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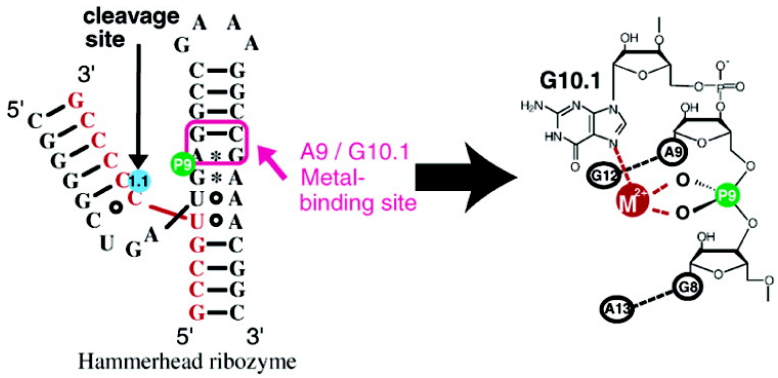

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NMR-Based Reappraisal of the Coordination of a Metal Ion at the Pro-*R*p Oxygen of the A9/G10.1 Site in a Hammerhead Ribozyme

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Abstract: In the identification of a metal-binding site within enzymes, kinetic analyses based on thio-effects and Cd^{2+}-rescues are widely used. In those analyses, kinetic studies using a phosphorothioate have been discussed on the premise that the substitution by a sulfur atom does not change the conformation of a ribozyme. However, our present NMR structural analysis demonstrates the change of the conformation at the metal-binding site by ***R*p**-sulfur but not by ***S*p**-sulfur substitution and warns against incautious interpretations of thio-effects and rescue phenomena in kinetic studies using a phosphorothioate. Our analysis further demonstrates that, in solution, a Cd^{2+} ion can interact with an ***R*p**-phosphorothioate (in support of the controversial McKay's structure, *Nature* **1994**, *372*, 68−74) and with an ***S*p**-phosphorothioate (in support of the controversial Scott's structure, *Cell* **1995**, *81*, 991−1002) at the metal-binding A9/G10.1 site and that, in the former case, the bound Cd^{2+} ion can return the ribozyme to an active conformation and rescue its enzymatic activity.

The hammerhead ribozyme is a self-cleaving RNA that is found in small RNA plant pathogens, and it catalyzes the sequence-specific cleavage of RNA. Metal ions play an important role in the catalytic cleavage of the phosphodiester bond in an RNA by hammerhead ribozymes and the reaction yields a 2′,3′-cyclic phosphate.[1] Studies by X-ray crystallography have facilitated analysis of the mechanism of action of these ribozymes.[2] The crystal structure obtained by McKay's group revealed a metal-binding site, A9/G10.1, located in the vicinity of domain II, which forms a continuous stack between stem II and stem III.[2a] In this crystal structure, an Mn^{2+} ion binds between the pro-***R*p** oxygen of the phosphate group of A9 (P9 oxygen) and the N7 atom of G10.1. Even though this metal-binding site (A9/G10.1 site) within the crystal structure is located approximately 20 Å from the scissile phosphodiester bond, this metal-binding site is thought to play a crucial role in achieving maximal cleavage activity for the following reasons. Replacement of the pro-***R*p**-phosphoryl P9 oxygen atom by a sulfur atom results in a dramatic decrease in Mg^{2+}-dependent catalytic activity.[3] Furthermore, replacement of G10.1 by a pyrimidine also results in a substantial decrease in the ribozyme's activity.[4] Moreover, the addition of a low concentration of Cd^{2+} ions, which are thiophilic, to a solution of the ribozyme with an ***R*p**-phosphorothioate linkage returns the rate of the catalytic reaction to the control value.[5]

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The results of crystallographic and kinetic studies have indicated that binding of a metal ion at the pro-***Rp*** oxygen of A9/G10.1 site is of critical importance for enzymatic catalysis by hammerhead ribozymes.[2a,5] However, this conclusion remains controversial. For example, in the crystal structure obtained by Scott's group, the metal ion does not bind at the pro-***Rp*** oxygen but, instead, it binds at the pro-***Sp*** oxygen. To be specific, $Mg(H_2O)_5^{2+}$ binds directly to the pro-***Sp*** oxygen of A9 and is associated, via the hydration shell, with the exocyclic oxygen O6 of G8, with N7 of G10.1, and with N2 of G12.[2b] This potential $Mg(H_2O)_5^{2+}$-binding site corresponds to the Mn^{2+}-binding site identified by McKay's group in their crystal structure of a hammerhead ribozyme. Furthermore, ^{31}P NMR studies using phosphorothioates indicated that a metal ion binds to both the pro-***Sp*** and pro-***Rp*** oxygen. The ^{31}P NMR signals from ***Rp***- and ***Sp***-phosphorothioates at A9 in hammerhead ribozymes moved 2−3 ppm upfield shift upon the addition of one to two molar equivalents of Cd^{2+} ions.[6] Finally, in studies of the metal-binding motifs GA10***Sp***S and GA10***Rp***S, we found that binding of a metal ion was supported at both the pro-***Sp*** and the pro-***Rp*** position.[7]

The oligomers designated GA10***Sp***S and GA10***Rp***S are derivatives of the well-characterized GA10 oligomer,[8] whose sequence is shown in Figure 1, and they have a phosphorothioate group at A6, which corresponds to the A9 metal-binding site of a hammerhead ribozyme. Even though GA10 corresponds to the structure of only part of a hammerhead ribozyme-substrate complex (R32−S11; Figure 1), GA10 is analogous to the A9/G10.1 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 demonstrated that the GA10 molecule is sufficient to allow capture of divalent cations in the absence of any of the other conserved residues that are found in hammerhead ribozymes.[8b,c,d]

In our previous study, to clarify the functions of the pro-***Rp*** and the pro-***Sp*** positions at the cleavage site and the A9/G10.1 metal-binding site of the hammerhead ribozyme, we employed GA10***Sp***S and GA10***Rp***S as metal-binding motifs at the A9/G10.1 site.[7] The ^{31}P signals from both the ***Rp***- and the ***Sp***-phosphorothioate of GA10***Sp***S and of GA10***Rp***S moved considerably upfield upon the addition of Cd^{2+} ions to solutions of GA10***Rp***S and GA10***Sp***S. At 9 molar equiv of Cd^{2+} ions, the extent of perturbations of the phosphorothioate signals were close to the maximum, indicating saturation by Cd^{2+} ions of both GA10***Rp***S and GA10***Sp***S. At saturation (9 molar equiv Cd^{2+} ions), the chemical shifts were 10 and 6 ppm higher than those of GA10***Rp***S and GA10***Sp***S, respectively, in the absence of Cd^{2+} ions. Our previous studies with GA10***Sp***S and GA10***Rp***S also demonstrated that a Cd^{2+} ion is able to bind to both the ***Sp*** and the ***Rp*** sulfur atom in the absence of domain I (catalytic domain, formation by C3, U4, G5, A6, and C17 in Figure 1) of the hammerhead ribozyme. However, the nature and the conformation of the metal-binding sites in the presence of ***Rp***- and ***Sp***-phosphorothioates and in the absence of bound metal ions remain to be investigated. The exact effects of metal ions at the catalytically significant P9 site and their roles in the catalytic activity of hammerhead ribozymes remain to be clarified.

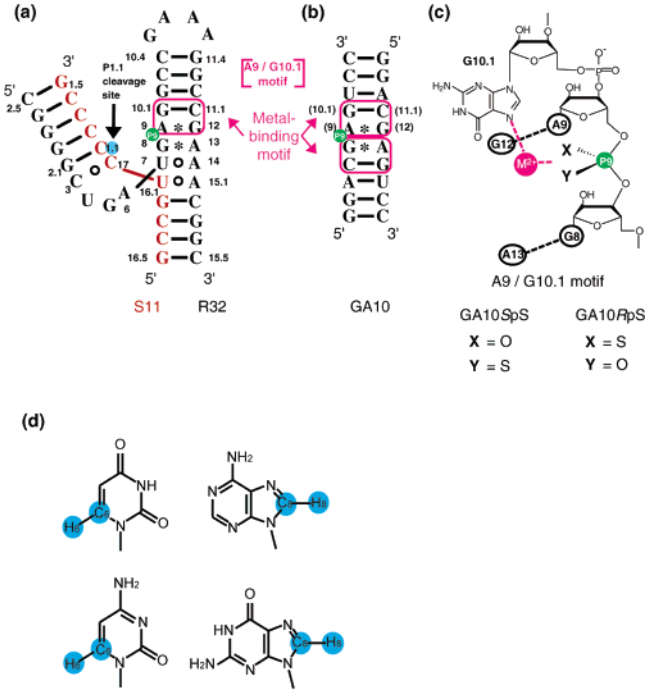

Figure 1. Sequences and secondary structures of (a) a hammerhead ribozyme (R32) and its substrate (S11). The black arrow indicates the cleavage site. (b) Sequence and secondary structure of GA10. The metal-binding motifs (the A9/G10.1 motif of the hammerhead ribozyme) are surrounded by magenta lines. In panels (a) and (b), Watson−Crick base pairs, non-Watson−Crick base pairs, and sheared-type G:A pairs are indicated by bars, open circles, and asterisks, respectively. GA10***Sp***S and GA10***Rp***S each have a phosphorothioate moiety at the A6 position, which corresponds to the A9 position of the hammerhead ribozyme. (c) Schematic representation of the A9/G10.1 motif. (d) Atoms in nucleobases that were monitored in natural-abundance $^1H-^{13}C$ HSQC.

Previous NMR investigations on the effects of a phosphorothioate on RNA structure led to two different conclusions.[9] One group observed a conformational change within an RNA hairpin by the introduction of an ***Rp***-phosphorothioate at the binding site of the phage MS2 capsid protein.[9a] The other group did not observe any structural changes by an ***Sp***-phosphorothioate modification at the metal binding site of yeast U6 RNA.[9b] The corresponding detailed structural study has not been performed for the hammerhead ribozyme with a phosphorothioate at the metal binding site, although limited ^{31}P NMR studies were reported.[6,7] In the present analysis, to elucidate the mechanism of binding of metal ions in the vicinity of the phosphate group at A9/G10.1, we examined the physicochemical properties of GA10***Sp***S and GA10***Rp***S by ^1H NMR,^{31}P NMR, and $^1H-^{13}C$ HSQC spectroscopy. The ^1H, ^{13}C, and ^{31}P NMR signals of GA10***Sp***S in the absence and in the presence of Cd^{2+} ions were, respectively, similar to those of the unmodified parental RNA,

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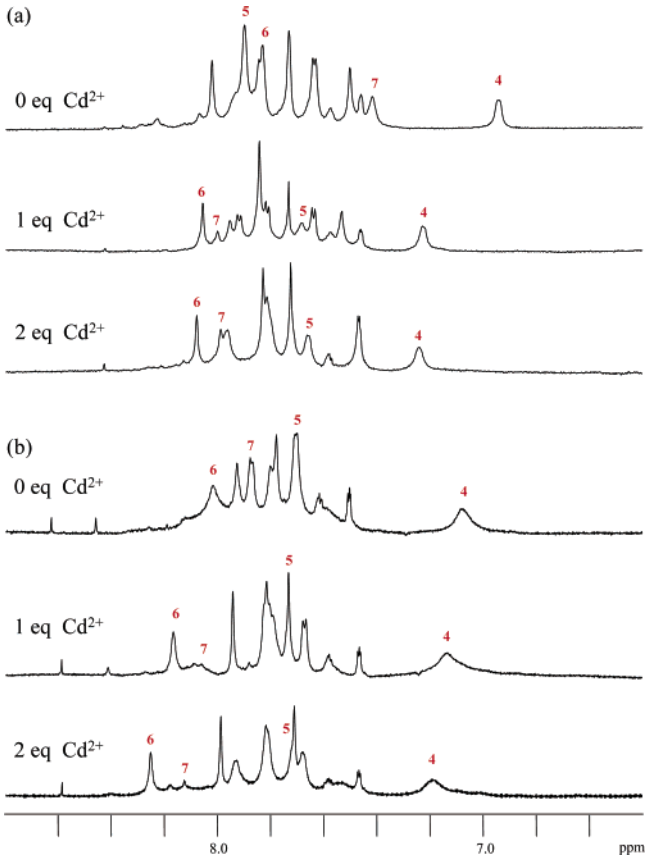

Figure 2. ^{1}H NMR spectra of GA10***Sp***S (a) and GA10***Rp***S (b). The spectra were recorded from samples dissolved in a solution of 40 mM $NaClO_4$ in 10 mM sodium cacodylate buffer at pH 7.6 in a 3-mm NMR tube at 40 °C. The concentrations, as duplexes, of GA10***Sp***S and GA10***Rp***S were 1.77 mM and 1.62 mM, respectively. From the top to the bottom in (a) and (b), the number of molar equivalents of $CdCl_2$ relative to the RNA was 0, 1, and 2, as indicated. Important signals of H8/H6 protons are labeled with respective residue numbers.

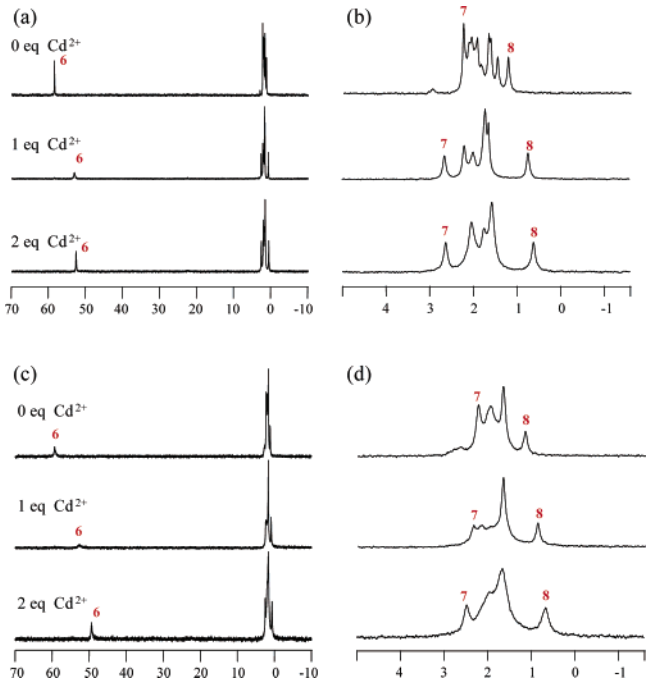

Figure 3. ^{31}P NMR spectra of GA10***Sp***S (a) and GA10***Rp***S (c). Expanded regions of the spectra are displayed in (b) and (d), respectively. The spectra were recorded from solutions of 40 mM $NaClO_4$ in 10 mM sodium cacodylate buffer at pH 7.6 in a 3-mm NMR tube at 40 °C. The concentrations, as a duplex, of GA10***Sp***S and GA10***Rp***S were 1.77 mM and 1.62 mM, respectively. From the top to the bottom in each group, the number of molar equivalents of $CdCl_2$ relative to the RNA was 0, 1, and 2, as indicated. With increasing concentrations of Cd^{2+} ions, signals from both the ***Rp***- and the ***Sp***-phosphorothioate (***Sp***, 58.5 ppm; and ***Rp***, 59.7 ppm, in the absence of Cd^{2+} ions) shifted toward a higher field. Important signals are labeled with their respective residue numbers.

namely GA10.[8] GA10***Sp***S can capture metal ions in the same manner as GA10. By contrast, the ^{1}H chemical shifts of GA10***Rp***S around the P9 phosphorothioate moiety were different from those of the parental GA10 in the absence of Cd^{2+} ions, suggesting that the introduction of a bulky sulfur atom at the P9 site had adversely affected the conformation. However, the shift of signals from GA10***Rp***S that was induced by Cd^{2+} ions was similar to that observed with GA10. Thus, the addition of Cd^{2+} ions caused a change in and a correction of conformation of GA10***Rp***S and the resultant, induced and "corrected" metal-binding form was identical to that of GA10. These result suggests that the introduction of a sulfur atom at the pro-***Rp*** position of A9/G10.1 can change the conformation of the ribozyme and that this conformational change has a negative effect on the hammerhead reaction, whereas the corresponding replacement at the pro-***Sp*** position does not influence the structure of the hammerhead ribozyme and does not affect the catalytic activity. Since the structure of a ribozyme with an ***Rp***-phosphorothioate in the absence of thiophilic metal ions is different from that of the natural ribozyme in the absence of those thiophilic metal ions, reactions catalyzed by these two different ribozymes in the presence of only hard metal ions can be quite different from each other.

While, it is still generally accepted, on the basis of the first crystal structure and kinetic studies, that coordination of a metal ion at the A9 pro-***Rp*** oxygen at A9/G10.1 is essential for hammerhead catalysis, our present analysis argues against the previous interpretation. Our data and previous structural and kinetic data can best be interpreted if we simply assume that the introduction of a sulfur atom at the ***Rp***-position but not at the ***Sp***-position deforms the active conformation of the ribozyme and that a Cd^{2+} ion can interact with either an ***Rp***- or an ***Sp***-phosphorothioate at the A9/G10.1 site to generate an active ribozyme. The reduced rate of cleavage by the ***Rp***-phosphorothioate appeared to be due to the negative effects of the structural change that were induced by the sulfur atom of the ***Rp***-phosphorothioate. Thus, care must be taken in the interpretation of thio-effects and Cd^{2+}-rescue effects in kinetic analyses.[1h,10]

Results

One-Dimensional (1D) ^{1}H and ^{31}P NMR and Two-Dimensional (2D) Natural-Abundance ^{1}H-^{13}C HSQC Spectroscopy. It has been reported that changes in chemical shifts of bases and phosphate groups provide evidence of the binding of metal ions and conformational changes.[8b,c,11] In this study, we examined chemical shifts in ^{1}H, ^{31}P, and ^{13}C NMR spectra in an effort to elucidate the structural changes in GA10***Sp***S and GA10***Rp***S that occur upon the addition of Cd^{2+} ions. The 1D ^{1}H and ^{31}P NMR spectra are shown in Figure 2 and Figure 3, respectively. It was impossible to detect signals from base carbons directly because of the low concentrations of our samples. We used natural abundance ^{1}H-^{13}C HSQC spectra

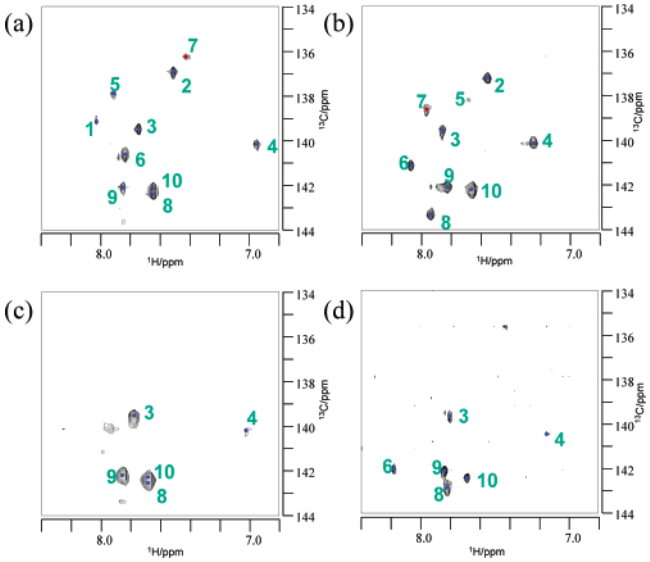

Figure 4. Natural-Abundance ^{1}H-^{13}C HSQC spectra of GA10***Sp***S in the absence of Cd^{2+} ions (a), of GA10***Sp***S in the presence of 1 molar equivalent of Cd^{2+} ions (b), of GA10***Rp***S in the absence of Cd^{2+} ions (c), and of GA10***Rp***S in the presence of 1 molar equivalent of Cd^{2+} ions (d). Intraresidue cross-peaks are labeled with their respective residue numbers.

for indirect detection of C6/C8 ^{13}C chemical shifts (Figure 1d and Figure 4).

The samples for NMR spectroscopy contained 1.77 mM GA10***Sp***S or 1.62 mM GA10***Rp***S as a duplex and 40 mM $NaClO_4$ at pH 7.6, plus $^{113}CdCl_2$, at various concentrations, namely, 0, 1.77 and 3.54 mM for GA10***Sp***S and 0, 1.62 and 3.24 mM for GA10***Rp***S. These concentrations of $CdCl_2$ corresponded to 0, 1 and 2 molar equivalents relative to GA10***Sp***S and GA10***Rp***S as a duplex. At each concentration of $CdCl_2$, we recorded the ^{1}H and ^{31}P NMR spectra and the ^{1}H-^{13}C HSQC spectra in D_2O at 40 °C.

Effects of the Sulfur Atom on the Structure of GA10*Sp*S and GA10*Rp*S in the Absence of Cd^{2+} ions. The signals due to the H6 and H8 protons of GA10***Sp***S and GA10***Rp***S in Figure 2 were assigned by reference to NOESY spectra. The H8 protons of G7 and G5 differed significantly between GA10***Sp***S and GA10***Rp***S. The chemical shift of the H8 proton of G7 (7.88 ppm) in GA10***Rp***S was significantly lower-field than that of GA10***Sp***S (7.42 ppm), suggesting a difference in conformation. The profile of the ^{31}P signals from GA10***Rp***S were broader than those from GA10***Sp***S. However, the ^{31}P chemical shifts for GA10***Sp***S and GA10***Rp***S were almost the same, as shown in Figure 3. Table 1 shows the assignments of ^{1}H and ^{31}P NMR signals in the absence of Cd^{2+} ions and the differences in chemical shifts relative to most of the parental GA10, which does not include a phosphorothioate moiety. The signals due to the protons of GA10***Sp***S were almost identical to those of GA10. The chemical shifts are different only by ±0.02 ppm.

The signals due to the protons of GA10***Rp***S were not the same as those of GA10. In particular, signals associated with C4, G5, A6, and G7 were very different from those of GA10. These results indicate that the effect of the sulfur atom on the structure of GA10***Sp***S was negligible but the effect of the sulfur atom on the structure of GA10***Rp***S was large, probably because the *R*p-sulfur atom is located toward the inside in the major groove that is a crowded position. The inserted sulfur atom in GA10***Rp***S apparently changed the structure from that of the parental GA10. Although the only difference between GA10***Rp***S and GA10 is the replacement of an oxygen atom by a sulfur atom in the former at the pro-***Rp*** position of A6, the conformation of GA10***Rp***S was not identical to that of GA10. Although the H8 proton of G2 in GA10***Rp***S is away from the metal binding site, the thiolation affected the chemical shift of this proton more than that of C4 though the origin of this shift is unknown (Table 1). However, the chemical shifts of ^{31}P signals were almost the same for GA10***Rp***S and GA10, suggesting that the conformation of the backbone of GA10***Rp***S was almost identical to that of GA10.[12] Thus, the effect of the sulfur atom in GA10***Rp***S appeared to be local and limited to the region around the A6 position, which is considered to be the metal-binding site.

Table 1. ^{1}H (H6/H8) and ^{31}P Chemical Shifts (ppm) of GA10*Sp*S and GA10*Rp*S at 40 °C in the Absence of Cd^{2+} Ions and Differences in Chemical Shifts from Those of GA10

	GA10*Sp*S				GA10*Rp*S			
	^{1}H (H6/H8)		^{31}P		^{1}H (H6/H8)		^{31}P	
base no.	ppm[a]	Δ[c]	ppm[b]	Δ[c]	ppm[a]	Δ[d]	ppm[b]	Δ[d]
1	8.02	0.01			8.02	0.01		
2	7.50	0.02	2.12	−0.08	7.62	0.14	2.23	0.03
3	7.73	−0.02	1.93	0	7.80	0.05	1.95[e]	0.02
4	6.94	−0.02	2.06	−0.03	7.07	0.11	1.95[e]	−0.14
5	7.90	0.01	1.46	−0.15	7.70	−0.19	1.66	0.05
6	7.83	−0.01	58.5	56.15	8.02	0.18	59.37	57.02
7	7.42	0	2.24	−0.03	7.88	0.46	2.23	−0.04
8	7.64	−0.02	1.22	0.06	7.70	0.04	1.16	0
9	7.85	0	1.66	−0.04	7.88	0.03	1.66[e]	−0.04
10	7.64	−0.01	1.62	−0.04	7.70	0.05	1.66[e]	0

[a] Relative to DSS. [b] Relative to 85% H_3PO_4. [c] The difference in chemical shifts (GA10***Sp***S−GA10). [d] The difference in chemical shifts (GA10***Rp***S−GA10). [e] Tentative assignment since the signal was broadening and no correlation could be obtained in the ^{1}H−^{31}P HMQC NOESY spectrum.

The ^{1}H-^{13}C HSQC spectrum (Figure 4) in the absence of Cd^{2+} ions was consistent with the results deduced from the 1D ^{1}H and ^{31}P spectra. The ^{1}H-^{13}C HSQC spectrum of GA10***Sp***S (Figure 4a) was almost identical to that of GA10,[8d] whereas the spectrum of GA10***Rp***S (Figure 4c) differed from that of GA10. The cross-peaks associated with G5, A6, and G7 were missing from the HSQC spectrum of GA10***Rp***S, suggesting that this metal-binding region around A6 might be associated with midrange chemical exchange on the NMR time-scale and might adopt multiple conformations locally. From the 1D ^{1}H and ^{31}P NMR spectra and the natural-abundance ^{1}H-^{13}C HSQC spectra, we deduced that the structure of GA10***Sp***S was almost identical to that of the parental and unmodified metal-binding motif, GA10, whereas the structure of GA10***Rp***S differed locally from the structure of GA10 around the metal-binding site in the absence of thiophilic Cd^{2+} ions. Furthermore, it appeared that midrange chemical exchange occurred around the metal-binding site of GA10***Rp***S.

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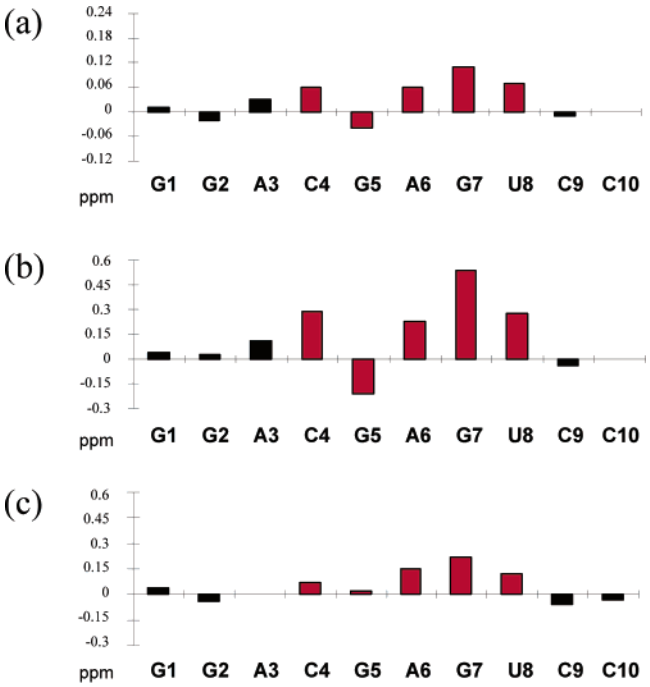

Figure 5. Changes in ^{1}H chemical shifts of H6/H8 protons of GA10 (a), GA10***Sp***S (b) and GA10***Rp***S (c) upon the addition of 1 molar equiv of Cd^{2+} ions.

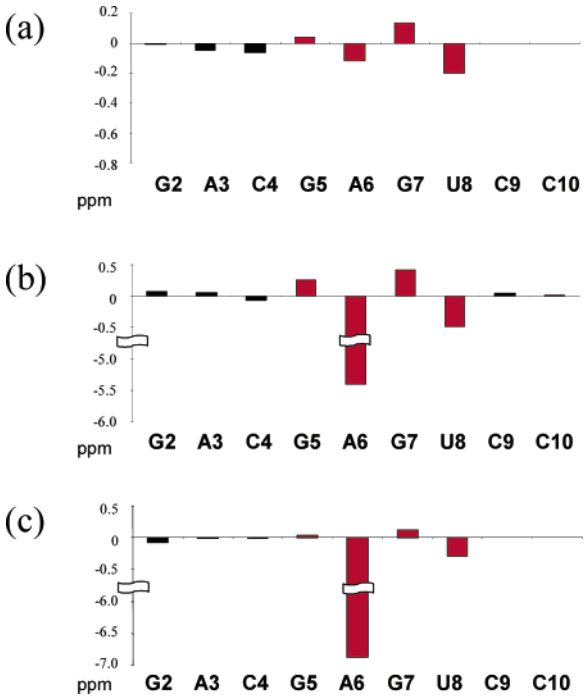

Figure 6. Changes in ^{31}P chemical shifts of phosphate signals of GA10 (a), GA10***Sp***S (b) and GA10***Rp***S (c) with the addition of one molar equiv of Cd^{2+} ions.

Perturbations Caused by Cd^{2+} Ions in GA10*Sp*S and GA10*Rp*S. The ^{1}H signals of GA10***Sp***S and GA10***Rp***S shifted upon the addition of Cd^{2+} ions (Figure 2). The changes of the chemical shifts are summarized in Figure 5 in a comparison with those for GA10. It should be noted that this decamer is self-complementary, thus, it contains two possible metal binding sites, and the addition of 1 molar equivalent Cd^{2+} will only occupy one site. However, because of the symmetry and fast-exchange, the observed ^{1}H NMR spectra for GA10***Sp***S and GA10***Rp***S were averaged singly metal-occupied structure. The changes in chemical shifts for GA10***Sp***S upon the addition of Cd^{2+} ions were similar to those for GA10. The proton signals associated with C4, A6, G7, and U8 moved largely downfield and the proton signal associated with G5 moved upfield upon the addition of Cd^{2+} ions to GA10***Sp***S (Figure 5b). These changes were almost identical to those of GA10 (Figure 5a). The signals for GA10***Sp***S were almost identical to those of GA10 not only in the absence of Cd^{2+} ions but also in the presence of Cd^{2+}, suggesting that these two motifs had similar conformations despite the introduction of a bulky sulfur atom at the ***S*p**-position of the P9 phosphate in GA10***Sp***S.

Although the movements of signals were not as large as those of signals from GA10***Sp***S, the actual number of shifted signals from GA10***Rp***S was similar to the number from GA10 (Figure 5c), suggesting that metal coordination occurred around the same region of GA10. Moreover, the proton signals associated with C4, A6, G7, and U8 also moved downfield. Thus, we can conclude, from the movement of ^{1}H signals, that the final form of GA10***Rp***S with bound metal ions was similar to that of GA10 with bound metal ions. In other words, although the conformation of GA10***Rp***S in the absence of Cd^{2+} ions was different from that of GA10, the added Cd^{2+} ions were able to induce a conformational change in GA10***Rp***S such that it adopted a conformation similar to that of GA10 with bound Cd^{2+} ions. Note that the vertical scales for GA10***Sp***S and GA10***Rp***S in Figure 5 are different from that for GA10. The changes in chemical shifts of GA10***Sp***S and GA10***Rp***S in ^{1}H NMR spectra were larger than those of GA10. The larger size of the changes in chemical shifts of GA10***Sp***S and GA10***Rp***S that were induced by the sulfur atom and Cd^{2+} ions can be explained by the *H*ard and *S*oft *A*cid *B*ase (HSAB) rule.[13] Thus, a 'soft acid', such as a Cd^{2+} ion, prefers to bind to a sulfur atom, which is a 'soft base' (within GA10***Sp***S and GA10***Rp***S) than to an oxygen atom, which is a 'hard base' (within GA10).

Figure 6 shows the changes in ^{31}P chemical shifts of signals from GA10***Sp***S and GA10***Rp***S upon the addition of Cd^{2+} ions, in a comparison with GA10. The changes in signals for GA10***Sp***S (Figure 6b) and GA10***Rp***S (Figure 6c) were similar to those for GA10 (Figure 6a). Since the sulfur atom of the phosphorothioate moiety binds more tightly to a Cd^{2+} ion than does an oxygen atom, the signals associated with A6 within GA10***Sp***S and GA10***Rp***S moved largely upfield. The shifts in the ^{31}P signal of the phosphorothioate were 5.4 and 6.87 ppm for GA10***Sp***S and GA10***Rp***S, respectively, upon the addition of 1 molar equiv of Cd^{2+} ions. The ^{31}P signals associated with G5 and G7 moved downfield and those of U8 moved upfield in all these oligomers. The directions of the movements of these respective signals from GA10***Sp***S and GA10***Rp***S were identical to those from GA10, indicating that the structures of GA10***Sp***S and GA10***Rp***S were changed by binding of Cd^{2+} ions and that the metal-bound forms of GA10***Sp***S and GA10***Rp***S were similar to that of GA10.[12]

The changes in ^{13}C chemical shifts for GA10***Sp***S also resembled those for GA10 (Figure 4).[8b] The cross-peaks associated with G5, A6, G7, and U8 moved considerably and the directions of movements of respective cross-peaks were identical to those for GA10 (Figure 4, parts a and b). In the

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case of GA10***Rp***S, although cross-peaks associated with G5 and G7 were not detected, the cross-peak associated with A6 emerged upon the addition of Cd^{2+} ions (Figure 4, parts c and d). The cross-peak associated with U8 in GA10***Rp***S shifted in the same direction as that in GA10. It appeared, from our analysis of the movements of ^{1}H, ^{31}P and ^{13}C resonances, that the structural changes in GA10***Sp***S and GA10***Rp***S that accompany coordination of a thiophilic metal ion resemble to those in GA10 and lead, eventually, to the similar active form upon the addition of appropriate metal ions.

Discussion

In this report, we described the characterization of the metal-binding forms of GA10***Sp***S and GA10***Rp***S, which correspond to the metal-binding motif of the A9/G10.1 site in a hammerhead ribozyme, by monitoring ^{1}H, ^{31}P, and ^{13}C NMR signals. GA10***Sp***S and GA10***Rp***S contained a phosphorothioate moiety at A6, which correspond to A9 in a hammerhead ribozyme and appear to be a metal-binding site. The ^{1}H and ^{13}C resonances of H6/H8 and C6/C8 atoms provided information about local structural changes around the A6 metal-binding sites of GA10***Sp***S and GA10***Rp***S. Comparisons of changes in chemical shifts with and without a sulfur atom at the A9/G10.1 site revealed the positional effect of the sulfur atom at P9 and the role of the pro-***Sp*** oxygen and the pro-***Rp*** oxygen at the A9/G10.1 site of hammerhead ribozymes.

Conformation of GA10*Sp*S and GA10*Rp*S in the Absence of Cd^{2+} Ions: The Effects of a Sulfur Atom at the pro-*Rp* and pro-*Sp* Positions on Structure. The effects of a sulfur atom on structure differed between GA10***Sp***S and GA10***Rp***S. The chemical shifts of the ^{1}H, ^{13}C, and ^{31}P signals of GA10***Sp***S were almost identical to those of GA10 (Figure 2a, 3a, 4a, and Table 1). These results imply that the tertiary structures of GA10***Sp***S and GA10 are similar and that the sulfur atom in GA10***Sp***S does not induce a structural change. By contrast to those of GA10***Sp***S, the ^{1}H and ^{13}C signals of GA10***Rp***S were different from those of GA10 in the absence of Cd^{2+} ions (Figure 2b, 4c, and Table 1). Although the line shape of ^{31}P signals from GA10***Rp***S was broadened (Figure 3, parts c and d), the chemical shifts were similar to those of signals from GA10 except at position 6, which corresponded to the phosphorothioate linkage (Table 1). Therefore, the results indicate that the sulfur atom of GA10***Rp***S induced a conformational change around the metal-binding site of A6 but the backbone structure of GA10***Rp***S was unaffected.[12] The radius of a sulfur atom is 1.85 Å and is 0.45 Å larger than that of an oxygen atom. The length of a P−S bond is 1.8 Å and is 0.3 Å longer than that of a P−O bond. Moreover, the pro-***Rp*** oxygen is located toward the inside in the major groove in the crystal structure and this location might not allow for any steric hindrance in the ribozyme's structure. The larger-radius sulfur atom, the longer P−S bond and the resultant steric hindrance by the pro-***Rp*** sulfur are likely to have induced a conformational change around the P9 metal-binding site and to have had a negative effect on the conformation of the ribozyme.

Binding of Metal Ions to GA10*Sp*S and GA10*Rp*S. In the previous study using a full-length hammerhead ribozyme, ^{31}P NMR signals of both ***Rp***- and ***Sp***-phosphorothioates shifted 2-3 ppm upfield upon the addition of 1 to 2 molar equiv of Cd^{2+} ions.[6] Similar changes were observed for the metal-binding motifs consisting solely of short GA10***Sp***S and GA10***Rp***S, although the magnitude of the upfield shift (5-7 ppm) upon addition of 1 equiv of Cd^{2+} was greater for the short oligomers probably because the full-length ribozyme might have more nonspecific interactions and/or specific interactions at site(s) other than the A9/G10.1 site between phosphates and Cd^{2+} ion-(s) that had increased the end-point by Cd^{2+} saturation. Nevertheless, since a Cd^{2+} ion can interact with ***Rp***- and ***Sp***-phosphorothioates in the metal-binding motif of both the full-length ribozyme and the shortened GA10***Sp***S and GA10***Rp***S and since complete assignments of ^{1}H, ^{13}C and ^{31}P signals were possible only for the short oligos, we in this study used GA10***Sp***S and GA10***Rp***S. Indeed, perturbations occurred not only in the ^{31}P signal from the inserted phosphorothioate (A6) but also in most of the ^{1}H, ^{13}C and ^{31}P signals, suggesting that there might have been a conformational change in both GA10***Sp***S and GA10***Rp***S as a result of the binding of Cd^{2+} ions. The perturbations in signals from GA10***Sp***S were almost identical to those from GA10. Thus, the Cd^{2+}-binding form in GA10***Sp***S was similar to that of GA10. In GA10***Rp***S, even though the chemical shifts in the ^{1}H NMR spectrum in the absence of metal ions, in particular at the H6 and H8 positions, were different from those in GA10, the perturbations induced by Cd^{2+} ions resembled the perturbations induced by Cd^{2+} ions in GA10 (Figures 5 and 6). It is noteworthy that the conformation not only of GA10***Sp***S with a bound metal ion but also of GA10***Rp***S with a bound metal ion was similar to that of GA10 with a bound metal ion, even though, in the absence of metal ions, the conformation of GA10***Rp***S was different from that of GA10.

Does a metal ion bind directly or indirectly to N7 and the phosphate oxygen at the A9/G10.1 metal-binding site? In a previous study, using ^{15}N-labeled GA10, we did not detect any coupling between the N7 atom at G7 and $^{113}Cd^{2+}$, even though signal perturbation occurred at this N7 atom and the C8 atom of G7.[8c] The binding of metal ions by GA10***Sp***S and GA10***Rp***S was stronger than that by GA10 because the phosphorothioate moiety contained a sulfur atom at the metal-binding site. The HSAB rule determines that a 'hard acid', such as a Mg^{2+} ion, prefers to bind to an oxygen atom. Before the titration of the phosphorothioate with Cd^{2+} ions, we expected that coupling might be evident, in the ^{31}P signal of A6 and the H8/C8 cross-peak of G7 in the ^{1}H-^{13}C HSQC spectra of GA10***Rp***S and GA10***Sp***S in the presence of one molar equivalent of Cd^{2+} ions, assuming that binding of metal ions would involve direct coordination.[14] No coupling between ^{31}P of A6 and ^{113}Cd (Figure 3, parts a and c) or between the H8 atom of G7 and ^{113}Cd (Figure 2, parts a and b) was detected in this study, even though large perturbations in chemical shifts were noted in the ^{31}P and ^{1}H NMR spectra.

Theoretical calculations of spin coupling constants and chemical shifts related to the binding of a divalent metal ion to guanine were reported recently.[15] The calculated chemical shift of the N7 atom of guanine for an inner-shell binding complex of Mg^{2+} ion with guanine is similar to the chemical shift detected experimentally by our group.[8d] The theoretical calcula-

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tions supported the direct coordination of a metal ion to the N7 atom of guanine,[15] suggesting the same conclusion as the one that we had reached on the basis of our experiments.[8d] The theoretical calculations, indicated that the change in chemical shift of the signal due to the C8 atom of G7 in the ^{1}H-^{13}C HSQC spectrum of GA10***Sp***S (Figure 4, parts a and b) implies the direct coordination of a Cd^{2+} ion to the N7 atom of G7,[15] although outer-sphere coordination cannot completely be ignored.[16] The coupling was absent in the present study, in which we used a sulfur atom and ^{113}Cd ions, because of rapid exchange of Cd^{2+} ions at the metal-binding site, although we cannot completely exclude the possibility of coupling between $^{113}Cd^{2+}$ and H8 of G7 in the case of the full length hammerhead ribozyme.

Pro-*R*p Phosphorothioate at A9 of a Hammerhead Ribozyme. Kinetic studies of constructs with a phosphorothioate moiety at the A9/G10.1 site of hammerhead ribozymes suggested the important metal-binding role of the pro-***R*p** oxygen but not of the pro-***S*p** oxygen since the pro-***R*p** sulfur-substituted ribozyme was inactive in Mg^{2+}-mediated reactions but was rescued by the addition of thiophilic Cd^{2+} ions. The corresponding pro-***S*p** sulfur-substituted ribozyme was active even in Mg^{2+}-mediated reactions. However, this interpretation requires the assumption that the sulfur atom of the phosphorothioate does not affect the initial conformation of the hammerhead ribozyme, nor the corresponding structures in the presence of hard Mg^{2+} ions. If the inserted sulfur atom were to influence the conformation of the hammerhead ribozyme, then we would have to consider the effects of this conformation change in our interpretation of the kinetic results obtained with the phosphorothioate. In other words, the conformational change caused by insertion of sulfur might disrupt the ribozyme reaction if the conformation of the phosphorothioate-containing ribozyme is not an active conformation.

We demonstrated in the present study that, in the absence of metal ions, a conformational change does indeed occur as a result of the sulfur in GA10***R*p**S but it does not occur in GA10***S*p**S. Therefore, we can postulate that the conformation of the hammerhead ribozyme that contains a phosphorothioate moiety at the pro-***R*p** of A9 is likely to be different from that of the wild-type ribozyme, whereas the hammerhead ribozyme that contains a phosphorothioate moiety at the pro-***S*p** of A9 retains the conformation of the wild-type ribozyme. The decreased rate of the reaction catalyzed by the ribozyme that contains a phosphorothioate moiety at the pro-***R*p** site means that the conformational change induced by the phosphorothioate has a negative effect on the ribozyme reaction. It appears that the rescue by Cd^{2+} ions in kinetic studies is due to the recovery of an active metal-associated form that resembles the metal-associated form of the wild-type ribozyme (remember that the changes in chemical shifts induced by Cd^{2+} ions in GA10***R*p**S were similar to those induced by Cd^{2+} ions in GA10).

In summary, we examined conformational changes upon the interaction of Cd^{2+} ions with the A9/G10.1 metal-binding motif of a hammerhead ribozyme. Our analysis demonstrated that the effects of the introduction of a phosphorothioate linkage at a specific position depend on whether the sulfur is at the pro-***R*p** or the pro-***S*p** position. Since the effects at the two positions are not the same, the resultant conformers are likely to be at different energy levels or, in catalytic nucleic acids, they are likely to have different activities, a possibility that has been ignored in the past. Indeed, two observations can be most simply and best explained by this conclusion. The first of these two observations is that the pro-***R*p** sulfur-substituted ribozyme is inactive in Mg^{2+}-mediated reactions but its activity can be rescued by the addition of thiophilic Cd^{2+} ions, whereas the corresponding pro-***S*p** sulfur-substituted ribozyme is active even in Mg^{2+}-mediated reactions. The second observation is that the conformation of GA10***S*p**S is similar to that of the parental GA10, whereas the conformation of GA10***R*p**S is different from that of the parental GA10 in the absence of Cd^{2+} ions, even though all oligomers yield similar Cd^{2+}-bound forms. Thus, the introduction of sulfur at the pro-***R*p** position but not at the pro-***S*p** position disturbs the active conformation of the ribozyme. Our analysis clearly warns against incautious interpretations of thio-effects and rescue phenomena because the previous assumption that the two different conformers have the same effects, other than the effects of position, is clearly not always valid.

Experimental Section

Preparation of Samples. A crude mixture of the GA10***R*p**S and GA10***S*p**S phosphorothioate isomers was purchased from Genset Corporation (France). The two components of the mixture were separated and purified as described previously.[7] Assignments of isomers were made after digestion by snake venom phosphodiesterase and nuclease P1.[17] 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, USA) 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 total 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, Japan) with a gradient 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% to 100% B over the course of 45 min). Retention times under these conditions 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.

Resonance Assignments. Samples for NMR spectroscopy were prepared by dissolving purified oligomers in 60 μL of a

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solution, prepared in D_2O, of 40 mM $NaClO_4$ and 10 mM sodium cacodylate buffer at pH 7.6 in a 3-mm NMR tube (Shigemi, Japan). The concentrations of GA10***Sp***S and GA10***Rp***S, each as a duplex were 1.77 mM and 1.62 mM, respectively. Concentrated solutions of $CdCl_2$ were added directly to each sample to give desired concentration. All NMR spectra were acquired on an INOVA 600 MHz NMR spectrometer (Varian, USA) operated at 40 °C, with a *z*-axis pulsed-field gradient probe. The chemical shifts of protons were determined relative to the signal from the internal standard, sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). The ^{31}P chemical shifts were referenced to an external 85% solution of H_3PO_4. Aromatic ^1H and ^{13}C resonances were assigned from ^1H-^1H NOESY, natural-abundance ^1H-^{13}C HSQC and ^1H-^{31}P HMQC NOESY spectra. Typical ^1H-^1H NOESY spectra were recorded with 4096 × 1024 complex points for a spectral width of 5071.0 Hz; 64 scans were averaged; and the mixing time was 300 ms. Natural-abundance ^1H-^{13}C HSQC spectra were recorded with 2048 × 160 complex points for a spectral width of 4743.8 × 6034.1 Hz, and 1408 scans were averaged. 2D ^1H-^{31}P HMQC NOESY spectra were recorded with 2048 × 32 complex points for a spectral width of 4821.6 × 1457.1 Hz; 2048 scans were averaged; and the mixing time for NOESY was 300 ms.

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