Mn^{2+}−Nitrogen Interactions in RNA Probed by Electron Spin−Echo Envelope Modulation Spectroscopy: Application to the Hammerhead Ribozyme

Susan R. Morrissey,[†] Thomas E. Horton,[†] Christopher V. Grant,[‡] Charles G. Hoogstraten,[‡] R. David Britt,*[,‡] and Victoria J. DeRose*[,†]

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

Received June 24, 1999. Revised Manuscript Received August 11, 1999

Abstract: We report application of electron spin−echo envelope modulation (ESEEM) spectroscopy to the problem of metal coordination environments in structured RNA molecules. ESEEM has been used in conjunction with ^{15}N-guanosine labeling to identify nitrogen ligation to a Mn^{2+} site in a hammerhead ribozyme and in Mn^{2+}−model guanosine monophosphate (GMP) complexes. Hammerhead ribozyme complexes consisting of a 34-nucleotide RNA enzyme strand annealed to a 13-nucleotide DNA substrate strand were poised in 1 M NaCl as a 1:1 complex with Mn^{2+}, conditions previously determined to populate a single high-affinity Mn^{2+} site (Horton, T. E.; Clardy, R. D.; DeRose, V. J. *Biochemistry* **1998**, *51*, 18094−18108). Significant modulation of the electron spin−echo from several low-frequency features is detected for the natural-abundance, ^{14}N-hammerhead samples. At 3600 G, the main hammerhead three-pulse ESEEM features arise at 0.6, 1.9, 2.5, and 5.2 MHz and are nearly identical for a Mn^{2+}−GMP complex under the same conditions. For a ribozyme having ^{15}N-guanosine incorporated into the enzyme strand, as well as for an ^{15}N-labeled Mn^{2+}−GMP complex, the modulation is completely altered and consists of one main feature at 3.4 MHz and a smaller feature at the $\nu_n(^{15}N)$ Larmor frequency of 1.6 MHz. Preliminary analysis of the ESEEM data reveals an apparent hyperfine coupling of $A(^{14}N) \sim 2.3$ MHz, similar to previously reported values for Mn^{2+} directly coordinated to histidine and imidazole. These data demonstrate the potential for ESEEM as a spectroscopic tool for metal ligand determination in structured RNA molecules.

Introduction

Metal ions are critical to structure and function in RNA molecules,[1] but defining precise metal ion coordination environments in RNA under solution conditions has been challenging. Extensive studies in protein systems have established that for paramagnetic metals, EPR-based spectroscopic techniques provide significant metal−ligand structural information.[2] These techniques have not yet been broadly applied to nucleic acids. Here we report the successful application of electron spin−echo envelope modulation (ESEEM) spectroscopy to the detection of a guanosine-derived nitrogen ligand to Mn^{2+} in the hammerhead ribozyme.

The hammerhead ribozyme, a chemically active RNA molecule commonly constructed as hybridized "enzyme" and "substrate" strands (Figure 1), performs a site-specific phosphodiester bond cleavage reaction that is highly activated by divalent cations.[3−5] Both Mg^{2+} and Mn^{2+} support hammerhead activity by a combination of structural and chemical roles. We have reported Mn^{2+} binding isotherms for the hammerhead

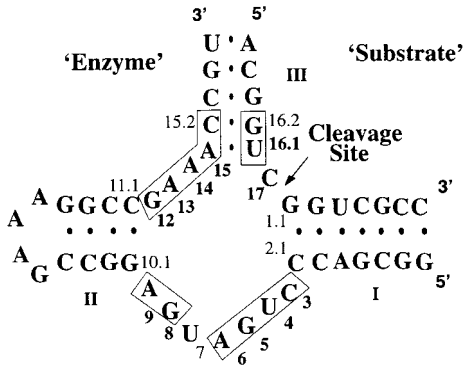

Figure 1. Hammerhead ribozyme model with "enzyme" and "substrate" strands. Nucleotides required for activity are indicated by rectangles. Phosphodiester bond cleavage occurs between positions C17 and G1.1 (arrow). ESEEM experiments were performed on a complex consisting of an unlabeled DNA substrate strand hybridized to an RNA enzyme strand which was either unlabeled or labeled with ^{15}N at all G positions.

* Address correspondence to these authors.
† Texas A&M University.
‡ University of California.

(1) (a) Cech, T. R. In *The RNA World*; Gesteland, R., Atkins, J., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1993; pp 239−270. (b) Pan, T.; Long, D. M.; Uhlenbeck, O. C. In *The RNA World*; Gesteland, R., Atkins, J., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1993; pp 271−302. (c) Pyle, A. M. *Science* **1993**, *261*, 709−714. (d) Narlikar, G. J.; Herschlag, D. *Annu. Rev. Biochem.* **1997**, *66*, 19−59. (e) Yarus, M. *FASEB J.* **1993**, *7*, 31−39.

(2) (a) Hoffman, B. M.; DeRose, V. J.; Doan, P. E.; Gurbiel, R. J.; Houseman, A. L. P.; Telser, J. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum: New York, 1993; Vol. 13, pp 151−218; DeRose, V. J.; Hoffman, B. M. *Methods in Enzymology* **1995**, *246*, 554−589. (b) Britt, R. D. In *Advances in Photosynthesis: Biophysical Techniques in Photosynthesis*; Hoff, A. J., Amesz, J., Eds.; Kluwer Academic Publishers: Amsterdam, 1995; pp 235−253.

(3) Reviewed in: (a) Bratty, J.; Chartrand, P.; Ferbeyre, G.; Cedergren, R. *Biochem. Biophys. Acta* **1993**, *1216*, 345−359. (b) McKay, D. B. *RNA* **1996**, *2*, 395−403. (c) Wedekind, J. E.; McKay, D. B. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 475−502.

10.1021/ja9921571 CCC: $18.00 © 1999 American Chemical Society
Published on Web 09/11/1999

ribozyme demonstrating at least two classes of Mn^{2+} affinities whose characteristics depend on the concentration of monovalent ions.[5] In 1 M NaCl, a single high-affinity Mn^{2+} binding site with $K_d < 10$ μM is populated at low Mn^{2+} concentrations. Population of a single paramagnetic Mn^{2+} site provides the opportunity for ESEEM spectroscopy to examine the metal ion ligation environment, as this pulsed EPR technique can detect interactions between unpaired electron spins and nuclear spins in close ($<\sim$6 Å) proximity.[2] Specifically, in the case of a Mn^{2+} binding site in RNA, ESEEM could be used to detect couplings between the spin $S = 5/2$ Mn^{2+} ion and the spin $I = 1$ ^{14}N nucleus of a base ligand. In such a case, analysis of the ^{14}N modulation will provide detailed information about the ligand hyperfine and quadrupole interactions, and specific ^{15}N labeling will provide unambiguous assignment of the ligand species.

Materials and Methods

The 34-nucleotide RNA "enzyme" (Dharmacon Research, Inc., Boulder, CO) and 13-nucleotide DNA "substrate" strands (Integrated DNA Technologies) were purified as described.[5] Hammerhead hybrids were formed by adding equimolar amounts of "enzyme" and "substrate" oligomers, heating to 90 °C for 3 min, and cooling slowly at room temperature in 5 mM triethanolamine pH 7.8, 1.0 M NaCl (Alfa Aesar, puratronic). $MnCl_2$ (American Analytical, Ultrapure grade) and ethylene glycol (20% v/v) were added following hybrid formation. GMP samples were prepared in the identical buffer. ESEEM samples were 1 mM hammerhead ribozyme hybrid:1 mM Mn^{2+} or 10 mM GMP:1 mM Mn^{2+}.

^{15}N-labeled GMP and GTP were prepared from *E. coli* grown on $(^{15}NH_4)_2SO_4$ (98%, Cambridge Isotopes) using slight modifications from published procedures.[6,7] Nucleotides were analyzed by HPLC (Vydac) as >85% pure. ^{15}N-GTP was incorporated into the 34-nucleotide enzyme strand by *in vitro* synthesis with T7 RNA polymerase,[5,8] using unmodified GMP to initiate transcription.[8]

Mn^{2+} binding to the hammerhead complex was monitored by X-band EPR spectroscopy (Bruker ESP 300 equipped with an Oxford liquid helium cryostat). No Mn^{2+} EPR signal was detected at room temperature in the 1:1 Mn^{2+}-hammerhead samples, consistent with all Mn^{2+} being bound to the biomolecule.[5] The low-temperature X-band EPR signal exhibited small but reproducible features that are diagnostic of Mn^{2+} in this hammerhead ribozyme site.[9] Ethylene glycol was chosen as an appropriate cryoprotectant based on activity and Mn^{2+}-binding assays.[9]

X-band ESEEM experiments were performed on a spin−echo instrument described previously,[10] with samples immersed in liquid helium (4.2 K). Three pulse ESEEM experiments measure the T dependence of the stimulated echo amplitude from the $\pi/2-\tau-\pi/2-T-\pi/2-$echo pulse sequence.[11] Frequency domain spectra were obtained from Fourier analysis of the time domain modulation patterns following the deadtime-backfill method described by Mims.[11]

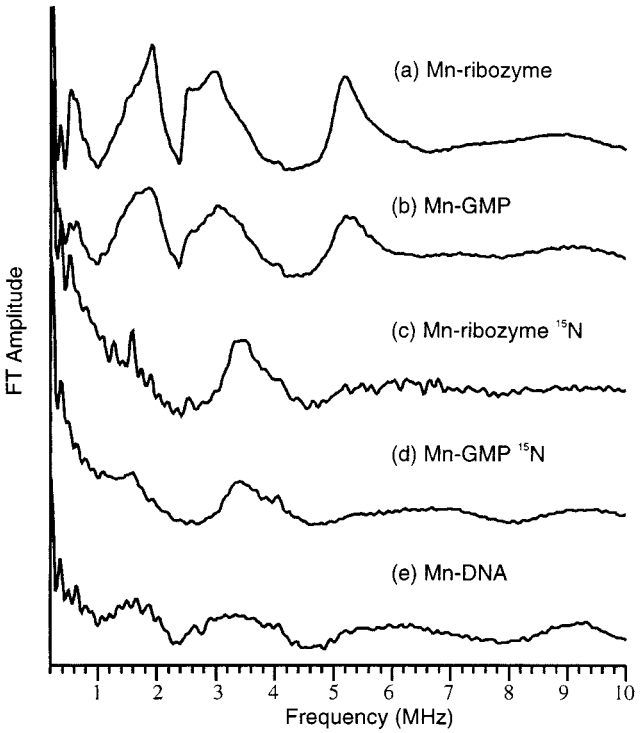

Figure 2. Three-pulse ESEEM Fourier transform spectra of Mn^{2+}−nucleic acid complexes: (a) natural abundance hammerhead; (b) natural abundance GMP; (c) hammerhead labeled with ^{15}N-G in enzyme strand; (d) ^{15}N-GMP; and (e) DNA substrate strand. Experimental conditions were as follows: temperature = 4.2 K; microwave frequency = 10.2337 GHz; magnetic field = 3600 G; pulse length = 11 ns; microwave power = 3.2 W.

Results

The 3-pulse ESEEM spectrum obtained for Mn^{2+} bound to a hammerhead ribozyme complex consisting of an RNA "enzyme" and DNA "substrate" strand exhibits significant modulation whose frequency-domain Fourier transform spectrum is shown in Figure 2a. Low-frequency features at <6 MHz are in the regime expected for ^{14}N coupled to Mn^{2+}.[13−16] A probable source of ^{14}N ligation in nucleic acids is the N7 position of purine nucleotides and, as a comparative model, ESEEM spectra were obtained on samples of Mn^{2+} with guanosine monophosphate (GMP) (Figure 2b). The natural abundance ^{14}N Mn^{2+}−GMP complex exhibits modulation frequencies identical with those observed for the hammerhead sample. On the basis of X-ray crystallography, in the GMP complex the Mn^{2+} coordination environment is expected to include a direct ligand from N7 of guanine.[17] These results therefore suggest that the modulation identified in the hammerhead ribozyme sample arises from direct Mn^{2+} coordination to a purine N7 nitrogen.

To further identify the source of ESEEM in the ribozyme, a hammerhead enzyme strand was prepared by *in vitro* transcription using ^{15}N-labeled GTP. The resulting hammerhead complex

(4) (a) Dahm, S. C.; Derrick, W. B.; Uhlenbeck, O. C. *Biochemistry* **1993**, *32*, 13040−13045. (b) Lott, W. B.; Pontius, B. W.; von Hippel, P. H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 542−547. (c) Zhou, D. M.; Kumar, P. K. R.; Zhang, L. H.; Taira, K. *J. Am. Chem. Soc.* **1996**, *118*, 8969−8970. (d) Dahm, S. C.; Uhlenbeck, O. C. *Biochemistry* **1991**, *30*, 9464−9469.

(5) Horton, T. E.; Clardy, D. R.; DeRose, V. J. *Biochemistry* **1998**, *37*, 18094−18101.

(6) Batey, R. T.; Battiste, J. L.; Williamson, J. R. *Methods Enzymol.* **1995**, *261*, 300−322.

(7) Nikonowicz, E. P.; Sirr, A.; Legault, P.; Jucker, F. M.; Baer, L. M.; Pardi, A. *Nucleic Acids Res.* **1992**, *20*, 4507−4513.

(8) Milligan, J. F.; Uhlenbeck, O. C. *Methods Enzymol.* **1989**, *180*, 51−62.

(9) Morrissey, S. R.; Horton, T. E.; DeRose, V. J. Submitted for publication.

(10) Sturgeon, B. E.; Britt, R. D. *Rev. Sci. Instrum.* **1992**, *63*, 2187−2192.

(11) Mims, W. B.; Peisach, J. In *Biological Magnetic Resonance*; Berliner, L. J., Ruben, J., Eds.; Plenum Press: New York, 1981; pp 213−263.

(12) Mims, W. B. *J. Magn. Reson.* **1984**, *59*, 291−306.

(13) LoBrutto, R.; Smithers, G. W.; Reed, G. H.; Orme-Johnson, W. H.; Tan, S. L.; Leigh, J. S., Jr. *Biochemistry* **1986**, *25*, 5654−5660.

(14) McCracken, J.; Peisach, J.; Bhattacharyya, L.; Brewer, F. *Biochemistry* **1991**, *30*, 4486-4491.

(15) Espe, M. P.; Hosler, J. P.; Ferguson-Miller, S.; Babcock, G. T.; McCracken, J. *Biochemistry* **1995**, *34*, 7593−7602.

(16) Buy, C.; Girault, G.; Zimmermann, J.-L. *Biochemistry* **1996**, *35*, 9880−9891.

(17) DeMeester, P.; Goodgame, D. M. L.; Jones, T. J.; Skapski, A. C. *Biochem. J.* **1974**, *139*, 791−792.

contains an enzyme strand labeled with ^{15}N only in guanosines, and an unlabeled substrate strand. The 3-pulse ESEEM observed for this Mn^{2+}−(^{15}N)G hammerhead sample is completely altered from that of the unlabeled hammerhead, showing a broad dominant peak centered at 3.4 MHz and a peak of smaller amplitude at the 1.6 MHz ^{15}N Larmor frequency (Figure 2c). For comparison, the frequency-domain ESEEM spectrum for Mn^{2+} coordinated to ^{15}N-GMP is shown in Figure 2d. This sample exhibits the same frequencies as found for the ^{15}N-G labeled hammerhead model. Finally, a spectrum of Mn^{2+} combined 1:1 with the isolated 13-nucleotide DNA substrate strand, which contains five guanosines in its sequence, exhibits only shallow modulation, and the Fourier transform lacks the distinct peaks observed in the hammerhead and GMP complexes (Figure 2e). This is consistent with the lack of a specific Mn^{2+} binding site in the substrate alone.[5]

Discussion

Comparison of the RNA-derived Mn^{2+}−guanosine nitrogen ESEEM signals reported here with those previously reported for Mn^{2+}−histidine[14,15] and imidazole[14] coordination reveals a set of ESEEM features with similar characteristics but slightly different frequencies, as expected for subtle differences between the nitrogen-containing heterocycles. For example, despite small frequency differences, the detailed ESEEM line shapes of the Mn^{2+}−hammerhead complex are almost identical to those reported by McCracken et al.[14] for modulation assigned to the directly coordinated ^{14}N of a histidine ligand to Mn^{2+} in a crystallographically defined site in the concanavalin A protein.[18] Having ESEEM data for both stable nitrogen isotopes in the hammerhead and GMP samples aids in further spectral assignment. The fact that the large 3.4 MHz peak in the spectra of the ^{15}N-labeled GMP and hammerhead samples is close to twice the ^{15}N Larmor frequency at this field ($\nu_n(^{15}N) = 1.6$ MHz) indicates that the measurements are obtained in a "cancellation" regime where the magnitudes of hyperfine and external fields are similar.[19] Using the familiar first-order perturbation expression $\nu_{\pm,\,\mathrm{endor}} = |\nu_n \pm A/2|$ to determine the hyperfine coupling constant, A, results in a calculated coupling of $A(^{15}N) = 3.6$ MHz [$A(^{14}N) = 2.6$ MHz]. For this cancellation regime in which $A/2 \sim \nu_n$, the ^{14}N ESEEM frequencies in a 3-pulse experiment generally consist of the three possible transitions between the three nuclear quadrupole sublevels, and a higher frequency double quantum $\Delta m_I = \pm 2$ feature.[19,20] Assuming this situation,[20] the simplest analysis of the lowest frequencies shown in Figure 2a gives nuclear quadrupole parameters of $e^2qQ \sim 2.9$ MHz and $\eta \sim 0.4$. This value of e^2qQ is reduced somewhat from the $e^2qQ = 3.26$ MHz value obtained for the N7 ^{14}N of guanine monohydrate,[21] a reduction expected upon metal coordination.[22] From the frequency of the 5.2 MHz double quantum feature, we estimate a hyperfine coupling of $A(^{14}N) \sim 2.3$ MHz,[20] in close agreement with the value obtained independently from the ^{15}N ESEEM analysis.

Comparing the ESEEM arising from Mn^{2+}−guanine coordination to Mn^{2+}−imidazole and histidine complexes,[14] it is seen that the putative double quantum features are similar and the differences arise mainly as slight shifts of the three lowest NQR-derived frequencies. This is consistent with a similar Mn^{2+}−nitrogen hyperfine coupling, but slightly different nuclear quadrupole parameters for the ligands. Indeed, for the uncoordinated ligands the reported nuclear quadrupole values (e^2qQ, η) differ slightly between the imino ^{14}N of imidazole (3.3 MHz, 0.13)[22] and N7 of guanine (3.1 MHz, 0.31).[21] The small peaks at the ^{15}N Larmor frequency in the two ^{15}N samples arise from the other nitrogens of the guanine ligand that are more distant than the coordinated nitrogen, and the small feature in all spectra at 4 MHz arises from weakly coupled ^{23}Na ($\nu_n(^{23}Na) = 4.06$ MHz).[19] It is also interesting to note that the ^{14}N ESEEM of the Mn^{2+}−hammerhead ribozyme shows better resolution than the Mn^{2+}−GMP model sample, consistent with less disorder in the ligand binding of the macromolecular RNA complex.

The ESEEM signals observed here set the stage for identification of RNA-derived metal ligands through site-specific isotopic labeling and further detailed analysis of the spectroscopic signals, both currently in progress. Several different metal sites have been predicted by X-ray crystallography of the hammerhead,[23−27] the population and detailed ligation environments of which remain to be tested under solution conditions. Of the common crystallographically predicted sites, two involve metal−nitrogen coordination by a guanine. One potential site in Stem II of the ribozyme involves N7 of guanine G10.1 (the A9/G10.1 site). Despite a distance of >10 Å from the cleavage site in the crystallographic models, activity studies have implicated metal binding at this site to be required for hammerhead activity.[28,29] All published structures involving divalent cations predict this site to be populated, but differences exist in the details of the ligation environments. A second site populated in X-ray crystallographic studies, also distant from the cleavage site but affecting activity,[27,30] involves nitrogen coordination from position G5 in the "U-turn" of the molecule. While these predictions from X-ray crystallography are a

(18) Becker, J. W.; Reeke, G. N.; Cunningham, B. A.; Edelman, G. M. *J. Biol. Chem.* **1975**, *250*, 1513−1524.

(19) (a) Lai, A.; Flanagan, H. L.; Singel, D. J. *J. Chem. Phys.* **1988**, *89*, 7161−7166. (b) Flanagan, H. L.; Singel, D. J. *J. Chem. Phys.* **1987**, *87*, 5606−5616. (c) A preliminary magnetic field tracking experiment (Hoogstraten, C.; Grant, C. V.; Horton, T. E.; DeRose, V. J.; Britt, R. D. In preparation) shows that both ^{14}N and ^{15}N modulations decrease dramatically in the "wings" of the absorption spectra on either side of the six peaks corresponding to the central $m_s = -1/2 \leftrightarrow +1/2$ transitions. This suggests that the "exact cancellation" condition at this field value occurs for the $m_s = \pm 1/2$ manifolds, rather than for one of the $m_s = \pm 3/2$ or $\pm 5/2$ derived manifolds that contribute more relative intensity in the spectral wings. Preliminary magnetic field tracking and buffer substitution experiments also support assignment of the small feature at 4 MHz to weakly coupled ^{23}Na.

(20) Assigning features at 0.6, 1.9, and 2.5 MHz to the quadrupolar transitions ν_0, ν_-, and ν_+, respectively, and the peak at 5.2 MHz to ν_{dq}, and using the following expressions to relate these frequencies to A, e^2qQ, and η: $\nu_0 = 2K\eta$, $\nu_- = K(3 - \eta)$, $\nu_+ = K(3 + \eta)$, $\nu_{dq} = 2[(\nu_N + A/2)^2 + K^2(3 + \eta^2)]^{1/2}$ where $K = e^2qQ/4$, one obtains values of $e^2qQ = 2.9$ MHz, $\eta = 0.4$, and $A(^{14}N) = 2.3$ MHz. Preliminary experiments tracking ESEEM peak frequencies as a function of field were consistent with this assignment of ^{14}N quadrupolar and double quantum transitions. However, an $S = 1/2$, $I = 1$ ESEEM simulation does not adequately reproduce the detailed line shapes of the 3-pulse spectra, and the above assignments of the quadrupolar peak frequencies are somewhat arbitrary. It is clear that a more detailed treatment of ESEEM effects caused by the $S = 5/2$ Mn^{2+} is required.

(21) (a) Garcia, M. L. S.; Smith, J. A. S. *J. Chem. Soc., Perkin Trans. 2* **1983**, 1401−1408. (b) Palmer M. H. *Z. Naturforsch.* **1996**, *51a*, 479−488. It should be noted that e^2qQ does not differ substantially between guanine N7 and N3; our assumption of ligation at N7 is based on it being the most commonly observed site.

(22) Ashby, C. I. H.; Cheng, C. P.; Brown, T. L. *J. Am. Chem. Soc.* **1978**, *100*, 6057−6067.

(23) Pley, H. W.; Flaherty, K. M.; McKay, D. B. *Nature* **1994**, *372*, 68−74.

(24) Scott, W. G.; Finch, J. T.; Klug, A. *Cell* **1995**, *81*, 991−1002.

(25) Scott, W. G.; Murray, J. B.; Arnold, J. R. P.; Stoddard, B. L.; Klug, A. *Science* **1996**, *274*, 2065−2069.

(26) Murray, J. B.; Terwey, D. P.; Maloney, L.; Karpeisky, A.; Usman, N.; Beigelman, L.; Scott, W. G. *Cell* **1998**, *92*, 665−673.

(27) Feig, A. L.; Scott, W. G.; Uhlenbeck, O. C. *Science* **1998**, *279*, 81−84.

(28) Peracchi, A.; Beigelman, L.; Scott, E. C.; Uhlenbeck, O. C.; Herschlag, D. *J. Biol. Chem.* **1997**, *272*, 26822−26826.

(29) Peracchi, A.; Beigelman, L.; Usman, N.; Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11522−11527.

(30) Fu, D. J.; Rajur, S. B.; McLaughlin L. W. *Biochemistry* **1993**, *32*, 10629−10637.

reasonable starting point in identifying the Mn^{2+} site under current study, it also is entirely possible that a previously unidentified site will be found populated under solution conditions.

In summary, these Mn^{2+} ESEEM experiments in the hammerhead ribozyme and the GMP model system demonstrate the potential of ESEEM spectroscopy as a powerful tool for decisively assigning metal coordination environments in structured RNA molecules in solution.

Acknowledgment. This work was supported by the National Science Foundation (CAREER to V.J.D.), the National Institutes of Health (GM58096 to V.J.D., GM48242 to R.D.B.), the Robert A. Welch Foundation (V.J.D.) and the Texas Higher Education Coordinating Board (V.J.D.). V.J.D. is a Cottrell Scholar of the Research Corporation.

JA9921571