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Nature of the Chemical Bond Formed with the Structural Metal Ion at the A9/G10.1 Motif Derived from Hammerhead Ribozymes

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Abstract: We have studied the interaction between metal ions and the metal ion-binding motif in hammerhead ribozymes, as well as the functions of the metal ion at the motif, with heteronuclear NMR spectroscopy. In this study, we employed model RNA systems which mimic the metal ion-binding motif and the altered motif. In $Co(NH_3)_6$(III) titrations, we observed large ^{1}H and ^{31}P chemical shift perturbations for the motif and found that outer-sphere complexation of $Co(NH_3)_6$(III) is possible for this motif. From the reinvestigation of our previous ^{15}N chemical shift data for Cd(II) binding, in comparison with those of organometallic compounds, we conclude that Cd(II) can form an inner-sphere complex with the nucleobase in the motif. Therefore, the A9/G10.1 site was found to accept both inner-sphere and outer-sphere complexations. The Mg(II) titration for a slightly different motif from the A9/G10.1 site (G10.1−C11.1 to A10.1−U11.1) revealed that its affinity to Mg(II) was drastically reduced, although the ribozyme with this altered motif is known to retain enzymatic activities. This observation suggests that the metal ion at these motifs is not a catalytic center of hammerhead ribozymes.

Introduction

Biologically active RNA molecules utilize metal ions to fold into specific conformation or to form a catalytic center. In the case of hammerhead ribozymes, there is a metal ion-binding motif (the A9/G10.1 site) in its conserved core region (Figure 1).[1−6] To understand the mechanism of hammerhead ribozymes, it is important to analyze the interaction between metal ions and the consensus sequences of hammerhead ribozymes.[7−16]

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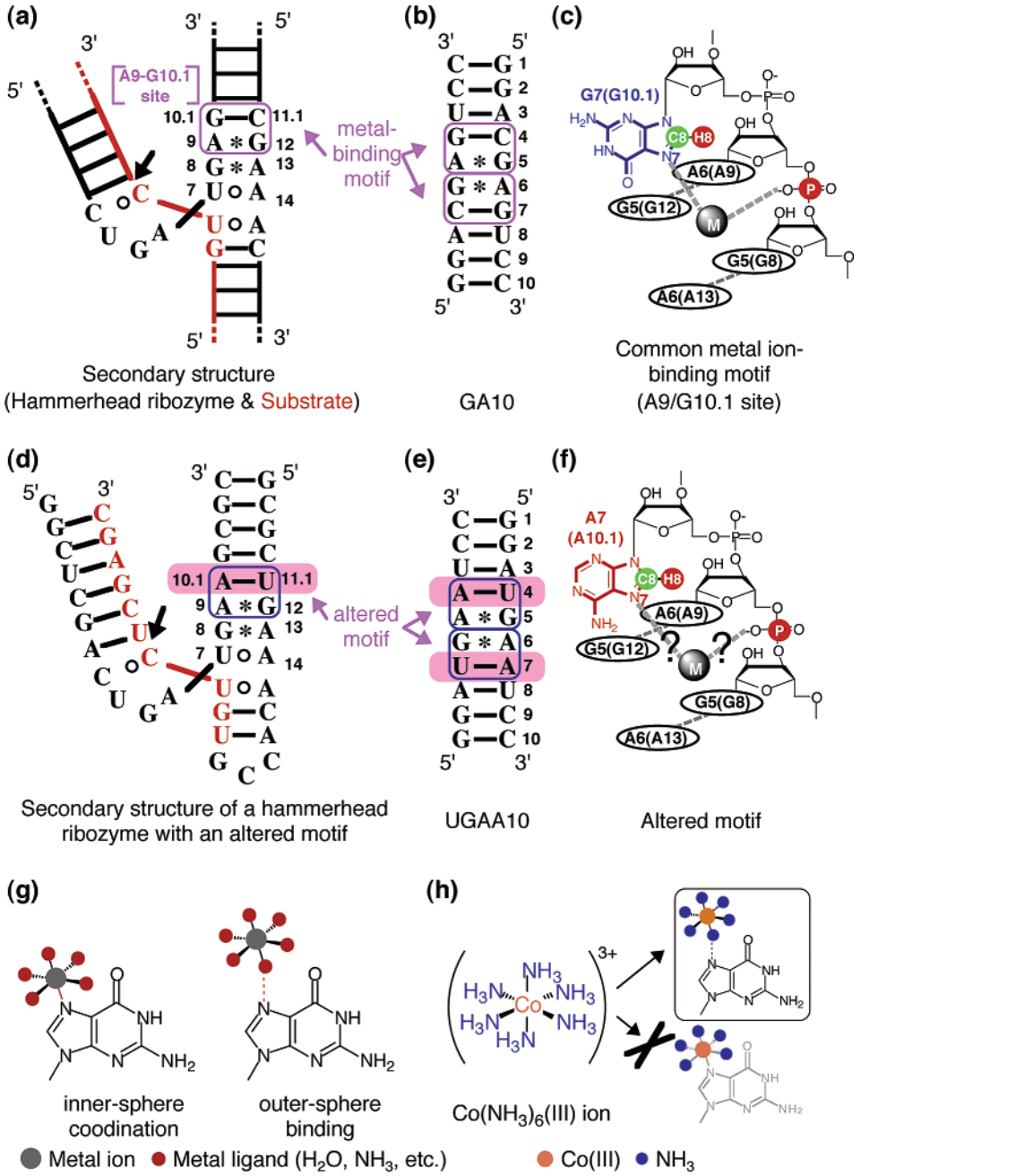

Figure 1. Sequences and metals. (a) Common sequences and a secondary structure of hammerhead ribozymes. (b) GA10 with numbering systems. (c) Schematic representation of the common metal ion-binding motif (the A9/G10.1 site) of hammerhead ribozymes. The residue numbers in GA10 are shown, and the residue numbers in hammerhead ribozymes are also shown in parentheses. (d) Sequences and the secondary structure of the hammerhead ribozyme with A10.1−U11.1, studied by Ruffner et al.[7] (e) UGAA10: A GA10 analogue with an altered motif. (f) Schematic representation of the altered motif. In (a), (b), (d), and (e), the common metal-binding motif (the A9/G10.1 site) and the altered motif are surrounded by magenta and blue lines, respectively. The substituted sequences are highlighted with magenta background. In (a) and (d), enzyme and substrate strands are shown in black and red, respectively, and the cleavage site is indicated by a black arrow. In (c) and (f), a metal ion is shown in a gray circle with a character "M" and is linked to binding sites via dashed lines. The tandem G−A pairs are indicated by black dashed lines with residue names. Watson−Crick base pairs, non-Watson−Crick base pairs, and sheared-type G−A pairs are indicated in bars, open circles, and asterisks. The nuclei monitored by 1D ^{31}P NMR spectra and 2D natural-abundance ^{1}H−^{13}C HSQC spectra are highlighted (P, red; C8, green; H8, red). In (g), metal ion recognition modes are illustrated (inner-sphere coordination and outer-sphere binding [hydrogen bonding through metal ligands]). In (h), $Co(NH_3)_6$(III) binding modes are illustrated. In the case of $Co(NH_3)_6$(III), an inner-sphere coordination of the Co(III) center to nucleobases is inhibited; however, outer-sphere binding is allowed because of exchange-inert metal ligands (NH_3).

Specifically, it is becoming important whether the metal ions at the A9/G10.1 site are catalytic metals (a catalytic center) or structural metals (structural constituents), since this metal ion is the most frequently observed metal ion throughout the crystal structures of hammerhead ribozymes.[1−6,8,11−16] It is also known that this metal ion-binding motif and very similar motifs appear in ribosomal RNAs.[17] Therefore, it is obviously important to study the interaction between metal ions and the metal ion-binding motif.

Recently, studies on the metal−biomacromolecule interaction became one of the topics in biology, and there have been several

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Table 1. Chemical Shift Perturbations upon Additions of Metal Ions[a]

sequences	metals	residue	C8/ppm	H8/ppm	ref.
r(GGACGA**G**UCC)$_2$ [GA10]	$MgCl_2$	G7	+0.5 (2.0 equiv)	+0.12 (9.0 equiv)	4
r(GGACGA**G**UCC)$_2$ [GA10]	$CdCl_2$	G7	+2.3 (5.0 equiv)	+0.38 (9.0 equiv)	5 and this work
r(GGACGA**G**UCC)$_2$ [GA10]	$Co(NH_3)_6Cl_3$	G7	+0.1 (2.0 equiv)	+0.25 (3.0 equiv)	this work
r(GGACGA**G**UCC)$_2$ [GA10]	$NaClO_4$	G7	+0.1 (230 mM)[b]	+0.12 (800 mM)[b]	this work
d(ATG**G**GTACCCAT)$_2$ [12mer]	$ZnCl_2$	G4	+2.5 (8.0 equiv)	+0.20 (8.0 equiv)	42
d(AT**G**GGTACCCAT)$_2$ [12mer]	$ZnCl_2$	G3	+1.5 (8.0 equiv)	+0.05 (8.0 equiv)	42
d(TG**G**T)	Pt(en)Cl_2[c]	G3	+1.1 (1.0 equiv)	+1.04 (1.0 equiv)	43
d(T**G**GT)	Pt(en)Cl_2[c]	G2	+0.2 (1.0 equiv)	+0.26 (1.0 equiv)	43

[a] Chemical shift perturbations were listed in ppm with molar equivalencies to duplexes (GA10 and 12mer) or a single strand of d(TGGT). Plus values indicate lower field shift. [b] Chemical shift perturbations from the basal solutions are listed. [c] Pt(en)Cl_2 (en = ethylenediamine) forms covalent complex d(TGGT)·Pt(en) with the two successive guanosines of d(TGGT).[43,44]

studies on this topic using solution NMR techniques. In the case of nucleic acids, there have been studies on the interaction between metal ions and nucleic acids.[4−6,18−23] DeRose and coworkers demonstrated that in hammerhead ribozymes there is at least one nitrogen atom which is coordinated to Mn(II), with the EPR spectrum measurements of Mn(II), and they suggested the Mn(II) coordination site on the ribozyme.[24−26] The solution NMR technique provides us with a more rigid answer for a metal ion-binding site in the RNA molecule.[4−6,18−23] Despite those extensive works, there is no direct evidence for what kind of chemical bonds were formed between metal ions and nucleobases in ribozymes. In the case of organometallic complexes for organic synthesis, it has been demonstrated that metal ligands dramatically influence reactivities, catalytic activities, and regio- and stereoselectivities of chemical reactions.[27] Thus, for understanding the mechanism of the action of hammerhead ribozymes, it is important to know not only what ligands are on the metal center, but also what kinds of chemical bonds are formed between the metal center and ligands. In our previous studies using heteronuclear multidimensional NMR spectroscopy, we used GA10 as a model RNA oligomer mimicking the metal ion-binding motif of hammerhead ribozymes (Figure 1) and found that Cd(II) specifically bound to the N7 nuclei of the G7 residue [N7(G7)].[4,5] However, we could not definitely conclude whether the binding of Cd(II) to N7(G7) was direct coordination or not.[4,5] This is because we could not detect the scalar coupling between ^{113}Cd(II) and ^{15}N7(G7),[5] as were the cases for other nucleoside−metal ion systems.[28,29] For further information, it is interesting to introduce several kinds of metal ion sources with different characters, such as $Co(NH_3)_6Cl_3$, $NaClO_4$, $MnCl_2$, $EuCl_3$, and $TbCl_3$.

The $Co(NH_3)_6$(III) ion is known as a model for a hexahydrated Mg(II) ion, since the ionic radius of $Co(NH_3)_6$(III), including NH_3 ligands, is very close to that of hexahydrated magnesium ions $[Mg(H_2O)_6]$.[30] In addition, $Co(NH_3)_6$(III) ions are ligated with exchange-inert NH_3 ligands, and then nucleobases cannot be directly coordinated to the Co(III) center of this complex (Figure 1g,h).[30] Therefore, it can be used for a control metal ion that cannot form an inner-sphere coordination.[30] Sodium ion is used to monitor the degree of ionic strength-dependent chemical shift changes. The Mn(II) ion is used to demonstrate whether metal ions are surely located near the nucleobase, since its paramagnetic property causes signal broadening around the binding site in a distance-dependent manner.[18−20] The Eu(III) and Tb(III) ions were also used as a fluorescent probe. Using these data as well as those of Cd(II) and Mg(II), we tried to reveal what kinds of chemical bonds are formed between a metal ion and the metal ion-binding motif in hammerhead ribozymes. The role of the metal ion at this motif was also investigated using a nucleotide-substituted RNA. Ruffner et al. indicated that the hammerhead ribozyme with the A10.1−U11.1 pair retained 30% cleavage activity relative to that with a common G10.1−C11.1 pair.[7] If the metal ion at the A9/G10.1 site is a catalytic metal of hammerhead ribozymes, the altered motif with A−U pair at the 10.1 and 11.1 positions should capture a metal ion. To clarify this point as well as the sequence requirements for the metal ion recognition, we performed titration experiments of the altered motif with Mg(II), the most probable cofactor in physiological conditions.[31]

Results

Titration Experiments with ^{1}H and ^{31}P NMR Spectra. In previous studies, we have demonstrated that divalent cations, such as Mg(II) and Cd(II), were able to bind to the metal ion-binding motif through the N7 nucleus of G7 in GA10 using ^{1}H, ^{13}C, and ^{15}N chemical shift perturbation (Tables 1 and 2).[4,5] In those studies, large chemical shift perturbations were observed for N7, C8, and H8 resonances of the G7 residues (−19.6, 2.3, and 0.38 ppm, respectively) upon the addition of Cd(II) ions (Tables 1 and 2).[4,5] Therefore, it was suggested that Cd(II) and Mg(II) ions directly coordinated to N7(G7). To clarify this situation in detail, we employed $Co(NH_3)_6$(III) ions, since exchange-inert NH_3 ligands prevent an inner-sphere coordination of the Co(III) center to nucleobases. If the direct coordination of a metal center is a prerequisite for the metal ion binding to the motif, $Co(NH_3)_6$(III) ions cannot bind to the motif. Contrary

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Table 2. Summary of ^{15}N NMR Data

metal ligands (ref.)	[ligand]/mM	metals	residue	$\Delta^{15}N^a$/ppm	$^1J_{N-M(II)}{}^b$/Hz
r(GGACGA**G**UCC)$_2$ [GA10] (5)	0.45	$CdCl_2$	G7	−19.6 (6.0 equiv)	not detected
guanosine monomer (28)	500	$Zn(NO_3)_2$	n.a.[c]	−20.1 (1.0 equiv)	n.a.
guanosine monomer (28)	500	$Hg(NO_3)_2$	n.a.	−20.5 (1.0 equiv)	not reported
inosine monomer (29)	1000	$Zn(NO_3)_2$	n.a.	−15.2 (0.7 equiv)	n.a.
inosine monomer (29)	1000	$Hg(NO_3)_2$	n.a.	−4.8 (0.75 equiv)	not detected
1-methylimidazole[d] [MI] (33)	240	$Cd(NO_3)_2$	n.a.	−28.7[d] (0.2 equiv)	not reported
1-methylimidazole[d] [MI] (33)	350	$Zn(NO_3)_2$	n.a.	−46.0[d] (0.22 equiv)	n.a.
β-lactamase (35)	0.8	$CdCl_2$	His[e]	n.r.[f]	∼78−216
EDTA (36)	400	$CdCl_2$	n.a.	+3.3 (1.9 equiv)	81

[a] Chemical shift perturbations of N7(guanosine), N3(1-methylimidazole), and tertiary amine nitrogens (EDTA) from the unmetalated state. Minus and plus values indicate higher and lower field shifts, respectively. [b] *J*-coupling between metalated nitrogens (^{15}N) and metal ions ($I = 1/2$), such as ^{113}Cd and ^{199}Hg. Only absolute values are indicated. [c] Not applicable. [d] In the equilibrium system of 1-methylimidazole [MI] and metal ions [M(II)], the ^{15}N chemical shift of N3 reflects an average value of those from free, protonated, and metal complexes (M(II)-MI, M(II)-2·MI, M(II)-3·MI, M(II)-4·MI, and so on). Therefore, chemical shift values of 1-methylimidazole are average values of these species. [e] Four of seven histidines were metalated, but residue numbers in the amino acid sequence were not reported. [f] Not reported. However, in β-lactamase-Cd(II) system, ranges of ^{15}N chemical shifts of metalated and unmetalated residues were overlapped, and ^{15}N chemical shift perturbations due to a metalation were estimated to be small. For the interpretations of the ^{15}N NMR chemical shift, Alei and co-workers presented interesting data on the ^{15}N chemical shift of 1-methylimidazole.[33] Chemical structure of the five-membered ring of the guanosine base is the same as 1-methylimidazole, and N7 of guanosine corresponds to N3 of 1-methylimidazole (Figure 7). They indicated that the ^{15}N chemical shift of N3, a metal ion-coordination site of 1-methylimidazole, was perturbed toward high field (∼15−30 ppm) upon the complexation with Cd(II) in H_2O.[33] In this system, it seems difficult for an imidazole ring to form stable outer-sphere complexes with Cd(II) (the outer-sphere binding via water molecules of Cd(II) ligands), since 1-methylimidazole is a monodentate ligand. In other words, the observed complex is thought to be a coordination compound, and the higher field shift of the N3 resonance of 1-methylimidazole is due to an inner-sphere coordination of the N3 atom to Cd(II). Therefore, when the imidazole ring nitrogen coordinates to transition metal ions, such as Cd(II) and Zn(II), the ^{15}N chemical shift can be perturbed toward high field.

to this, if $Co(NH_3)_6$(III) ions can bind to the motif, it is deduced that this metal ion-binding motif allows the metal ion binding through the metal ligands, including water molecules. To estimate ionic strength-dependent chemical shift changes, titration experiments with $NaClO_4$ were also performed. On the basis of the above concepts, we performed titration experiments with $Co(NH_3)_6Cl_3$ and $NaClO_4$, using one-dimensional (1D) ^{1}H and ^{31}P NMR spectra (Figure 2), as well as those of $CdCl_2$ (an example for tight binding).

Contrary to our early prediction, titration data with 1D ^{1}H and ^{31}P NMR spectra indicated $Co(NH_3)_6$(III) ion binding to GA10, since large chemical shift perturbations were observed for the resonances in the motif (the H8 resonance of the G7 residue [H8(G7)], phosphorus of the A6 residue [P(A6)], and the neighboring residues in GA10) (Figure 2). In contrast to the titrations for $Co(NH_3)_6$(III) and Cd(II), sodium ion titration curves of ^{1}H and ^{31}P resonances were almost linearly increased against the concentration of $NaClO_4$, except for those at the 5′ end (Figure 2). It was also found that ionic strength-dependent chemical shift perturbations for proton resonances were small (the maximum perturbation: 0.0002 ppm per 1 molar equivalent $NaClO_4$ for the H6 proton of the C4 residue) and negligible for the titration data of Cd(II) and $Co(NH_3)_6$(III). It was found that metal ion binding to the motif was not a simple electrostatic interaction, and sodium ions do not bind to the motif in a site-specific manner. This is consistent with the result by Herschlag's group that monovalent lithium ions did not bind to the motif during the catalytic reaction of hammerhead ribozymes.[16] Therefore, the chemical shift perturbations by Cd(II) and $Co(NH_3)_6$(III) titrations were due to their site-specific binding to GA10.

Intermolecular NOEs between GA10 and $Co(NH_3)_6$(III). We tried to exclude the possibility that the chemical shift perturbations for $Co(NH_3)_6$(III) titrations were due to the secondary effect from $Co(NH_3)_6$(III) binding to somewhere outside the motif. For this purpose, we measured two-dimensional (2D) ^{1}H−^{1}H NOESY spectra (90% H_2O, 10% D_2O). In the spectrum, several intermolecular NOEs were observed between protons of $Co(NH_3)_6$(III) ions and base protons of GA10 (Figure 3). Especially between the $Co(NH_3)_6$(III) ions and H8(G7) was one of the strongest intermolecular NOEs, and other NOEs were observed for the base protons and amino protons around the A9/G10.1 site (Figure 3a). In the upper panel of Figure 3a, possible NOE counterparts for $Co(NH_3)_6$(III) are indicated. A strong intermolecular NOE was also observed between the imino proton of the G7 residue and the NH_3 ligands (Figure 3b). It was surely demonstrated that the $Co(NH_3)_6$(III) ion bound to the A9/G10.1 site selectively.

^{1}H−^{13}C HSQC Spectra of GA10. We recorded 2D ^{1}H−^{13}C HSQC spectra with or without metal salts such as $Co(NH_3)_6Cl_3$, $CdCl_2$, and $NaClO_4$ (Figure 4). As previously shown,[4,5] Cd(II) ions significantly perturbed the C8(G7) resonance toward low field (Figure 4a,b). In contrast, $Co(NH_3)_6Cl_3$ and $NaClO_4$ only perturbed the C8(G7) resonance within an error range of absolute chemical shift values (Figure 4c−f). From the titration and NOE data for the GA10−$Co(NH_3)_6$(III) system, $Co(NH_3)_6$(III) is thought to bind to N7(G7) via NH_3 ligand-mediated hydrogen bonding (outer-sphere binding); however, this outer-sphere binding did not affect the chemical shift of C8(G7). In other words, the lower field shift of C8(G7) upon the addition of Cd(II) is not explainable without an inner-sphere coordination of a metal ion to N7(G7). It was also found that sodium ions did not affect the chemical shifts of C8 atoms of all residues significantly.

Effects of a Paramagnetic Metal Ion. To exclude the possibility that the chemical shift perturbations for C8(G7) upon addition of Cd(II) and Mg(II) merely reflect the conformational changes due to their binding to allosteric sites, we have monitored signal broadening due to paramagnetic Mn(II) ion binding. This technique indicates that Mn(II) ions surely locate near the site where the resonances were broadened since the signal broadening occurs in a distance-dependent manner.[18−20] It is also known that Mn(II) and Cd(II) ions belong to relatively soft acids, according to the hard and soft acids and bases (HSAB) rule, and kinetic and thermodynamic behavior of Mn(II) is similar to that of Cd(II) in the catalytic reaction of ham-

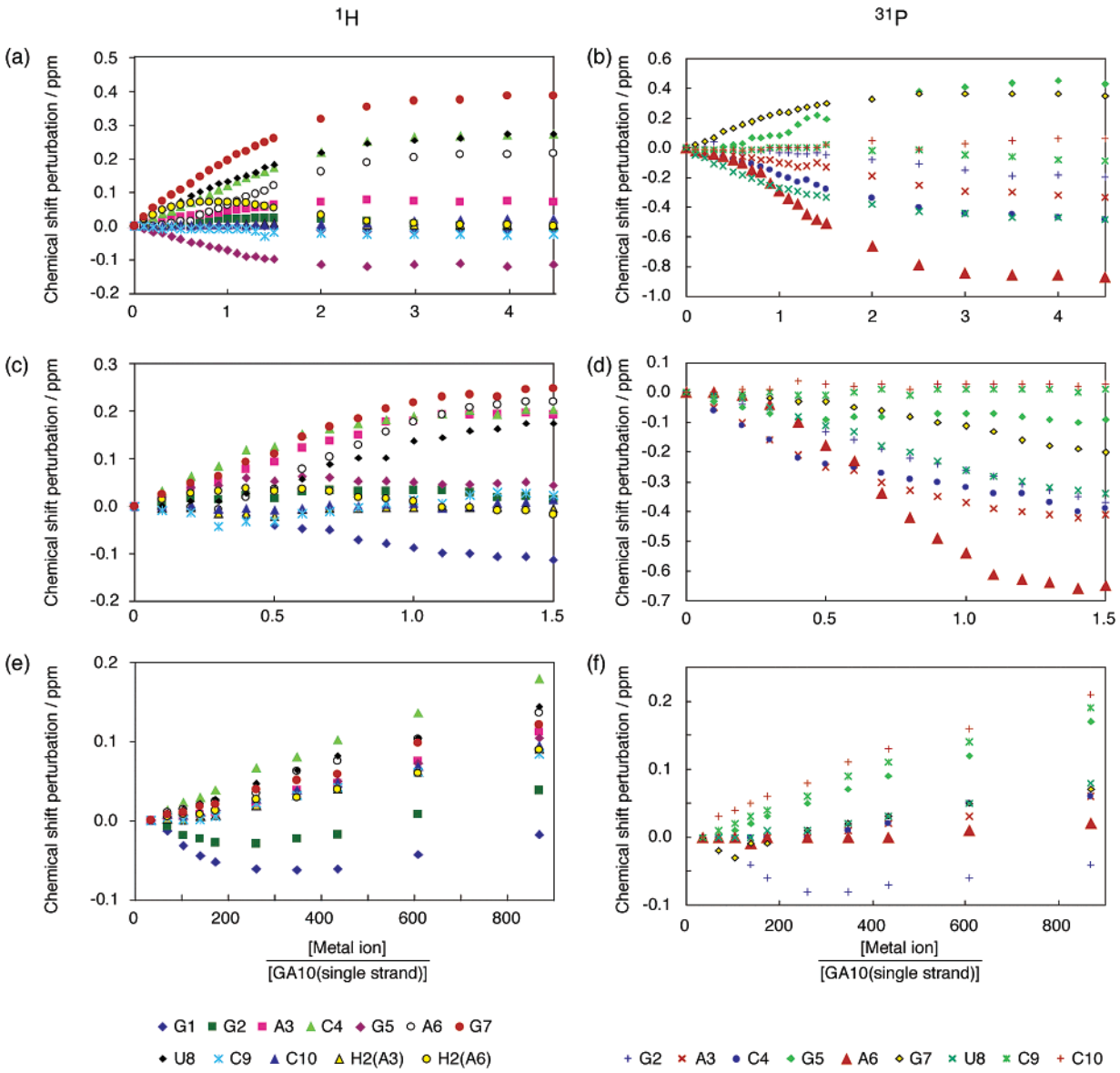

Figure 2. Chemical shift perturbations for base protons [H8(purine), H6(pyrimidine), and H2(adenosine)] against a molar ratio ([metal ion]/[GA10(single strand)]). In (a), (c), and (e), those of ^{1}H resonances were plotted for Cd(II), $Co(NH_3)_6$(III), and sodium ions, respectively. In (b), (d), and (f), those of ^{31}P resonances were plotted for Cd(II), $Co(NH_3)_6$(III), and sodium ions, respectively. For Cd(II) titrations, various concentrations of $CdCl_2$ were added to the solution containing 1.5 mM GA10(single strand) and 40 mM $NaClO_4$. For $Co(NH_3)_6$(III) titrations, various concentrations of $Co(NH_3)_6Cl_3$ were added to the solution containing 2.0 mM GA10(single strand) and 40 mM $NaClO_4$. For sodium titrations, various concentrations of additional $NaClO_4$ were added to the solution containing 1.1 mM GA10(single strand) and 40 mM $NaClO_4$. Typical 1D ^{1}H, ^{31}P NMR spectra were recorded on Bruker DMX 750 spectrometer at 313 K. For ^{1}H NMR spectra, spectral width was 10 000 Hz digitized into 16 384 complex points; 128 scans were averaged. For ^{31}P NMR spectra, spectral width was 10 000 Hz digitized into 16384 complex points; 1024 scans were averaged. We confirmed that resonance assignments were consistent with other spectra, such as 2D ^{1}H−^{1}H NOESY, ^{1}H−^{13}C HSQC, and ^{1}H−^{31}P HMQC NOESY spectra.

merhead ribozymes.[8−12,15,16] Accordingly, Mn(II) is regarded as a model of Cd(II). From this experiment, broadening of H8-(G7) was evident (Figure 5). It was demonstrated that Mn(II) was surely located near H8(G7). Therefore, Cd(II) is also thought to be located near N7(G7), and the chemical shift perturbation of H8, C8, and N7 of G7 should be due to the metal ion binding to N7(G7).

Sequence Requirements for the Metal Ion Binding. We have also tested whether the G7 residue (G10.1 in hammerhead ribozymes) can be replaced by adenosine without a loss of the affinities for divalent cations. In the metal ion-binding motif of GA10, N7(G7) was shown to be a Cd(II)-binding site, whereas O6(G7) was not.[5] Although there is one example where O6-(G10.1) is utilized as a metal ligand in the crystal structure of the hammerhead ribozyme−Co(II) complex,[3] it is not a definitive requirement for other systems.[1,2] Therefore, we thought that the guanosine residue at the G10.1 position is able to be substituted by an adenosine residue. Then, we synthesized the RNA oligomer, UGAA10:rGGA**U**UGA**A**UCC, and measured the NMR spectra. It had been demonstrated that UGAA10 forms a duplex with sheared type G−A pairs in tandem at the middle of the duplex, and the UGAA sequence took a similar structure to that of r(GGGC**U**GA**A**GCCU)$_2$ by Heus et al.[32] It is also known that the hammerhead ribozyme with A10.1−U11.1

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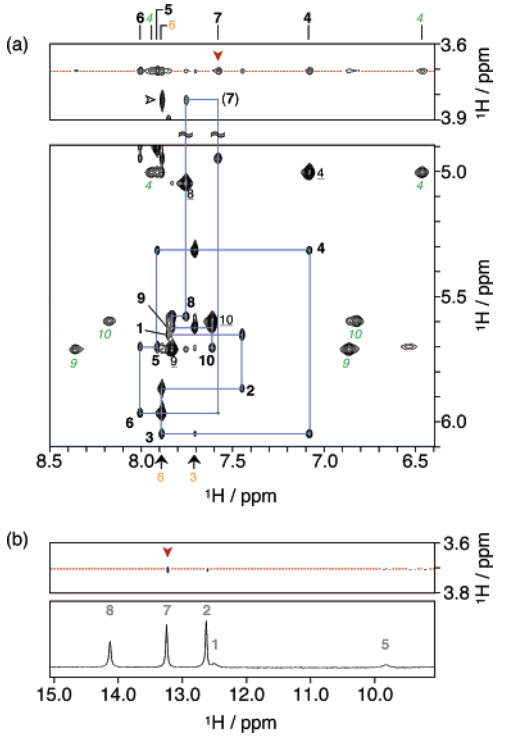

Figure 3. 2D ^{1}H−^{1}H NOESY spectrum in the presence of 1 molar equivalent of $Co(NH_3)_6Cl_3$ to the motif. The solution contained 3.4 mM GA10, 40 mM $NaClO_4$, and 3.4 mM $Co(NH_3)_6Cl_3$. The chemical shift of NH_3 ligands of $Co(NH_3)_6$(III) are indicated by red dashed lines. (a) NOE cross-peaks from $Co(NH_3)_6$(III) to GA10 (upper panel) and sequential NOE between base protons and anomeric protons (upper and lower panels) are presented. These intraresidue NOE cross-peaks were labeled with their residue number. The underlined numbers and green italic numbers are intraresidue H5−H6 cross-peaks and H5 amino protons cross-peaks for the corresponding residue number, respectively. Below the lower panel of (a), black arrows indicate the presence of H2 resonances on the corresponding chemical shift, with the residue numbers (orange numbers). NOE counterparts for $Co(NH_3)_6$(III) are also indicated above the upper panel of (a), and that of H8(G7) is indicated with a red arrowhead. We assigned the resonance at 3.81 ppm as H1′ of G7, since it is well-known that the H1′ resonances of the guanosine in the C−G pair, which is adjacent to a sheared type G−A pair in the same arrangement as the A9/G10.1 site, always resonated at an extraordinarily high field.[41] Katahira et al. assigned ^{1}H resonances of the same sequence as GA10 in the absence of $Co(NH_3)_6$(III) (H1′(G7) at 4.14 ppm for a solution containing 1.8 mM GA10, 20 mM sodium-phosphate buffer pH 7.0, and 150 mM NaCl, at 306 K).[41b] Heus and Pardi assigned ^{1}H resonances of the RNA oligomer r(GGGC*GCAA***G**CCUUAU), including *GCAA* loop with a *C*−***G*** closing base pair, adjacent to a sheared type G−A pair in the loop (H1′(G9) at approximately 3.7 ppm for a solution containing 1.9 mM RNA, 10 mM sodium phosphate buffer pH 6.8, and 100 mM NaCl at 298 K).[41a] It should be noted that in the absence of $Co(NH_3)_6$(III), H1′-(G7) of GA10 resonated at 3.99 ppm (1.2 mM GA10, 40 mM NaCl, pH 6 at 313 K).[39] Therefore, the chemical shift of H1′(G7) is significantly dependent on the measurement conditions, and slightly different chemical shifts for Katahira's conditions and ours are probably attributed to the differences in conditions. We have also confirmed this assignment by monitoring the NOE cross-peak (an open arrowhead) with H2(A6) (7.89 ppm), as Katahira et al. showed.[41b] This NOE cross-peak was observed in all 2D ^{1}H−^{1}H NOESY spectra in D_2O in the presence and absence of $CdCl_2$ and $Co(NH_3)_6Cl_3$.[39] In (b), 1D ^{1}H NMR spectrum of an imino proton region (lower panel) and NOE cross-peaks between $Co(NH_3)_6$(III) and imino protons are presented. In the lower panel of (b), residue numbers of imino protons are indicated. In the upper panel of (b), the cross-peak between imino proton of G7 and $Co(NH_3)_6$(III) is pointed to with a red arrowhead. In the NOESY spectrum, weak NOE cross-peaks from $Co(NH_3)_6$(III) were observed for nonspecific sites at this threshold. However, this phenomena might be a general feature of this experiment, since nonspecific NOEs from $Co(NH_3)_6$(III) were observed in other studies as well.[21,22] A 2D ^{1}H−^{1}H NOESY spectrum was recorded on Bruker DMX750 at 293 K; 4096*2048 complex points for a spectral width of 15 700*15 000 Hz.

instead of G10.1−C11.1 retains 30% cleavage activity, relative to the hammerhead ribozyme with the common G10.1−C11.1 pair.[7] This altered sequence motif is the same as that in UGAA10. Therefore, if the metal ion at A9/G10.1 and the corresponding site is a catalytic metal, this altered motif in UGAA10 should also capture divalent cations. However, chemical shift perturbations for UGAA10 were much less than those observed for GA10 upon the addition of 1 molar equivalent of $MgCl_2$ to the metal ion-binding motif (Figure 6). The maximum chemical shift perturbation of base protons in UGAA10 was observed for H8(G2) (0.03 ppm), the distal site from the putative metal ion-binding site (A7) (Figure 6). In addition, this perturbation is much smaller than those due to the specific binding of metal ions to GA10 (0.38 ppm for H8(G7) in Cd(II) titrations). Therefore, it is plausible that the chemical shift perturbations of the proton resonances in UGAA10 merely reflect changes in surrounding environments and cannot be attributed to specific binding of Mg(II). It is also known that nonspecific binding of divalent cations to backbone phosphates as counterions can occur at this concentration.[5] Therefore, the affinity between the motif in UGAA10 and Mg(II) is comparable to or lower than that between backbone phosphates and counterions of divalent cations. Accordingly, we can conclude that this altered motif with an adenosine residue at 10.1 position was not sufficient to capture divalent cations. From this result, in conjunction with sequence requirements for the cleavage reaction of hammerhead ribozymes,[7] it was suggested that the role of the metal ion at the A9/G10.1 site is not a catalytic metal, but a structural metal for the folding of hammerhead ribozymes.

Discussion

Investigations for $Co(NH_3)_6$(III) Binding. Intermolecular NOEs between $Co(NH_3)_6$(III) ions and the motif surely indicated that the $Co(NH_3)_6$(III) ion binds to the metal ion-binding motif (the A9/G10.1 site) (Figure 3). In addition, the binding isotherms of ^{1}H resonances for $Co(NH_3)_6$(III) titrations indicated biphasic (two-step) transitions, which means that two $Co(NH_3)_6$(III) ions were captured by GA10 at the metal ion-binding motif (Figure 2). This phenomenon is the same as in the GA10−Cd(II) system in which the binding isotherms are biphasic, and two Cd(II) ions bind to a GA10 duplex.[5] It should be noted that $Co(NH_3)_6$(III) titration curves for H8(A6) and H2(A6) significantly deviated from a monophasic transition curve and cannot be explained without biphasic transitions, as was the case for GA10−Cd(II) system.[5] It was also found that the overall binding isotherms of ^{1}H- and ^{31}P resonances for Cd(II) and $Co(NH_3)_6$(III) titrations were similar to each other. The resonances for the specific sites (H8(G7) and P(A6)) were especially similar to each other, although degrees of perturbations for the above resonances and titration curves for some resonances were different to some extent (Figure 2).

These data indicate that the binding sites of both $Co(NH_3)_6$-(III) and Cd(II) ions are similar or the same. Unfortunately, we could not completely exclude the possibility that $Co(NH_3)_6$(III) bound to O6(G7) or neighboring sites except for N7(G7). However, it is plausible that one of the binding sites for $Co(NH_3)_6$(III) ions is N7(G7) because of many similarities of the binding isotherms for both $Co(NH_3)_6$(III) and Cd(II) ions at this moment. Therefore, $Co(NH_3)_6$(III) is thought to be able to bind to the motif through the ligand-mediated hydrogen

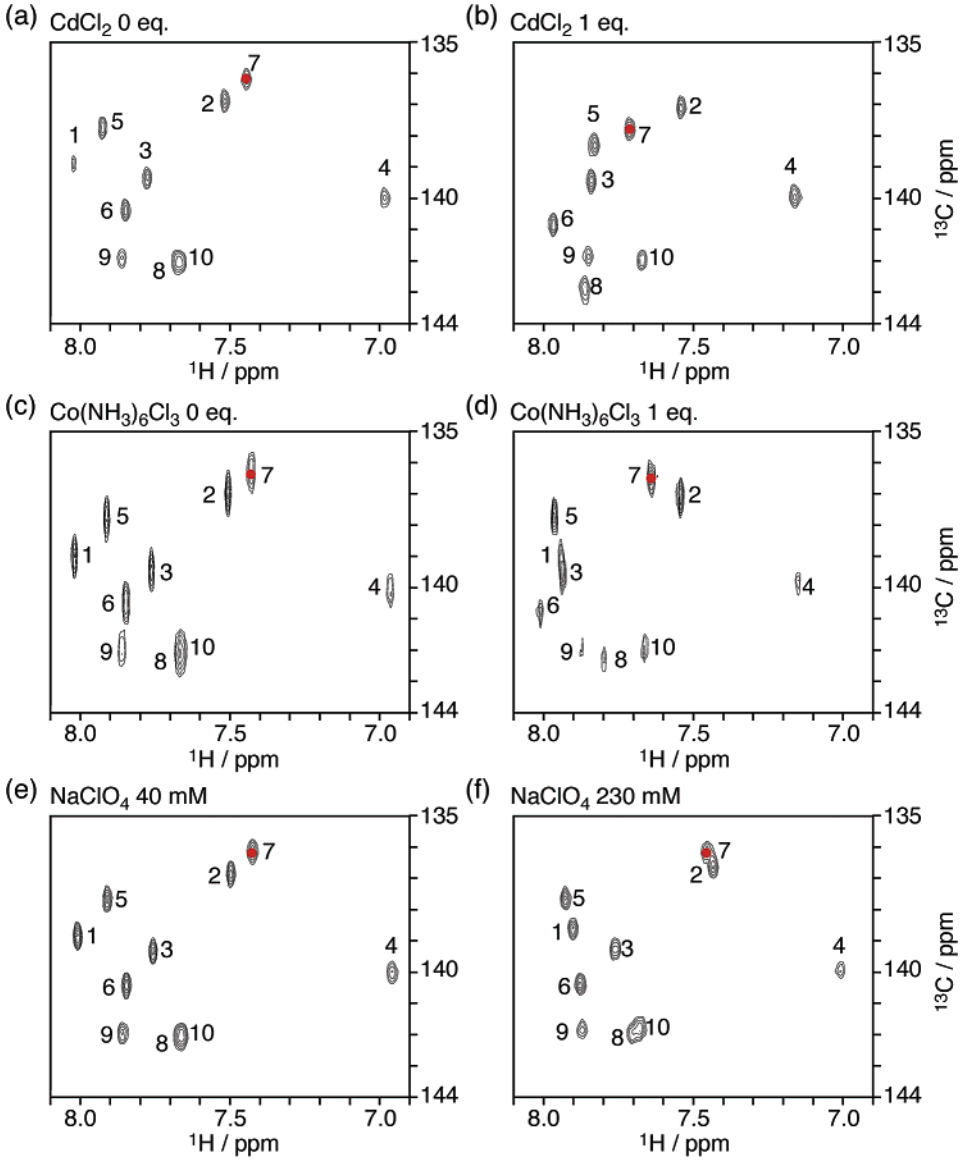

Figure 4. 2D $^{1}H-^{13}C$ HSQC spectra with or without metal ions. Panels (a) and (b) indicate spectra of basal solution (2.4 mM GA10(single strand), 40 mM $NaClO_4$) and that in the presence of $CdCl_2$ (2.4 mM GA10(single strand), 40 mM $NaClO_4$, and 2.4 mM $CdCl_2$). Panels (c) and (d) indicate spectra of basal solution (2.0 mM GA10(single strand), 40 mM $NaClO_4$) and that in the presence of $Co(NH_3)_6Cl_3$ (2.0 mM GA10(single strand), 40 mM $NaClO_4$, and 2.0 mM $Co(NH_3)_6Cl_3$). Panels (e) and (f) indicate spectra of basal solution (3.5 mM GA10(single strand), 40 mM $NaClO_4$) and that in the presence of excess $NaClO_4$ (3.5 mM GA10(single strand) and 230 mM $NaClO_4$). Intraresidue cross-peaks between H8 and C8 nuclei are labeled with their residue numbers, and that between H8(G7) and C8(G7) are indicated by red circles. In (a), (b), (e), and (f), spectra were recorded on Bruker DMX750 at 313 K; 1024*128 real points for a spectral width of 7003*5659 Hz. In (c) and (d), the point resolution was reduced to 1024*64 real points for the same spectral width since spectra were broadened upon the addition of 1 molar equivalent of $Co(NH_3)_6Cl_3$.

bonding (outer-sphere binding) (Figures 2−4). On the other hand, crystal structures indicate that several metal ions, such as Mn(II) and Co(II), can bind to the motif via an inner-sphere coordination to N7(G10.1) of hammerhead ribozymes.[1,3] Accordingly, the A9/G10.1 site is a motif which allows two kinds of binding modes, i.e., an inner-sphere coordination[1,3] and outer-sphere binding via metal ligands.

More importantly, the chemical shift of C8(G7) was not affected by the $Co(NH_3)_6$(III) ion binding (Figure 4c,d and Table 1), which means that hydrogen bonding between NH_3 ligands and the N7(G7) did not perturb the resonance of C8(G7) or other carbon resonances. In contrast, chemical shift values of both C8(G7) and H8(G7) were selectively perturbed upon the addition of Cd(II) and Mg(II) (Figure 4a,b and Table 1),[4] which can potentially coordinate to N7(G7). Together with the above data, simultaneous large lower field shift of C8 and H8 resonances of G7 cannot be explained without an inner-sphere coordination of a metal ion to N7(G7), although the resonance of C8(G7) for $Co(NH_3)_6$(III) titrations might be unchanged due to some cancellation by chance.

Reinvestigations of ^{15}N NMR Data. In our previous publication, we observed a higher field shift by 19.6 ppm for the ^{15}N resonance of N7(G7) without a detectable *J*-coupling between ^{15}N and ^{113}Cd(II) (Table 2).[5] The same phenomena were observed for the cases of guanosine−^{199}Hg(II) complex in dimethyl sulfoxide (DMSO) solution (Table 2).[28] In those studies, it was thought that guanosine and inosine were the ligands for the metal ions, and these nucleosides directly coordinated to the metal ions.[28,29] However, there has been no direct evidence that indicates the formation of the coordination

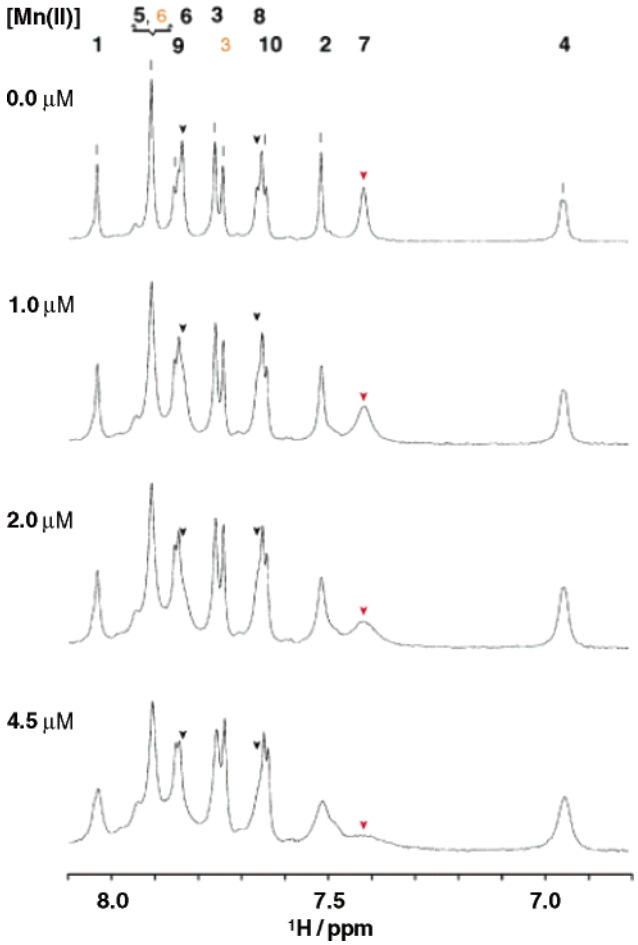

Figure 5. 1D ^{1}H NMR spectra with increasing $MnCl_2$ concentration. Concentration of $MnCl_2$ for each spectrum is indicated on the left of each spectrum. Signal broadening was observed for H8(G7) (red arrowhead). Signal intensities of H8(A6) and H6(U8) (black arrowheads) seemed to be decreased as well. Assignments of base protons are indicated with their residue numbers, and small orange characters indicate H2 resonances of the corresponding residue.

compound for the above systems. We then tried to detect what kind of complex is formed between Cd(II) and a guanosine monomer in DMSO by means of a specially designed mass spectrometer using an electrospray interface.[34] In this mass spectrometry, we can detect charged clusters that are present beforehand in solution.[34b] For example, even solvated ions such as lithium ion−methanol and cesium ion−methanol clusters were detected with this system.[34b]

In the DMSO solution of Cd(II) and guanosine (the Cd(II)−guanosine−DMSO system), we detected several complexes of three components (Cd(II), guanosine, and DMSO). The major complexes of Cd(II)−guanosine−DMSO were 1:1:3 (*M*/*Z*: 315.4), 1:1:4 (*M*/*Z*: 354.4), and 1:1:5 (*M*/*Z*: 393.4) complexes, which may possibly indicate Cd(II) complexes with the ligands in tetrahedral, square pyramidal, and octahedral geometries, respectively (unpublished data). Although other ternary complexes with different molar ratios were detected, their populations were much less than the 1:1:3, 1:1:4, and 1:1:5 complexes. It was indicated that guanosine directly coordinates to a soft

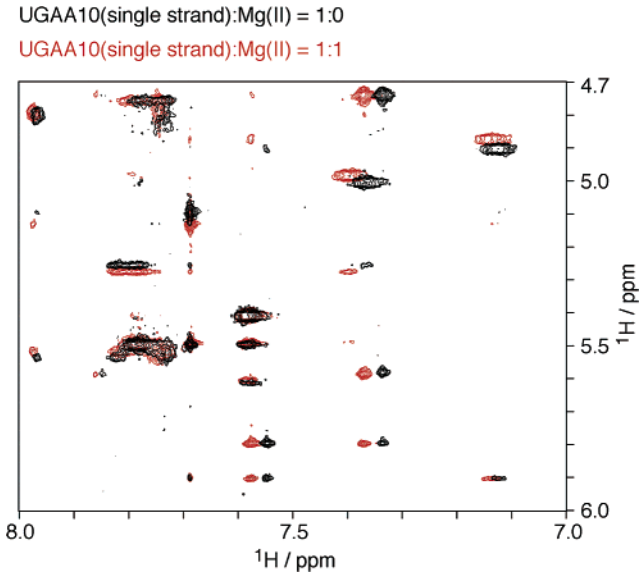

Figure 6. 2D ^{1}H−^{1}H NOESY spectra of UGAA10. Black spectrum: 2 mM UGAA10(single strand) without $MgCl_2$ (UGAA10(single strand): $MgCl_2$ = 1:0 mol/mol). Red spectrum: 2.0 mM UGAA10(single strand) with 2.0 mM $MgCl_2$ (UGAA10(single strand): $MgCl_2$ = 1:1). Spectra were recorded on Bruker DMX500 at 300 K; 4096*2048 complex points for a spectral width of 4006*4006 Hz.

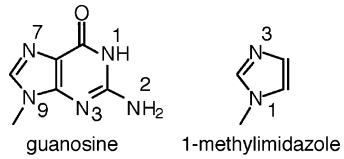

Figure 7. Chemical structures of guanine and 1-methylimidazole with numbering systems.

Lewis acid, Cd(II), in DMSO. Accordingly, the higher field shift (approximately 20 ppm) of the N7 resonance of the guanosine−Hg(II) or −Zn(II) in DMSO[28,29] is thought to be due to a direct coordination of guanosine to Hg(II) and Zn(II), since these metal ions belong to soft Lewis acids. In analogy, the higher field shift (19.6 ppm) of the N7(G7) in GA10 (Table 2)[5] indicates that the G7 residue directly coordinates to Cd(II) through N7. The lack of *J*-coupling between ^{113}Cd(II) and ^{15}N7(G7) is likely due to the fast exchange of the association−dissociation process of the Cd(II) binding to N7(G7).

On the other hand, in the cases for β-lactamase−Cd(II)[35] and ethylenediaminetetraacetic acid (EDTA)−Cd(II)[36] systems, *J*-coupling between ^{113}Cd and ^{15}N ($^1J_{N-Cd}$) were observed with only a slight chemical shift perturbation (Table 2). Although metalation sites of β-lactamase are imidazole rings of histidines and several imidazole rings surely coordinated to Cd(II), metalation did not affect ^{15}N chemical shifts of imidazole rings very much. This is quite different from Cd(II)−GA10 and Hg(II)−guanosine systems (no detectable $^1J_{N-M(II)}$ and large chemical shift perturbations). Unfortunately, we could not conclude the origins of the discrepancies at this moment. However, it was found that there are at least two patterns of appearance for ^{15}N NMR spectra which indicate an inner-sphere coordination of nitrogen to Cd(II). Therefore, caution must be

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taken for the interpretation of ^{15}N NMR data when we determine the sort of chemical bonds from ^{15}N NMR data. For more definitive results, solid-state NMR with crystal powder of Cd(II)−guanosine complex might provide the definitive answer for the chemical bond formed between Cd(II) and N7 of guanosine, since in the crystal lattice association−dissociation dynamics is suppressed. In such a system, the Cd(II)−N7(G) bond can be determined by crystallography, and intrinsic *J*-coupling between $^{113}Cd(II)-^{15}N7(G)$ can be derived from solid-state NMR, and then ^{15}N and ^{13}C chemical shifts can be confirmed as well.

Roles of the Metal Ion at the A9/G10.1 Site. We have demonstrated that the altered motif in UGAA10, with an adenosine residue instead of a guanosine, was not sufficient to capture divalent cations from 2D $^1H-^1H$ NOESY spectra of UGAA10 (Figure 6). We had shown that nonspecific binding of metal ions was observed in the titration curves of GA10 at "mM" order, as well.[5] Thus, the altered motif is no longer thought to be a metal ion-binding motif. Interestingly, Sigel et al. indicated that N7 of adenosine is a much less efficient acceptor of metal ions than that of guanosine by means of potentiometric titrations at the nucleoside level.[37] They also demonstrated that basicities of nitrogen atoms were well-correlated with affinities of the nitrogen atoms to divalent cations, i.e., the order of affinities of N7 to divalent cations is guanosine > adenosine and the order of the basicities of N7 is guanosine > adenosine.[37] Therefore, we think that this effect is one of the major reasons for the reduced affinity of the altered motif to Mg(II). For more detailed physicochemical characterizations of this altered motif, titration experiments with $Co(NH_3)_6$(III) and Cd(II) are also required.

From the aspect of the roles of metal ions at the A9/G10.1 site, the above titration data of UGAA10 indicate a more important thing. Kinetic data by Ruffner et al. indicated that hammerhead ribozymes with the A10.1−U11.1 base pair (the altered motif) still possess 30% catalytic activities relative to those with the common sequence which includes the G10.1−C11.1 base pair.[7] Furthermore, in the sequence of the wild-type hammerhead ribozyme from the newt, A10.1−U11.1 is included instead of a G10.1−C11.1.[38] These data indicate that G10.1−C11.1 is not a strict requirement for the reaction catalyzed by hammerhead ribozymes, and G10.1−C11.1 can be replaced with A10.1−U11.1 without a loss of functions, although this base pair substitution significantly reduces a metal ion-binding ability of the motif (Figure 6). Therefore, if we assume that the metal ion at the A9/G10.1 site is a catalytic metal, reduced (almost loss of) metal ion-binding ability of UGAA10 is inconsistent with this assumption. Accordingly, it was suggested that the metal ion at the A9/G10.1 site is not a catalytic metal, although there might be a possibility that other conserved sequences might support metal ion binding to the altered motif upon conformational changes. Eckstein's group and Lilley's group indicated that hammerhead ribozymes changed their conformation in a divalent cation concentration-dependent manner.[9] The metal ion at the A9/G10.1 motif might be one of the structural cofactors for the formation of the γ-shape of hammerhead ribozymes.

Experimental Section

Sample Preparations. RNA oligomers were chemically synthesized, purified, and quantitated as described previously.[5,39] Synthesized RNA oligomers are the following:

GA10:r(GGACGAGUCC)

UGAA10:r(GGAUGAAUCC)

The basal solutions for NMR measurements contained 40 mM $NaClO_4$ and various concentrations of metal salts ($CdCl_2$, $Co(NH_3)_6Cl_3$, $MnCl_2$, and $NaClO_4$). To avoid precipitation of the metal−buffer complex and the resulting pH changes, no buffer was added to the solution used in the NMR measurements.[4,5,39] Instead, the pH of each solution was adjusted to 6 with the direct titration as described previously.[4,5,39] All titration experiments were performed at 313 K, unless stated in legends to the figures. Other spectral parameters are presented in legends to the figures.

Fluorescence Spectroscopy. We measured fluorescence spectroscopy using Tb(III) and Eu(III) as fluorescence probes. It is expected that fluorescence-detected circular dichroism (FDCD) can be observed if the above ions are recognized by the motif via an inner-sphere coordination, since a chirality is generated around a fluorescent metal center in such a case. In the GA10−Tb(III) system, a canonical-sensitized emission was observed (data not shown), and we found that Tb(III) was able to bind to GA10. However, a sensitized emission was not observed for the GA10−Eu(III) system (data not shown). Unfortunately, in such a case, the sensitized emission of Tb(III) might be arisen from a weak dipole−dipole (through-space) interaction,[40] which means that energy transfer can occur even if a nucleobase does not coordinate to Tb(III). Therefore, we could not get critical evidence for an inner-sphere coordination of metal ions to the motif (the A9/G10.1 site) from fluorescence spectroscopy and stopped further experiments with fluorescence spectroscopy, although the sensitized emission from the GA10−Tb(III) complexes is still interesting. Solution conditions for fluorescence spectroscopy were 1 μM GA10, 50 mM PIPES pH 6.5, 50 mM NaCl, and various concentrations (0.6, 2.6, and 4.6 μM) of $TbCl_3$ and $EuCl_3$. Spectra were recorded on a JASCO spectrofluorometer FP-750.

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