Well-Conserved Tandem G·A Pairs and the Flanking C·G Pair in Hammerhead Ribozymes Are Sufficient for Capture of Structurally and Catalytically Important Metal Ions

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Received March 21, 2000

Abstract: An important metal ion in reactions catalyzed by hammerhead ribozymes is the so-called P9 metal ion (the metal ion captured by the phosphorus atom of A9 (P9) and N7 of G10.1 [P9−G10.1 motif]). Hammerhead ribozymes have catalytically important tandem G·A pairs in the core region, and the P9−G10.1 motif captures the P9 metal ion, which is most probably a Mg^{2+} ion under physiological conditions. In this study, we used ^{1}H, ^{31}P, and ^{13}C NMR spectroscopy to examine whether this motif by itself, in the absence of other catalytic loops, might be sufficient to capture structurally and catalytically important metal ions in solution. We deduced that the P9−G10.1 motif was able to capture a Mg^{2+} ion in solution in the absence of any other part of a hammerhead ribozyme, because the chemical shift values of the phosphorus atom of A6 (P/A6), C8 of G7 (C8/G7), and H8 of G7 (H8/G7) in the P9−G10.1 motif of a model duplex, designated GA10, were selectively perturbed during titration up to a 1:1 molar ratio of Mg^{2+} ions and the P9−G10.1 motif. We next studied the binding of a Cd^{2+} ion to the P9−G10.1 motif and found that, in agreement with recent kinetic studies (Yoshinari and Taira, *Nucleic Acids Res.* **2000**, *28*, 1730−1742), a Cd^{2+} ion also bound to this motif. Finally, we conclude that the P9−G10.1 motif (a sheared-type G·A pair with a guanine residue on the 3′ side of the adenine residue) with several flanking base pairs is sufficient for capture of divalent cations such as Mg^{2+} and Cd^{2+} ions and that hammerhead ribozymes utilize this intrinsic metal-binding property of the P9−G10.1 motif for catalysis.

Hammerhead ribozymes are metalloenzymes, at least under physiological conditions,[1] and X-ray crystallography has revealed that metal ions bind to core sequences that include tandem G·A pairs[2] (Figure 1). In most of the crystal structures that were analyzed, metal ions bound at the tandem G·A pairs through the phosphate of A9 (P9) and N7 of G10.1 (P9−G10.1 motif, enclosed by the magenta lines in Figure 1a,e).[2] Furthermore, in the crystal structure of an RNA dodecamer with sheared-type G·A pairs (Figure 1d), Mg^{2+} ions bound to the G·A pairs (Figure 1d) in a manner similar to the binding to G·A pairs in a hammerhead ribozyme (Figure 1e).[3] NMR spectroscopy of a hammerhead ribozyme in solution also revealed another metal-binding site, namely, the phosphate of A13 (Figure 1a).[4] This site has structural features that are similar to those of the P9−G10.1 motif of a hammerhead ribozyme (enclosed by green line in Figure 1a), and it was suggested that the sheared-type G·A pair with a purine residue on the 3′ side of the adenine might be a metal-binding motif. Furthermore, it was suggested that a metal ion bound to the P9−G10.1 motif might be a catalytic metal ion,[1c] and it is obviously important to characterize this motif and its properties.[1j,k] However, it is not yet clear whether the P9−G10.1 motif can, by itself, support the binding of the structurally and catalytically important Cd^{2+}

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Published on Web 11/04/2000

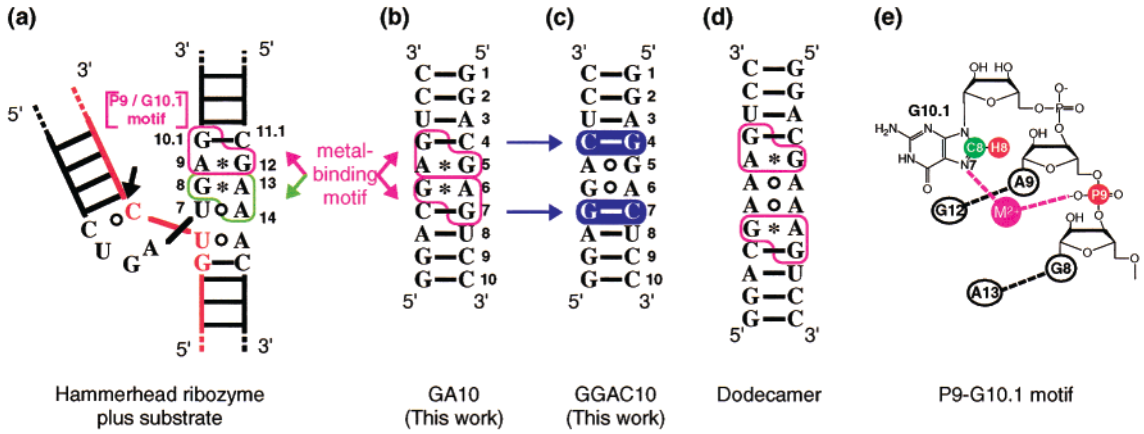

Figure 1. Sequences and secondary structures of (a) a hammerhead ribozyme,[2] (b) GA10, (c) GGAC10 (negative control sequence), (d) RNA dodecamer,[3] with numbering systems. The metal-binding motifs (P9−G10.1 motif of hammerhead ribozymes[2] and the corresponding sequences of RNA dodecamer[3] and GA10), and the motif as revealed by solution NMR spectroscopy[4] are surrounded by magenta and green lines, respectively. In panel a, enzyme and substrate strands with conserved sequences are shown in black and red, respectively, and the cleavage site is indicated by a black arrow. In panels a−d, Watson−Crick base pairs, non-Watson−Crick base pairs, and sheared-type G·A pairs are indicated in bars, open circles, and asterisks, respectively. (e) Schematic representation of the P9−G10.1 motif, as revealed by X-ray crystallography.[2,3] A divalent metal ion is shown in magenta and is linked to binding sites via magenta dashed lines. The tandem G·A pairs are indicated by black dashed lines with residue names. The nuclei monitored by 2D ^{1}H−^{31}P HMQC−NOESY and natural-abundance ^{1}H−^{13}C HSQC spectra are highlighted (P, red; C8, green; H8, red).

ion as well as other general divalent cations, nor is it clear how this motif might recognize metal ions in solution.

To investigate the P9−G10.1 motif, we used the oligomer designated GA10, whose sequence is shown in Figure 1b. This oligomer was originally studied, by means of NMR spectroscopy, as a model sequence that includes the well-conserved G·A pairs (P9−G10.1 motif) of hammerhead ribozymes.[5] GA10 provides a good model for studies of structural features of the P9−G10.1 motif of hammerhead ribozymes for the following reasons. (i) It was confirmed that the guanine (G5) and adenine (A6) residues form sheared-type G·A pairs in the duplex, just as they do in the core region of a hammerhead ribozyme.[5] (ii) Both GA10 and the corresponding regions of hammerhead ribozymes have two putative metal-binding motifs in tandem (Figure 1a,b). (iii) Our two-dimensional (2D) ^{1}H−^{1}H NOESY analysis of GA10 indicated that sequential assignments of protons and the resultant deduced global folding of GA10 were essentially the same as those previously reported[5] (Figure S1 in Supporting Information). Thus, GA10 appears suitable for studies of the interaction of metal ions with the P9−G10.1 motif of hammerhead ribozymes. We synthesized the model RNA oligomer GA10 and examined whether and how the P9−G10.1 motif might, by itself, recognize Mg^{2+} and Cd^{2+} ions in titration experiments with heteronuclear 2D NMR spectroscopy.

Results

In general, a phosphate group and N7 of a purine base have the potential to bind a metal ion,[3,6] and these sites were used as metal-binding sites in the case of the P9−G10.1 motif in hammerhead ribozymes as well.[2] In such cases, the chemical shift values of the nuclei and the neighboring sites are expected to be perturbed considerably.[7] Actually, large chemical shift perturbations were observed for the base carbon (C8) (2.5 ppm)[7c] and base proton (H8) (1 ppm),[7d] which are located next to N7 of each metal-binding site, and the phosphorus atom (P) (2 ppm).[7b] Because these signals can be monitored in samples without isotope enrichment, we monitored the chemical shift values of the indicated base protons (H8 and H6), base carbons (C8 and C6), and phosphorus atoms in GA10 by using 2D ^{1}H−^{31}P HMQC−NOESY and natural-abundance ^{1}H−^{13}C HSQC spectra (Figure 2) to analyze the recognition of a metal ion by the P9−G10.1 motif. We performed titration experiments using a solution that contained 3 mM GA10 (single stand) and 40 mM NaCl at pH 6 and increasing concentrations of $MgCl_2$ (0, 1.5, 3.0, and 13.5 mM). These concentrations of $MgCl_2$ correspond to 0, 1, 2, and 9 molar equivalents relative to GA10 (duplex), and at 13.5 mM $MgCl_2$, Mg^{2+} ions neutralize the net charge of the phosphates of the oligomer. At each titration point, we recorded 2D ^{1}H−^{31}P HMQC−NOESY and ^{1}H−^{13}C HSQC spectra in D_2O at 32 °C and definitively assigned all the resonances of phosphorus, H8, and C8 atoms (Figure 2). The 2D ^{1}H−^{31}P HMQC−NOESY spectra were particularly useful for the assignment of phosphorus resonances in the duplex (Figure 2). Unfortunately, at the final point in titration of Mg^{2+} ions (13.5 mM $MgCl_2$), we could not definitely determine the chemical shift values because most of the cross-peaks were broadened out from 2D ^{1}H−^{31}P HMQC−NOESY and ^{1}H−^{13}C HSQC spectra at 32 °C due to a microaggregation. Therefore, the chemical shift values corresponding to the final titration point were not included in the analysis (Figures 2 and 3).

As shown in Figure 2, the chemical shift values of P/A6 (the phosphorus atom of A6), C8/G7 (the C8 atom of G7), and H8/G7 (the H8 atom of G7) in the P9−G10.1 motif of GA10 were selectively perturbed until $MgCl_2$ had saturated the two P9−G10.1 motifs of the duplex. Although some irregularities associated with the terminal residues were also observed, chemical shift perturbations were concentrated at the P9−G10.1 motif of GA10 and related sites (C4, which forms a base pair with G7, and U8, the residue on the 3′ side of G7). If these

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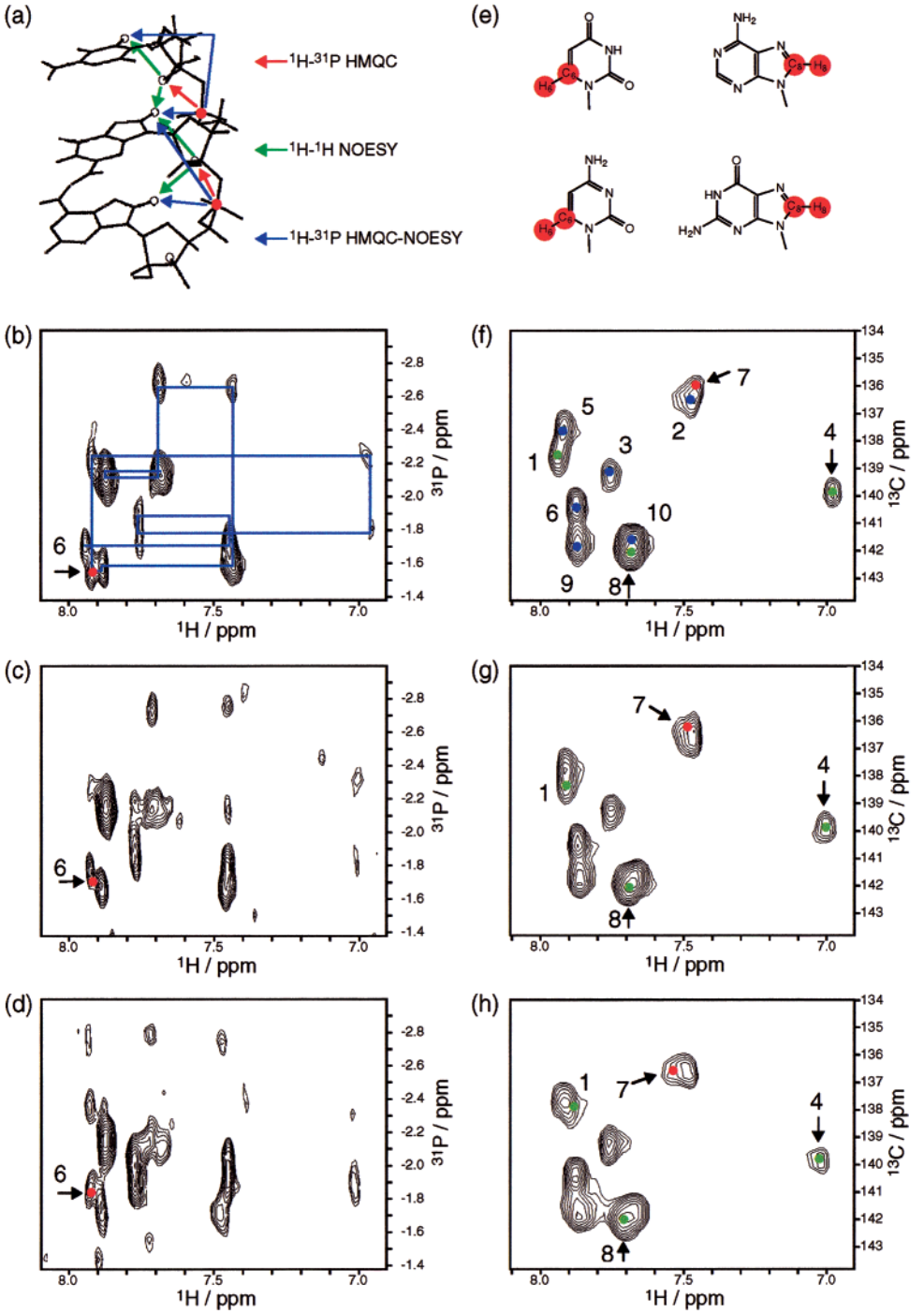

Figure 2. Results of titrations with $MgCl_2$. In panels a and e, correlations observed in 2D $^1H-^{31}P$ HMQC−NOESY and $^1H-^{13}C$ HSQC spectra, respectively, are indicated. Panels b−d show 2D $^1H-^{31}P$ HMQC−NOESY spectra, for (b) 0 mM $MgCl_2$ (GA10 duplex:$MgCl_2$ = 1:0 mol/mol), (c) 1.5 mM $MgCl_2$ (GA10 duplex:$MgCl_2$ = 1:1), and (d) 3.0 mM $MgCl_2$ (GA10 duplex:$MgCl_2$ = 1:2). Natural-abundance $^1H-^{13}C$ HSQC spectra are shown (f) for 0 mM $MgCl_2$, (g) for 1.5 mM $MgCl_2$, and (h) for 3.0 mM $MgCl_2$. In panel b, the results of sequential assignments are depicted by blue lines, and in panels b−d, the cross-peaks between P/A6 and H8/G5 are depicted as red circles with arrows and the residue number of A6. In panel f, assignments of all intraresidue cross-peaks between base carbon atoms and base protons are shown with their residue numbers. In f−h, cross-peaks of C8/G7−H8/G7 are depicted as red circles with arrows; those of C8/G1−H8/G1, C6/C4−H6/C4, and C6/U8−H6/U8 are depicted as green circles with arrows.

perturbations were simply due to conformational changes, signals at other sites would also have to be shifted. However, the latter signals were not shifted, and thus, the perturbations must have been due to the binding of Mg^{2+} ions. It should also be noted that chemical shift values of C8/G7 and H8/G7 were shifted to a lower field during the titration (Figures 2 and 3, Table 1), as observed for the metalation sites in previous titration experiments,[7c,d] which suggested that a similar linkage might be also generated for GA10. We can conclude that the P9−G10.1 motif (a sheared-type G·A pair with a guanine residue on the 3′ side of the adenine, with several flanking base pairs) can function, by itself, as a Mg^{2+} ion-binding motif without other conserved residues that are found in hammerhead ribozymes.

To make this conclusion more rigid, we prepared a negative control sequence designated GGAC10, r(GGA**G**GA**C**UCC)

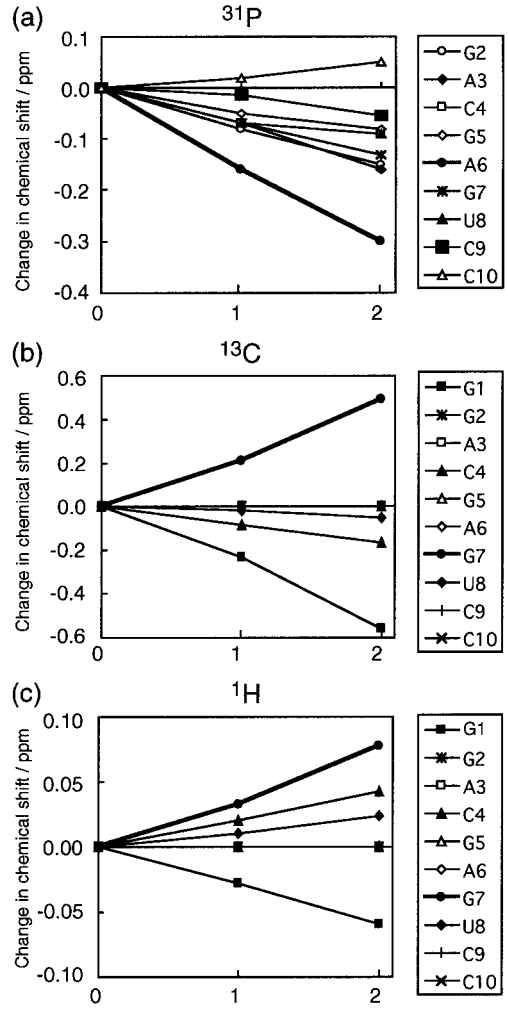

Figure 3. Plots of chemical shift perturbations for (a) ^{31}P, (b) ^{13}C (C8/C6), and (c) ^{1}H (H8/H6). Horizontal axes show molar ratios of [$MgCl_2$]/[GA10 (duplex)]. In the case of phosphorus resonances, chemical shift values of P/A6 and other phosphorus atoms, except for that at the 3′ terminal residue were shifted to a higher field, but the chemical shift perturbation of P/A6 was significantly larger than those of other signals and experimental errors [signal width at half-height, 20 Hz (0.1 ppm) on average]. In the case of proton resonances, chemical shift values of H8/G7, H6/C4 and H6/U8 were shifted to a lower field and that of H8/G1 at the 5′ terminus was shifted to a higher field. The other proton resonances were unchanged. However, the chemical shift perturbation of H8/G7 was significantly larger than those of other protons. The experimental errors of protons (signal width at half-height) were 20 Hz (0.04 ppm) on average. Likewise, in the case of carbon resonances, we observed changes in the chemical shift values of C8/G7, C6/C4, C6/U8, and C8/G1, even though the extent of perturbations was similar or slightly larger than the extent of experimental errors (signal width at half-height, 37 Hz (0.3 ppm) on average). However, it is interesting that only the resonance of C8/G7 was shifted to a lower field, although those of C6/C4, C6/U8, and C8/G1 were shifted to a higher field, whereas the other resonances (C8/G2, C8/A3, C8/G5, C8/A6, C6/C9, and C6/C10) were unchanged, and they are overlapped on the horizontal axis (see also Figure 2f−h). Likewise, proton resonances of H8/G2, H8/A3, H8/G5, H8/A6, H6/C9, and H6/C10 are overlapped on the horizontal axis.

(Figure 1c), which is expected to lose a metal-binding property with a minimum sequence alteration from GA10, r(GGA**C**-GA**G**UCC). In the GGAC10, C4−G7 pairs of P9−G10.1 motifs in GA10 were substituted by G4−C7 pairs and, as a result, the discrete N7/G7 of P9−G10.1 motif for the metal-binding was deleted. In Figure 4, ^{1}H−^{13}C HSQC spectra of GGAC10 with and without $MgCl_2$ ions are presented. We emphasize that lower-field shifts of C8-carbon nuclei of purine residues in GGAC10, which can be a metalation residue, were not detected through the titration experiments with Mg^{2+} ions, although chemical shift perturbations of protons were observed for several residues. In addition, the lower-field shift of C8/G7 (+0.5 ppm) of the P9−G10.1 motif in GA10 was significantly larger than the maximum lower-field shift observed for GGAC10 (+0.2 ppm for C6/C7).

Table 1. Chemical Shift Perturbations[a]

sample	residue	H8 (ppm)	C8 (ppm)	ref
DNA−Zn	G4	+0.20	+2.5	7c
	G3	+0.05	+1.5	7c
DNA−Pt	G3	+1.04	+1.1	7d
	G2	+0.26	+0.2	7d
GA10−Mg	G7	+0.08	+0.5	this work
	C4	+0.04	−0.1	this work
	U8	+0.02	−0.1	this work
	G1	−0.06	−0.5	this work
GA10−Cd	G7	+0.08	+0.8	this work
	C4	+0.05	±0.0	this work
	G5	−0.08	±0.0	this work
	A6	+0.05	+0.5	this work
	U8	+0.06	+0.3	this work

[a] The sequences and references are as follows: DNA−Zn refers to the titration of $ZnCl_2$ against d(ATGGGTACCCAT)$_2$.[7c] DNA−Pt represents covalent complex *d*(TGGT)−Pt(ethylenediamine).[7d] GA10−Mg represents the titration of $MgCl_2$ against *r*(GGACGAGUCC)$_2$ (this work). GA10−Cd represents the titration of $CdCl_2$ against *r*(GGACGAGUCC)$_2$ in the presence of 13.5 mM $MgCl_2$ (this work). In the case of the titration of Pb^{2+} ions against leadzyme, the considerable chemical shift perturbation for the carbon atom (C2/A25), by 1.4 ppm, was also observed.[7e] However, it was not listed here because the residue, A25, is known to have an irregular pK_a value for N1,[7e] and it is obscure whether this perturbation is due to metal-binding or a deprotonation at N1/A25. It should be noted that, as in the cases of the titration of $ZnCl_2$ against DNA dodecamer[7c] and the formation of the DNA−Pt covalent complex,[7d] chemical shift values of C8/G7 and H8/G7 of GA10 were shifted to a lower field upon titration with Mg^{2+} ion (see also Figures 2 and 3). Therefore, it was suggested that a Mg^{2+} ion might be directly coordinated to N7/G7 or, at least, that a hydrated Mg^{2+} ion was captured by the P9−G10.1 motif through a water-mediated hydrogen bond. In the cases of sites adjacent to G7, such as H6/C4, C6/C4, H6/U8, and C6/U8, chemical shift values of protons were shifted to a lower field but those of carbon atoms were shifted to a higher field, unlike the result for metal-binding sites during the course of titration with Mg^{2+} ion (see also Figures 2 and 3). This phenomena can be explained, in all likelihood, by conformational changes due to the binding of a Mg^{2+} ion to the P9−G10.1 motif because there is no metal-binding site near H6/C4, C6/C4, H6/U8, and C6/U8. In the case of H8/G1 and C8/G1, both chemical shift values were shifted to a higher field. In general, salts increase a stacking interaction, and a duplex is stabilized by $MgCl_2$. Therefore, increases in stacking interactions and resultant ring current shifts might also provide an explanation. All of the data presented here are consistent with the conclusion that a Mg^{2+} ion binds to the P9−G10.1 motif, irrespective of whether the binding of the Mg^{2+} ion involves direct coordination. In the case of the titration with $CdCl_2$ in the presence of 13.5 mM $MgCl_2$, large chemical shift perturbations were observed at the P9−G10.1 motif and neighboring residues, such as C4 and U8 (see also Figure 5). In particular, large lower-field shifts for both protons and carbon atoms were observed for residues G7 and A6. By analogy to the case of titration with $MgCl_2$, these significant lower-field shifts for both proton and carbon resonances suggest direct coordination of a metal ion or formation of a hydrogen bond to N7 of a purine base. The large lower-field shifts for C8/G7 and H8/G7 might be due to direct coordination or hydrogen bonding of a Cd^{2+}-coordinated water molecule to N7/G7. In the case of A6, N7/A6 is involved in the hydrogen bond of a sheared-type G·A pair, and the strengthening of this hydrogen bond might explain the lower-field shifts. In the case of the other cross-peaks for C4, G5, and U8, chemical shift perturbations for ^{13}C were relatively smaller, and no metalation site exists near C6/C4 and C6/U8. Therefore, chemical shift perturbations of base protons (H6/C4, H8/G5, and H6/U8) might have been due to structural changes caused by the replacement of a Mg^{2+} ion by a Cd^{2+} ion at the P9−G10.1 motif.

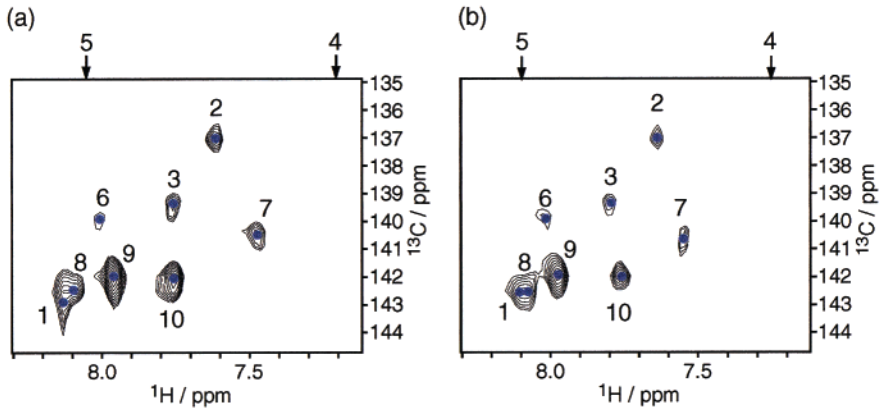

Figure 4. Natural-abundance $^1H-^{13}C$ HSQC spectra of GGAC10 (a) in the absence of $MgCl_2$ or (b) in the presence of 2.5 mM $MgCl_2$. 2.5 mM $MgCl_2$ corresponds to 1:1 molar equivalent to single strand of GGAC10. Under both conditions, intraresidue cross-peaks of H8/G4−C8/G4 and H8/G5−C8/G5 were not observed, because their proton signals were broad in the 1D ^1H NMR spectra. Therefore, their chemical shifts are indicated by arrows with their residue number. Assignments of ^1H resonances and the formation of a duplex for GGAC10 were also confirmed by the 1D ^1H NMR spectra and 2D $^1H-^1H$ NOESY spectra under both conditions. Higher-field shifts of both ^1H and ^{13}C nuclei due to incresed stacking interaction were observed for C8/G1 and H8/G1 of GGAC10, as observed in GA10. Although lower-field shifts were observed for C6/C7 of GGAC10, it was deduced to be due to conformational changes, because there is no metal-binding site near C6/C7.

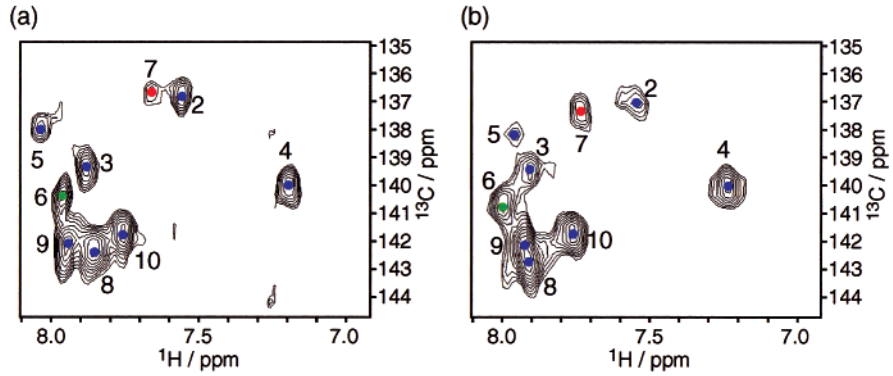

Figure 5. Natural-abundance $^1H-^{13}C$ HSQC spectrum in the presence of background 13.5 mM $MgCl_2$, recorded in the absence of $CdCl_2$ (a), and in the presence of 3 mM $CdCl_2$ (b). Intraresidue cross-peaks are labeled with their residue number.

It should also be noted that pyrimidine residues do not have a metalation site near the C6-carbon nuclei. Thus, chemical shift perturbations (lower-field shift of C8/G7) which were observed for the P9−G10.1 motif of GA10 were much larger than the maximum lower-field shift in chemical shift values due to a nonspecific interaction between metal ions and an RNA duplex. From these results, we conclude that the chemical shift perturbations of the P9−G10.1 motif were significant and should be due to metalation of the P9−G10.1 motif.

We continued our study of the metal-binding properties of the P9−G10.1 motif by using $CdCl_2$. We added $CdCl_2$ (final concentration, 3.0 mM) to a solution of 3.0 mM GA10 (single strand), 40 mM NaCl, and 13.5 mM $MgCl_2$, as background, at pH 6 (the ratio of Cd^{2+} ions to P9−G10.1 motif was kept at 1:1). In other words, $CdCl_2$ was added to determine whether Cd^{2+} would replace a preexisting Mg^{2+} ion and occupy the P9−G10.1 motif in the presence of 13.5 mM $MgCl_2$ ions, as we observed in the hammerhead ribozyme-catalyzed reactions.[1e] Spectra in the presence and absence of $CdCl_2$ were recorded at 40 °C to avoid microaggregation. Figure 5 shows the natural abundance $^1H-^{13}C$ HSQC spectra recorded in the presence and in the absence of 3.0 mM $CdCl_2$, both in the presence of 13.5 mM background Mg^{2+} ions. The cross-peak between H8/G7 and C8/G7 was extraordinarily perturbed toward a lower field for both the proton and carbon resonances upon the addition of $CdCl_2$ (Figure 5 and Table 1). Thus, we confirmed that the P9−G10.1 motif could also act as a Cd^{2+}-binding motif and that the Mg^{2+} ion was replaced by a Cd^{2+} ion at a ratio of Cd^{2+} ions (3.0 mM) to the P9−G10.1 motif of 1:1 even in the presence of an excess of $MgCl_2$ (13.5 mM). These observations are in agreement with the results of our recent kinetic studies which showed that the P9−G10.1 motif within hammerhead ribozyme−substrate complexes can capture several kinds of divalent cations, such as Mg^{2+} and Cd^{2+} ions.[1e] In other words, binding of the structurally and catalytically important divalent cations to the motif within hammerhead ribozyme−substrate complexes reflects intrinsic metal-binding properties of the P9−G10.1 motif.

Discussion

We showed here that the 2D $^1H-^{31}P$ HMQC−NOESY spectrum is a powerful tool for the assignments of phosphorus atoms (Figures 2b−d). In an RNA duplex, such spectra exhibit sequential correlations between phosphorus atoms and base protons that are similar to those between base protons and anomeric protons in 2D $^1H-^1H$ NOESY spectra. In other words, each phosphorus atom generates two cross-peaks, one between a phosphorus atom and the intraresidue base proton and another between the phosphorus atom and the 5′-adjacent base proton, as in the case of 2D $^1H-^{31}P$ TOCSY−NOESY spectra.[8] The redundant information that was generated is useful for the accurate assignment of resonances. Furthermore, the assignment

of phosphorus resonances by a 2D $^1H-^{31}P$ HMQC−NOESY spectrum can be achieved without any information related to the result of the assignment of proton resonances. This type of assignment is not possible with $^1H-^{31}P$ HETCOR spectra and related spectra[9] because in such cases, resonance assignments of H3′ protons are required for the assignment of the phosphorus resonances. In addition, a 2D $^1H-^{31}P$ HMQC−NOESY spectrum is useful for studies of metal−RNA interactions because it provides chemical shift values for atoms that are very sensitive to the binding of a metal ion (Figure 1e).

We also demonstrated here that the P9−G10.1 motif recognized both Mg^{2+} and Cd^{2+} ions, and that the P9−G10.1 motif (sheared-type G·A pair with a guanine residue on the 3′ side of adenine, with several flanking base pairs) can function by itself as a metal-binding motif without other conserved residues of hammerhead ribozymes. These results suggested that the P9−G10.1 motif can capture a variety of divalent cations. According to the concept of HSAB, the Mg^{2+} ion is a canonical hard acid, whereas the Cd^{2+} ion is a canonical soft acid.[1e] Although both ions are divalent cations, the orders of their affinities for Lewis bases are different. Thus, the affinity of a Mg^{2+} ion for sulfur and oxygen is S < O, while the affinity of a Cd^{2+} ion for sulfur and oxygen is S > O. Nevertheless, the P9−G10.1 motif recognized both ions and appears to be a binding motif for general divalent cations. Furthermore, although the GA10 duplex has two identical P9−G10.1 motifs, the signals from Mg^{2+} ion-binding and nonbinding nuclei in the P9−G10.1 motif were averaged and not separated under metal-unsaturated conditions (P9−G10.1 motif:Mg^{2+} ion = 2:1) in all of the spectra that we recorded. However, slight broadening of the signal from H8 of G7 in the P9−G10.1 motif was observed in the 1D spectrum for ^1H under metal-unsaturated conditions. These data indicate that the exchange rate for association and dissociation of Mg^{2+} ions to the motif is relatively fast or, possibly, similar to the NMR time scale.

We considered here whether the binding of a divalent cation to the P9−G10.1 motif is a direct coordination or not. In cases of previously observed, large chemical shift perturbations, namely, 2 ppm (P) in the titration of $MgCl_2$ against fully ionized ATP,[7b] 2.5 ppm (C8) in the titration of $ZnCl_2$ against DNA dodecamer,[7c] and 1 ppm (H8) in the formation of a covalent complex between Pt and DNA,[7d] the extraordinarily large perturbations were probably due to a direct salt bridge between a Mg^{2+} ion and phosphate, direct coordination of a Zn^{2+} ion to the N7 of a purine base, and to the formation of a covalent bond between Pt and the N7 of a purine base. By contrast, in the titration experiment with GA10 and $MgCl_2$, the chemical shift perturbations of P/A6 (−0.3 ppm), C8/G7 (+0.5 ppm) and H8/G7 (+0.08 ppm) in the P9−G10.1 motif were smaller than the above perturbations[7b−d] (Figures 2 and 3, Table 1). Similarly, in the titration experiment with GA10 and $CdCl_2$, the chemical shift perturbations were +0.8 ppm for C8/G7 and +0.08 ppm for H8/G7 (Figure 5, Table 1). Accordingly, as far as the extent of chemical shift perturbations were concerned, we could not at present obtain any evidence for direct coordination of a divalent cation to the P9−G10.1 motif. Our data does not, however, exclude the possibility that a divalent cation is directly coordinated to the motif, because chemical shift perturbations due to conformational changes might compensate for those due to direct coordination of a divalent cation. Alternatively, the maximum chemical shift perturbations of the base carbon and proton might only correspond to the above-mentioned low values, even if a divalent cation were directly coordinated to the motif. However, it should be noted that chemical shift values of C8/G7 and H8/G7 were shifted to a lower field during titration experiments (Figures 2, 3, and 5; Table 1), as observed for the metalation sites in previous titration experiments.[7c,d] Therefore, a Mg^{2+} ion might be directly coordinated to N7/G7 or, at least, a hydrated Mg^{2+} ion was captured by the P9−G10.1 motif through a water-mediated hydrogen bond.

Most 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 oxygen) and the N7 atom of G10.1.[2] Even though this P9−G10.1 motif 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. The substitution of the *pro-R*p phosphoryl P9 oxygen by a sulfur atom results in a decrease in Mg^{2+}-dependent catalytic activity.[1f] Furthermore, replacement of G10.1 by a pyrimidine also results in a substantial decrease in the ribozyme's activity.[1g,h] Moreover, from their kinetic studies of ribozymes with a phosphorothioate modification at the P9 phosphate, Uhlenbeck's group and Herschlag's group reported that an *R*p phosphorothioate linkage reduced the cleavage rate by a factor of 10^3.[1i] However, the rate returned to the control value after the addition of a low concentration of Cd^{2+} ions, which are thiophilic.[1i] A noteworthy aspect of such studies is that the affinity of Cd^{2+} ions was, indeed, higher for the P9−*R*pS ribozyme−substrate complex than for the wild-type ribozyme−substrate complex [$K_{d_{app}}$ (25 μM) for the P9−*R*pS ribozyme-substrate complex is smaller than $K_{d_{app}}$ (220 μM) for the wild-type ribozyme−substrate complex].[1e,i] Thus, it appears that a metal ion coordinates directly with the *pro-R*p oxygen of the P9 phosphate and this *pro-R*p oxygen is one of the metal-binding sites that is required for efficient catalysis. Therefore, these results demand that in hammerhead ribozyme-catalyzed reactions, a metal ion binds directly with the P9 phosphate rather than via a water-mediated hydrogen bonding.

The above metal ion rescue experiments[1] are well correlated with our present NMR data, which demonstrated that the P9−G10.1 motif can capture, by itself, divalent cations, such as Mg^{2+} and Cd^{2+} ions (Figures 2, 3 and 5). Data of EPR spectroscopy by DeRose and co-workers[10] also supported this result. They found that there is a single high-affinity site in hammerhead ribozymes for a Mn^{2+} ion ($K_d \sim 10$ μM) in the presence of 1 M NaCl[10a] and that a Mn^{2+} ion binds to a particular nitrogen atom.[10b] They proposed that the P9−G10.1 motif of a hammerhead ribozyme−substrate complex might be a possible Mn^{2+}-binding site.[10b] Thus, those observations, together with our present NMR data, strongly suggest that the P9−G10.1 motif can capture a divalent cation, irrespective of whether the P9−G10.1 motif is included in hammerhead ribozyme−substrate complexes or is extracted from ribozymes. Accordingly, metal-binding properties of the P9−G10.1 motif within hammerhead ribozyme−substrate complexes reflect the

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intrinsic physicochemical properties of the motif, although the affinity of the cation to the motif might be changed in the transition state of hammerhead ribozyme-catalyzed reactions.[1c,e] All data support that a divalent cation can bind selectively to the P9−G10.1 motif in solution.

Several studies have been carried out to investigate metal−RNA interactions in solution.[7e,10,11] However, this paper is the report in which proton, carbon, and phosphorus resonances of RNA (P9−G10.1 motif) were monitored simultaneously as a function of the concentration of a divalent cation. Simultaneous monitoring of several nuclei within an RNA strand was useful to discriminate between chemical shift changes due to metal-binding and those due to conformational changes and also provided model-independent insights into metal-binding sites on RNAs. In this sense, our data are complementary to those reported by DeRose's group,[10] who focused on the metal ion ($MnCl_2$). Furthermore, we performed titration experiments with $MgCl_2$, which is the most probable cofactor for the P9−G10.1 motif under physiological conditions. Therefore, the phenomena observed here can be expected to occur in biological systems. Gutell and co-workers reported that neighboring base pairs of a G·A mismatch in ribosomal RNAs exhibit considerable bias.[12] Their data suggest the extraordinarily frequent occurrence in ribosomal RNAs of the same sequence as that of the P9−G10.1 motif. Such motifs in ribosomal RNAs would be expected to function as metal-binding sites, to fold into specific structures, or to make catalytic pockets because the P9−G10.1 motif itself has metal-ion-binding ability in the absence of other conserved sequences of hammerhead ribozymes (Figures 2, 3, and 5; Table 1). Accordingly, the P9−G10.1 motif does not appear to be a hammerhead ribozyme-specific motif, but it might be a universal metal-binding motif.

In conclusion, we have been able to definitively assign phosphorus resonances in GA10 from 2D $^1H-^{31}P$ HMQC−NOESY spectra and have shown that the P9−G10.1 motif is sufficient for the capture of the structurally and catalytically indispensable divalent cations, such as Mg^{2+} and Cd^{2+} ions, in the absence of any of the other conserved residues that are found in hammerhead ribozymes. More importantly, the metal-binding properties of the P9−G10.1 motif within hammerhead ribozyme−substrate complexes reflect the intrinsic physicochemical properties of the motif. Finally, our data suggest that in hammerhead ribozymes the P9−G10.1 motif acts as a discrete functional module.

Experimental Section

The RNA oligomers which we used in this study, GA10 and GGAC10, were 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.[13] 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. The oligomer was eluted with 2 M NaCl to make Na^+ the counterion. Finally, excess NaCl was removed by desalting on a Gel filtration column (TSK-GEL G3000PW; TOSO, Japan) with MILLI-Q water as the mobile phase. The final solution contained only the oligomer and the counterion (Na^+ ion). However, some of the Na^+ counterions had been removed by desalting and had been replaced by protons. Such a solution of an oligomer always becomes weakly acidic (∼ pH 4) after gel filtration and, therefore, the pH of the 3 mM solution of GA10 was adjusted to 6 by direct titration with a solution of NaOH. To avoid precipitation of a metal−buffer complex and a resulting change in pH upon titration with $MgCl_2$, no buffer was added. Instead, the pH at each titration point was measured. We should emphasize that pH at each titration point was confirmed to be constant throughout each experiment. Prior to recording of 2D $^1H-^1H$ NOESY spectra and titration experiments, we also determined the concentration of NaCl (40 mM) that gave the best signal separation on one-dimensional (1D) spectra for ^1H. Each oligomer was quantitated by UV absorbance at 260 nm after digestion by nuclease P1.

Spectra of GA10 for the titration of $MgCl_2$ were recorded at 32 °C using a Bruker DMX500 NMR spectrometer. Typical 2D $^1H-^{31}P$ HMQC NOESY spectra were recorded with 1024*32 complex points for a spectral width of 5040.32*404.913 Hz; 4096 scans were averaged, and the mixing time for NOESY was 150 ms. Natural-abundance $^1H-^{13}C$ HSQC spectra were recorded with 1024*128 complex points for a spectral width of 8012.82*7546.36 Hz, and 1024 scans were averaged. A stock solution of $MgCl_2$ in D_2O was evaporated, and the residue was dissolved in D_2O several times to minimize contamination of H_2O. The natural-abundance $^1H-^{13}C$ HSQC spectra for the titration of $CdCl_2$ were recorded at 40 °C using a Bruker DMX500 NMR spectrometer, with 1024*128 complex points, for a spectral width of 8012.82*8804.10 Hz, at 40 °C, in D_2O; 1024 scans were averaged. Prior to the assignments of the cross-peaks between base protons and carbons, proton resonances with or without $CdCl_2$ were assigned from 2D $^1H-^1H$ NOESY spectra. In $^1H-^{13}C$ HSQC spectra, the cross-peaks of H8/G1−C8/G1 were not observed, because H8/G1 was replaced by deuterium, due to the long period of NMR measurements in D_2O (5 months from the first titration experiment). This replacement was also confirmed by 2D $^1H-^1H$ NOESY spectra and 1D spectra for ^1H, which were recorded just before and after titration experiments of $CdCl_2$, because H8/G1 was not detected in these spectra as well. These 2D $^1H-^1H$ NOESY spectra also indicated that GA10 formed a duplex during the titration experiments with $CdCl_2$, because the spectra showed sequential correlations of H8/H6−H1′ and H8/H6−H2″ which were observed for duplexes (data not shown). We have not obtained any evidence for the formation of minor conformers throughout titrations with $CdCl_2$ as well as those with $MgCl_2$. GA10 formed a duplex during the experiments presented here.

In the case of GGAC10, its solution condition for titration experiments was deduced to be 2.5 mM GGAC10, 40 mM NaCl, at pH 7 without additional buffer. Because microaggregation occurs for GGAC10 at lower temperatures, we performed titration experiments at 40 °C with 1D spetra for ^1H and 2D $^1H-^{13}C$ HSQC spectra. Here we also confirmed that GGAC10 formed a duplex as we designed through the titration experiments, because sequential NOEs between base protons and sugar protons (H1′ and H2″) were observed.

Acknowledgment. We would like to thank Prof. Uesugi and Dr. Katahira at Yokohama National University, Dr. Kojima at

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Tokyo Metropolitan University, Drs. Yoshinari, Nonoyama, and Sawata at NAIR, and Dr. Kodama at Osaka University for helpful comments.

Supporting Information Available: Two-dimensional ^{1}H−^{1}H NOESY spectrum showing sequential assignments of GA10 is included in Supporting Information. This material is available free of charge via Internet at http://pubs.acs.org.

Note Added in Proof: Maderia et al.[16] reported recently that Cd^{2+} ions bind to the modified P9−G10.1 motif with a phosphorothioate linkage at the P9 phosphate.

JA001001U

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