Identification and characterization of a novel high affinity metal-binding site in the hammerhead ribozyme

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ABSTRACT

A novel metal-binding site has been identified in the hammerhead ribozyme by ³¹P NMR. The metal-binding site is associated with the A₁₃ phosphate in the catalytic core of the hammerhead ribozyme and is distinct from any previously identified metal-binding sites. ³¹P NMR spectroscopy was used to measure the metal-binding affinity for this site and leads to an apparent dissociation constant of 250–570 μM at 25 °C for binding of a single Mg²⁺ ion. The NMR data also show evidence of a structural change at this site upon metal binding and these results are compared with previous data on metal-induced structural changes in the core of the hammerhead ribozyme. These NMR data were combined with the X-ray structure of the hammerhead ribozyme (Pley HW, Flaherty KM, McKay DB. 1994. *Nature* 372:68–74) to model RNA ligands involved in binding the metal at this A₁₃ site. In this model, the A₁₃ metal-binding site is structurally similar to the previously identified A₉ metal-binding site and illustrates the symmetrical nature of the tandem G•A base pairs in domain 2 of the hammerhead ribozyme. These results demonstrate that ³¹P NMR represents an important method for both identification and characterization of metal-binding sites in nucleic acids.

Keywords: ³¹P NMR; hammerhead ribozyme; metal binding

INTRODUCTION

Divalent metal ions play key roles in the folding and function of RNA and are critical to catalytic activity of most ribozymes. The global folding and catalytic activity of the hammerhead ribozyme is dramatically influenced by metal ion type and concentration (Dahm & Uhlenbeck, 1991; Koizumi & Ohtsuka, 1991; Dahm et al., 1993; Bassi et al., 1995, 1996, 1997), and high-affinity metal binding has been proposed to affect two conformational transitions in the hammerhead (Bassi et al., 1996, 1997). A number of specific metal-binding sites have been observed by biophysical, X-ray crystallographic, and biochemical techniques (see Fig. 1) (Ruffner & Uhlenbeck, 1990; Pley et al., 1994; Scott et al., 1995, 1996; Menger et al., 1996; Knoll et al., 1997; Feig et al., 1998; Horton et al., 1998; Murray et al., 1998; Feig & Uhlenbeck, 1999). However, except for two examples involving unusual metal ions [the study

of Cd²⁺ binding to a modified hammerhead containing a single phosphorothioate (Peracchi et al., 1997) and a Tb³⁺-binding site that was characterized biochemically and crystallographically (Feig et al., 1998)], it has not been possible to obtain binding affinities for specific sites within the hammerhead ribozyme. In the current study, ³¹P NMR techniques have been used *both* to identify a novel metal-binding site associated with the A₁₃ phosphate in the catalytic core of the hammerhead ribozyme and to determine the Mg²⁺ ion-binding affinity of this site.

RESULTS AND DISCUSSION

The ³¹P NMR spectra of the hammerhead ribozyme at substoichiometric concentrations of Mn²⁺ reveal a single ³¹P resonance at 0.95 ppm that broadens before any other resolved resonances in the spectrum (Fig. 2). The paramagnetic Mn²⁺ ion causes distance-dependent (1/*r*⁶) relaxation of NMR-active nuclei near the metal-binding site (Bertini & Luchinat, 1986). These data provide unambiguous evidence for participation of this phosphate in a metal-binding site. This ³¹P resonance was assigned to the A₁₃ phosphate by the incorpora-

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FIGURE 1. Summary of metal-binding sites in the catalytic core of the hammerhead ribozyme. The sequence, secondary structure, and numbering scheme for hammerhead ribozyme studied here are shown. The 34-nt ribozyme was synthesized by *in vitro* transcription with T7 RNA polymerase as described (Milligan et al., 1987; Nikonowicz et al., 1992), and the 13-nt noncleavable substrate was chemically synthesized as described (Wincott & Usman, 1997). Potential Mg^{2+} -binding sites identified by phosphorothioate studies are marked with an open circle, the A_{13} site identified here is marked by a filled circle, sites observed by X-ray crystallography are marked with an X (Pley et al., 1994; Scott et al., 1995, 1996; Feig et al., 1998), the uranyl photocleavage site (Bassi et al., 1995) is marked with an asterisk, and the cleavage site is marked by an arrow. The substrate used in this study has a dA at position 17 to prevent cleavage. The shaded area indicates the so-called domain 2 in the hammerhead (Pley et al., 1994) that contains the tandem G•A base pairs. The dashed lines indicates formation of the sheared G•A base pairs.

tion of a nonbridging ^{17}O at position A_{13} (not shown) in a chemically synthesized hammerhead ribozyme using ^{17}O enriched H_2O (~40% ^{17}O , ~30% ^{16}O , ~30% ^{18}O H_2O , Cambridge Isotopes, Inc.) in the oxidation step with aqueous iodine (Wincott & Usman, 1997). Site specific incorporation of ^{17}O has been previously shown to be a general method for assigning ^{31}P resonances in chemically synthesized nucleic acids (Connolly & Eckstein, 1984; Petersheim et al., 1984), because the ^{17}O efficiently broadens the attached ^{31}P resonance by quadrupolar relaxation (Tsai, 1984) without affecting the conformation of the molecule. This assignment was confirmed by specific substitution of A_{13} with a phosphorothioate in a chemically synthesized ribozyme (Wincott & Usman, 1997) that shifts the associated ^{31}P resonance downfield by 50 ppm (not shown) (Tsai, 1984). Although the Mn^{2+} titration shown in Figure 2 can identify the metal-binding site, it cannot be used to determine metal-binding affinity. This is because rapid exchange of the metal ion means that a single Mn^{2+} ion can cause relaxation-induced broadening of phosphates on multiple RNAs (Bertini & Luchinat, 1986; Ott et al., 1993). Thus even though the highest ratio of Mn^{2+} to hammerhead is 0.02:1.0, the A_{13} ^{31}P resonances on all hammerhead molecules are affected by the Mn^{2+} (see Fig. 2). As seen in the titration in Figure 2, resonances at ~0.0 and -1.6 ppm are broadened by Mn^{2+} at lower concen-

FIGURE 2. ^{31}P NMR spectra of the hammerhead-substrate complex as a function of added $MnCl_2$. The A_{13} resonance is highlighted with an arrow, and the sharp peak at ~0.72 ppm is from inorganic phosphate that arises from hydrolysis of the 5'-terminal triphosphate on the ribozyme. The sample conditions are ~0.8 mM hammerhead ribozyme:substrate complex, 100 mM NaCl, 25 mM sodium succinate, 0.05% NaN_3 , 20% D_2O , pH 5.5, 25°C, and the Mn^{2+} concentration was increased by the addition of aliquots of 0.55 mM $MnCl_2$. The spectra were acquired on a Varian VXR-500 MHz spectrometer using proton decoupling during the 0.55-s acquisition time and with a sweep width of 30,000 Hz, a 1.0-s recycle delay, and 40,000 scans. The NMR data were processed using Felix95 (MSI, Inc.) applying a 2-Hz exponential linebroadening window prior to Fourier transformation. The spectra were scaled to the most upfield resonance and referenced externally to DSS.

trations than the bulk of the ^{31}P peaks. These resonances have been assigned to the GAAA tetraloop (Legault & Pardi, 1994) in the hammerhead (Fig. 1) and indicate that there is also a Mn^{2+} binding site in the GAAA tetraloop.

To determine metal-binding affinity for the A_{13} site, a Mg^{2+} titration was performed on the hammerhead ribozyme (Fig. 3). The diamagnetic Mg^{2+} ion does not cause enhanced relaxation of the phosphates, thus the effect of Mg^{2+} ions on specific resonances is stoichiometric and can be used to measure apparent binding affinities of Mg^{2+} to specific sites in the hammerhead. At the high concentration of RNA used for the NMR

FIGURE 3. ^{31}P NMR spectra of the hammerhead ribozyme–substrate complex as a function of free Mg^{2+} ion. The A_{13} resonance is highlighted with an arrow, and the sharp peak at ~ -0.72 ppm is from inorganic phosphate that arises from hydrolysis of the 5'-terminal triphosphate on the ribozyme. The sample conditions and NMR acquisition and processing parameters are the same as those used in Figure 2 except that the samples were extensively dialyzed to the designated free Mg^{2+} concentrations. Data points were collected in ascending order of Mg^{2+} concentration: 0, 25, 50, 100, 150, 200, and 300 μM MgCl_2 , and then 75-, 50-, 25-, and 0- μM MgCl_2 ; spectra were recollected in descending order to determine experimental reproducibility. The initial two data points were collected with 20,000 scans with the number of transients doubled for all subsequent data points to achieve better signal-to-noise as the A_{13} ^{31}P resonance broadened.

experiments (~ 0.85 mM), the free concentration of Mg^{2+} does not equal the total Mg^{2+} ion concentration (as can generally be assumed in biochemical experiments performed at much lower RNA concentrations). Thus extensive dialysis of the hammerhead sample at each data point is required to achieve a known concentration of free Mg^{2+} ion, which is then used in determining metal-binding affinities by NMR. The effects of free Mg^{2+} concentration on this site are depicted in Figure 3, where the A_{13} ^{31}P resonance shifts and broadens as a function of Mg^{2+} concentration. The broadening of the A_{13} resonance indicates that it is undergoing chemical exchange on the NMR chemical shift timescale between

the metal-free and metal-bound states (Gutowsky & Holm, 1956; Lian & Roberts, 1993). The equilibrium constant for metal binding can be determined from line-shape analysis using the chemical shifts of the free and bound states and the lifetime for exchange, assuming a single metal binding to this site (Gutowsky & Holm, 1956; Lian & Roberts, 1993). The populations and exchange lifetime were fit from the experimentally determined chemical shift for the free state, and by using a range of chemical shifts for the bound state. The chemical-shift change of the ^{31}P resonance depends primarily upon the populations of the two states, whereas the increased linewidth of the resonance is a function of the exchange lifetime. The chemical shift of the free A_{13} resonance is easily assigned; however, upon addition of Mg^{2+} , the A_{13} resonance shifts into the crowded region of the ^{31}P spectrum; thus it was not possible to determine precisely the chemical shift of the bound state (Fig. 3). The bound-state ^{31}P resonance must be within the unresolved region of the spectrum (between -0.3 and -1.3 ppm in Fig. 3) and this uncertainty in the chemical shift leads to the error in the calculated equilibrium constant. This lineshape analysis for the ^{31}P spectra of A_{13} in Figure 3 leads to an apparent dissociation constant of 250–570 μM at 25 °C for binding of a single Mg^{2+} ion to the A_{13} phosphate in the hammerhead.

Biophysical studies have previously been used to measure Mg^{2+} ions binding to the hammerhead with apparent dissociation constants of 100 μM to >1 mM (Koizumi & Ohtsuka, 1991; Menger et al., 1996; Bassi et al., 1997). Considering temperature and ionic strength effects, the Mg^{2+} binding affinity for the A_{13} site measured here is one of the higher affinity Mg^{2+} binding sites in the hammerhead ribozyme. Lilley and coworkers have used gel electrophoresis and fluorescence resonance energy transfer to show that the hammerhead ribozyme undergoes two distinct metal-induced structural transitions (Bassi et al., 1995, 1996, 1997). They modeled the high-affinity transition to binding of a single metal ion with an apparent dissociation constant of 100–125 μM at 4 °C and 80 mM Tris, 25 mM NaCl, pH 8.5. This transition has been linked to the purine track, $\text{G}_{12}\cdot\text{A}_{14}$, through mutational studies and is proposed to involve formation of the tandem sheared $\text{G}\cdot\text{A}$ base pairs leading to coaxial stacking of stems II and III in the hammerhead (Bassi et al., 1996). Our NMR data also show evidence for a structural change in this region of the ribozyme. As seen in Figure 3, the A_{13} ^{31}P resonance shifts upfield upon addition of Mg^{2+} , which is in contrast to the titration of GTP with Mg^{2+} where the ^{31}P resonances shift downfield as a result of deshielding by the divalent metal ion (Son et al., 1975). This upfield shift of the ^{31}P resonance indicates a Mg^{2+} -induced structural change occurring within the ribozyme, because an electrostatic interaction between the metal and the phosphate would lead to a downfield

shift. It is not possible to determine from the ^{31}P NMR data if this is a local structural change, involving only the phosphate of A_{13} , or if this is part of a globular conformational transition such as the high-affinity metal-induced structural change observed by fluorescence energy transfer experiments (Bassi et al., 1996, 1997).

Recent electron paramagnetic resonance studies on the hammerhead ribozyme have revealed two classes of Mn^{2+} -binding sites in the hammerhead, a set of ~ 4 sites with dissociation constants of $\sim 4 \mu\text{M}$ and another ~ 5 sites with dissociation constants of $\sim 460 \mu\text{M}$ in 100 mM NaCl, pH 7.8 (Horton et al., 1998). These apparent binding constants for Mn^{2+} are much tighter than the dissociation constant for Mg^{2+} binding to the A_{13} site determined here, or the "high-affinity" Mg^{2+} site observed by Lilley and coworkers. Unfortunately, it is not possible to determine binding affinities for Mn^{2+} sites from an NMR titration such as that given in Figure 2, because, as discussed above, a single paramagnetic Mn^{2+} ion leads to relaxation-induced broadening for resonances on multiple RNA molecules in the sample. Thus additional studies are needed to understand if these large differences in metal-binding affinities are simply a result of switching from the hard metal Mg^{2+} to the softer metal Mn^{2+} .

There are additional data suggesting that the A_{13} phosphate is a potential metal-binding site in the hammerhead. Phosphorothioate substitution experiments show that replacement of the pro-Rp oxygen with a sulfur at any one of the C_{17} , A_9 , A_{13} , or A_{14} phosphates leads to inactive ribozyme (Ruffner & Uhlenbeck, 1990; Knoll et al., 1997). The activity of the C_{17} or A_9 phosphorothioate substituted molecules can be rescued with thiophilic metals such as Mn^{2+} and Cd^{2+} , but Mn^{2+} is unable to rescue activity for the A_{13} or A_{14} phosphorothioate substitutions (Ruffner & Uhlenbeck, 1990; Knoll et al., 1997; Peracchi et al., 1997; Scott, 1997). The A_9 phosphate is part of a metal-binding site observed in the original X-ray structure of the hammerhead (Pley et al., 1994), where a Mn^{2+} ion is ligated by the pro-Rp

oxygen of A_9 and the N7 of $\text{G}_{10.1}$. As pointed out by McKay and coworkers, the tandem $\text{G}_8\cdot\text{A}_{13}$, $\text{A}_9\cdot\text{G}_{12}$ base pairs in domain 2 of the hammerhead, which includes the A_9 metal site, form a pseudo-symmetric structure where the A_{13} phosphate could potentially form a metal-binding site similar to that observed for A_9 (Pley et al., 1994). The NMR data unambiguously identify a metal-binding site involving the A_{13} phosphate and Figure 4 shows a proposed model for metal binding to this site. The A_{13} metal site (green sphere) was modeled after the pseudo-symmetric A_9 metal-binding site (red sphere) while also including the pro-Rp oxygen of A_{14} as one of metal ligand interactions. In the X-ray structure of the hammerhead, the Mn^{2+} is 1.89 and 2.05 Å from the pro-Rp oxygen of A_9 , and the N7 of $\text{G}_{10.1}$, respectively (Pley et al., 1994). An essentially identical metal-binding site was observed in the X-ray structure of a RNA duplex containing an internal loop with $\text{G}\cdot\text{A}$ and $\text{A}\cdot\text{A}$ base pairs (Baeyens et al., 1996), indicating that this site may represent a general metal-binding motif in RNA. In our modeled A_{13} metal-binding site (Fig. 4), it is not possible to place a metal such that it has as short distances to both the corresponding pro-Rp oxygen and N7 atoms as is observed for the A_9 site. Thus the ligands for the metal cannot be uniquely determined, and one or more of the metal-RNA interactions are likely water mediated (Feig & Uhlenbeck, 1999). Possible candidates for these metal ligands are the pro-Rp oxygen of A_{13} , N7 of A_{14} , and the pro-Rp oxygen of A_{14} , where some or all of these atoms could form water-mediated interactions with the metal. Cedergren and coworkers calculated the electrostatic surface of domain 2 in the X-ray structure of the hammerhead, and the region around the A_{13} metal-binding site has a very high negative potential (Chartrand et al., 1997). They also modeled metal binding in this region where instead of the metal interaction with the N7 of A_{14} proposed here (Fig. 4), the metal was positioned to interact with the N1 of G_8 . Westhof and coworkers have used Brownian-dynamics simulations to model metal-binding

FIGURE 4. A stereoview illustrating the tandem sheared $\text{G}\cdot\text{A}$ base pairs in domain 2 in the hammerhead ribozyme. The red sphere highlights the Mn^{2+} at the A_9 phosphate binding site observed in the original X-ray structure of the hammerhead (Pley et al., 1994). The green sphere represents the modeled metal-binding site involving the A_{13} phosphate identified here. There are several ways to model a metal-binding site for the A_{13} phosphate and only one possible set of RNA ligands is shown here (see text). The G and A residues in the tandem $\text{G}\cdot\text{A}$ base pair are colored red and purple, respectively, to help illustrate the symmetrical properties of the A_9 and A_{13} metal-binding sites in domain 2.

sites in several RNAs (Hermann & Westhof, 1998), and they also proposed a metal-binding site in the region around A₁₃ and A₁₄ phosphates.

One curious aspect of the A₁₃ metal-binding site is that no metal is found in this region of any of the X-ray structures of the hammerhead ribozyme (Pley et al., 1994; Scott et al., 1995, 1996), even though this appears to be one of the tighter metal-binding sites in the molecule (Fig. 2). One possible reason is that the high concentrations of monovalent ions used in the crystallization (Pley et al., 1994) may compete away divalent ions from this site. Another possibility is that there may be multiple ways in which a metal could bind at this site. For example, part of the time the metal could interact directly with one RNA ligand (for example, the purine N7) and other times this particular interaction would be water mediated and a different ligand may directly interact with the metal. Such heterogeneity of binding interactions would lead to a low occupancy of any of the individual binding motifs, thus smearing out the electron density and preventing identification of the metal in the X-ray structure. This type of heterogeneity of binding interactions would be predicted to be more entropically favorable than an interaction where there is only one set of metal–RNA ligand interactions. Thus a model involving heterogeneous metal–ligand interactions is thermodynamically plausible.

In conclusion, the NMR data presented here reveal a high-affinity metal-binding site involving the A₁₃ phosphate in the hammerhead ribozyme. This site was not previously observed by X-ray or biophysical studies, but was previously predicted to occur based on the symmetrical nature of tandem G•A base pairs in domain 2 in the hammerhead ribozyme (Pley et al., 1994). These results demonstrate that ³¹P NMR represents an important method for both identification and characterization of metal-binding sites in nucleic acids.

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