Studies of the Catalytic-Metal-Binding Site in the Hammerhead Ribozyme Using the Phosphorothioate Approach

Lynette A. Cunningham Final Seminar July 29, 1999

Ribonucleic acid (RNA) molecules can fold into complex three-dimensional structures called "ribozymes" which are capable of binding and cleaving RNA target sequences. The finding that RNA can function as an enzyme resulted in the awarding of the 1989 Nobel Prize in chemistry to Sidney Altman and Thomas Cech.[1,2] Because of their potential ability to specifically base pair with and cleave viral RNA, ribozymes are currently being investigated as potential anti-viral drugs.[3] In addition, ribozymes obtained from *in vitro* selection experiments can catalyze a variety of reactions, including peptide bond formation, Diels Alder cycloaddition and porphyrin metallation.[4]

Ribozymes represent a new class of metalloenzymes, requiring divalent metal ions like Mg^{2+} for cleavage activity.[5] Small ribozymes, like the hammerhead ribozyme (Figure 1A), catalyze a transesterification reaction in which a 2' OH group in the RNA substrate attacks the adjacent phosphate to generate 2',3'-cyclic phosphate and 5'-OH products in the presence of millimolar concentrations of Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+} or Cd^{2+}.[6] Based on analogy with protein phosphoryl transfer enzymes, a two-metal-ion mechanism has been proposed for ribozyme-catalyzed cleavage (Figure 1B).[7,8] One metal ion can act as a Lewis acid and facilitate deprotonation of the attacking 2' OH through direct coordination. Another metal ion may stabilize the developing negative charge on the leaving group. Both metal ions can facilitate nucleophilic attack by pulling electron density away from the phosphorus center via coordination to one of the nonbridging phosphate oxygens at the cleavage site. The number of metal ions involved, the metal-ligand geometry, and whether direct metal coordination to the 2' OH, non-bridging pro-R oxygen and 5' oxygen groups occurs remain in question.

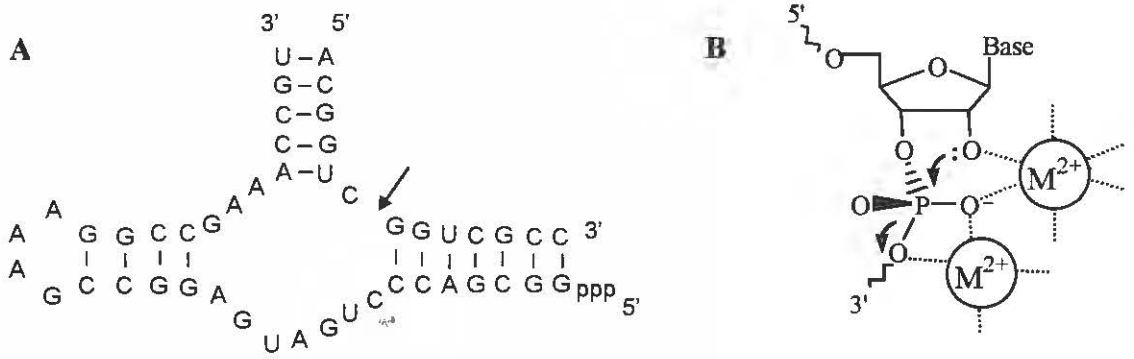

Figure 1

In the same way that metal ions have been excellent probes of metalloprotein active sites,[9] spectroscopic study of metal-binding sites in ribozymes can provide detailed structural and mechanistic information about RNA catalysis. However, the low metal-binding affinity typical of ribozymes and lack of an efficient method to purify large quantities of RNA makes it difficult to study the role of metal ions in RNA catalysis using spectroscopy. We have overcome these obstacles by (1) developing a preparative-scale purification method that integrates the high capacity and automation of column chromatography with the high resolution of gel electrophoresis;[10,11] and (2) using the phosphorothioate approach which involves selectively placing a phosphorothioate (Figure 2) in the ribozyme active site and probing the interaction of the phosphorothioate sulfur with thiophilic metal ions, such as Hg^{2+}. Using this approach, we can specifically increase the metal-binding affinity at the active site in the ribozyme and probe the site with spectroscopy.

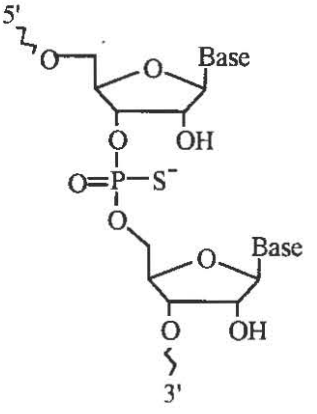

Figure 2

Our UV-vis, ^{31}P NMR and EXAFS studies provide evidence for the interaction of Hg^{2+} with the phosphorothioate sulfur in the hammerhead ribozyme. Addition of $HgCl_2$ to the phosphorothioate RNA substrate resulted in a UV-vis spectrum containing bands at 244 nm (ε = 13,000 $M^{-1}cm^{-1}$) and 323 nm (ε = 2,500 $M^{-1}cm^{-1}$).[12] Thiolate sulfur-to-Hg(II) charge-transfer 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.[13] Thus, the absorptions we observed are most likely due to sulfur-to-Hg(II) charge-transfer. ^{31}P NMR of the ribozyme bound to its DNA phosphorothioate inhibitor substrate revealed an 18 ppm upfield shift of the phosphorothioate phosphorus signal upon addition of Hg(II). Further support for binding of Hg(II) to the phosphorothioate sulfur comes from L-edge mercury EXAFS of the ribozyme bound to the DNA phosphorothioate inhibitor substrate which can be fit with a model containing two Hg(II) ions bridged by the phosphorothioate sulfur with an average Hg(II)-S bond length of 2.52 Å.

The phosphorothioate approach has been widely used in combination with activity assays in the literature to establish an interaction of metal ions with phosphate oxygens in the ribozyme structure.[14] If an interaction of a phosphate oxygen with Mg^{2+} is important, replacement of that phosphate oxygen with sulfur decreases ribozyme activity. An enhancement or "rescue" in cleavage rate upon addition of a softer metal ion than Mg^{2+}, such as Mn^{2+}, is taken as evidence for coordination of the metal ion to the phosphorothioate sulfur and by analogy, an interaction of Mg^{2+} with oxygen in the native ribozyme. We obtained HPLC evidence for the unexpected discovery that soft metal ions like Hg^{2+} and Mn^{2+} are capable of catalyzing desulfurization, isomerization and cleavage of the phosphorothioate RNA at rates similar to that of ribozyme-catalyzed cleavage (Figure 3). The latter result is significant because it suggests that the observed rescue in rate observed in previous phosphorothioate studies may be due in part to ribozyme-catalyzed cleavage of the native (phosphate) substrate produced from metal-catalyzed desulfurization rather than the phosphorothioate-substituted substrate itself.

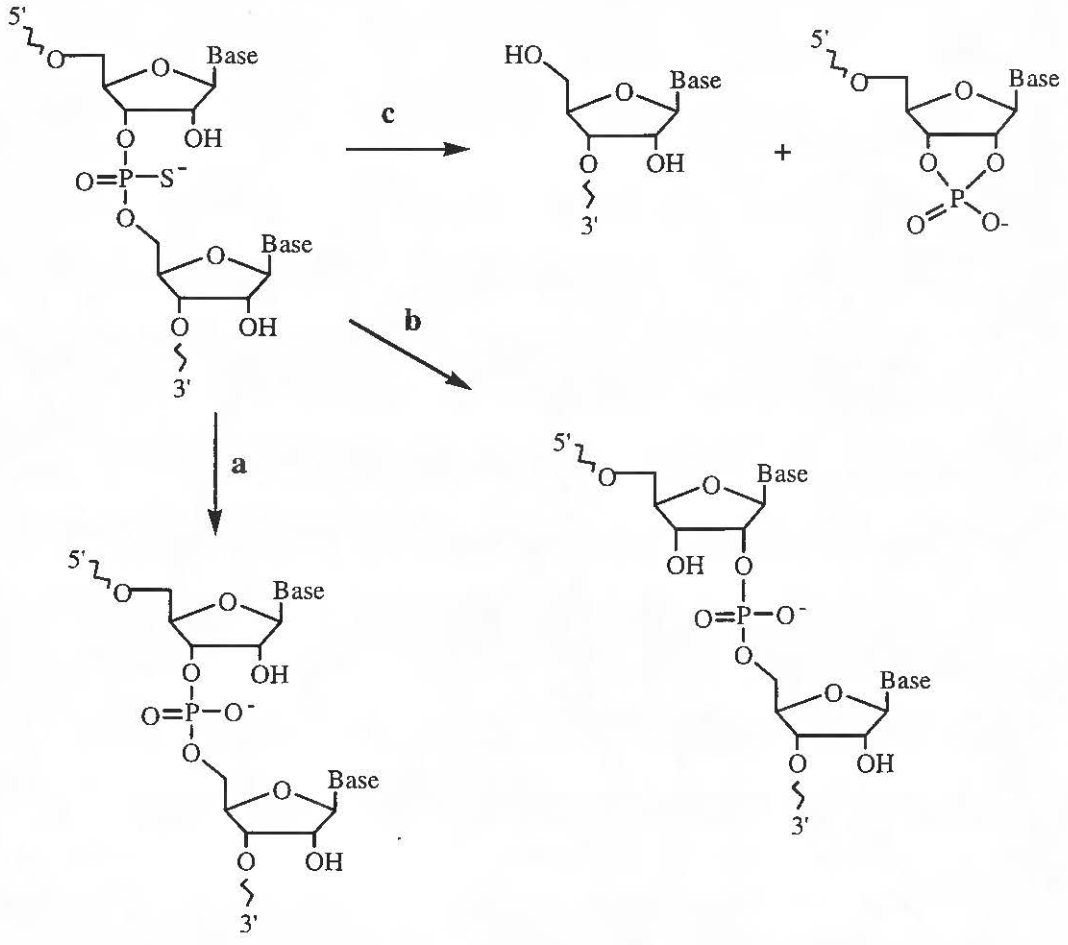

Figure 3

References

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