Detection of RNA nucleobase metalation by NMR spectroscopy

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Nucleic acids possess several metal cation recognition sites, including phosphates, nucleobases and possibly riboses. This article focuses on the detection of nucleobase–metal interactions by NMR spectroscopy.

Introduction

Metal-related biomolecular reactions are interesting targets for chemistry, since all fields of chemistry are required for precise understanding of their mechanisms of action. In addition, this aspect is indispensable for understanding biological phenomena, since huge numbers of biological responses are performed by metal–biomolecular complexes.[1] In biological systems, the counterparts for metals are mainly proteins and RNAs, such as the self-splicing introns of rRNAs, heme-proteins, metallo-proteases, some nucleases, DNA/RNA polymerases, calmodulins.

Historically, protein–metal interactions have been characterized by several methods, such as crystallography, spectroscopy, calculations.[1] These chemical studies of metallo-proteins have provided deep chemical insights into the electronic structures of metals and ligands, and the resulting mechanisms of action of metallo-proteins. In contrast, analytical methodologies for RNA–metal interactions are only now being developed, although metal cations are indispensable for the activities of RNA molecules.

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Metal cations have several roles with regard to RNA molecules, and can act as 1) a counter cation, 2) a structural cofactor (structural metal), and 3) a catalytic center (catalytic metal) (Fig. 1).[1,2] Regarding the first case (acting as a counter cation), RNA molecules are negatively charged polyelectrolytes (each residue has one negative charge at the backbone phosphate), and thus counter cations are required not only for neutralization of these multiple charges, but also for double helix formation (neutralization of repulsive forces between complementary strands).[2,3] For the second case (acting as a structural metal), some metal cations are known to assist the formation of three-dimensional structures, such as the magnesium ions of transfer RNAs (tRNAs). These metal ions bind to the loop regions of tRNAs, and assist their folding.[1,2,4] In the third case (acting as a catalytic metal), metal ions are utilized as real catalysts, such as the divalent metal cations in group I self-splicing introns[1,5] and possibly hammerhead ribozymes.

As discussed above, metal ions are indispensable for the structure formation and resulting functionalization of RNA molecules. Nevertheless, RNA–metal interactions have not been studied as extensively as protein–metal interactions, and this may be due to the reasons outlined below.

First, metallo-proteins possess well-organized metal-binding pockets, and the metal is captured by several ligands from the

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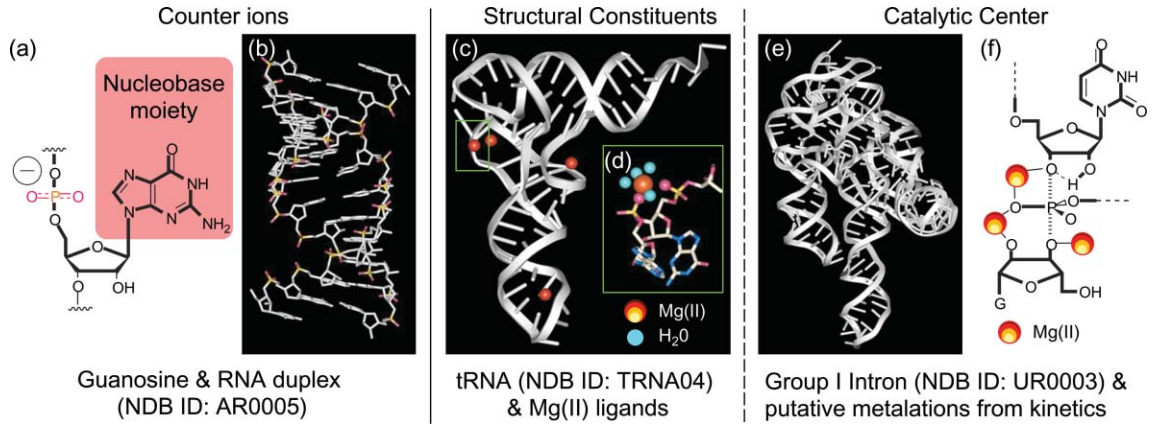

Fig. 1 Modes of RNA–metal interactions. (a) Chemical structure of a guanosine residue in an oligomer. (b) Crystal structure of an RNA duplex,[40] in which the phosphorus and phosphate oxygen atoms are colored orange and pink, respectively, as shown in Fig. 1a. (c) Crystal structure of phenylalanyl transfer RNA ($tRNA^{Phe}$).[4] (d) Magnified view of the Mg(II) binding site of $tRNA^{Phe}$, in which the ligand atoms are depicted as balls. (e) Crystal structure of the group I self-splicing intron.[41] (f) Putative metal cation binding sites in the group I intron from kinetics data.[5]

protein, most likely protein side chains (Fig. 2).[1,6] Such rigid platforms are conserved among several phases, such as the crystal and solution phases.[6,7] As a result, experimental data can be explained based on crystal structures (the environments of the metals in crystals). Regarding RNA molecules, however, there are only a few motifs in which multiple functional groups of an RNA molecule directly coordinate with a metal cation (Figs. 1c & 1d).[4] To date, it has not even been demonstrated whether or not the RNA–metal interaction modes in crystals are the same as those in solution.

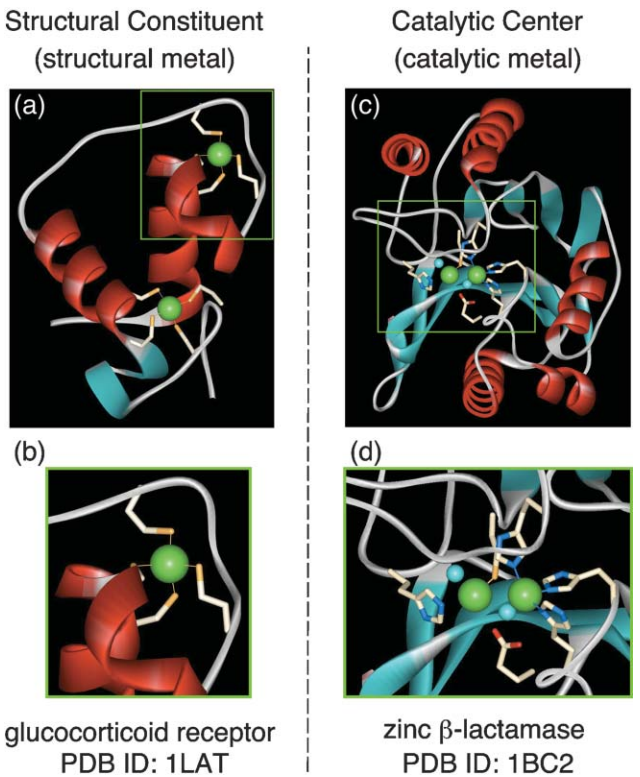

Fig. 2 Crystal structures of metallo-proteins. (a) & (b) Crystal structure of a zinc finger protein, an example of a protein with a structural metal: (a) that of the glucocorticoid receptor,[6a] (b) magnified view of the metal binding site. (c) & (d) Crystal structure of a metallo-enzyme, an example of a protein with a catalytic metal: (c) that of β-lactamase,[6b] (d) magnified view of the metal binding site, in which the metals and the ligand water molecules are depicted as green and light blue balls.

Second, the exchange rate of the metal cation in metallo-proteins is very slow, relative to the NMR timescale, due to the multiple coordinations (Fig. 2). Therefore, through NMR spectroscopy, one can observe the *J*-coupling between the metal and the coordination site in several protein–Cd(II) complexes, which indicates the formation of the inner-sphere coordination of the metal. In fact, *J*-coupling was observed for the proteins shown in Fig. 2.[7] However, no *J*-coupling has been observed between RNAs and metals, except for the covalent linkage of the Pt–guanine complex observed by Barbarella *et al.*,[8] probably due to exchange processes. To date, there is no rigid way to discriminate inner-sphere coordination (one or several ligands arising from RNA molecules) or outer-sphere binding (all the metal ligands are water molecules) for RNA–metal systems (Fig. 3). In addition, we do not know whether inner-sphere coordination of metal cations is possible for RNA molecules in solution.

As mentioned above, investigations into RNA–metal interactions are still in the early stage, relative to those of

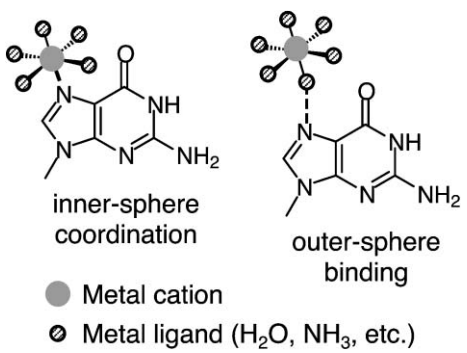

Fig. 3 Two kinds of metal cation binding modes. (a) Inner-sphere coordination. (b) Outer-sphere binding.

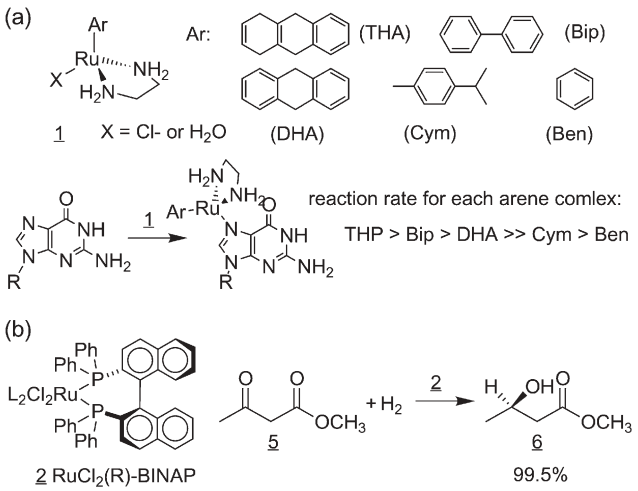

Fig. 4 Organometallic reagents. (a) Chemical structures of Ru(II) arene anticancer complexes (**1**) with arene groups, and their reactions with guanosine in water. (b) Chemical structure of $RuCl_2$(R)–BINAP (**2**) and its catalytic asymmetric reaction in the organic phase.

metallo-proteins. More critically, the nature of RNA–metal interactions is much more complicated than that of protein–metal interactions. In particular, this second issue is troublesome, and always renders any conclusions weaker. Accordingly, in order to elucidate precise pictures of RNA–metal interactions, we need to clarify the above uncertainties.

From the above descriptions, it is obviously important to devise methods for detecting metal ion binding and discriminating between inner- and outer-sphere interactions in RNA–metal systems. For chemical reactions of organometallic reagents, it is well known that metal ligands dramatically influence the reactivities, such as stereo-selectivities or reaction rates (Fig. 4).[9–11] In other words, metal–ligand identifications represent the chemical basis for mechanistic studies on metal complexes. Therefore, we herein describe our recent results for the above topics in RNA–metal systems.[12]

Hammerhead ribozyme and its metal ion binding motif

In order to study RNA–metal interactions, hammerhead ribozymes[13] were chosen for the following reasons. First, their crystal structures strongly suggest that they possess a metal ion binding motif that is rarely observed in RNA molecules.[14] From these crystal structures, the motif itself may be a bidentate ligand for a metal cation (a nucleobase nitrogen and a phosphate oxygen) (Fig. 5). Second, the three-dimensional coordinates of hammerhead ribozymes are available. Third, the hammerhead ribozyme is a biologically interesting molecule. It is a **ribo**nucleic acid en**zyme** (a catalytic RNA molecule) which promotes a site-specific cleavage of RNA strands.

Since we aimed to establish chemically definitive methods for the detection and discrimination of inner- and outer-sphere interactions in solution, we needed an RNA motif that formed inner-sphere coordinations in solution. Among previously reported metal ion binding motifs, the coordination sites for metal cations were the phosphates, even among tRNAs (Figs. 1c & 1d).[4] Such interactions may be classified as a salt bridge with very low covalency between the metal cation and the RNA. In contrast, the metal ion binding motif in hammerhead ribozymes was reported to contain a nucleobase as the recognition site (the N7 atom of the G10.1 residue) for the first time (Figs. 5d, 5e & 5f), and much higher covalency and resulting functionalization of the metal cations and the

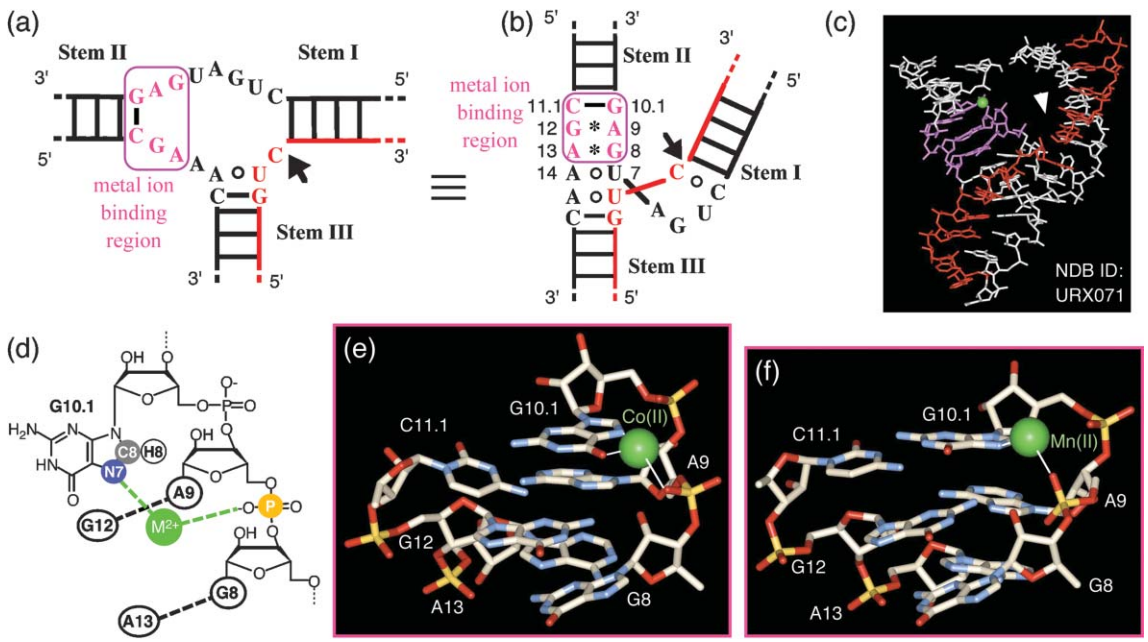

Fig. 5 Summary for hammerhead ribozymes. (a) Secondary structure of hammerhead ribozymes showing the hammerhead shape. (b) Topology of hammerhead ribozymes based on the crystal structures (γ-shape). (c) Crystal structure of a hammerhead ribozyme,[14d] in which the substrate and metal cation binding site are colored in red and magenta, respectively, as indicated in Fig. 5a & 5b. (d) Schematic representation of the binding site. (e) Magnified view of the crystal structure of the Co(II)-motif complex.[14d] (f) Magnified view of the crystal structure of the Mn(II)-motif complex.[14c]

RNAs themselves are expected, as observed for metalloproteins. Therefore, we considered that this binding motif was the most suitable one currently available for our detailed chemical analyses.

Hammerhead ribozymes are structured RNA molecules, and derive their name from their secondary structure which resembles a hammerhead (Fig. 5a). From crystal structure analyses, their three-dimensional structures were found to adopt a γ-shape (Figs. 5b & 5c).

The motif consists of sheared type G*A pairs and a normal C–G pair (Fig. 6), namely G12*A9, G8*A13 and C11.1–G10.1 pairs, which are included in the consensus sequences of hammerhead ribozymes (Fig. 5).[15] Due to the sequence conservation of the motif and repeated observations of a metal bound to the motif in crystals, catalytic roles for the metal have been suspected,[16] and much attention has been paid to the motif.[16,17] This is also because hammerhead ribozymes are supposed to be metallo-enzymes under physiological conditions,[18] although it was recently found that hammerhead ribozymes do not necessarily acts as metallo-enzymes under extreme conditions.[19] Therefore, the metal ion binding motif is an interesting target for studies on RNA–metal interactions, from both the chemical and biological aspects.

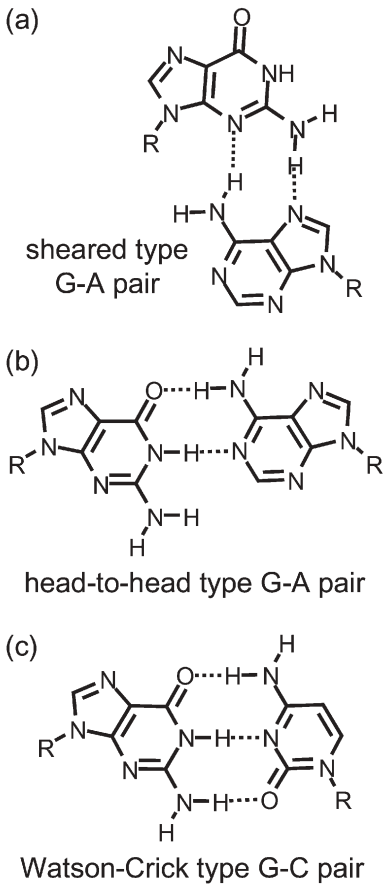

Fig. 6 Base-pairing modes of the G–A mismatch. (a) Sheared type G*A pair. (b) Head-to-head type G–A pair. (c) Watson–Crick type G–C pair.

Working hypothesis

In Fig. 5, the metal ion binding motif is located in stem II, and consists of tandem G–A mismatches and an adjacent C–G base-pair. In the sequence context of hammerhead ribozymes and the oligomer duplex GA10 (Fig. 7), the G–A mismatches form sheared type G*A pairs.[20] The secondary structure around G–A mismatches in hammerhead ribozymes is almost identical to that in GA10 (Fig. 7). Therefore, we considered that GA10 could be used as an analog of the metal ion binding motif in hammerhead ribozymes. It should be mentioned that a GA10 duplex contains two metal ion binding motifs in a symmetric manner, and thus two molar equivalents of metal cation [M(II)] to one GA10 duplex are required.

In general, a larger molecular weight causes broadening of NMR signals, and thus using GA10 is spectroscopically preferable to using a whole hammerhead ribozyme. Furthermore, in order to study the intrinsic properties of the metal ion binding motif, it is better to exclude other conserved sequences. For the above reasons, we therefore employed the small oligomer sequence GA10 for the following studies. It should also be mentioned here that if the binding motif in GA10 can act as a metal ion binding motif, then it may be regarded as an independent functional module of hammerhead ribozymes.

Historically, for nucleic acids, most of the detection of metal ion binding by NMR analysis has been performed using proton resonances,[21] which are located at distal sites from the metalated atom, although there have been several pioneering works using hetero-atom signals.[8b,22] Since proton chemical shifts reflect not only metal ion binding, but also structural changes, proton resonances are not suitable for definitive identification of metalated sites at the atomic level. In order to detect metal ion binding to nucleobases directly, it is indispensable to monitor the signal from the metalated atom. As shown in Fig. 7, the metal binding nucleus is nitrogen. Therefore, we chemically synthesized a site-specific ^{15}N-labeled GA10 series, as shown below.

GA10G1L: **G**GACGA*G*UCC
GA10G2L: G**G**ACGA*G*UCC
GA10G5L: GGAC**G**A*G*UCC
GA10G7L: GGACGA***G***UCC

The guanosines shown in bold are uniformly ^{15}N- and ^{13}C-labeled guanosines, and the italicized residues indicate the metalated sites. Therefore, GA10G7L is the target sequence for the detection of metalation, and the other sequences can be used as control sequences.

The labeled samples have several advantages. First, direct information can be drawn from the metalated nucleus. If a specific chemical shift perturbation of a certain ^{15}N nucleus is observed, the metal binding atom can be specified (Fig. 7e). In addition, the electronic states of metal–nucleobase complexes can be estimated in combination with molecular orbital calculations. Second, the inner-sphere coordination between the metal [M(II)] and nitrogen nuclei can be detected using M(II)–^{15}N *J*-coupling ($^{1}J_{M(II)–N}$), in a favorable case, since there is covalency in a coordination bond. In other words, if $^{1}J_{M(II)–N}$ is observed between a metal and a nucleobase, the

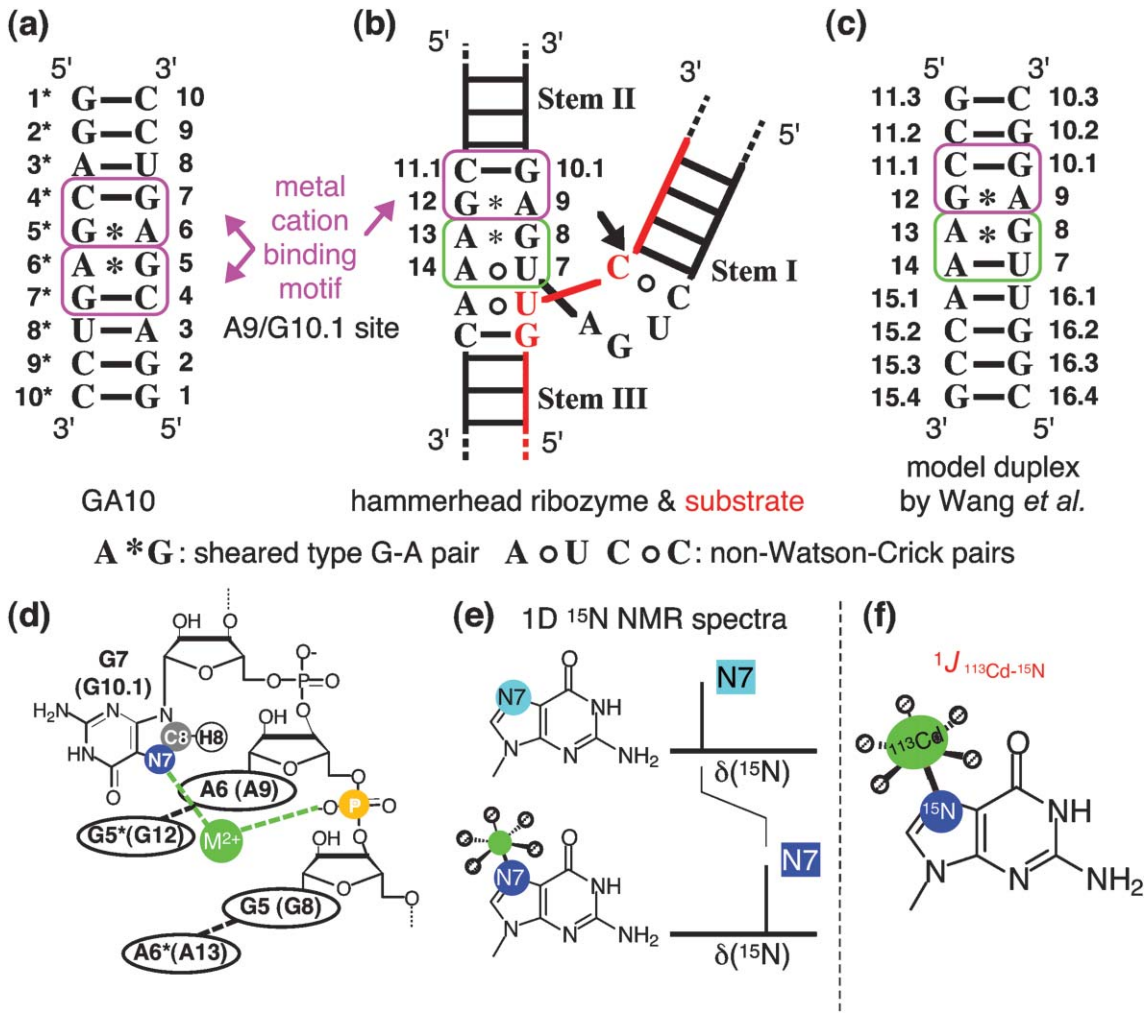

Fig. 7 Model duplexes and working hypotheses. (a) Duplex modelled on a metal cation binding motif, GA10. (b) Secondary structure of hammerhead ribozymes. (c) Duplex modelled on a metal cation binding motif in Wang *et al.*[25] (d) Schematic representation of the binding motif. (e) Expected phenomenon for ^{15}N chemical shifts upon metal cation binding. (f) Expected phenomenon for *J*-coupling upon metal cation binding.

metal–nitrogen bond can be concluded to be a coordination bond (Fig. 7f).

Therefore, titration experiments of labeled RNA oligomers were initially performed with Cd(II), whose spin quantum number is 1/2 (^{113}Cd). Cd(II) was chosen because metals with spin quantum numbers other than 1/2 or 0 cause broadening (and resultant disappearance) of the resonances of the metalated sites. In addition, several metal cations of soft Lewis acids bind to the metal ion binding motif. It should also be mentioned that hammerhead ribozymes are more active in the presence of both soft [Cd(II) and Mn(II)] and hard [Mg(II) and Ca(II)] Lewis acids than in the presence of a hard Lewis acid alone.[17e,g] The use of Cd(II) is reasonable and suitable for studies on RNA–metal interactions.

^{15}N NMR titration experiments

We recorded one-dimensional (1D) ^{15}N NMR spectra of all the labeled oligomers, namely GA10G1L, GA10G2L, GA10G5L and GA10G7L, in the presence of various concentrations of Cd(II). Fig. 8 only shows the titration spectra for GA10G7L (the oligomer with an isotope-labeled guanosine at the seventh residue). Since a guanosine possesses five kinds of nitrogens (N1, N2, N3, N7 and N9), five ^{15}N resonances of the G7 residue were seen in the spectra (Fig. 8). Next, the N7 chemical shifts of four guanosine residues, namely G1, G2, G5 and G7, were plotted against the molar equivalency of the Cd(II) ion (Fig. 8c). An extraordinarily large perturbation was observed for the N7 resonance of G7, N7(G7), among all the nitrogen atoms (19.6 ppm higher-field shift at the molar ratio of [CdCl2]/[GA10(duplex)] = 6.0) (Fig. 8 and Table 1). On the other hand, the perturbations of the N7 resonances for the other guanosines (G1, G2 and G5 residues) were not shifted significantly (Fig. 8). In addition, much smaller perturbations were observed for the other kinds of nitrogens, relative to the N7 resonances.[12b] Accordingly, Cd(II) ion binding is N7(G7)-specific.

At this point, two conclusions were derived. One is that metal ion binding to RNA in solution is detectable and the binding atom can be clearly specified using ^{15}N NMR spectroscopy. The other is that the metal ion binding motif is an independent functional module from hammerhead

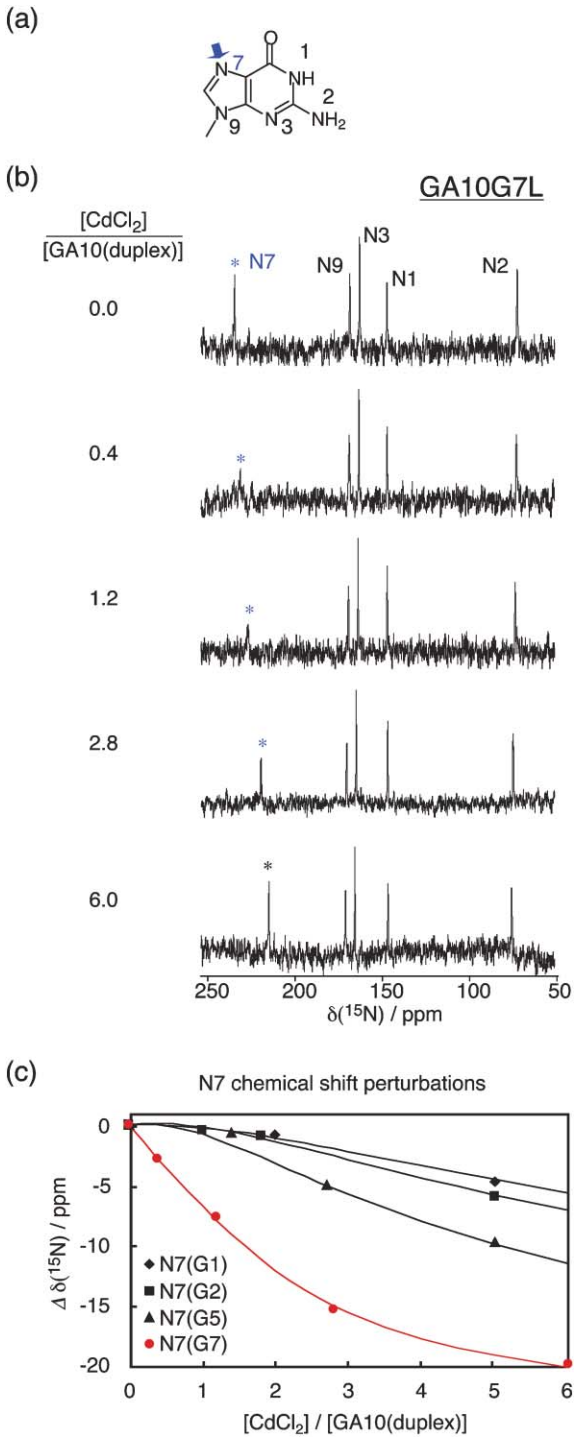

Fig. 8 1D ^{15}N NMR spectra and a plot of the N7 chemical shifts. (a) Chemical structure of guanosine, in which the metalated atom is indicated by a blue arrow. (b) 1D ^{15}N NMR spectra of GA10G7L. Five ^{15}N resonances of the G7 residue seen in each spectrum are labeled with their numbers. (c) Plot of the N7 chemical shifts of four guanosine residues (G1, G2, G5 and G7). The chemical shifts for the metalated site are shown in red. In the experiments, we used the guanosine nucleoside with all carbon and nitrogen atoms labeled. It took 12 to 24 hours to record the above spectra (simple 1D ^{15}N spectra) for our samples.

ribozymes, since the motif sequences themselves were found to be the minimum requirement for metal ion binding.

Next, we further investigated the 1-bond *J*-coupling between ^{113}Cd(II) and ^{15}N ($^{1}J_{Cd(II)–N}$) in 1D ^{15}N NMR spectra. However, $^{1}J_{Cd(II)–N}$ was not detected under the conditions used for the measurements. Similar phenomena were observed for guanosine nucleoside–metal systems in Buchanan *et al.*,[22a,b] and these results are summarized in Table 1. These authors considered that the exchange processes between the metal-bound and dissociated states obliterated the *J*-coupling between the metal and N7. At least in our case, a unique N7(G7) resonance was observed under metal unsaturated conditions, indicating that such exchange processes do indeed exist. Therefore, we also consider that the chemical exchange obliterates the *J*-coupling in our case. At that time, we were not able to conclude what kinds of chemical bonds are formed between Cd(II) and N7(G7) definitively, although the inner-sphere coordination is the most plausible.

DeRose and co-workers indicated that identification of metal ligands and 3D structure calculations for metal–ligand complexes can be performed by EPR spectroscopy.[23,24] From their structure calculations of the binding motif of hammerhead ribozymes, the metal cation, Mn(II) in their case, directly coordinated to N7 of the guanosine residue in the motif (G10.1). Both Mn(II) and Cd(II) belong to the relatively soft Lewis acids, and have considerable affinities for nitrogen. Based on the results from EPR data, the N7 resonance perturbation of approximately 20 ppm (Fig. 8) would be an indication of the inner-sphere coordination of Cd(II) to N7(G7).

Wang *et al.* recently published ^{15}N NMR data for the same binding motif (Fig. 7c).[25] They performed titration experiments of the metal cation binding motif of hammerhead ribozymes with several metal cations.[25] Their titration data with Cd(II) are in good agreement with our data (Table 1). Interestingly, they also presented ^{15}N chemical shift perturbations for Mg(II), Zn(II) and $[Co(III)(NH_3)_6]^{3+}$ titrations (Table 1). These ^{15}N chemical shift data and that of GA10 provide a chemical basis for the detection of metal cation binding to nucleobases.

Theoretical calculations for metal–guanosine systems were also reported at the beginning of 2004 by Sychrovskey *et al.*[26] Through their molecular orbital calculations, N7 metalation causes a higher-field shift of N7, irrespective of the metal species (Table 1).[26] Regarding Zn(II) (a relatively soft Lewis acid), the direction of the N7 perturbation and the order of magnitude for the theoretical value (data in Sychrovskey *et al.*[26]) are consistent with the experimental values (data in Wang *et al.*[25]) (Table 1). Although there is still a significant difference in the chemical shift perturbations between the theoretical and experimental data for Mg(II) (a hard Lewis acid) binding,[25,26] this difference may arise from a difference in the metal cation binding mode. From the theoretical aspect, it is necessary to elucidate what kind of interactions can explain the experimental value for Mg(II) binding.

Considering the Zn(II) data again, the consistency between the experimental and theoretical values indicates that the approximately 20 ppm higher-field shift of N7(G10.1) in Wang's duplex for Zn(II) titrations should be due to the

Table 1 Summary of the ^{15}N NMR data

Metal ligand	Metal cation	Residue	^{15}N[a]/ppm	$^{1}J_{N-M(II)}$[b]/Hz	Ref.
r(GGA*CGA***G**UCC)$_2$ [GA10][c]	Cd(II)	G7	−19.6 (3.0 eq)	not detected	12*b*
r(CGGUU*GA***G**GC)•r(GC*CGA*AACCG)[c]	Cd(II)	G10.1	∼−20 (6.0 eq)	N.D.	25
r(CGGUU*GA***G**GC)•r(GC*CGA*AACCG)[c]	Zn(II)	G10.1	∼−20 (4.0 eq)	n.a.	25
r(CGGUU*GA***G**GC)•r(GC*CGA*AACCG)[c]	Mg(II)	G10.1	−6.5 (10.0 eq)	n.a.	25
r(CGGUU*GA***G**GC)•r(GC*CGA*AACCG)[c]	Co(NH$_3$)$_6$(III)	G10.1	<−1 (6.0 eq)	n.a.	25
Guanosine[d]	Zn(II)	n.a.	−20.1 (1.0 eq)	n.a.	22*a*
Guanosine[d]	Hg(II)	n.a.	−20.5 (1.0 eq)	N.D.	22*a*
Inosine[d]	Zn(II)	n.a.	−15.2 (0.7 eq)	n.a.	22*b*
Inosine[d]	Hg(II)	n.a.	−4.8 (0.75 eq)	not detected	22*b*
β-lactamase[e]	Cd(II)	His	N.D.	78∼216	7*b*
Guanosine (calculation)	Zn(II)	n.a.	−14.8	−36.5[f]	26
Guanosine (calculation)	Mg(II)	n.a.	−15.3	5.6[f]	26

[a] Chemical shift perturbations of N7(guanosine) upon the metalation. Negative and positive values indicate higher- and lower-field shifts, respectively. The numbers in parentheses beside the perturbation values indicate the molar ratios of [CdCl2]/[ligand]. [b] *J*-coupling between metalated nitrogens (^{15}N) and metal ions (I = 1/2), such as ^{113}Cd and ^{199}Hg. [c] For these RNA oligomers, the metal cation binding motifs are written in italic, and the metalated guanosines are shown in boldface. [d] Titration experiments were performed in dimethyl sulfoxide (DMSO). [e] Four of seven histidines were metalated, but the residue numbers in the amino acid sequence were not reported. In the β-lactamase–Cd(II) system, the ranges of the ^{15}N chemical shifts of metalated and non-metalated residues overlapped, and the ^{15}N chemical shift perturbations due to metalation of histidines were estimated to be small. [f] The quadrupole moments of ^{67}Zn and ^{25}Mg are so large that experimental *J*-coupling values are not available currently. [g] n.a.: not applicable. N.D.: not determined.

inner-sphere coordination of Zn(II) to N7(G10.1). In addition, the degree of ^{15}N perturbations and the binding isotherms for Zn(II) and Cd(II) titrations were almost identical.[25] and both Zn(II) and Cd(II) are relatively soft Lewis acids. Taking the data together, the extraordinary higher-field shift of ^{15}N resonance for N7(G7) in GA10 should also be due to the inner-sphere coordination of Cd(II) to N7(G7).

^{1}H and ^{13}C chemical shift perturbations

We also monitored ^{1}H and ^{13}C chemical shift perturbations upon Cd(II) addition. Due to the recent development of hardware such as cryo-probes, ^{13}C resonances (1-bond ^{1}H–^{13}C correlation peaks) have become detectable in samples without isotope enrichments. Therefore, the ^{13}C chemical shift of the carbon atom adjacent to the metalated site could be a conventional probe for metal ion binding, if the ^{13}C resonance shows different behavior from those of other control sites.

Since the metalated site is N7 of the guanosine in this case, we focused on the chemical shifts of C8 in guanosine, which is adjacent to N7. In titration experiments, an unusual lower-field shift was observed for the C8 resonance of the metalated residue, G7 (+2.3 ppm as a limiting shift) (Table 2). C8(G7) was perturbed significantly more than the other non-specific sites of G1, G2 and G5,[12*a*,*d*] thus demonstrating that the chemical shifts of C8 in guanosines can be used as a probe for metal ion binding.

Next, the H8 chemical shifts of guanosines and H2 chemical shift of A6 were plotted against molar ratios of [Cd(II)]/[GA10(duplex)] (Fig. 9). As was the case for the N7 and C8 resonances, the H8 resonance of G7, H8(G7), was perturbed (0.38 ppm lower-field shift as a limiting shift) (Fig. 9 and Table 2). As mentioned earlier, proton chemical shifts are not very good probes for metal ion binding, and it is therefore better to monitor both H8 and C8 resonances simultaneously for specification of the metalated site.

However, proton chemical shifts provide precise information on the solution equilibrium, such as the number of bound metal ions, from the shapes of the binding isotherms.[12*b*,*d*,21] In fact, from the biphasic transition for the H2 resonance of the A6 residue, H2(A6), the number of bound cations was determined to be two cations per GA10 duplex (two binding motifs) for Mg(II), Cd(II) and $[Co(III)(NH_3)_6]^{3+}$.[12*b*,*d*] Unfortunately, due to the polyelectrolyte nature of nucleic acids,[3] the dissociation constants derived from the binding

Table 2 Summary of the ^{13}C and ^{1}H NMR data[a]

			C8/ppm		H8/ppm		
Metal ligand	Metal salt	Residue	1:1(M(II) :motif)	Limiting shifts	1:1(M(II) :motif)	Limiting shifts	Ref.
r(GGACGA***G**UCC)$_2$ [GA10]	$MgCl_2$	G7	+0.5 (2.0eq)	N.D.	+0.08 (2.0eq)	+0.12 (9.0eq)	12*a*,*d*
r(GGACGA***G**UCC)$_2$ [GA10]	$CdCl_2$	G7	+1.6 (2.0eq)	+2.3 (5.0eq)	+0.19 (2.0eq)	+0.38 (9.0eq)	12*b*,*d*
r(GGACGA***G**UCC)$_2$ [GA10]	$Co(NH_3)_6Cl_3$	G7	+0.1 (2.0eq)	N.D.	+0.22 (2.0eq)	+0.25 (3.0eq)	12*d*
r(GGACGA***G**UCC)$_2$ [GA10]	$NaClO_4$	G7	n.a.	+0.1 (230 mM)[b]	n.a.	+0.12 (800 mM)[b]	12*a*,*d*
d(ATG***G**GTACCCAT)$_2$ [12mer]	$ZnCl_2$	G4	+1.0 (1.0eq)	+2.5 (8.0eq)	N.D.	+0.20 (8.0eq)	35
d(AT***G**GGTACCCAT)$_2$ [12mer]	$ZnCl_2$	G3	+0.5 (1.0eq)	+1.5 (8.0eq)	N.D.	+0.05 (8.0eq)	35
d(TG***G**T)	$Pt(en)Cl_2$[c]	G3	+1.1 (1.0eq)	+1.1 (1.0eq)	+1.04 (1.0eq)	+1.04 (1.0eq)	36
d(T***G**GT)	$Pt(en)Cl_2$[c]	G2	+0.2 (1.0eq)	+0.2 (1.0eq)	+0.26 (1.0eq)	+0.26 (1.0eq)	36

[a] N.D.: not determined. n.a.: not applicable. Chemical shift perturbations are listed in ppm with molar equivalencies to duplexes (GA10 and 12mer) or a single strand of d(TGGT). Positive values indicate a lower-field shift. [b] Chemical shift perturbations for the basal solution (50 mM $NaClO_4$) are listed. [c] $Pt(en)Cl_2$ (en = ethylenediamine) forms the covalent complex d(TGGT)•Pt(en) with the two successive guanosines of d(TGGT).[36–39]

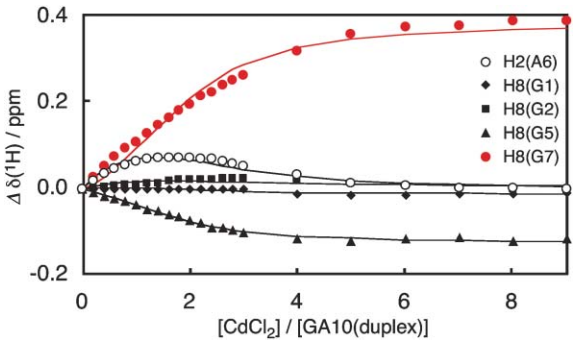

Fig. 9 Plot of the H8 chemical shifts of four guanosine residues (G1, G2, G5 and G7) and the H2 chemical shifts of A6, H2(A6). The H8 chemical shifts for the metalated residue are shown in red.

isotherms merely indicate the apparent values ($K\mathrm{d}_{\mathrm{app}}$). Establishing how to determine the intrinsic *K*d values for RNA–metal systems is one of the next targets, since it requires statistical mechanical treatments of the distributions of cations around polyelectrolytes. It is also interesting to study hydrogen-binding between a carbonyl group and metal ligands, using ^{13}C chemical shifts of carbonyl carbons, since such hydrogen-binding was suggested for a guanosine–cisplatin complex.[22g,h]

Interactions with other metal cations

We performed further titration experiments of GA10 with several metal cations of different natures, such as Mg(II), $[Co(III)(NH_3)_6]^{3+}$ and sodium ions. Mg(II) is one of the abundant divalent cations in living organisms, and was also used as a canonical hard Lewis acid. Furthermore, we used $[Co(III)(NH_3)_6]^{3+}$ as a control metal ion that can only form outer-sphere binding, since it is ligated to kinetically exchange-inert amines, and nucleobases cannot directly coordinate with the Co(III) center of this complex in general.[27] Finally, sodium ions were used to monitor the degree of ionic strength-dependent chemical shift changes.

The results of the titration experiments are summarized in Table 2. In the sodium titrations, the H8 and C8 resonances of the G7 residue, H8(G7) and C8(G7), respectively, were not significantly perturbed, indicating that the metal–nucleobase interactions are not just simple electrostatic interaction. In contrast, simultaneous lower-field shifts of the H8 and C8 resonances were observed for the Mg(II) titrations, as was the case for the Cd(II) titrations. However, the derived perturbations were not limiting shifts, and their limiting shifts are therefore larger than the experimental values. Hence, the limiting shifts for Mg(II) titrations need to be clarified experimentally. In the case of the $[Co(III)(NH_3)_6]^{3+}$ titrations, H8(G7) was considerably perturbed, suggesting that $[Co(III)(NH_3)_6]^{3+}$ was hydrogen-bonded to the binding motif of GA10 through amines (outer-sphere binding). Binding of $[Co(III)(NH_3)_6]^{3+}$ was also confirmed by the inter-molecular NOEs between GA10 and $[Co(III)(NH_3)_6]^{3+}$ and the binding isotherms from ^{1}H chemical shifts.[12d] On the other hand, C8(G7) was not perturbed, which may indicate that the outer-sphere binding (ligand-mediated hydrogen bonding) did not alter the chemical shift of C8(G7). Strictly speaking, there are currently several ambiguities in the interpretation of the data for the $[Co(III)(NH_3)_6]^{3+}$ titrations, including which atom was the preferential binding site and whether the ligand exchanges occurred as reported by Ennifar *et al.*[28] However, the data are quite interesting, since C8 chemical shifts may provide a way to discriminate between inner- and outer-sphere interactions.

Comparisons with crystal structures

There are several crystal structures of hammerhead ribozymes with metal cations at the binding motif. Regarding the G10.1–metal interaction, both inner- and outer-sphere interactions were observed. In the case of transition metals, which are softer Lewis acids than alkali earth metals, inner-sphere coordination was observed for Mn(II) and Co(II).[14,29] On the other hand, outer-sphere interaction with the guanosine was suggested for the canonical hard Lewis acid Mg(II), which is an alkali earth metal,[14b] although this does not mean that inner-sphere coordination of Mg(II) is completely excluded.

In our NMR data, the chemical shift of C8(G7) in GA10 was significantly perturbed upon addition of the d10-metal Cd(II). This observation is consistent with the crystal structures, since transition metals tend to form an inner-sphere coordination with G10.1 in crystals. On the other hand, a moderate perturbation was observed for the same site upon addition of the alkali earth metal Mg(II). There are three possible explanations for these data. The first is that the moderate shift is due to outer-sphere binding of Mg(II). The second is the data arise from chemical shift averaging of Mg(II)-coordinated and dissociated states. The third is that the inner-sphere coordination of Mg(II) may not perturb C8(G7) in GA10 very much, due to the low covalency between the hard Lewis acid Mg(II) and the relatively soft Lewis base N7(G7). Even when the Mg(II) titration data in the ^{15}N NMR data of Wang *et al.*[25] are taken into consideration, it is difficult to discriminate among these three possibilities.

Sequence requirements for metal ion binding

First, we would like to present the working hypothesis for our studies on the sequence requirements. The G7 residue in GA10, which corresponds to G10.1 in hammerhead ribozymes, was found to be metalated at N7 (Fig. 7), and the other purine base of adenosine also possesses N7 (Fig. 10). Hence, we considered that an adenosine residue could be replaced with a metalated guanosine residue. We therefore synthesized the RNA oligomer UGAA10: rGGAUGAAUCC, which represents a GA10 analog with a Watson–Crick U4–A7 pair instead of the C4–G7 pair (Fig. 10). We also tested the metal ion binding ability of another GA10 analog, GGAC10: rGGAGGACUCC, with a Watson–Crick G4–C7 pair instead of the C4–G7 pair (Fig. 10).

In the sequence context of UGAA10, tandem G–A mismatches are known to form sheared type G*A pairs, and the metalated site of N7 is conserved. On the other hand, in the sequence context of GGAC10, G–A mismatches are known to form head-to-head (Watson–Crick type-like) type G–A pairs, and the metalated N7 atom is absent due to the replacement of guanosine with cytosine. As a consequence, Mg(II), the most

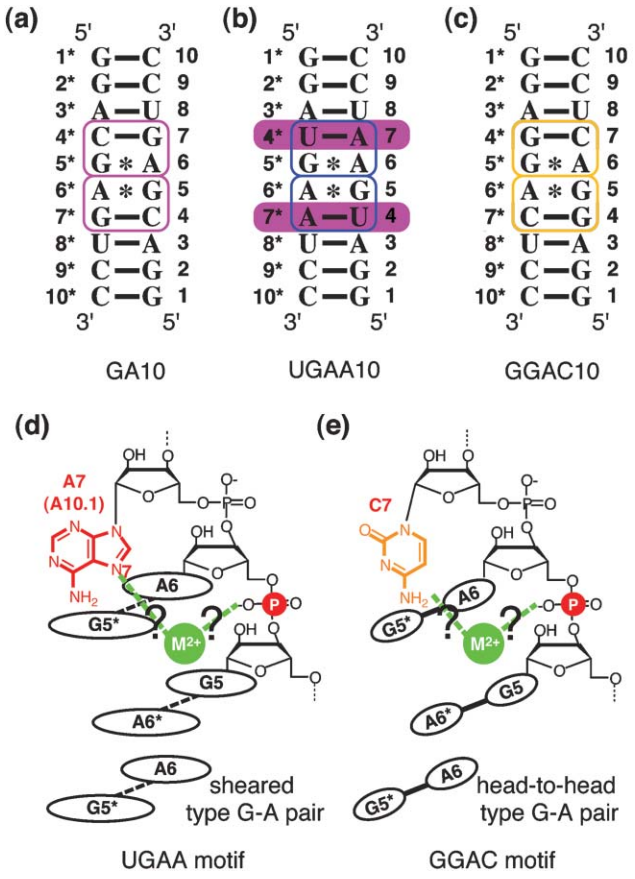

Fig. 10 Original metal cation binding motif and mutated motifs. (a) RNA duplex with the original binding motif. (b) RNA duplex with a mutated binding motif containing A7–U4 pairs in place of G7–C4 pairs, designated UGAA10. (c) RNA duplex with a mutated binding motif containing C7–G4 pairs in place of G7–C4 pairs, designated GGAC10. (d) Schematic representation of the mutated motif of UGAA10. (e) Schematic representation of the mutated motif of GGAC10.

probable cofactor in living organisms, may bind to the motif of UGAA10 (UGAA motif), but should not bind to that of GGAC10 (GGAC motif) (Fig. 10).

As expected, the GGAC motif did not capture Mg(II) around G–A mismatches.[12a] Either the formation of the sheared type G*A pairs or the guanosine residue in the binding motif therefore appears to be important for the metal ion recognition, although additional studies are required to reach a definitive conclusion. On the other hand, the UGAA motif, which was expected to capture Mg(II), did not capture it, since the chemical shift perturbations of all the base protons and anomeric protons were very small after the addition of Mg(II).[12d] It was found that the adenosine residue in the UGAA motif was not suitable for the metal ligand.

It was reported that N7 of adenosine is a much less efficient acceptor of metal cations than that of guanosine at the nucleoside level, by means of potentiometric titrations.[30] It was also reported that the basicities of nitrogen atoms are well correlated with their affinities for divalent cations.[30] Therefore, we consider that these effects might be the possible reasons for the reduced affinity of the UGAA motif for Mg(II).

Baeyens *et al.* reported that a very similar sequence motif was able to act as a metal cation binding motif in the crystal structure of a dodecamer (Fig. 11).[29] The arrangement of a sheared type G*A pair (G5–A8*) and a Watson–Crick type C–G pair (C4–G9*) was the same as the binding motif of hammerhead ribozymes. However, in the case of the dodecamer, the opposite site of the G*A pair to the C–G pair was an A–A mismatch (A6–A7*), instead of the G*A pair in hammerhead ribozymes and GA10 (Figs. 7 & 11). Together with our data, the prerequisites for metal cation binding are a sheared type G*A pair and a guanosine residue at the 3′ side of the adenosine of the G*A pair.

The importance of G–A mismatches in hammerhead ribozymes was pointed out by Uesugi and Katahira,[20b,c] and the base pairing patterns of G–A mismatches in various sequence contexts have been extensively studied by Turner's group (Pardi and Heus, as well).[31] Although the sequence requirements for a sheared type G*A pair have not been completely resolved, one of the possible roles of the G*A pair in hammerhead ribozymes is to capture a metal cation with the resulting formation of a catalytically suitable structure. Frequent observations of G–A mismatches in ribosomal RNAs may also indicate the use of G*A pairs as a metal cation binding motif.[32] Recently, Khvorova *et al.* reported that ribozymes with extra loop–loop interaction sites, so-called

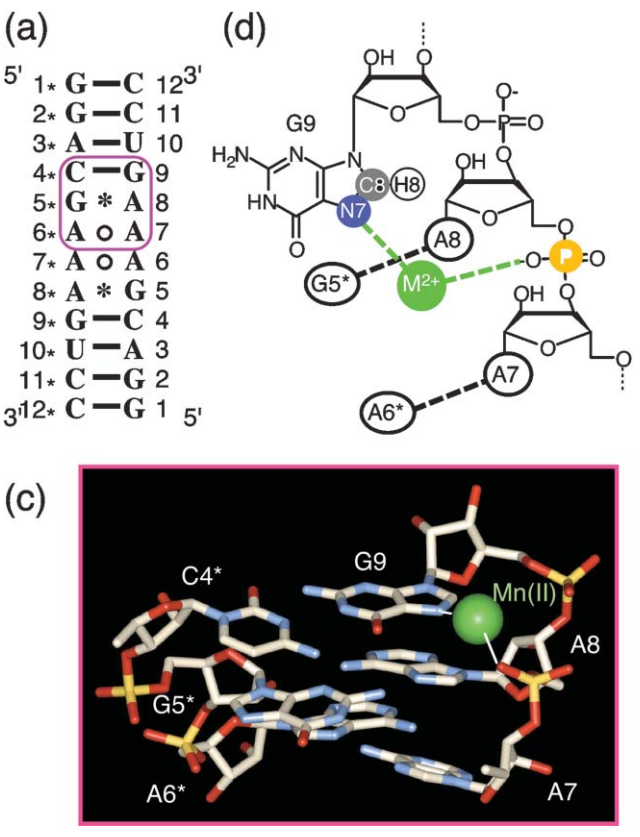

Fig. 11 Crystal structure of the RNA dodecamer in Baeyens *et al.*[29] (a) Sequence and base-pairing of the dodecamer. (b) Schematic representation of the metal cation binding to the motif. (c) Magnified view of the crystal structure of the binding motif and Mn(II).

extended ribozymes, showed much higher catalytic activities than ribozymes with minimum consensus sequences (minimum hammerhead).[33] Therefore, the precise roles of the G–A mismatches may be revealed by analyses of extended ribozymes.

Roles of the metal cation at the motif

Next, we discuss the roles of the metal cation at the binding motif, in regard to whether the metal acts as a catalytic center or just a structural constituent. Kinetic data have indicated that hammerhead ribozymes with the A10.1–U11.1 base pair (the same motif as UGAA10) still possess 30% catalytic activity relative to those with the common sequence that includes the G10.1–C11.1 base pair.[15] Furthermore, in the sequence of the wild-type hammerhead ribozyme from the newt, A10.1–U11.1 is included instead of G10.1–C11.1.[34]

These data indicate that G10.1–C11.1 can be replaced by A10.1–U11.1 without loss of functions, although this base pair substitution significantly reduces the metal cation binding ability of the motif.[12d] Therefore, if we assume that the metal ion at the binding motif is a catalytic center, the reduced (almost lack of) metal cation binding ability of UGAA10 is inconsistent with this assumption. Accordingly, it is strongly suggested that the metal ion at the motif is not a catalytic center, although it is possible that other conserved sequences may support metal cation binding to the mutated motif with the A10.1–U11.1 pair.

As mentioned above, extended ribozymes showed higher activities than minimum hammerheads. In combination with our experimental data on the sequence requirements, the possible role of this metal cofactor might be to facilitate stem–stem interactions through charge screening between two helices, in place of the loop–loop interactions of extended ribozymes.

Concluding remarks

In summary, when Cd(II) directly coordinates with N7 of a guanosine, chemical shift perturbations at related sites occur as follows: N7: a higher-field shift; C8: a lower-field shift; and H8: a lower-field shift. In order to elucidate the states of metal–nucleobase complexes, monitoring of ^{15}N resonances or simultaneous monitoring of ^{13}C and ^{1}H resonances is required. Here, we have established guidelines for specifying the metalated site in nucleobases and discriminating between inner- and outer-sphere interactions with nucleobases. We have also emphasized that either chemical shift perturbations of hetero atoms or metal–ligand *J*-coupling is applicable to many types of metal complexes, including organometallic reagents.

We have also provided some evidence for the roles of the metal cation at the binding motif of hammerhead ribozymes. Intrinsically, the mutated motif with an A–U pair in place of the G–C pair was no longer able to act as a metal cation binding motif. These experimental data strongly suggest that the metal cation at the binding motif is a structural constituent.

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References

1 S. J. Lippard and J. M. Berg, in *Principles of Bioinorganic Chemistry*, University Science Books, California, 1996.
2 W. Saenger, in *Principle of Nucleic Acid Structures*, Springer-Verlag, New York, 1984.
3 (*a*) J. Marmur and P. Doty, *J. Mol. Biol.*, 1962, **5**, 109–118; (*b*) W. F. Dove and N. Davidson, *J. Mol. Biol.*, 1962, **5**, 467–478; (*c*) M. T. Record, Jr., *Biopolymers*, 1967, **5**, 975–992; (*d*) V. A. Bloomfield, D. M. Crothers and I. Tinoco, Jr., *Physical Chemistry of Nucleic Acids*, Harper & Row, Publisher, New York, 1974; (*e*) C. F. Anderson and M. T. Record, Jr., *Ann. Rev. Biophys. Biophys. Chem.*, 1990, **19**, 423–465; (*f*) Mg(II): V. K. Misra and D. E. Draper, *J. Mol. Biol.*, 1999, **294**, 1135–1147.
4 J. L. Sussman, S. R. Holbrook, R. W. Warrant, G. M. Church and S.-H. Kim, *J. Mol. Biol.*, 1978, **123**, 607–630.
5 S.-O. Shan, A. V. Kravchuk, J. A. Piccirilli and D. Herschlag, *Biochemistry*, 2001, **40**, 5161.
6 (*a*) D. T. Gewairth and P. B. Sigler, *Nat. Struct. Biol.*, 1995, **2**, 386; (*b*) S. M. Fabiane, M. K. Sohi, T. Wan, D. J. Dayne, J. H. Bateson, T. Mitchell and B. J. Sutton, *Biochemistry*, 1998, **37**, 12404.
7 (*a*) E. Kellenbach, B. A. Maler, K. R. Yamamoto, R. Boelens and R. Kaptein, *FEBS Lett.*, 1991, **291**, 367–370; (*b*) C. Damblon, C. Prosperi, L.-Y. Lian, I. Barsukov, R. P. Soto, M. Galleni, J.-M. Frere and G. C. K. Roberts, *J. Am. Chem. Soc.*, 1999, **121**, 11575–11576.
8 (*a*) G. Barbarella, A. Bertoluzza, M. A. Morelli, M. R. Tosi and V. Tuglioni, *Gazz. Chim. Ital.*, 1988, **118**, 637; (*b*) E. Sletten and N. Å. Frøystein, in *Metal Ions in Biological Systems*, ed. A. Sigel and H. Sigel, Marcel Dekker, Basel, 1996, vol. 32, p. 397.
9 (*a*) R. Noyori, in *Asymmetric Catalysis in Organic Synthesis*, John Wiley and Sons, New York, 1984, pp. 16–94; (*b*) M. Uchiyama, M. Kameda, O. Mishima, N. Yokoyama, M. Koike, Y. Kondo and T. Sakamoto, *J. Am. Chem. Soc.*, 1998, **120**, 4934–4946; (*c*) Y. Kondo, M. Shilai, M. Uchiyama and T. Sakamoto, *J. Am. Chem. Soc.*, 1999, **121**, 3539–3540.
10 (*a*) H. Chen, J. A. Parkinson, R. E. Morris and P. J. Sadler, *J. Am. Chem. Soc.*, 2003, **125**, 173–186; (*b*) H. Chen, J. A. Parkinson, O. Novakova, J. Bella, F. Y. Wang, A. Dawson, R. Gould, S. Parsons, V. Brabec and P. J. Sadler, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 14623–14628; (*c*) H. Chen, J. A. Parkinson, S. Parsons, R. A. Coxall, R. O. Gould and P. J. Sadler, *J. Am. Chem. Soc.*, 2002, **124**, 3064–3082.
11 (*a*) J. L. Rutherford and D. B. Collum, *J. Am. Chem. Soc.*, 2001, **123**, 199–202.
12 (*a*) Y. Tanaka, E. H. Morita, H. Hayashi, Y. Kasai, T. Tanaka and K. Taira, *J. Am. Chem. Soc.*, 2000, **122**, 11303–11310; (*b*) Y. Tanaka, C. Kojima, E. H. Morita, Y. Kasai, K. Yamasaki, A. Ono, M. Kainosho and K. Taira, *J. Am. Chem. Soc.*, 2002, **124**, 4595–4601; (*c*) K. Suzumura, K. Yoshinari, Y. Tanaka, Y. Takagi, Y. Kasai, M. Warashina, T. Kuwabara, M. Orita and K. Taira, *J. Am. Chem. Soc.*, 2002, **124**, 8230–8236; (*d*) Y. Tanaka, Y. Kasai, S. Mochizuki, A. Wakisaka, E. H. Morita, C. Kojima, A. Toyozawa, Y. Kondo, M. Taki, Y. Takagi, A. Inoue,

K. Yamasaki and K. Taira, *J. Am. Chem. Soc.*, 2004, **126**, 744–752; (*e*) K. Suzumura, Y. Takagi, M. Orita and K. Taira, *J. Am. Chem. Soc.*, 2004, **126**, 15504–15511.
13 (*a*) C. J. Hutchins, P. D. Rathjen, A. C. Forster and R. H. Symons, *Nucleic Acids Res.*, 1986, **14**, 3627–40; (*b*) J. M. Buzayan, A. Hampel and G. Bruening, *Nucleic Acids Res.*, 1986, **14**, 9729–43; (*c*) O. C. Uhlenbeck, *Nature*, 1987, **328**, 596–600.
14 (*a*) H. W. Pley, K. M. Flaherty and D. B. McKay, *Nature*, 1994, **372**, 68–74; (*b*) W. G. Scott, J. T. Finch and A. Klug, *Cell*, 1995, **81**, 991–1002; (*c*) W. G. Scott, J. B. Murray, J. R. Arnold, B. L. Stoddard and A. Klug, *Science*, 1996, **274**, 2065–2069; (*d*) J. B. Murray, D. P. Terwey, L. Maloney, A. Karpeisky, N. Usman, L. Beigelman and W. G. Scott, *Cell*, 1998, **92**, 665–673.
15 D. E. Ruffner, G. D. Stormo and O. C. Uhlenbeck, *Biochemistry*, 1990, **29**, 10695–10702.
16 (*a*) A. Peracchi, L. Beigelman, N. Usman and D. Herschlag, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 11522–11527; (*b*) A. Peracchi, L. Beigelman, E. C. Scott, O. C. Uhlenbeck and D. Herschlag, *J. Biol. Chem.*, 1997, **272**, 26822–26826; (*c*) E. C. Scott and O. C. Uhlenbeck, *Nucleic Acids Res.*, 1999, **27**, 479–484; (*d*) S. Wang, K. Karbstein, A. Peracchi, L. Beigelman and D. Herschlag, *Biochemistry*, 1999, **38**, 14363–14378.
17 (*a*) D. E. Ruffner and O. C. Uhlenbeck, *Nucleic Acids Res.*, 1990, **18**, 6025–6029; (*b*) T. Tuschl and F. Eckstein, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 6991–6994; (*c*) D. M. J. Lilley, *Current Opinion Struct. Biol.*, 1999, **9**, 330–338; (*d*) J. B. Murray and W. G. Scott, *J. Mol. Biol.*, 2000, **296**, 33–41; (*e*) K. Yoshinari and K. Taira, *Nucleic Acids Res.*, 2000, **28**, 1730–1742; (*f*) M. Warashina, Y. Takagi, W. J. Stec and K. Taira, *Curr. Opin. Biotechnol.*, 2000, **11**, 354–362; (*g*) Y. Nakamatsu, T. Kuwabara, M. Warashina, Y. Tanaka, K. Yoshinari and K. Taira, *Genes Cells*, 2000, **5**, 603–612; (*h*) M. Warashina, T. Kuwabara, Y. Nakamatsu, Y. Takagi, Y. Kato and K. Taira, *J. Am. Chem. Soc.*, 2004, **126**, 12291–12297.
18 J.-M. Zhou, D.-M. Zhou, Y. Takagi, Y. Kasai, A. Inoue, T. Baba and K. Taira, *Nucleic Acids Res.*, 2002, **30**, 2374–2382.
19 (*a*) J. B. Murray, A. A. Seyhan, N. G. Walter, J. M. Burke and W. G. Scott, *Chem. Biol.*, 1998, **5**, 587–595; (*b*) J. L. O'rear, S. Wang, A. L. Feig, L. Beigelman, O. C. Uhlenbeck and D. Herschlag, *RNA*, 2001, **7**, 537–545; (*c*) E. A. Curtis and D. P. Bartel, *RNA*, 2001, **7**, 546–552; (*d*) Y. Takagi and K. Taira, *J. Am. Chem. Soc.*, 2002, **124**, 3850–3852; (*e*) Y. Takagi, A. Inoue and K. Taira, *J. Am. Chem. Soc.*, 2004, **126**, 12856–12864.
20 (*a*) J. SantaLucia, Jr. and D. H. Turner, *Biochemistry*, 1993, **32**, 12612–12623; (*b*) M. Katahira, H. Sato, K. Mishima, S. Uesugi and S. Fujii, *Nucleic Acids Res.*, 1993, **21**, 5418–5424; (*c*) M. Katahira, M. Kanagawa, H. Sato, S. Uesugi, S. Fujii, T. Kohno and T. Maeda, *Nucleic Acids Res.*, 1994, **22**, 2752–2759.
21 (*a*) J. S. Kieft and I. Tinoco, Jr., *Structure.*, 1997, **5**, 713–721; (*b*) M. Katahira, M. H. Kim, T. Sugiyama, Y. Nishimura and S. Uesugi, *Eur. J. Biochem.*, 1998, **255**, 727–733; (*c*) R. L. Gonzalez, Jr. and I. Tinoco, Jr., *J. Mol. Biol.*, 1999, **289**, 1267–1282; (*d*) G. Colmenarejo and I. Tinoco, Jr., *J. Mol. Biol.*, 1999, **290**, 119–135; (*e*) S. Rüdisser and I. Tinoco, Jr., *J. Mol. Biol.*, 2000, **295**, 1211–1223; (*f*) M. Schmitz and I. Tinoco, Jr., *RNA*, 2000, **6**, 1212–1225.
22 (*a*) G. W. Buchanan and J. B. Stothers, *Can. J. Chem.*, 1982, **60**, 787–791; (*b*) G. W. Buchanan and M. J. Bell, *Can. J. Chem.*, 1983, **61**, 2445–2448; (*c*) P. Legault, C. G. Hoogstraten, E. Metlitzky and A. Pardi, *J. Mol. Biol.*, 1999, **284**, 325–335; (*d*) S. E. Butcher, F. H.-T. Allain and J. Feigon, *Biochemistry*, 2000, **39**, 2174–2182; (*e*) M. R. Hansen, J.-P. Simorre, P. Hanson, V. Mokler, L. Bellon, L. Beigelman and A. Pardi, *RNA*, 1999, **5**, 1099–1104; (*f*) S. Steinkopf, Q. L. Liu, E. Sletten and N. Å. Frøystein, *Acta Chem. Scand.*, 1992, **46**, 446–450; (*g*) F. Reeder, Z. J. Guo, P. D. Murdoch, A. Corazza, T. W. Hambley, S. J. Berners-Price, J. C. Chottard and P. J. Sadler, *Eur. J. Biochem.*, 1997, **249**, 370–382; (*h*) S. J. Berners-Price, K. J. Barnham, U. Frey and P. J. Sadler, *Chem. Eur. J.*, 1996, **2**, 1283–1291; (*i*) J. Flinders and T. Dieckmann, *J. Mol. Biol.*, 2004, **341**, 935–949.
23 (*a*) T. E. Horton, D. R. Clardy and V. J. DeRose, *Biochemistry*, 1998, **37**, 18094–18101; (*b*) S. R. Morrissey, T. E. Horton, C. V. Grant, C. G. Hoogstraten, R. D. Britt and V. J. DeRose, *J. Am. Chem. Soc.*, 1999, **121**, 9215–9218; (*c*) S. R. Morrissey, T. E. Horton and V. J. DeRose, *J. Am. Chem. Soc.*, 2000, **122**, 3473–3481; (*d*) L. M. Hunsicker and V. J. DeRose, *J. Inorg. Biochem.*, 2000, **80**, 271–281; (*e*) C. G. Hoogstraten, C. V. Grant, T. E. Horton, V. J. DeRose and R. D. Britt, *J. Am. Chem. Soc.*, 2002, **124**, 834–842.
24 At the current time, this technique provides us with the most reliable solution structures of metal–ligand complexes. It should also be mentioned that discrimination of the metal–ligand bond is performed with the calculated structure, which satisfies the EPR spectra.
25 G. Wang, B. L. Gaffney and R. A. Jones, *J. Am. Chem. Soc.*, 2004, **126**, 8908–8909.
26 V. Sychrovsky, J. Sponer and P. Hobza, *J. Am. Chem. Soc.*, 2004, **126**, 663–672.
27 J. A. Cowan, *J. Inorg. Biochem.*, 1993, **49**, 171–175.
28 E. Ennifar, P. Walter and P. Dumas, *Nucleic Acids Res.*, 2003, **31**, 2671–2682.
29 K. J. Baeyens, H. L. DeBondt, A. Pardi and S. R. Holbrook, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 12851–12855.
30 L. E. Kapinos, A. Holy, J. Günter and H. Sigel, *Inorg. Chem.*, 2001, **40**, 2500–2508.
31 (*a*) H. A. Heus and A. Pardi, *Science*, 1991, **253**, 191–194; (*b*) J. SantaLucia, Jr. and D. H. Turner, *Biochemistry*, 1993, **32**, 12612–12623; (*c*) M. Wu and D. H. Turner, *Biochemistry.*, 1996, **35**, 9677–9689; (*d*) M. Wu, J. SantaLucia, Jr. and D. H. Turner, *Biochemistry.*, 1997, **36**, 4449–4460; (*e*) H. A. Heus, S. S. Wijmenga, H. Hoppe and C. W. Hilbers, *J. Mol. Biol.*, 1997, **271**, 147–158.
32 D. Gautheret, D. Konings and R. R. Gutell, *J. Mol. Biol.*, 1994, **242**, 1–8.
33 A. Khvorova, A. Lescoute, E. Westhof and S. D. Jayasena, *Nat. Struct. Biol.*, 2003, **10**, 708–712.
34 (*a*) A. C. Forster and R. H. Symons, *Cell*, 1987, **50**, 9–16; (*b*) L. M. Pabon-Pena, Y. Zhang and L. M. Epstein, *Mol. Cell Biol.*, 1991, **11**, 6109–6115.
35 X. Jia, G. Zon and L. G. Marzilli, *Inorg. Chem.*, 1991, **30**, 228–239.
36 S. Mukundan, Jr., Y. Xu, G. Zon and L. G. Marzilli, *J. Am. Chem. Soc.*, 1991, **113**, 3021–3027.
37 S. E. Sherman, D. Gibson, A. H.-J. Wang and S. J. Lippard, *Science*, 1985, **230**, 412–417.
38 S. E. Sherman and S. J. Lippard, *Chem. Rev.*, 1987, **87**, 1153–1181.
39 W. I. Sundquist and S. J. Lippard, *Coord. Chem. Rev.*, 1990, **100**, 293–322.
40 Y. Tanaka, S. Fujii, H. Hiroaki, T. Sakata, T. Tanaka, S. Uesugi, K. Tomita and Y. Kyogoku, *Nucleic Acids Res.*, 1999, **27**, 949–955.
41 B. L. Golden, A. R. Gooding, E. R. Podell and T. R. Cech, *Science*, 1998, **282**, 259–264.