Spectroscopic Evidence for Inner-Sphere Coordination of Metal Ions to the Active Site of a Hammerhead Ribozyme

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The discovery of catalytic RNA[1] (ribozymes) has led to an intense interest in understanding the structure and mechanism used by RNA to carry out enzymatic functions once thought unique only to proteins. Of special interest is the divalent metal ion requirement for ribozyme activity.[2] Proposed mechanisms for ribozyme-catalyzed cleavage by an internal nucleophile include one- and two-metal-ion models (Figure 1A).[3] In both models, one metal ion coordinates to a nonbridging oxygen at the scissile phosphate, making the phosphorus center more electrophilic and susceptible to attack by the 2′ OH.

The small hammerhead ribozyme (Figure 1B) is an ideal target for studying the role of metal ions in ribozyme catalysis. Its three-dimensional structure has been solved,[4] and the kinetic parameters have been determined.[5] Phosphorothioate replacement experiments performed on the hammerhead ribozyme suggest that a Mg^{2+} ion coordinates specifically to the pro-R oxygen at the cleavage site in the substrate.[6] Replacement of the pro-R oxygen with sulfur (Rp) results in a reduced cleavage rate in the presence of Mg^{2+} while the Sp isomer cleavage rate is comparable to that of the unmodified phosphodiester. However, the cleavage rate of the Rp isomer can be enhanced or "rescued" to a level close to that of the native enzyme by addition of "softer" Mn^{2+} ions. Thus, it was concluded that the pro-R position must be coordinated directly to a metal ion in order for cleavage to occur. This conclusion was recently disputed by Zhou et al.[7] who point out that although the thio effect ($k_{Mg,oxy}/k_{Mg,thio}$) is higher for the Rp isomer, the rescue value ($k_{Mn,thio}/k_{Mg,thio}$) is small and approximately the same for each phosphorothioate isomer. In addition, the unmodified substrate is cleaved 20-fold faster in the presence of Mn^{2+} compared to Mg^{2+}. Thus, Zhou et al. concluded that the similar magnitude of the rescue values is the result of a slightly lower pK_a of Mn^{2+}-bound H_2O and that direct coordination to the pro-R oxygen is unlikely. On the other hand, a Mg^{2+} ion was found 2.2 to 2.4 Å away from the pro-R phosphate oxygen in the recent crystal structure of a hammerhead conformational intermediate,[4c] which supports direct coordination. However, this Mg^{2+} ion is only observable in the crystal at high pH (pH 8.5) and the structural configuration shown cannot explain the reaction mechanism without further conformational change.

Spectroscopy is an ideal tool for addressing this question of whether direct coordination of metal ions to the pro-R oxygen occurs in ribozyme catalysis. Unlike X-ray crystallography, most spectroscopic techniques require no crystals, use smaller quantities of RNA, and allow one to probe metal-binding sites in solution under physiological conditions. However, in contrast to the tremendous success in small metal complexes and metalloproteins,[8] spectroscopy has not been applied to probe the metal-binding sites in ribozymes. One of the main obstacles is the low metal-binding affinity ($K_d \sim 0.1-10$ mM),[9] making it difficult to probe the metal ion(s) at the active site because metal ions at other locations in the ribozyme often dominate the spectrum.

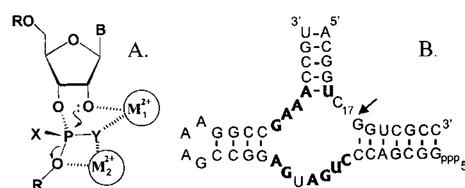

Figure 1. (A) The proposed model for the catalytic divalent metal ions involved in ribozyme-mediated cleavage.[3] X and Y denote the pro-S and pro-R oxygens, respectively. (B) Sequence of a 34-mer hammerhead ribozyme complexed to its all-RNA 13-mer substrate. Conserved residues are shown in bold and the cleavage site designated with an arrow.

We have overcome this obstacle by taking advantage of the high affinity of soft divalent metal ions such as Hg(II) for the phosphorothioate sulfur atom.[10,11] Phosphorothioates have been used successfully to elucidate structural and mechanistic properties of both proteins[12] and ribozymes.[6,7,13] Hg(II) has been substituted into a variety of thiolate-containing metalloproteins.[14,15] The position and intensity of its thiolate ligand-to-metal charge-transfer bands in UV−vis spectra and its chemical shifts in ^{199}Hg NMR are sensitive to the number and identity of the ligands, and the geometry of the site.[15] Hg(II)-substituted proteins have also been studied by X-ray absorption spectroscopy[16] and X-ray crystallography.[17] In addition, the interaction of Hg(II) with mononucleotide[18] and polynucleotide[19] bases and phosphorothioates[20] has been established. By placing the phosphorothioate at the cleavage site and using soft metal ions as spectroscopic probes, we can (a) increase the metal-binding affinity at the active site

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(10) A phosphorothioate derivative of the hammerhead ribozyme's substrate which contains a single sulfur substitution in place of either the pro-R or pro-S nonbridging phosphate oxygen at the cleavage site (Figure 1B) was synthesized (Oligos, Etc., Wilsonville, OR) and purified by strong anion exchange HPLC. The substrate Rp and Sp isomers were separated by reverse phase HPLC and identified by nuclease digestion according to ref 6b. The ribozyme was prepared by in vitro transcription with T7 RNA polymerase and purified using a novel method developed in our laboratory (Cunningham, L.; Kittikamron, K.; Lu, Y. *Nucleic Acids Res.* **1996**, *24*, 3647−3648).

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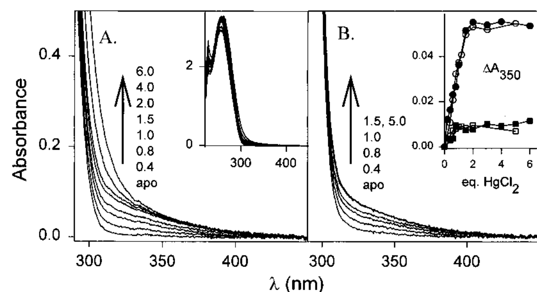

Figure 2. UV−vis spectra resulting from titration with $HgCl_2$. The numbers in the spectra represent the increasing equivalents of $HgCl_2$ added (1 equiv = 28 μM $HgCl_2$). (A) S_{thio} (28 μM phosphorothioate substrate). The inset shows the full-scale spectrum. (B) ES_{thio} (40 μM ribozyme complexed to 28 μM phosphorothioate substrate). The inset shows the change in absorbance at 350 nm of S_{thio} (●), ES_{thio} (○), S_{oxy} (■), ES_{oxy} (□) upon addition of $HgCl_2$. The substrate (S_{thio}) used in all UV−vis titrations is a roughly equal mixture of Rp and Sp isomers. All solutions were buffered at pH 7 with 200 mM PIPES.

without affecting metal-binding affinity at other sites and (b) focus on the study of the target metal-binding site.

The high affinity of mercury for sulfur is evident in the UV−vis absorption spectra (Figure 2) of $HgCl_2$ titration of the phosphorothioate substrate (S_{thio}) alone and ribozyme−substrate complex (ES_{thio}). Addition of a subequivalent of $HgCl_2$ results in a broad absorption from 300 to 400 nm. Titrations of the unmodified substrate (S_{oxy}) and native ribozyme complex (ES_{oxy}) under identical conditions show no such band (Figure 2B, inset), indicating that the 300−400 nm band is a result of mercury's interaction with the phosphorothioate sulfur atom.[21,22]

To determine if the observed Hg(II)-binding contributes to ribozyme activity, single turnover activity assays were performed. Cleavage activity is not supported by Hg^{2+} alone. However, addition of $HgCl_2$ to a solution containing 15 mM $MgCl_2$ results in a rescue of k_{obs} if Rp S_{thio} is the substrate and no rescue if Sp S_{thio} substrate is used. When 5 equiv of $HgCl_2$ (500 nM) was used, a 9-fold rescue of k_{obs} was observed.[23−25] The rescue value for the Rp isomer is close to the value of 18 obtained for 10 mM Mn^{2+} in the rescue study by Dahm et al.[6a] It is remarkable that, rather than being an inhibitor of the enzyme, Hg^{2+} is capable of rescuing the cleavage activity at sub-micromolar concentrations whereas millimolar concentrations of Mn^{2+} are required.[26] This high metal-binding affinity is due to the unparalleled thiophilicity of Hg(II), enabling us to separate a metal interaction involved in the cleavage step from the other metal−RNA interactions present in the ribozyme. To further probe the mechanism, the 5′-cleavage product was characterized using high-resolution polyacrylamide gel electrophoresis.[13f] The 5′-product migrates at the same position as the 2′,3′-cyclic phosphate product standard in all of the following cases: $ES_{oxy} + Mg^{2+}$, $ES_{thio} + Mn^{2+}$, and $ES_{thio} + Mg^{2+} + Hg^{2+}$. This strongly suggests that the Hg(II) "rescue" reaction follows the same cleavage mechanism as the Mn(II) "rescue" reaction and, in turn, the same mechanism used by the native ribozyme in the presence of Mg(II).

The nature of the broad 300−400-nm absorption band is still uncertain. Phosphorothioate S-to-Hg(II) charge-transfer (CT) bands have not been reported in the literature. Thiolate S-to-Hg(II) CT bands in metalloproteins and model complexes typically range from 230 to 300 nm, with a strong absorption around 245 nm and a weaker absorption around 290 nm.[14,15] It is possible that the absorption we observed around 300 nm is a phosphorothioate S-to-Hg(II) CT band since it falls into the range for thiolate S-to-Hg CT absorption.[27] To further support this assignment, we performed titrations of Hg(II) with low concentrations (8 μM) of substrates S_{thio} and S_{oxy}. Subtraction of S_{oxy} from S_{thio} resulted in a difference spectrum with one band at 244 nm (ϵ = 13 000 M^{-1} cm^{-1}) and another around 323 nm (ϵ = 2500 M^{-1} cm^{-1}). Additional studies of Hg(II)−phosphorothioate complexes are underway to establish how the CT band's intensity and position changes with coordination number.

In summary, our study represents the first UV−vis spectroscopic study of the metal-binding site in a hammerhead ribozyme, and our results provide clear evidence for inner-sphere coordination of Hg(II) to the nonbridging sulfur at the cleavage site in an active phosphorothioate hammerhead derivative. This direct coordination is responsible for mercury's ability to rescue the cleavage activity of a phosphorothioate ribozyme. Our method of combining the high thiophilicity of Hg(II) with selective placing of phosphorothioates in ribozymes can overcome the low metal-binding affinity, allowing spectroscopic study of the catalytic metal-binding sites in ribozymes.

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Supporting Information Available: Text describing the experimental procedures used, a plot of the activity timecourse, autoradiogram of high-resolution PAGE analysis, and a UV−vis difference spectrum (4 pages print/PDF). See any current masthead page for ordering information and Web access instructions.

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(21) The UV absorbance at 350 nm for Hg(II) with S_{thio} and ES_{thio} is similar in magnitude which may indicate that the coordination geometry is not particularly different between the two systems.

(22) Preliminary Hg(II) titrations using Rp ES_{thio} and Sp ES_{thio} showed that the 300−400 nm absorption band was observed in both isomers, although the intensity of the band differed for each isomer. This result indicates that both nonbridging positions are accessible for coordination even though only Rp ES_{thio} possesses the correct structural configuration for cleavage activity (see footnotes 23 and 26).

(23) The k_{obs} values ($\times$ 10^{-3} min^{-1}) determined were the following: 10 (Rp ES_{thio}), 112 (Sp ES_{thio}), and 250 (ES_{oxy}) in the presence of 15 mM $MgCl_2$ and 90 (Rp ES_{thio}), 116 (Sp ES_{thio}), and 225 (ES_{oxy}) with 15 mM $MgCl_2$ and 5 equiv of $HgCl_2$. All assays were carried out at 25 °C in 50 mM PIPES, pH 7. Addition of 5 equiv of $HgCl_2$ alone to S_{thio} or ES_{thio} does not result in any significant cleavage above the background level.

(24) A titration of 50 μM R_p S_{thio} substrate complexed with 70 μM ribozyme was performed in which $MgCl_2$ was added first followed immediately by $HgCl_2$. The 300−400 nm region showed no change from the apo ES_{thio} absorbance when 15 mM $MgCl_2$ was added, but upon subsequent addition of 1 equiv (50 μM) of $HgCl_2$, an immediate appearance of the broad 300-nm band was observed.

(25) Rescue was also observed at the RNA concentrations used in UV−vis titrations. Maximum rescue (23% more product) was seen when 1 equiv of (28 μM) $HgCl_2$ was added to a solution containing 15 mM $MgCl_2$, 28 μM Rp S_{thio}, and 40 μM ribozyme (in 200 mM PIPES).

(26) The magnitude of rescue observed with Rp ES_{thio} remains constant from 5 to 50 equiv of Hg(II). However, the cleavage activity of Sp ES_{thio} is inhibited by Hg(II) above 5 equiv (0.5 μM), probably due to disruption of the active site.

(27) Electronic absorptions around 300 nm attributable to Hg(II) interaction with either a base nitrogen,[18a−c] or the C-5 of cytosine and uracil[18d] have been observed. Hg(II) binding to base nitrogens can be reversed by addition of Cl^- ion.[18a] The absorption increase for S_{oxy} (see inset of Figure 2B) can be reversed by addition of 280 mM NaCl (data not shown), and therefore is attributable to Hg(II)−nitrogen interaction. On the other hand, the 350 nm absorption increase for S_{thio} is largely unaffected by the addition of NaCl. This result indicates that the 350 nm absorption of S_{thio} and ES_{thio} must have a significant component arising from coordination to the phosphorothioate sulfur. Furthermore, there is evidence against Hg(II) binding to the C-5 position of C or U under the conditions used in our study. According to Dale et al.,[18d] (a) $HgCl_2$ is completely inactive toward C-5 mercuration and (b) the rate of mercuration of heteropolynucleotides using HgOAc, the best reagent for C-5 mercuration, is considerably slower than that of mononucleotides and requires high temperature and prolonged incubation.