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A Reappraisal, Based on ^{31}P NMR, of the Direct Coordination of a Metal Ion with the Phosphoryl Oxygen at the Cleavage Site of a Hammerhead Ribozyme

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Abstract: It has been generally accepted, on the basis of kinetic studies with phosphorothioate-containing substrates and analyses by NMR spectroscopy, that a divalent metal ion interacts directly with the pro-*R*p oxygen at the cleavage site in reactions catalyzed by hammerhead ribozymes. However, results of our recent kinetic studies (Zhou, D.-M.; Kumar, P. K. R.; Zhang. L. H.; Taira, K. *J. Am. Chem. Soc.* **1996**, *118*, 8969−8970. Yoshinari, K.; Taira, K. *Nucleic Acids Res.* **2000**, *28*, 1730−1742) demonstrated that a Cd^{2+} ion does not interact with the sulfur atom at the *R*p position of the scissile phosphate (P1.1) in the ground state or in the transition state. Therefore, in the present study, we attempted to determine by ^{31}P NMR spectroscopy whether a Cd^{2+} ion binds to the P1.1 phosphorothioate at the cleavage site in solution. In the case of the R32-S11S (ribozyme−substrate) complex, neither the *R*p- nor the *S*p-phosphorothioate signal from the S11S substrate at the cleavage site was perturbed (the change was less than 0.1 ppm) upon the addition of Cd^{2+} ions (19 equiv) at pH 5.9 and 8.5. By contrast, we detected the significant perturbation of the P9 phosphorothioate signal from another known metal-binding site (the A9/G10.1 metal-binding motif). The *R*p-phosphorothioate signal from A9/G10.1 was shifted by about 10 ppm in the higher field direction upon the addition of Cd^{2+} ions. These observations support the results of our kinetic analysis and indicate that a Cd^{2+} ion interacts with the sulfur atom of the phosphorothioate at the A9/G10.1 site (P9) but that a Cd^{2+} ion does not interact with the sulfur atom at the *R*p- or at the *S*p-position of the scissile phosphate (P1.1) in the ground state.

Hammerhead ribozymes, which are catalytic RNA molecules, cleave a specific phosphodiester bond to generate a 2′,3′-cyclic phosphate and 5′-hydroxyl product in solutions with millimolar concentrations of divalent metal ions or molar concentrations of monovalent cations.[1,2] Hammerhead ribozymes are also relatively small, and X-ray crystallography has facilitated studies of their mechanism of action.[3] Considerable controversy exists, however, as to the role of metal ions, which are of critical importance in the folding of RNA and in catalysis.[1−4] Replacement of phosphate by phosphorothioate groups has been a popular approach to the elucidation of the binding sites and functions of metal ions in various kinetic studies. In such an analysis of the scissile phosphate (P1.1; Figure 1a) of a model hammerhead ribozyme, the rate of cleavage of the *R*p-phosphorothioate stereoisomer was drastically reduced, as compared to that of the unmodified substrate (the so-called thio effect), whereas the rate of cleavage of the *S*p-isomer was unchanged.

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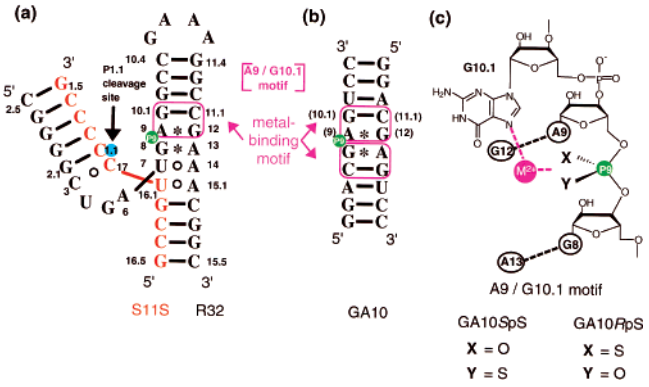

Figure 1. Sequences and secondary structures of (a) a hammerhead ribozyme (R32) and the uncleavable substrate with phosphorothioate at the cleavage site (S11S). A black arrow indicates the cleavage site. (b) Sequence and secondary structure of GA10. The metal-binding motifs (the A9/G10.1 motif of hammerhead ribozymes) are surrounded by magenta lines. In panels a and b, Watson−Crick base pairs, non-Watson−Crick base pairs, and sheared-type G:A pairs are indicated in bars, open circles, and asterisks, respectively. (c) Schematic representation of the A9/G10.1 motif.

However, addition of trace amounts of a thiophilic metal ion (Cd^{2+}) enhanced the rate of cleavage of the *R*p-phosphorothioate.[1f] This phenomenon is known as "Cd^{2+} rescue" and has been explained by the HSAB (hard and soft, acid and base) rule.[5] According to the HSAB rule, a "hard acid", such as a Mg^{2+} ion, prefers to bind to a "hard base" oxygen atom rather than to a "soft base" sulfur atom. By contrast, a "soft acid", such as a Cd^{2+} ion, prefers to bind to a "soft base" sulfur atom. On the basis of the experimental results, it was proposed that the *R*p-sulfur substitution disrupted the coordination to the *R*p-position of a hard metal ion, a Mg^{2+} ion, that is in some way essential for cleavage. This proposal is supported by the fact that the crystallographic structure of a freeze-trapped intermediate revealed a Mg^{2+} ion in close proximity to the pro-*R*p oxygen of the phosphate (P1.1) at the cleavage site.[6] This proposal is, however, somewhat controversial. In a recent kinetic study of the effects of Cd^{2+} ions on a background of Ca^{2+} ions, we concluded that a Cd^{2+} ion does not interact directly with the sulfur atom at the *R*p-position of the scissile phosphate either in the ground state or in the transition state.[7] In a titration experiment with Cd^{2+} ions in which we monitored the ribozyme-catalyzed cleavage of native and phosphorothioate substrates, we found that the rate of the ribozyme-mediated cleavage of the *R*p-phosphorothioate substrate was different (significantly lower in the presence of lower concentrations of Cd^{2+} ions) from that of both the unmodified and the *S*p-phosphorothioate substrate. However, the apparent K_D of the *R*p-phosphorothioate substrate for Cd^{2+} ions was identical with that of the unmodified and the *S*p-phosphorothioate substrate, indicating that the affinity for Cd^{2+} ion of each ribozyme−substrate complex was identical, regardless of whether the cleavage site included a phosphate or a phosphorothioate group. This result suggested, furthermore, that the sulfur in the phosphorothioate group at the cleavage site did not interact specifically with a Cd^{2+} ion.

In the present study, to clarify the involvement of metal ions in the vicinity of the phosphate group at the cleavage site, we examined the physicochemical properties of a hammerhead ribozyme−substrate complex (R32-S11S; Figure 1a) by ^{31}P NMR spectroscopy. The substrate, S11S, contained a phosphorothioate group at the C1.1 residue and was a diastereomeric mixture of *R*p- and *S*p-phosphorothioates. R32 is a 32-mer hammerhead ribozyme (Figure 1a) that has been studied in considerable detail.[7,8] Analysis of ^{31}P NMR spectra facilitates investigation of interactions between a metal ion and a phosphorothioate group since the chemical shift of the phosphorothioate signal is clearly separate from the signals due to phosphodiesters, which appear within a crowded narrow region of the spectrum. Thus, the perturbations of the chemical shift of the phosphorothioate signal can easily be recognized.[9−11] In the present study, we also analyzed the A9/G10.1 metal-binding motif by separating two diastereomeric isomers, GA10*S*pS and GA10*R*pS (Figure 1b,c), and monitoring their ^{31}P NMR spectra. This system provided a positive control for changes in chemical shifts since the well-characterized GA10 sample contained a phosphorothioate moiety at P9, as described below.[10]

An important metal-binding site within the R32 hammerhead ribozyme, A9/G10.1, is located around domain II (U7-A9 pairs with G12-A14), which forms a continuous stack between stem II (G10.1-C10.4 pairs with C11.1-G11.4) and stem III (A15.1-C15.5 pairs with U16.1-G16.5) (Figure 1a). Many studies of crystal structures of hammerhead ribozymes have identified a metal-binding site between the pro-*R*p oxygen of the phosphate of A9 (P9 phosphate) and the N7 atom of G10.1.[3] Moreover, Uhlenbeck's group and Herschlag's group reported that their kinetic studies of ribozymes with a phosphorothioate group instead of the A9 phosphate indicated that an *R*p-phosphorothioate linkage reduced the cleavage rate by a factor of 10^3, with the rate returning to the control value after the addition of Cd^{2+} ions.[1d,f] Thus, it appears that the nonbridging oxygen of the phosphate at the A9 residue is one of the metal-binding sites that is required for efficient catalysis even though this metal-binding site, within the crystal structure, is located approximately 20 Å from the scissile phosphodiester bond. Although this metal ion at the A9 residue that is 20 Å away from the cleavage site was suggested to be a catalytic metal ion,[1d,g] recent molecular dynamics analysis denied such a possibility,[1h] leading to a controversy.

In the present study, we examined GA10*S*pS and GA10*R*pS, which are derivatives of the well-characterized GA10 molecule,[10] whose sequence is shown in Figure 1b. Although GA10 corresponds only to the structure of part of a hammerhead ribozyme, GA10 is comparable to the A9/10.1 motif of the hammerhead ribozyme since GA10 includes a sheared-type tandem G12-A9 pair as a metal-binding site in the duplex and it has been shown that the GA10 molecule is sufficient to allow

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capture of divalent cations in the absence of any of the other conserved residues that are found in hammerhead ribozymes.[10] Thus, GA10 plays a role as a metal-binding site by itself and it is for this reason that we used GA10*R*pS and GA10*S*pS, which correspond to GA10 with a phosphorothioate group at A9, as a positive control for a metal-binding site in our comparisons of perturbations of chemical shifts.

Both the P1.1 and the P9 sites have recently been analyzed by ^{31}P NMR.[9−11] All the results of such analyses are consistent with the conclusion that a metal ion interacts with the nonbridging phosphoryl oxygen at the P9 site. It was also reported that a Cd^{2+} ion interacts with the cleavage-site phosphate (P1.1) of a hammerhead ribozyme.[11] In the cited study, the ^{31}P NMR signals from *R*p- and *S*p-phosphorothioates at the cleavage site were slightly perturbed upon the addition of up to 10 equiv of Cd^{2+} ions: the *R*p-signal was shifted to a higher field by about 0.6 ppm and the *S*p-signal was shifted to a lower field by about the same amount. These results appear to argue for a specific interaction between a Cd^{2+} ion and the *R*p-sulfur at the P1.1 cleavage site. It is still generally accepted that a metal ion coordinates directly with the cleavage-site pro-*R*p oxygen at P1.1. Nonetheless, our present results and analysis argue against the previous interpretation and our data can best be interpreted if we assume that a Cd^{2+} ion does not interact with either an *R*p- or an *S*p-phosphorothioate at the cleavage site. The present conclusions, based on NMR analysis, are relevant to our previous discussions of the results of our kinetic analyses.[7,8]

Results

Extent of Perturbations of ^{31}P NMR Signals from Phosphorothioates during Interactions with Metal Ions. The ^{31}P NMR signals from normal phosphodiester bonds are usually observed around 0 ppm (relative to the signal from external trimethyl phosphate)[12] and signals due to phosphorothioate linkages are usually found at approximately 55 ppm.[13] It is easy to monitor phosphorothioate signals and to investigate interactions between phosphorothioate groups and metal ions because the chemical shifts of signals due to phosphorus in phosphorothioates are located far from normal phosphodiester signals. The effects of metal ions on adenine thionucleotides were studied by Cleland's group, who found that ADPαS and ATPβS (adenine nucleotides prepared with phosphorothioates groups) yielded a higher field shift of 6.0 ppm than the controls upon addition of Cd^{2+} ions, an indication that binding of metal ions induced a large change in chemical shift.[14]

Before initiating our study of a hammerhead ribozyme, we examined two kinds of diribonucleotide, each of which contained a phosphorothioate, namely CpsC and GpsA, as negative controls. These dimers did not show any significant perturbation of signals (less than 0.1 ppm) upon addition of 19 equiv of Cd^{2+} ions (data not shown), indicating that chemical shifts of phosphorus are not perturbed just by the presence of Cd^{2+} ions and that perturbations occur only where there are specific and direct interactions between metal ions and sulfur atoms. In other words, there is no specific interaction between Cd^{2+} ions and the sulfur atoms of these diribonucleotides. Since the dinucleotides do not act, by themselves, as ligands with a metal ion-binding pocket, this negligible perturbation of the signal from the dimer provides an indication of nonspecific interactions (if any) between Cd^{2+} and the sulfur atoms of the phosphorothioates. These results suggested that it should be possible to examine the binding of a metal ion at the cleavage site of a hammerhead ribozyme by ^{31}P NMR analysis of titration of phosphorothioates with Cd^{2+}.

Absence of Any Change in ^{31}P NMR Signals from Phosphorothioate at the P1.1 Cleavage Site upon Addition of Excess Cd^{2+} Ions. We performed titration experiments with the R32-S11S complex, focusing on the cleavage site of the ribozyme−substrate complex, using a solution that contained 0.6 mM S11S (substrate containing a phosphorothioate at the P1.1 site; Figure 1a), R32 (the hammerhead ribozyme; Figure 1a), 100 mM NaCl, and 10 mM $MgCl_2$, at pH 5.9 and 8.5, with various concentrations of $CdCl_2$, namely, 0, 1.8, 6, and 11.4 mM. These concentrations of $CdCl_2$ corresponded to 0, 3, 10, and 19 molar equiv relative to R32-S11S. At each concentration of $CdCl_2$, we recorded the ^{31}P NMR spectrum in H_2O/D_2O (molar ratio, 9:1) at 30 °C. Figure 2 shows the ^{31}P NMR spectra of the R32-S11S complex. Since S11S was a mixture of *R*p- and *S*p-phosphorothioate diastereomers, two phosphorothioate signals were detectable even in the absence of $CdCl_2$. These two signals were identified by reference to a previous study.[11,13] In the absence of $CdCl_2$ at pH 5.9, the signal at 51 ppm corresponded to the *S*p-isomer and the signal of 54 ppm corresponded to the *R*p-isomer (Figure 2a).

We checked the pH before each measurement and, even though the final concentration of Cd^{2+} ions was equal to 19 molar equiv, the two phosphorothioate signals were perturbed by less than 0.1 ppm. Changes of this magnitude reflect nonspecific environmental changes, as noted above. Thus, the addition of $CdCl_2$ did not induce any significant changes in the chemical shifts of the two phosphorothioate signals at pH 5.9. With increases in the concentration of $CdCl_2$, a new signal emerged at 17 ppm, corresponding to the signal from the product of hydrolysis, namely, a cyclic phosphate. We repeated the titration experiments at pH 8.5 because in a study reported by DeRose and co-workers, while there was no Cd^{2+}-induced perturbation of the signal due to the cleavage-site phosphorothioate at pH 5.5, a perturbation was observed at pH 8.5.[11] However, in our analysis, the chemical shifts of the two signals from the phosphorothioate of S11S did not change (Figure 2b). The data can best be interpreted if we assume, on the basis of earlier kinetic analyses,[7,8a,c] that Cd^{2+} ions do not interact with the *R*p- or the *S*p-phosphorothioate at the cleavage site.

Significant Perturbations of ^{31}P NMR Signals from Phosphorothioate at the P9 Metal-Binding Site within the A9/G10.1 Motif upon Addition of Excess Cd^{2+} Ions. To examine the perturbations of chemical shifts caused by binding of metal ions to the A9/G10.1 motif, we next examined GA10*S*pS and GA10*R*pS by ^{31}P NMR spectroscopy. We separated the two stereoisomers by reversed-phase HPLC. The resonances of imino protons of GA10*S*pS and GA10*R*pS were nearly identical

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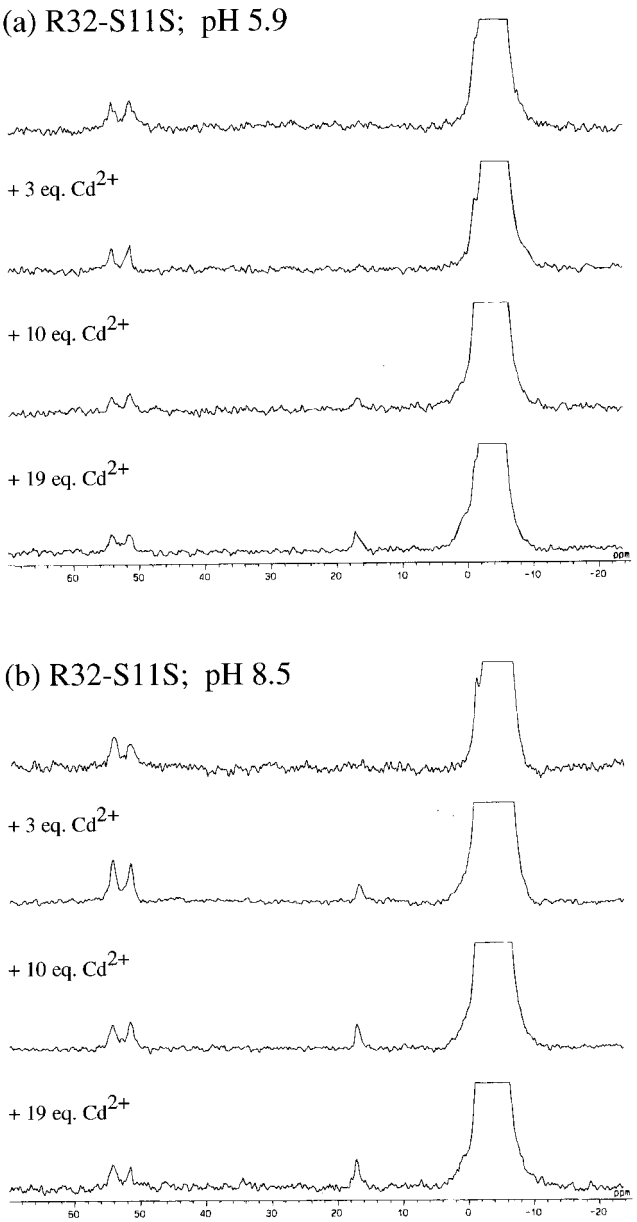

Figure 2. ^{31}P NMR spectra of R32-S11S. The spectra were recorded from a solution of 0.1 M NaCl in 10 mM succinate-d_4 buffer at pH 5.9 (a) and 8.5 (b) at 30 °C with an acquisition time of 0.33 s, a sweep width of 25 000 Hz, and a 1.5 s recycle delay. The concentration of R32-S11S was 0.6 mM in each case. From the top to the bottom in panels a and b, the molar equivalence of $CdCl_2$ to the ribozyme was 0, 3, 10, and 19, respectively. At both pH 5.9 and 8.5, the chemical shifts of the phosphorothioate of S11S were perturbed by less than 0.1 ppm upon addition of the titration of $CdCl_2$.

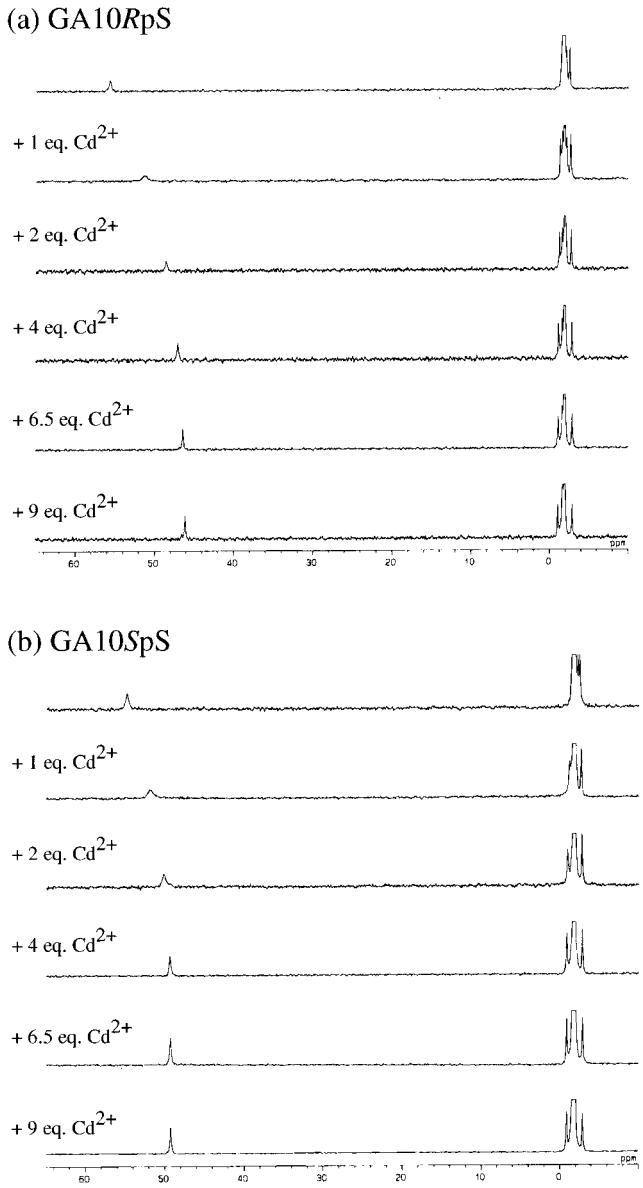

Figure 3. ^{31}P NMR spectra of GA10*R*pS (a) and GA10*S*pS (b). The spectra were recorded from a solution of 0.1 M NaCl in 10 mM succinate-d_4 buffer (pH 5.9) at 40 °C with an acquisition time of 0.33 s, a sweep width of 25 000 Hz, and a 1.5 s recycle delay. The concentrations of GA10*R*pS and of GA10*S*pS were 0.5 mM (as a duplex) in all cases. From the top to the bottom in panels a and b, the molar equivalence of $CdCl_2$ relative to the duplex was 0, 1, 2, 4, 6.5, and 9, respectively. With increasing concentrations of Cd^{2+} ions, signals from both *R*p- and *S*p-phosphorothioates (*R*p, 56 ppm; *S*p, 55 ppm, in the absence of Cd^{2+} ions) shifted in the higher field direction. The profile of each phosphorothioate signal was also changed by $CdCl_2$, as discussed in the text.

with those of GA10, which forms a double-stranded structure (data not shown).[15] Therefore, it appeared that GA10*S*pS and GA10*R*pS also formed double-stranded structures. Figure 3 shows the ^{31}P NMR spectra obtained in experiments in which GA10*R*pS and GA10*S*pS were exposed to increasing concentrations of Cd^{2+} ions. We performed these titration experiments using a solution that contained 1 mM GA10*R*pS or GA10*S*pS (single strand), 100 mM NaCl, and 2 mM $MgCl_2$ at pH 5.9 with concentrations of $CdCl_2$ of 0, 0.5, 1, 2, 3.25, and 4.5 mM. These concentrations of $CdCl_2$ corresponded to 0, 1, 2, 4, 6.5, and 9 molar equiv relative to GA10*R*pS and GA10*S*pS (with respect to each duplex). At each concentration of Cd^{2+} ions, we recorded the ^{31}P NMR spectra in H_2O/D_2O (molar ratio, 9:1) at 40 °C.

In the absence of Cd^{2+} ions, signals due to normal phosphodiester bonds were detected around 0 ppm and signals due to phosphorothioate linkages were detected at 56 (in the case of purified GA10*R*pS) and 55 ppm (in the case of purified GA10*S*pS). Upon addition of Cd^{2+} ions to the solutions of GA10*R*pS and GA10*S*pS, the signals from both *R*p- and *S*p-phosphorothioates shifted considerably in the higher field direction (Figures 3 and 4). At 9 equiv of Cd^{2+} ions, the

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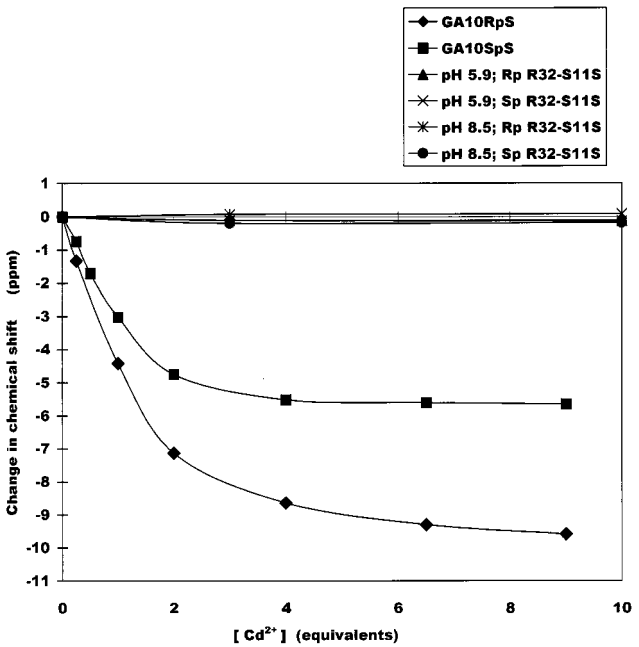

Figure 4. Changes in chemical shifts of phosphorothioate signals in ^{31}P NMR spectra of GA10*R*pS, GA10*S*pS, and R32-S11S with increasing concentrations of Cd^{2+} ions. It should be noted that the binding isotherm does not reflect the intrinsic K_D values, and the estimated K_D value is only an apparent one.[10b,21]

perturbations of phosphorothioate signals were close to maximal, indicating saturation by Cd^{2+} ions of both GA10*R*pS and GA10*S*pS. At that point (9 equiv Cd^{2+} ion), the chemical shift was 10 ppm higher than that from GA10*R*pS in the absence of Cd^{2+} ions. In the case of GA10*S*pS, the signal shifted by about 6 ppm at 9 equiv of $CdCl_2$ (Figures 3 and 4). The change in chemical shift of the signal from GA10*R*pS that was induced by $CdCl_2$ was larger than that from GA10*S*pS. Thus, the order of the chemical shifts of the signals from the phosphorothioate groups of the two isomers was reversed upon addition of $CdCl_2$ (Figure 3).

Discussion

In this report, we described the characterization, by monitoring ^{31}P NMR signals from phosphorothioates, of two possible metal-binding sites, the P9 site in the A9/G10.1 metal-binding motif and the P1.1 site at the cleavage site, of a hammerhead ribozyme. The *R*p- and *S*p-phosphorothioate signals of GA10*R*pS and GA10*S*pS (A9/G10.1 motif) moved dramatically in the higher field direction with increasing concentrations of Cd^{2+} ions in the presence of Mg^{2+} ions (Figure 3). However, the *R*p- and *S*p-signals at the P1.1 cleavage site of the R32-S11S complex remained unchanged upon addition of Cd^{2+} ions, as was also the case for two diribonucleotides (CpsC and GpsA), which lack metal-binding sites. It follows from the difference, in terms of a change in chemical shift, between the A9/G10.1 motif and the P1.1 cleavage site that the presence of phosphorothioate and Cd^{2+} ions does not necessarily, by itself, cause a change in chemical shift, and this observation implies that a change in chemical shift reflects a specific interaction between the sulfur atoms of phosphorothioates and Cd^{2+} ions. Therefore, as we postulated initially, titration with Cd^{2+} ions of phosphorothioate groups allows analysis of interactions between Cd^{2+} ions and phosphorothioates and investigations of metal-binding pockets.

The Large Perturbation of the Chemical Shift in the Case of the *R*p-Isomer with a Phosphorothioate at the P9 Site. In an earlier titration experiment with GA10 (which does not include a phosphorothioate group), perturbations in chemical shifts of P9 (−0.3 ppm), C8/G10.1 (+0.5 ppm), and H8/G10.1 (+0.08 ppm) occurred upon addition of $MgCl_2$.[10a] The extents of such changes in chemical shifts were relatively small and so we could not judge whether the coordination of a divalent cation to the A9/G10.1 motif was direct or indirect. In the present study, by contrast, the large (10 ppm) change in chemical shift of the phosphorothioate signal from GA10*R*pS (Figure 4) appears to imply direct coordination of Cd^{2+} to the sulfur atom of the phosphorothioate.

The change in chemical shift of the signal from GA10*R*pS induced by $CdCl_2$ was larger than that from GA10*S*pS, and, thus, the order of the chemical shifts of the signals from phosphorothioate groups of the two isomers was reversed. In the absence of $CdCl_2$, the chemical shift of the phosphorothioate signals from GA10*R*pS (56 ppm) was larger than that of GA10*S*pS (55 ppm). With the addition of 9 equiv of $CdCl_2$, the signal from GA10*R*pS was observed at 46 ppm and that from GA10*S*pS was observed at 49 ppm. In general, the chemical shift of the *R*p-isomer of a phosphorothioate is larger than that of the *S*p-isomer, and this reversal of the order of chemical shifts upon the addition of Cd^{2+} ions is unusual.[13b−d]

The profiles of phosphorothioate signals were also affected by the addition of $CdCl_2$ to the solution of each isomer. With the addition of 1 equiv of $CdCl_2$, the signal from both RNAs broadened (Figure 3) but further addition of $CdCl_2$ sharpened the signals conspicuously. These results reflect the transition from a metal-free form to a metal-bound form. Thus, at 1 equiv of $CdCl_2$, metal-bound and metal-free states were presented in solution and medium-range exchange between the two species resulted in the broadening of phosphorothioate signals on the NMR time scale.

The changes in chemical shifts and in profiles of signals from phosphorothioates suggest the binding of Cd^{2+} ions at the phosphorothioates. These observations are in agreement with those of DeRose's group. They studied ribozyme−substrate complexes in which the hammerhead ribozyme contained a phosphorothioate at the P9 site and reported the shifting of phosphorothioate signals in the higher field direction upon addition of Cd^{2+} ions.[11] In our studies and those of DeRose's group, the perturbation caused by Cd^{2+} ions of the chemical shift for the *R*p-isomer was significantly greater than that for the *S*p-isomer (it should be mentioned here that the assignment of *R*p- and *S*p-isomers was reversed in their paper[11,16]). The larger perturbation in the case of the *R*p-isomer as compared to the *S*p-isomer suggests that the interaction between the Cd^{2+} ion and the *R*p-sulfur atom might be preferred over that between the Cd^{2+} ion and the *S*p-sulfur atom at the P9 site.

Evidence Against Direct Coordination of a Metal Ion with the P1.1 Phosphoryl Oxygen at the Cleavage Site in Both the Ground State and the Transition State. The mechanism of the cleavage reaction of the hammerhead ribozyme has not been fully elucidated and, in particular, the conformation at the reaction site is a major source of controversy, because the crystal structure suggests that the cleavage bond does not adopt a conformation that would allow in-line attack by the 2′-OH of

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nucleotide 17 at the cleavage site and, thus, further rearrangement is required for cleavage of the bond.[3a−c,f] Moreover, current research is reshaping basic theories about the roles of metal ions in reactions catalyzed by hammerhead ribozymes and, in the opinion of many researchers, such ribozymes should no longer be viewed as true metalloenzymes.[1j,k,3e] The activity of a hammerhead ribozyme in the presence of monovalent ions has been used to argue against the hypothesis that metal ions can induce the deprotonation of 2′-OH or stabilize the leaving group directly or indirectly. The activity of a hammerhead ribozyme in the presence of $Co(NH_3)_6^{3+}$ also revealed that inner-sphere coordination is not a prerequisite for catalysis.[1k] Despite the variations in the properties of divalent metal ions, monovalent metal ions, exchange-inert metal ions, and even ammonium ions, all such ions have positive charge in common. In general, it is becoming more and more accepted that the presence of a relatively dense positive charge, rather than the presence of any particular metal ions, is the general fundamental requirement. Whether or not the positive charge plays a role in the chemical process now seems less important. However, our recent kinetic analysis indicates that the role of monovalent ions can be assigned to a specific chemical step.[2]

Moreover, the results of our kinetic studies of phosphorothioates indicated that Cd^{2+} ions do not interact with the sulfur atom at the *R*p-position of the scissile phosphate in the ground state and in the transition state.[7] We performed titrations with Cd^{2+} ions of the ribozyme-catalyzed cleavage of native and phosphorothioate substrates with 100 mM Ca^{2+} ions as background [50 mM MES-Na (pH 6), 37 °C]. The rate of the ribozyme-mediated cleavage of *R*p-phosphorothioate substrate was 3 to 4 orders of magnitude lower than that of the unmodified and *S*p-phosphorothioate substrates. However, the apparent K_D for Cd^{2+} ions was identical for all three substrates, indicating that, regardless of whether the cleavage site includes a regular phosphate or a phosphorothioate, there is no difference in the affinity for Cd^{2+} ions. This observation suggests that the sulfur of the phosphorothioate at the cleavage site does not interact with Cd^{2+} ions specifically, and the apparent K_D for Cd^{2+} ions reflects Cd^{2+} ion-binding to the metal ion-binding motif (A9/G10.1 motif) that is distal from the scissile phosphate. Indeed, in the present study, the addition of Cd^{2+} ions did not perturb the chemical shift of the P1.1 signal, indicating that, in the ground state, Cd^{2+} ions did not interact with the sulfur atom at the *R*p-position of the scissile phosphate. Although Maderia et al.[11] observed the chemical shift perturbations at the phosphorothioates at the scissile phosphate, the degrees of perturbation were much smaller than those due to specific interactions. The difference in the degrees of perturbation between our sample and that of Maderia et al.[11] seems to reflect the difference in the ribozyme sequence and NMR measurement conditions within solution equilibrium.

The question that remains to be answered is whether the hammerhead ribozyme is a metalloenzyme. Our recent studies suggest that more than one channel might exist for reactions catalyzed by the *R*32 hammerhead ribozyme and that the role of metal ions can be assigned to a specific chemical process.[2,17] The putative existence of different channels in hammerhead-catalyzed reactions is strongly supported by the result of analyses of intrinsic isotope effects.[2] Kinetic solvent−isotope effects suggest that proton transfer occurs in reactions catalyzed by the hammerhead ribozyme in the presence of high concentrations of monovalent NH_4^+ ions in the absence of any metal ions. By contrast, no such proton transfer apparently occurs in reactions catalyzed by R32 in the presence of divalent metal ions.[7c,8a,18] It is clear, moreover, that the divalent-metal-ion-catalyzed reaction is significantly more efficient than the monovalent-metal-ion-catalyzed reaction. Thus, extremely high concentrations of metal ions are required for the monovalent-metal-ion-catalyzed reactions. Therefore, it is likely that, under physiological conditions, hammerhead ribozymes use divalent ions as the catalytic cofactor and that they act as true metalloenzymes in vivo. However, as demonstrated in the present study, it is unlikely that the divalent metal ion coordinates directly with the P1.1 phosphoryl oxygen.

Experimental Section

The hammerhead ribozyme R32 was synthesized by the phosphoramidite method on an automated DNA/RNA synthesizer (model 394; PE Biosystems, USA). All of the reagents for RNA synthesis were purchased from Glen Research (USA). Each oligomer was purified by reversed-phase column chromatography as described elsewhere.[19] For the exchange of counterions, each oligomer was adsorbed onto an anion-exchange column (mono-Q; Pharmacia Biotech, Uppsala, Sweden). The column was washed with more than 10 column volumes of MILLI-Q water (MILLIPORE, USA) to wash out triethylammonium ions, which were replaced by Na^+ by elution of the oligomer with 2 M NaCl. Finally, excess NaCl was removed by desalting on a gel-filtration column (TSK-GEL G3000PW; TOSOH, Japan) with MILLI-Q water as the mobile phase. The final solution contained only the oligomer and the counterion (Na^+ ion). A crude sample of diribonucleotides (CpsC and GpsA) and the uncleavable pseudosubstrate S11S, which has a 2′-OMe group and a phosphorothioate at the cleavage site (C17) of the normal substrate, were purchased from Genset Corporation (France). S11S was purified by polyacrylamide (20%) gel electrophoresis. In this study, we did not separate S11S into its phosphorothioate diastereomeric isomers. Thus, S11S gave two *R*p- and *S*p-phosphorothioate signals in its ^{31}P NMR spectrum. A crude mixture of the GA10*R*pS and GA10*S*pS phosphorothioate isomers was purchased from Genset Corporation. The two compounds of the mixture were separated by reversed-phase HPLC (Hypersil BDS C18 5 μm; length, 250 mm; i.d., 4.6 mm; Alltech, USA), with elution with a linear gradient of CH_3CN (2%-6%, v/v) in 0.1 M CH_3COONH_4 buffer (pH 7.0). The retention times were 8.6 min for GA10*R*pS and 11.5 min for GA10*S*pS. The preparations of separated isomers were desalted on a column of Sephadex G-25 (Fast Desalting column; Pharmacia Biotech Inc.). Assignments of isomers were made after digestion by snake venom phosphodiesterase and nuclease P1.[13a,b,20−21] For digestion by snake venom phosphodiesterase, an aliquot of each thio-substituted oligonucleotide (0.5 nmol) was incubated for 8 h at 37 °C with snake venom phosphodiesterase (0.1 μg; Sigma-Aldrich) and calf alkaline phosphatase (6.0 μg; Takara, Japan) in 0.05 M Tris-HCl (pH 9.0), 0.3 mM DTT, and 1 mM $MgCl_2$ in a reaction volume of 150 μL. The products were analyzed directly by HPLC on a reversed-phase column (TSK-GEL ODS-80TM; length, 250 mm; i.d., 4.6 mm; TOSOH) with a gradient

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of buffer A, namely, 0.1 M triethylammonium acetate (pH 7.0), and buffer B, which consisted of 60% buffer A and 40% acetonitrile (5% B for 15 min followed by 5−100% B over the course of 45 min). Retention times were as follows: cytidine, 2.86 min; uridine, 5.89 min; guanosine, 8.02 min; and adenosine, 12.31 min. The products of digestion of the later-eluting isomer generated a peak at 25.87 min that corresponded to *S*p-GpsA. For digestion by nuclease P1, an aliquot of each thio-substituted oligonucleotide (0.5 nmol) was digested with nuclease P1 (2.0 μg; Sigma-Aldrich) in distilled water (120 μL) for 1 h at 37 °C. The solution was buffered with 16 μL of 50 mM Tris-HCl (pH 9.0) and digested with calf alkaline phosphatase (6.0 μg; Takara) for 1 h at 37 °C. The products were analyzed by reversed-phase HPLC as described above. The products of digestion of the earlier-eluting isomer generated a peak at 24.46 min that corresponded to *R*p-GpsA.

Samples for NMR spectroscopy were prepared by dissolving purified oligomers in 0.12 mL of a solution, prepared in 90% H_2O and 10% D_2O, of 0.1 M NaCl and 10 mM succinate-d_4 buffer at pH 5.9 in a 3 mm NMR tube (Shigemi, Japan). A solution of R32-S11S was also prepared at pH 8.5. Each sample was heated to 90 °C and then allowed to cool slowly over the course of 30 min. Concentrated solutions of $MgCl_2$ and $CdCl_2$ were added directly to the sample. For studies in the presence of metal ions, by contrast, the oligomers were not heated to 90 °C because Mg^{2+} ions at high concentrations can destroy RNA oligomers at high temperature. All ^{1}H-decoupled ^{31}P NMR spectra were recorded on a spectrometer (ALPHA-500; JEOL, Japan) operated at 202 MHz with an acquisition time of 0.33 s and a sweep width of 25000 Hz, a 1.5 s recycle delay, and 9000−60000 scans. The ^{31}P chemical shifts were referenced to external trimethyl phosphate (2.0 ppm; 10% solution in ethanol). The concentrations of GA10*S*pS and of GA10*R*pS were 0.5 mM (as duplexes) and spectra from both samples were recorded at 40 °C. The concentration of the ribozyme−substrate complex, R32-S11S, was 0.6 mM (as a complex) and spectra were recorded at 30 °C.

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