **2001**, *98*, 5503–5508.

(40) Hansen, M. R.; Simorre, J.-P.; Hanson, P.; Mokler, V.; Bellon, L.; Beigelman, L.; Pardi, A. *RNA* **1999**, *5*, 1099–1104.

(41) Sigel, H.; Massoud, S. S.; Corfu, N. A. J. *Am. Chem. Soc.* **1994**, *116*, 2958–2971.

(42) Hunsicker, L. M.; DeRose, V. J. *J. Inorg. Biochem.* **2000**, *80*, 271–281.

and II results in a ribozyme that is active in much lower concentrations of  $Mg^{2+}$ .<sup>44–47</sup> The interacting loops may promote docking of Stems I and II, stabilizing an HHRz conformation that is closer to its active form. Thiophosphate interference studies<sup>47</sup> indicate that, even in this context, the A9/G10.1 site is still populated and important for activity. Consistent with this, it has recently been shown that a high-affinity  $Mn^{2+}$  binding site with similar <sup>14</sup>N ESEEM spectra is present in an extended HHRz sequence.<sup>48</sup> A very recent X-ray crystal structure of an extended HHRz sequence has been reported that indeed shows the predicted docked conformation and a much closer approach of  $\sim 5$  Å between the A9- and cleavage-site phosphodiester groups. No divalent cations were localized in that structure.<sup>52</sup>

The A9/G10.1 metal ion tunes activity but is not the only

metal ion that influences catalysis in the truncated HHRz. In 1 M NaCl, the HHRz requires millimolar concentrations of  $Mg^{2+}$  or  $Mn^{2+}$  for activity. Since the A9/G10.1 site is occupied in very low  $Mg^{2+}$  concentrations, this means that other  $Mg^{2+}$ –RNA interactions are critical in addition to this very high affinity one.<sup>4,46,49–51</sup> The nature of these other interactions, and whether they include “site-bound” ions, is not yet known. The spectroscopic studies presented here demonstrate that the A9/G10.1 metal ion site is discretely populated with a defined geometry, even in 1 M NaCl. By analogy with protein-based metalloenzymes, these data support the tuning of HHRz catalysis by a coordinated metal ion cofactor.

**Acknowledgment.** We are grateful for support from the NIH (GM58096 to V.J.D. and BM61211 to R.D.B.) and the NSF Chemistry Division (CHE0111696 to V.J.D. and CHE0092010 for support of TAMU EPR facilities).

JA057035P

- (43) Heckman, J. E.; Lambert, D.; Burke, J. M. *Biochemistry* **2005**, *44*, 4148–4156.  
(44) Khvorova, A.; Lescoute, A.; Westhof, E.; Jayasena, S. D. *Nat. Struct. Biol.* **2003**, *10*, 708–712.  
(45) Penedo, J. C.; Wilson, T. J.; Jayasena, S. D.; Khvorova, A.; Lilley, D. M. *RNA* **2004**, *10*, 880–888.  
(46) Canny, M. D.; Jucker, F. M.; Kellogg, E.; Khvorova, A.; Jayasena, S. D.; Pardi, A. *J. Am. Chem. Soc.* **2004**, *126*, 10848–10849.  
(47) Osborne, E. M.; Schaak, J. E.; DeRose, V. J. *RNA* **2005**, *11*, 187–196.  
(48) Kisseleva, N.; Khvorova, A.; Westhof, E.; Schiemann, O. *RNA* **2005**, *11*, 1–6.

- (49) Rueda, D.; Wick, K.; McDowell, S. E.; Walter, N. G. *Biochemistry* **2003**, *42*, 9924–9936.  
(50) Kim, N. K.; Murali, A.; DeRose, V. J. *J. Am. Chem. Soc.* **2005**, *127*, 14134–14135.  
(51) Hampel, K. J.; Burke, J. M. *Biochemistry* **2003**, *42*, 4421–4429.  
(52) Martick, M.; Scott, W. G. *Cell* **2006**, *36*, 309–320.