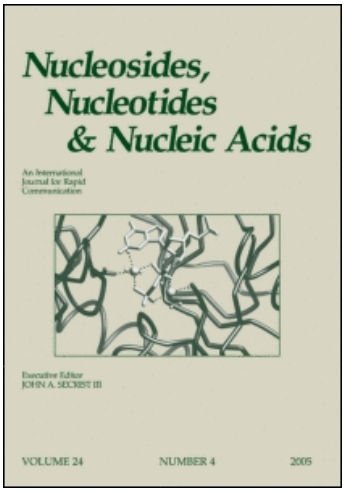

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

NMR Studies of a Lead Ribozyme and Its Non-Cleavable Analogue

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To cite this Article Katahira, Masato , Sugiyama, Takashi , Kanagawa, Mayumi , Kim, Mi Hee , Uesugi, Seiichi and Kohno, Toshiyuki(1996) 'NMR Studies of a Lead Ribozyme and Its Non-Cleavable Analogue', Nucleosides, Nucleotides and Nucleic Acids, 15: 1, 489 — 503
To link to this Article: DOI: 10.1080/07328319608002400
URL: http://dx.doi.org/10.1080/07328319608002400

NUCLEOSIDES & NUCLEOTIDES, 15(1-3), 489-503 (1996)

NMR STUDIES OF A LEAD RIBOZYME AND ITS NON-CLEAVABLE ANALOGUE

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ABSTRACT: The structure of a lead ribozyme, which consists of two RNA strands, at neutral pH has been studied by NMR. Nearly all resonances of imino protons, base protons (H2, H5, H6 and H8) and sugar protons (H1' and H2') were assigned sequentially. Interesting structural features which deviate from the standard structure were found for the residues at an active site which consists of an internal loop. No indication of stable G:A base pairs was found in the loop. The effect of addition of Pb^{2+} was studied by the use of a non-cleavable analogue in which the cytidine at a cleavage site is replaced by 2'-O-methylcytidine. It was suggested that Pb^{2+} binds close to the cleavage site and that the structural change induced by Pb^{2+} is moderate and localized.

INTRODUCTION

A lead ribozyme was discovered by in vitro selection experiments of $tRNA^{Phe}$.[1] The cleavage reaction is highly specific for Pb^{2+}. It does not need other ions such as Mg^{2+}.[2] Initially the lead ribozyme was discovered as a single RNA molecule, but later it was found that the lead ribozyme can be formed by a combination of two separate RNAs. The active site of the lead ribozyme consists of an asymmetric internal loop of six nucleotides.

Because the lead ribozyme was discovered recently, no structural information has been reported except for two reports,[3,4] where ^{13}C and ^{1}H chemical shifts of just three A residues and ^{31}P chemical shifts of the lead ribozyme at acidic pH were shown. Here we report the first com-

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#This paper is dedicated to Dr. Yoshihisa Mizuno on the occasion of his 75th birthday.

prehensive structural study of the lead ribozyme by means of NMR. FIG. 1(a) shows the sequence and numbering of the studied lead ribozyme which consists of two strands. It was found that specific cleavage occurs in the presence of Pb^{2+} at the site indicated with an arrow in FIG.1 and that the cleavage activity is higher at neutral pH than at acidic pH (Kim et al., unpublished results). The sequence of an RNA duplex which contains a partial sequence of hammerhead ribozymes, and has already been studied by NMR is also shown in FIG. 1.[5] The homologous sequences between the lead ribozyme and the RNA duplex are boxed. The non-cleavable analogue, in which the cytidine at the cleavage site is replaced by 2'-O-methylcytidine, was also studied in order to investigate the effect of Pb^{2+}.

MATERIALS AND METHODS

The two RNA oligomers for the lead ribozyme (FIG. 1(a)) were synthesized manually by the solid-phase phosphoramidite method using o-nitrobenzyl groups for 2'-OH protection and purified as described previously.[6] The non-cleavable substrate, in which the cytidine at the cleavage site (C6 in FIG. 1(a)) is replaced by the 2'-O-methylcytidine, was synthesized and purified as described previously.[6] Equimolar amounts of the two RNA oligomers were mixed and then annealed by heating up to 85 °C followed by slow cooling. The annealed sample was applied on a Sephadex G-50 column to remove single stranded materials and lyophilized. The sample of the non-cleavable analogue was also prepared in the same manner.

For NMR measurement of non-exchangeable protons of the lead ribozyme, the lyophilized sample was dissolved in 20 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The solution was lyophilized and dissolved in 0.17 ml of D_2O (99.96 %). The concentration of the lead ribozyme was 3.0 mM. For measurement of exchangeable protons, an H_2O-D_2O mixture (19:1) was substituted for D_2O. For the non-cleavable analogue, 15 mM MOPS buffer (pH 6.7) containing 30 mM NaCl and 2 mM sodium phosphate was used and two samples (3.0 mM and 0.5 mM) were prepared. DSS was used as an internal chemical shift reference.

NMR spectra were recorded at 10-50 °C with Bruker AMX-500 and AM-400 NMR spectrometers. Phase-sensitive NOESY[7], HOHAHA[8] and C-H HMQC[9] spectra were recorded by the time-proportional phase-increment method[10].

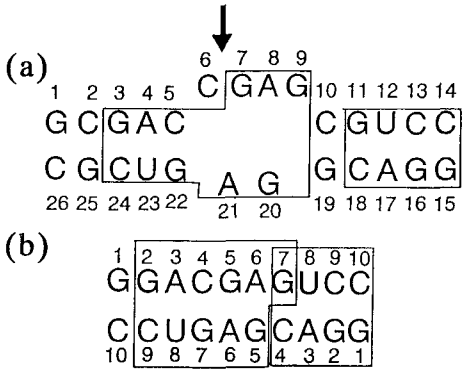

FIG. 1 The sequences and numberings of the lead ribozyme studied (a) and the RNA duplex which contains a partial sequence of hammerhead ribozymes, and was studied previously[3] (b). An arrow indicates the site of specific cleavage in the presence of Pb^{2+}. The homologous sequences between the lead ribozyme and the RNA duplex are boxed. In the case of the non-cleavable analogue, C6 is replaced by 2'-O-methylcytidine.

NOESY with jump-and-return pulse sequences[11] as three 90° pulses was used for 2D measurement of exchangeable protons. The mixing times for NOESY were 100, 120, 150 and 300 ms, and that for HOHAHA was 40 ms. The repetition delay was 2.0 s. NOESY and HOHAHA were recorded with 512 t_1 increments, 96-256 free induction decays of 2 K data points per increment being collected. C-H HMQC spectra were recorded with 154 t_1 increments, 600 free induction decays of 2 K data points per increment being collected. The t_1 and t_2 data were apodized with a $\pi/3$-shifted sine-bell function for NOESY and HOHAHA in D_2O, and with an exponential function with 10 Hz of a broadening factor for NOESY in H_2O and C-H HMQC. One-dimensional spectra in H_2O were accumulated with a 1-1 pulse sequence,[12] and NOE difference spectra were obtained as described previously.[13] A concentrated $Pb(CH_3COO)_2$ solution was added to the solution of the non-cleavable analogue in the Pb^{2+} titration experiments.

RESULTS

Assignments of the imino protons of the lead ribozyme.

One-dimensional ^1H NMR spectrum of the imino protons of the lead ribozyme at pH 7.0 is shown in FIG. 2(a). The resonances were assigned on the basis of sequential NOE cross peaks observed in the NOESY spec-

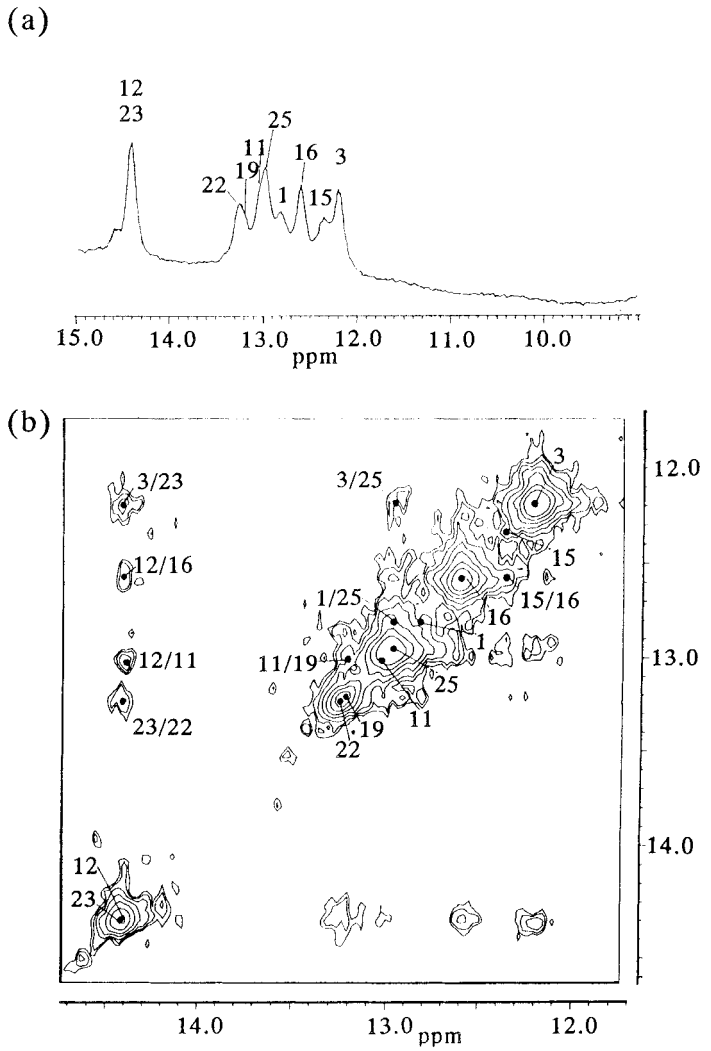

FIG. 2 (a) One-dimensional ^1H NMR spectrum of the lead ribozyme in H_2O at pH 7.0 and 25 °C, with the assignments of the imino protons indicated by the residue numbers. (b) NOESY spectrum of the lead ribozyme in H_2O with a mixing time of 120 ms at pH 7.0 and 25 °C, with the assignments and the sequential cross peaks labelled.

trum shown in FIG. 2(b). The assignments were further confirmed by the following observations: the intra- and inter-base-pair NOEs between AH2 and imino protons,[13,14] the intra-base-pair NOE between CH5 and an imino proton of G[15] and the inter-base-pair NOEs between H1's and an imino proton of G.[15] They are all consistent with the assignments.

Assignments of the non-exchangeable protons of the lead ribozyme.

The resonances of non-exchangeable protons of the lead ribozyme were assigned sequentially by analysis of two-dimensional NOESY, HOHAHA and C-H HMQC spectra in D_2O in the same way as reported for other DNA and RNA,[5,13-17] using the previously established methods.[9,15,18-26] FIG. 3 shows an expansion of the NOESY spectrum, indicating the sequential assignments of H1' and H6/H8 through the H1'(i-1)-H6/H8(i)-H1'(i) connectivities. The intraresidue H5-H6 cross peaks of the pyrimidines are also labelled for reference. The assignments were confirmed further by H6/H8(i-1)-pyrimidine H5(i) NOEs (labelled with A-G in FIG. 3), AH2(i)-H1'(i+1) NOEs (labelled with a-c in FIG. 3) and H6/H8(i)-H6/H8(i+1) NOEs (data not shown). The sequential assignments are interrupted at the G9 residue, which will be discussed later. G9H8 is assigned tentatively on the basis of the potential C10H5-G9H8 cross peak. G9H1' is not assigned.

Most of the H1' resonances of the residues outside of the internal loop give no cross peak in HOHAHA spectrum (data not shown). This is a typical phenomenon for the A form structure, where the sugar puckering is C3'-endo, and thus the coupling constant between H1' and H2' is almost zero. The H1'-H2' distance is the shortest among the H1'-H2', H1'-H3' and H1'-H4' distances for virtually all puckering geometries of the sugar ring[27,28] (only for puckering around O4'-endo, is the H1'-H4' distance shorter than the H1'-H2' distance[27]). Particularly for the C3'-endo puckering suggested above, the H1'-H2' distance is much shorter than the other two distances[27]. Thus, H2' resonances of the residues outside of the internal loop were assigned on the basis of the strong H1'-H2' NOE cross peaks in the NOESY spectra (data not shown). Those assignments for H2' were further confirmed by the successful tracing of the sequential H2'(i-1)-H6/H8(i)-H2'(i) connectivities (data not shown). Generally, very strong interresidue H2'(i-1)-H6/H8(i) cross peaks and weaker intraresidue H2'(i)-H6/H8(i) cross peaks are observed. This is

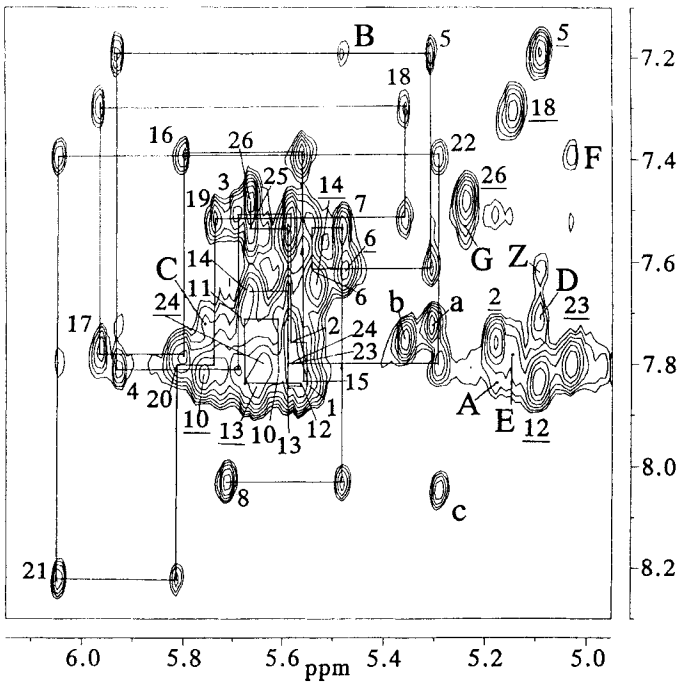

FIG. 3 Expansion of the NOESY spectrum of the lead ribozyme in D_2O with a mixing time of 300 ms at pH 7.0 and 37 °C. The lines show the H1'(i-1)-H6/H8(i)-H1'(i) connectivities. The intraresidue H6/H8-H1' cross peaks are indicated by their residue numbers. The intraresidue H5-H6 cross peaks of pyrimidines are indicated by underlined residue numbers. H6/H8(i-1)-pyrimidine H5(i) cross peaks are indicated by A-G (A, G1H8-C2H5; B, C5H6-C6H5; C, G9H8-C10H5; D, G11H8-U12H5; E, A17H8-C18H5; F, G22H8-U23H5; G, G25H8-C26H5). An unusual C5H5-C6H6 cross peak is indicated by Z. AH2(i)-H1'(i+1) cross peaks are indicated by a-c (a, A4H2-C5H1'; b, A17H2-C18H1'; c, A21H2-G22H1').

again a characteristics of the A form structure with C3'-endo sugar puckering.[15,18,23,24,27,28]

The H1' resonances of the residues in the internal loop give H1'-H2' HOHAHA cross peaks except for C6 (data not shown), which indicates deviation from C3'-endo sugar puckering, and thus their H2' resonances were assigned. G9H2' is not assigned because G9H1' is not assigned. The assignments of the non-exchangeable protons were consistent with the C-H HMQC spectrum.[9]

Assignments of the imino protons of the analogue.

Because the imino proton spectrum of the analogue (FIG. 4(a)), in which the C6 is replaced by 2'-O-methylcytidine, is very similar to that of the lead ribozyme, the assignments were made almost automatically by comparing the two spectra. The assignments were confirmed by one-dimensional NOE difference spectra carried out at the analogue concentration of 3.0 mM (data not shown), as described previously.[13] One of the two resonances originated from the terminal residues is absent and the other is broadened at the analogue concentration of 0.5 mM due to fraying (FIG. 4(a)), while they are observed clearly at the analogue concentration of 3.0 mM (TABLE 1).

All the assignments are summarized in TABLE 1. As shown in FIG. 1, the lead ribozyme contains the sequences which are homologous to those of the RNA duplex, $r(GGACGAGUCC)_2$. Characteristic patterns in the trace of H1'(i-1)-H6/H8(i)-H1'(i) connectivities in the NOESY spectra were commonly recognized for each boxed sequence. Chemical shifts of H2's of the lead ribozyme were generally close to those of the corresponding residues of the RNA duplex. These are indications of the reliability of the assignments of the lead ribozyme.

Change in the imino proton spectrum of the analogue on addition of Pb^{2+}.

The change of the imino proton spectrum of the analogue on addition of Pb^{2+} was traced. FIG. 4(b) shows the spectrum at Pb^{2+} concentration of 0.5 mM, where the molecular ratio of the analogue to Pb^{2+} is 1:1. The binding constant of Pb^{2+} to the analogue has been estimated by the use of the change of the CD spectrum of the analogue upon addition of Pb^{2+}, and it is expected that most of the analogue molecules are bound by Pb^{2+} under these conditions (Kim et al., unpublished results). The imino proton spectrum remains essentially the same. It is noted that the resonance G22 exhibits a larger shift than the other resonances. The signal-to-noise ratio of FIG. 4(b) is better than that of FIG. 4(a), because the number of FIDs accumulated for FIG. 4(b) is more than that for FIG. 4(a).

DISCUSSIONS

No indication of stable G:A base pairs in the internal loop at neutral pH.

Since the lead ribozyme contains two GA segments in the internal loop (FIG. 1(a)), it can be assumed that G:A base pairs are formed

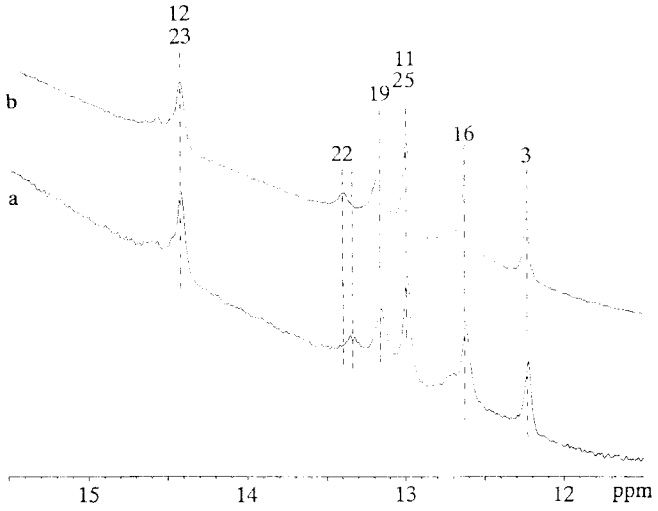

FIG. 4 One-dimensional ^1H NMR spectra of the non-cleavable analogue in which the C6 is replaced by 2'-O-methylcytidine in H_2O at pH 6.7 and 16 °C, with the assignments of the imino protons indicated by the residue numbers. The concentration of the analogue is 0.5 mM. (a) 0 mM Pb^{2+} and (b) 0.5 mM Pb^{2+}.

between G7 and A21 and between A8 and G20. Several different types of G:A base pairs have been observed: 'head to head' G(anti):A(anti),[29-33] 'sheared' G(anti):A(anti),[5,14,35-48] G(anti):A(syn)[49] and G(syn):A(anti).[32,33,50] Quite recently the 'sheared' G:A base pairs have been found in the crystal structure of a hammerhead ribozyme.[51]

As discussed below, all of G7, A8, G20 and A21 take an anti conformation. Thus 'head to head' and 'sheared' G:A base pairs are possible candidates, if G:A base pairs are formed.

When the 'head to head' G:A base pair is formed, the imino proton signal of G is observed at around 12.5-13.5 ppm.[29,30,32] When the 'sheared' G:A base pair is formed, the signal is observed at around 9.5-10.5 ppm.[5,14,35-39,44] In the case of $r(GGACGAGUCC)_2$ (FIG. 1(b)), for example, the imino proton signal of G5 forming the 'sheared' G:A base pair with A6 is observed at 9.89 ppm.[5] However, the imino proton signal of G in the internal loop is not observed at pH 7.0 (FIG. 2(a)), although it is difficult to completely rule out the possibility that the signal is hidden by overlapping with other signals in the 12.1-13.3

TABLE 1. ^{1}H chemical shifts for the lead ribozyme at 37 °C.[a]

RESIDUE	H6/H8	H2/H5	H1'	H2'	IMINO	IMINO(analogue)[b]
G1	7.84	-----	5.56	4.61	12.82	12.83[c]
C2	7.76	5.18	5.59	4.57	-----	-----
G3	7.51	-----	5.69	4.57	12.22	12.23
A4	7.82	7.73	5.93	4.53	-----	-----
C5	7.20	5.09	5.31	4.29	-----	-----
C6	7.62	5.48	5.56	4.18	-----	-----
G7	7.53	-----	5.49	4.20	n.o.[d]	n.o.[d]
A8	8.03	7.80	5.72	4.35	-----	-----
G9	7.72[e]	-----	n.a.[f]	n.a.[f]	n.o.[d]	n.o.[d]
C10	7.82	5.76	5.61	4.55	-----	-----
G11	7.71	-----	5.68	4.51	13.03	12.99
U12	7.84	5.10	5.56	4.50	14.40	14.42
C13	7.85	5.66	5.59	4.29	-----	-----
C14	7.67	5.54	5.67	4.04	-----	-----
G15	7.85	-----	5.56	4.72	12.36	12.38[c]
G16	7.38	-----	5.80	4.60	12.60	12.63
A17	7.78	7.76	5.96	4.52	-----	-----
C18	7.30	5.15	5.36	4.31	-----	-----
G19	7.52	-----	5.74	4.76	13.21	13.17
G20	7.78	-----	5.82	4.79	n.o.[d]	n.o.[d]
A21	8.23	8.06	6.05	4.81	-----	-----
G22	7.39	-----	5.29	4.52	13.25	13.34
U23	7.80	5.03	5.58	4.50	14.41	14.42
C24	7.80	5.64	5.59	4.52	-----	-----
G25	7.54	-----	5.66	4.44	12.96	12.99
C26	7.47	5.24	5.66	4.16	-----	-----

[a] At 25 °C for the imino protons of the lead ribozyme, and at 16 °C for those of the analogue.
[b] For the non-cleavable analogue in which C6 is replaced by 2'-O-methylcytidine at the analogue concentration of 0.5 mM and without Pb^{2+}.
[c] At the analogue concentration of 3.0 mM.
[d] Not observed.
[e] Tentatively assigned.
[f] Not assigned.

region of the FIG. 2(a). Therefore there is no indication of stable G:A base pair formation in the internal loop region at neutral pH.

When the pH was lowered to 5.5, broad signals appeared at around 10.5 and 10.9 ppm (data not shown). These signals could be those for the G:A base pairs because their chemical shifts are close to the shifts expected for sheared G:A base pairs, although they could also be the signals for non-base-paired G residues observable at low pH due to the

reduced exchange rate with H_2O. Because the lead ribozyme exhibits higher activity at neutral pH than that at acidic pH (Kim et al., unpublished results), base pairing at pH 5.5 was not investigated further.

The structure outside of the internal loop.

The structure outside of the internal loop is essentially A form. Except for G19, the H1'-H2' cross peak in the HOHAHA spectrum was not observed for the residues locating outside of the internal loop, indicating that the sugar puckering is C3'-endo characteristic of A form.[27,28] H2'(i-1)-H6/H8(i) cross peaks in NOESY spectra were generally strong for those residues, which also supports the A form structure.[27] Additionally, strong AH2(i)-H1'(i+1) cross peaks were observed in the NOESY spectra for A4 and A17 (labelled with a and b in FIG. 3), which again suggests the A form structure.[15,27]

Some distortion of the A form structure is noted for the residues locating close to the internal loop such as G19.

The structure of the internal loop.

As mentioned in RESULTS, H1'(i-1)-H6/H8(i)-H1'(i) connectivities are traced through out the two strands, except for the interruption at the residue G9. Successful tracing of the connectivities indicates that a right-handed helical structure is maintained not only in the stem region but also in the loop region.[27]

For G9, any cross peak which could correspond to either intraresidue G9H8-G9H1' or interresidue G9H8-A8H1' or C10H6-G9H1' was not observed in NOESY spectra. It is not likely that all of these cross peaks are hidden by overlapping with other cross peaks. One possible explanation is that the G9 residue is flipped out of the helical structure and that the interresidue distances are too large to give the NOEs. Enhanced mobility of the G9 residue resulting from the flipping out may also contribute to the absence of NOEs. The flipping out of G9 is also suggested from the A8H8-C10H5 NOE which indicates that A8 and C10 bases are close to each other (This NOE is observed when the level of the plot of FIG. 3 is lowered).

At the C6 residue, on the other hand, the H1'(i-1)-H6/H8(i)-H1'(i) connectivities are not interrupted, suggesting that C6 is accommodated into the helical structure, instead of flipping out. It is noted that the H6/H8(i)-H6/H8(i+1) NOE is observed for C5-C6 but not for C6-G7. It is also noted that an unusual C5H5-C6H6 NOE is observed[27] (indicated by

Z in FIG. 3), in addition to the usual[27] C5H6-C6H5 NOE (indicated by B in FIG. 3). These results suggest that the C6 residue is accommodated in the helical structure, being closer to the C5 residue than to the G7 residue.

All of G7, A8, G20 and A21 take an anti conformation at neutral pH, because their intraresidue H8-H1' cross peaks in the NOESY spectrum with a mixing time of 100 ms are not as strong as expected for a syn conformation[5,27] (data not shown). These four residues together with G19 give clear H1'-H2' cross peaks in the HOHAHA spectrum, indicating that their sugar puckering is not C3'-endo characteristic of A form. The H2'(i-1)-H6/H8(i) NOE is missing for C6-G7 and G7-A8, which is another indication of the deviation from the A form structure.

After all, it turns out that the structure of the internal loop which consists of six nucleotides deviates from the standard structure of RNA. The following interesting NOEs which could be used to construct the structure of the active site of the lead ribozyme were also observed in the NOESY spectra with a mixing time of 300 ms at neutral pH: strong A21H2-G22H1' (indicated by c in FIG. 3), and weak interstrand A8H2-G20H1', A8H2-A21H1', A21H2-G7H1', A21H2-A8H1' and A21H2-G7H8.

The site of Pb^{2+}-binding and its effect on the structure.

The imino proton spectrum of the non-cleavable analogue, in which C6 is replaced by 2'-O-methylcytidine, is very similar to that of the lead ribozyme as summarized in TABLE 1. As far as the imino proton spectra of the lead ribozyme and the analogue are compared, it is suggested that the replacement produces little effect on the structure, although further analysis of the analogue in D_2O is required for definite confirmation.

When Pb^{2+} was added, the imino proton spectrum remains essentially the same (FIG. 4(b)). Changes in the chemical shift are little for most resonances (< ca. 0.01 ppm). It is remarkable that a much larger change (c.a. 0.06 ppm) is observed exclusively for the imino proton signal of G22, which suggests that Pb^{2+} binds near the G22 residue. This positioning of Pb^{2+} is reasonable, because the Pb^{2+} can be located close to the cleavage site (FIG. 1(a)).

The drastic change of the imino proton spectrum, such as large shift of the resonances in the whole spectral region or appearance of

new resonances, does not occur upon addition of Pb^{2+}. Rather, a moderate shift is observed for the resonance of a particular residue. This indicates that the effect of Pb^{2+} on the structure is moderate and localized. It is assumed that the added Pb^{2+} gets accommodated into the pocket already formed by the two strands of the lead ribozyme, resulting in moderate and localized change of the structure of the active site, and that the specific cleavage occurs probably through extraction of a proton from the 2'-OH of the C6 residue to allow nucleophilic attack of the 2' oxyanion at the adjacent phosphodiester bond.[1] It is likely that the size of Pb^{2+} best fits to the pocket already formed by the two strands of the lead ribozyme and/or Pb^{2+} can be accommodated in the pocket in the best orientation to extract the proton, and that thus the cleavage of the lead ribozyme is specific for Pb^{2+}.

ACKNOWLEDGEMENTS

We wish to thank Professor H. Akutsu (Yokohama Natl. Univ.) and Dr. G. Kawai (Tokyo Univ.) for giving us the opportunity to use the NMR machines and for helpful discussions. We thank Mr. K. Moriyama and Mr. J. Saeki for their contribution in preparing mononucleotides and assignments, respectively. M.K. was supported by a Grant-in-Aid for Scientific Research on Priority Areas (#06276103 and #06276105) and a grant (#07680713) from the Ministry of Education, Science and Culture of Japan, and a grant from the Kanagawa Academy of Science and Technology.

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